ACTA CHEMICA SCANDINAVICA 13 (1959) 1—12

Determination of Free Protein Mobilities by Paper Electrophoresis with Evaporation *

I. Evaluation of the Buffer Flow Due to Evaporation and Electroosmosis

KURT SCHILLING and HEINZ WALDMANN-MEYER

From the Biological Institute, Carlsberg Foundation, Copenhagen, Denmark

A procedure was devised for the determination of electrophoretic mobilities by means of open-strip paper electrophoresis.

An equation covering evaporation and electroosmosis was derived. The fundamental premise is the constancy of the electric current during the experiment. Evaporation and electroosmosis are represented by the parameters f and e, respectively. The determination of these parameters may be omitted in the calculation of electrophoretic mobilities, since a correction for both evaporation and electroosmosis is also obtained by running a single aliquot of an uncharged substance parallel to the sample.

The equation was tested in an experimental series where current and concentration were systematically varied. It was thus confirmed that the migration takes place in a zone of increasing, but uniform, salt concentration. This was further substantiated by independent procedures. The migration of dextran spots placed evenly along the strip was found to be in agreement with the theoretical basis. Straight lines were invariably obtained when the migration distance was plotted against the position of the application points. When the migration distance was first divided by time and voltage, all the lines intersected the ordinate at the same point, and the slopes were proportional to the electric current, thus demonstrating the constancy of the parameters f and e.

Problems arising from the presence of evaporation in paper electrophoretic mobility determinations are discussed. It is emphasized that a satisfactory method is obtained by using constant electric current, instead of the constant voltage hitherto considered essential.

Since the appearance in 1937 of the first report on zone electrophoresis [1] numerous technical modifications, aiming at the achievement of greater reproducibility and precision, have been introduced by various authors in order

* Presented at the Meeting of the Kemisk Forening, Copenhagen, March 25th, 1958.

to raise the reliability of the method to the level attained by free electrophoresis.

Undoubtedly this object has been accomplished as far as fractionation and clinical analysis of proteins are concerned, where zone electrophoresis, especially on paper strips, has even proved superior in several aspects to the classical Tiselius technique. However, serious difficulties are encountered in determinations of the electrophoretic mobility since interfering factors unknown from the Tiselius method, *i. e.* the electroosmotic flow, the evaporation of the buffer due to heat development and the influence of the carrier medium, have to be taken into account.

On the other hand, paper electrophoresis offers so many advantages, one of them being the minimal amount of substance required, that it may be considered a matter of great interest to arrive at a satisfactory method for the determination of mobilities and isoelectric points by means of this technique.

The mobility obtained in free solution at a defined pH and temperature, although in some degree dependent on the type and concentration of the buffer used, may be considered a characteristic property of a protein and is referred to as *free mobility* in the following. In order to have any practical value, a method using paper electrophoresis would have to yield the same free mobilities.

Previous approaches are scarce and seem in several respects open to criticism. In the present study the effects of the interfering factors have been evaluated quantitatively and a procedure developed yielding results identical with those obtained by free electrophoresis. In the first part of this investigation the buffer flow caused by the combined effects of evaporation and electroosmosis has been studied, and it is shown that, when a constant current is used throughout the experiment, a simple expression can be derived for the mobility of a migrating substance.

MATERIALS AND METHODS

Apparatus. Horizontal open strip electrophoresis sets with buffer cells of about 1 000 ml capacity were employed, each holding two paper strips. The height of the buffer levels was equalized by means of leveling glass tubes prior to equilibration of the strip. The paper rested upon nylon threads between two 29 × 8.5 cm glass plates, kept 6 mm apart and tightened at the edges by plastic borders which held the nylon threads. At all times the strip was in strictly horizontal position. The length of the vertical paper section to the buffer surface was 4.5 cm. The voltage gradient over the paper was measured by secondary platinum electrodes fixed to two of the nylon threads at 15.0 cm distance from each other. The power supply was a valve rectifier in conjunction with an adjustable electronic current stabilizer, giving a constancy of about 0.1 %.

Solutions. Sodium acetate buffers of pH 5.10 and of 0.045 and 0.09 ionic strength.

4 % solutions of dextran (Pharmacia, Uppsala) in the buffer, prepared by gentle heating.

Procedure. Two 8 × 39 cm strips of Munktell paper No. 20/150 were used per cell unit. The strips were immersed in buffer for a few seconds and blotted on crepe-paper before being placed into the apparatus. After a minimum of 3 h equilibration, six 10 μl spots of dextran solution were applied at 3 cm distance from each other measured along the longitudinal axis of the paper. In order to avoid interference, they were placed alternately to the right and the left at 2.5 cm distance from the paper edge.

The current was switched on immediately after the application of the solution to the paper, and the experiments were run for 17 to 20 h at room temperature ranging from 20.8 to 21.5°C. Current and voltage were recorded at the beginning and conclusion of the experiment. Corrections for voltmeter resistance were introduced and a correction of 0.8 V (0.053 V · cm^{-1}), due to the polarization of the secondary platinum electrodes was added to all readings. The polarization was estimated by measuring the voltage between 3 platinum electrodes A, B, and C on the paper with constant current. The difference between the voltage readings AB + BC and AC would be equal to the polarization potential. The same value was obtained from rapid reversal of the electrode polarization.

After conclusion of the experiment, the vertical paper ends were cut off, and the strips dried horizontally in the air at room temperature, after which they were placed for 30 min in a hot air oven at 110°C and then stained for 25 min with a 1 % bromo phenolblue (Merck) solution in 95 % ethanol containing about 10 % (w/v) $HgCl_2$. Since the dextran spots appeared most clearly during the first few minutes of staining, their centers were marked with a pencil during the process. Finally, the strips were washed several times with methanol until the appearance of a white background, and the migration distance measured.

THEORY

The equations derived below are based on a few simple assumptions which, as well as the conclusions drawn from them, have been experimentally tested, as described in the next paragraph.

The symbols used will be defined below in connection with the explanations. Distances and velocities are considered positive in the direction anode to cathode, according to the usual convention.

It is assumed that the migration velocity ds/dt is made up of three separate components in the following way:

$$\frac{ds}{dt} = m \cdot V + e \cdot V + (a - s)\ \mathrm{L} \qquad \text{(I,1)}$$

The first term gives the migration rate due to the influence of the electric field on the migrating particle and is proportional to the potential gradient V. It appears as $m \cdot V$ in equation I,1. The factor m used for the mobility differs from the free mobility u on account of the retarding effects of the carrier medium. This problem will be treated in the subsequent communication [2].

The two other terms express the migration velocity caused by the buffer flow. $e \cdot V$ represents the electroosmotic flow, which too is approximately proportional to V. The last term of the equation gives the migration velocity due to evaporation. Assuming uniform evaporation along the strip and that the capillary forces keep the amount of solution in the paper constant, this component of the migration velocity will be proportional to the distance

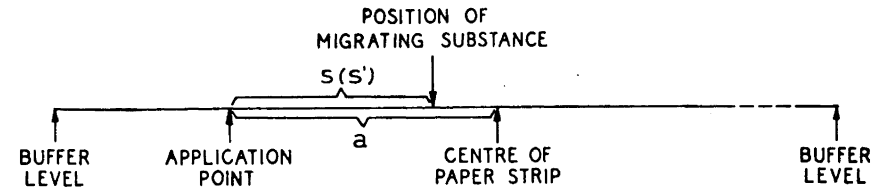


Fig. 1. Schematic presentation of the experimental arrangement.

between the sample and the center of the paper strip, as expressed by $(a—s)$, where a is the distance from the point of application to the paper center and s the distance of migration (Fig. 1).

L denotes the intensity of evaporation and may be assumed to be proportional to the wattage dissipation $(i \cdot V)$ in the paper:

$$L = f \cdot i \cdot V \tag{I,2}$$

The increase of the salt concentration caused by the evaporation from the strip produces a corresponding increase of the buffer conductivity. Working with constant current this results in a steadily decreasing voltage V. To obtain the correct time function we again consider Fig. 1. s' now represents the distance which the buffer has migrated by evaporation alone. The concentration has increased by the factor $\frac{a}{a—s'}$, and, assuming that the activity coefficients remain practically constant, as in the case of monovalent ions in the concentration range usual in electrophoresis, the voltage has dropped correspondingly from its initial value V_0:

$$V = V_0 \frac{a—s'}{a} \tag{I,3}$$

The migration velocity ds'/dt of the buffer as caused by evaporation will, in analogy to the third term of eqn. 1, be determined by

$$\frac{ds'}{dt} = (a—s')\ L \tag{I,4}$$

and substituting eqns. 2 and 3 we obtain

$$\frac{ds'}{dt} = fiV_0 \frac{(a—s')^2}{a} \tag{I,5}$$

Integration and combination with eqns. 2 and 3 result in

$$\frac{a}{a—s'} = 1 + fiV_0t \tag{I,6}$$

$$V = \frac{V_0}{1 + fiV_0t} \tag{I,7}$$

$$L = \frac{fiV_0}{1 + fiV_0t} \tag{I,8}$$

Substituting eqns. 7 and 8 in eqn. 1 we arrive at

$$\frac{ds}{dt} = \frac{m \cdot V_0}{1 + fiV_0t} + \frac{eV_0}{1 + fiV_0t} + \frac{(a—s)fiV_0}{1 + fiV_0t} \tag{I,9}$$

which after integration gives

$$s = (m+e+afi)\ \frac{V_0 t}{1 + fiV_0 t} \qquad \text{(I,10)}$$

A more convenient expression is obtained by substituting eqn. 7, resulting in

$$s = (m+e+afi)V_t t \qquad \text{(I,11)}$$

where the voltage V_t at the time t has to be used in the calculation.

When an uncharged substance such as dextran is used, eqn. 11 reduces to

$$s_d = (e+afi)V_t t \qquad \text{(I,12)}$$

When protein and dextran are run under identical conditions and applied at the same distance from the center, the difference in migration distance is given by

$$s_p - s_d = mV_t t \qquad \text{(I,13)}$$

obtained by subtracting eqn. 12 from eqn. 11.

It appears from this fundamental equation that by running an uncharged substance parallel to the protein, not only is it possible to correct for electroosmosis but also for the more complicated effects of evaporation. The voltage referred to in eqn. 13 is that measured at the end of the experiment, and a necessary condition for the validity of this equation is the use of a constant current during the whole run.

Once the parameters e and f have been determined, it should be possible to obtain mobility values corrected for evaporation and electroosmosis from eqn. 11, without the use of an uncharged indicator substance. However, since e and f may vary with the experimental conditions, we have preferred to apply dextran spots in each mobility determination, and to use eqn. 13 in the calculations.

EXPERIMENTAL VERIFICATION

The validity of the propounded equations was tested in a number of experiments in sodium acetate buffer of pH 5.1. To visualize the movement of the buffer solution, dextran spots were placed at various positions. In chromatographic experiments with the same buffer and the same paper [3] dextran yielded a R_F-value of approximately 1.0. It may thus be concluded that dextran follows the surrounding fluid and that its displacement closely reflects the movement of the buffer solution. This is further substantiated by voltage measurements described below. The slight electrophoretic mobility of dextran can be neglected, since it amounts to only $-0.16 \times 10^{-5} \cdot \text{cm}^2 \cdot \text{V}^{-1} \cdot \text{sec}^{-1}$ in pH 8.8 barbital buffer [4] and may be expected to be even less at pH 5.1.

When the migration distance of the dextran spots was plotted as function of their starting positions, straight lines were obtained (Fig. 4). Similar experiments have been performed by other authors [5,6,12] and they prove that the salt distribution remains uniform within the dextran zone. This was controlled in

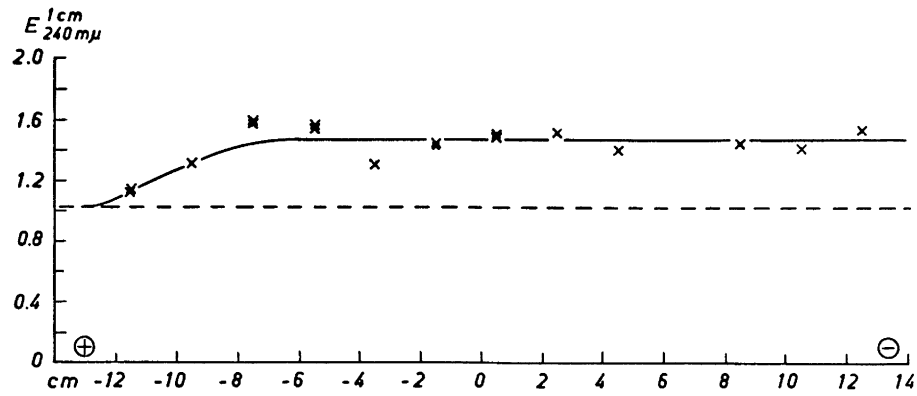


Fig. 2. The concentration increase caused by evaporation. The dotted line represents the original concentration. Determinations by U.V. absorption. pH 8.8 barbital buffer of $\Gamma/2$ 0.08 employed.

an experiment with 5 secondary platinum electrodes placed at various positions along the paper. The conductivity was measured at several time intervals. It rose twofold during the run, but was at any time uniform along the strip. Moreover, it was found by weighing that the amount of buffer in the paper — 27.5 μl/cm^2 — did not change during the experiment.

In an experiment with pH 8.6 barbital buffer, the paper strip was cut in sections perpendicular to the electric field after the current had passed for 19 h. The paper sections were eluted and the barbital concentration determined by measuring the extinction at 240 mμ. The results are shown in Fig. 2, and it is evident that there is a broad zone of increased, but uniform, concentration.

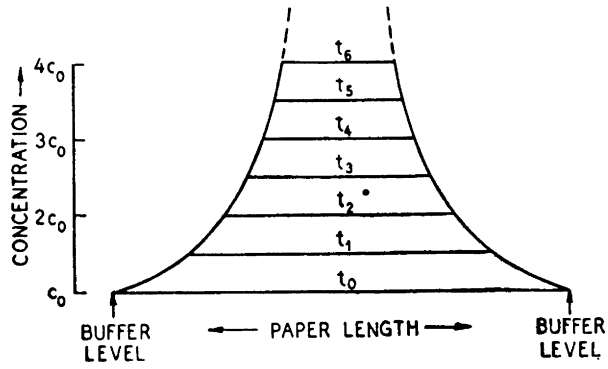


Fig. 3. Schematic presentation of the uniform concentration zone according to theory showing the shrinkage with time and the concentration increase owing to evaporation. The additional effect of electroosmosis will produce a certain skewness varying with the experimental conditions. The t values recorded are arbitrary.

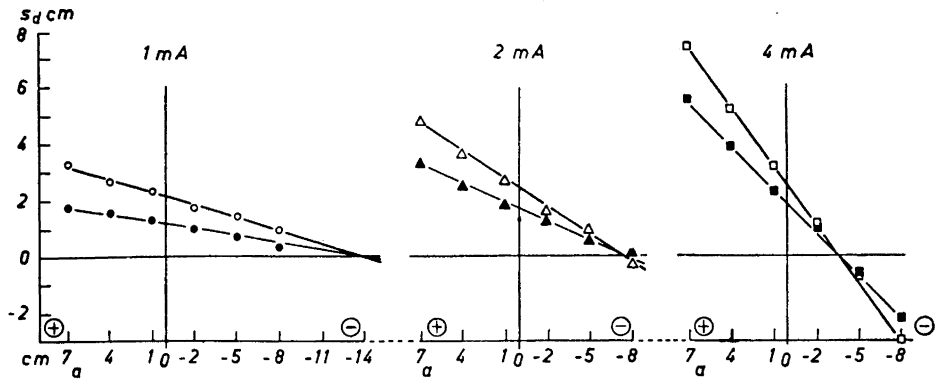


Fig. 4. Dextran migration with different currents and buffer concentrations. Migration distance (s_d) plotted against the position of the application points (a). The scale of the abscissa of this and the following figure reads from right to left because a is measured from application point to paper center.

	Sodium acetate pH 5.10	
mA	$\Gamma/2$ 0.045	$\Gamma/2$ 0.090
1.0	○	●
2.0	△	▲
4.0	□	■

The only possible explanation of all these findings is a homogeneous evaporation from the strip together with a fluid displacement resulting in uniform buffer distribution.

It is clear that toward each end of the paper we come to a point where the concentration begins to fall off until, at the buffer level, we reach the original concentration. This is seen in Fig. 2 and schematically presented in Fig. 3, where it is shown how the zone of uniform concentration shrinks with time.

It is essential that the substance, the mobility of which is to be determined, as well as the electrodes used for voltage measurements are within this zone. This condition can easily be fulfilled. In our mobility measurements [2] performed over 16—19 h with 2.5 mA and 2—3 V/cm, the evaporation amounted to about 30 % of the buffer solution in the paper with a corresponding reduction in length of the uniform concentration zone. In recent experiments even 10 mA/strip have been routinely employed for the same length of time. By increase of the strip length still wider ranges of current and time could be used.

Uniform buffer distribution and straight lines when plotting dextran migration against starting positions will be obtained regardless of any variations in voltage and current during the experiment. Under defined conditions, however, it is possible to calculate the position of the spots at a certain time. The assumptions made in the theoretical part thus led to eqn. 12. In order to test this equation, experiments were performed at different currents and buffer concentrations. Representative results are shown in Fig. 4.

The point of intersection of the dextran lines with the abscissa gives the position on the paper termed π by Macheboeuf *et al.*[7,8] At this point the buffer displacement caused by evaporation is exactly counterbalanced by the electro-osmotic flow. It is apparent that the lines obtained at different ionic strengths but identical current intersect the abscissa at the same point. This is in accordance with eqn. 12. With $s_d = 0$, also $(e+afi)$ must be equal to zero, and therefore the corresponding application distance

$$a_0 = -\frac{e}{fi} \qquad \text{(I,14)}$$

As e and f are constants, a_0 will be constant with constant current, irrespective of variations in the other conditions. It follows further from eqn. 14 that a_0 will be inversely proportional to i. Comparison of the values of a_0 obtained from Fig. 4 at 1.0, 2.0 and 4.0 mA demonstrates that this is indeed the case.

To evaluate the slopes of the various dextran lines, we write eqn. 12 in the form

$$\frac{s_d}{V_t t} = e+afi \qquad \text{(I,12a)}$$

In Fig. 5, $\frac{s_d}{V_t t}$ *viz.* the migration distance divided by time and final voltage, is plotted against a.

As to be expected from eqn. 12a, lines from experiments with the same current coincide, and the slopes are proportional to the current applied. Further, the intersection with the ordinate is very nearly the same with all currents, indicating the constancy of e, and thereby the validity of the second term of eqn. 1.

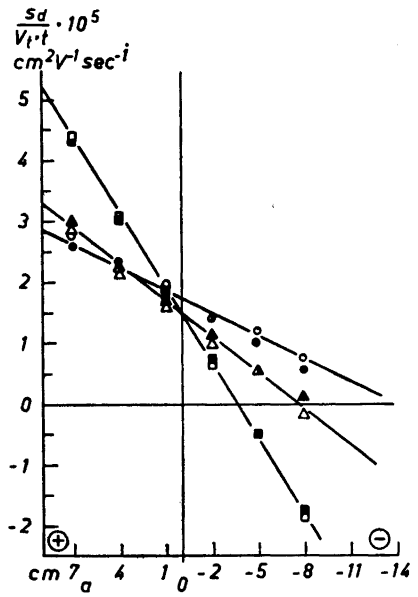


Fig. 5. Dextran migration distance divided by time and final voltage as function of the position of the application points.

	Sodium acetate pH 5.10	
mA	$\Gamma/2$ 0.045	$\Gamma/2$ 0.090
1.0	○	●
2.0	△	▲
4.0	□	■

The value of e can be obtained from Fig. 5. It is found to be about 1.6 $cm^2 \cdot sec^{-1} \cdot V^{-1}$ and shows the magnitude of the electroosmotic flow under the experimental conditions. The small deviations observed in these and similar experiments indicate that the electroosmotic flow is rather sensitive to minor experimental variations.

It has been claimed that the electroosmotic flow may vary along the length of the paper strip. With uniform evaporation and buffer distribution, as demonstrated by our experiments, it follows from obvious reasons that the electroosmotic flow must be uniform even if the driving force varies along the paper. The constant amount of buffer held in the paper by capillary forces behaves like a fluid in a tube with a uniform cross sectional area.

The concentration being uniform in the central part of the strip, the evaporation can be followed by the voltage decrease as well as through the slope of the dextran lines. Comparisons made according to eqn. 3 revealed a scattering of a few per cent, but on an average the agreement between V_0/V_t and the "dextran shrinkage" (ratio of distance between starting positions to distance between final positions) was excellent.

The fact that the slopes in Fig. 5 are indeed proportional to the current demonstrates that the factor f in eqn. 2 is constant with different currents. Experiments covering a 50 fold variation of the initial watt input showed only minor deviations in the value of f.

It follows from eqn. 2 that f indicates the fraction of the watt input consumed by evaporation. When the buffer has moved the distance s' (Fig. 1), an amount of water equal to $w \cdot s'$ has evaporated, w being the amount of water present in 1 cm length of paper. $585\ w \cdot s'$ cal or $585 \times 4.18\ w \cdot s'$ W sec. are used for evaporation, and the dissipation of electrical energy is determined by $\int (a - s')\ iV dt$. After substituting eqn. 6 and integrating, the ratio is found to be $585 \times 4.18\ w \cdot f$. w was determined separately by weighing in a number of experiments and found to be 0.22 g at the employed paper width of 8 cm.

Taking $f = 1.1 \times 10^{-3}$ cm/watt · sec. from the dextran experiments, we find that about 60 % of the electrical input is consumed for evaporation within wide limits of current and voltage. This value probably will vary considerably with the type of apparatus and the ambient temperature. It was estimated at about 70 % by Macheboeuf *et al.* in their experiments [8].

According to eqn. 7, the reciprocal voltage plotted against time should result in a straight line. A typical example is shown in Fig. 6 where a perfectly straight line is obtained. Only in the beginning a greater increase in $1/V$ is noted. This undoubtedly is caused by an initial temperature increase. Extrapolation of the straight line to the ordinate gives the initial conductivity value at equilibrium temperature and the difference from the measured value indicates the temperature increase which here amounts to $\Delta T = 1.75$°C. It is apparent that temperature equilibrium is established within one hour after starting. The straight line of Fig. 6 proves the time relationship expressed in eqn. 7, which was used in the derivation of the final equations.

DISCUSSION

In developing a procedure for the determination of free mobilities on paper strips, the most important requirement is to establish a quantitative relation which makes it possible to correct for the effects of evaporation. The evapora-

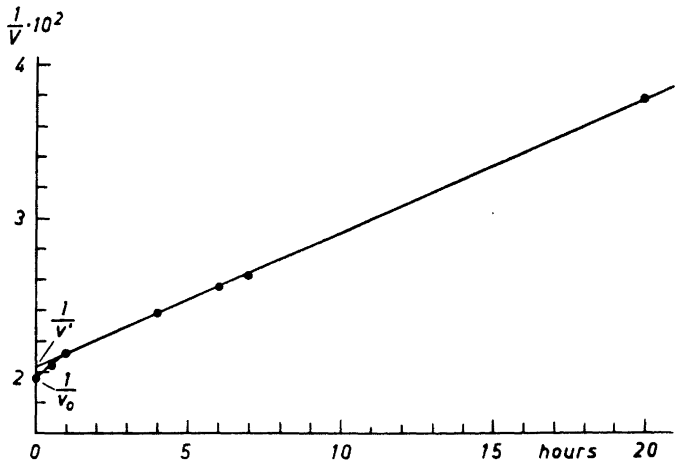


Fig. 6. Voltage decrease due to evaporation as function of time. Experiment carried out in $\Gamma/2$ 0.09 sodium acetate, pH 5.10. Current 3.0 mA; V: Voltage over 15 cm.

tion from the paper causes a continuous change in the salt concentration on the strip and thereby of the voltage gradient acting on the migrating substance, even if the voltage on the apparatus as a whole is kept constant. Furthermore, evaporation causes a flow toward the center of the paper. Since the velocity of this flow increases with the distance from the center its effect on the migration distance cannot be simply corrected for by comparison with that of an uncharged substance, because specimens differing in their migration velocities will be under the influence of different flow rates. Finally, the intensity of evaporation will change during the run because of variations in voltage and current.

In fact, most authors are of the opinion that a reliable method can only be worked out by suppressing evaporation. Low temperatures and very low currents or the addition of substances able to reduce the vapor pressure, such as glycerol [9] have been used to this effect. Even thus it is difficult to eliminate the evaporation to a degree where it may be fully neglected. The closed strip apparatus operated at low temperatures [4], in which the paper is compressed between glass plates and, in most cases, made air-tight by chemical seals, seems to be the best way to avoid distillation from the paper surface.

On the other hand, the open strip technique with free evaporation is claimed to give a much clearer resolution of the protein pattern [11] aside from preventing contact of the specimens with glass plates or alien substances used as seals, which might modify their migration rates. Also in experiments which employ the tracer technique, a contact with the enclosing plates should preferably be avoided. Any method which makes it possible to determine free mobilities using an open strip in the presence of evaporation would therefore be of great value.

An attempt to correct for the effects of evaporation was made by Woods and Gillespie [6] who, although working with a closed strip apparatus, found evaporation to be present in their experiments. The correction applied, however, is empirical and does not appear convincing. Moreover, as shown in the following communication [2], their mobility value for albumin in pH 8.6 barbital buffer is about 25 % lower than the corresponding free mobility.

An interesting approach to the problem of mobility determination with open paper strips was made by Macheboeuf *et al.*[7,8] This method even takes advantage of evaporation for the calculation of free mobility values. It had previously been pointed out by Durrum [10] that in the presence of evaporation a substance will finally reach an equilibrium position where the migration in the electric field is counterbalanced by the buffer flow caused by evaporation and electroosmosis. A necessary condition is that the evaporation remains constant with time. The time for the attainment of this equilibrium position is theoretically infinite and, as the duration of the experiment is limited on account of the increasing buffer concentration, satisfactory results cannot always be obtained. In Macheboeuf's method the substance is placed as a diagonal streak across the paper and the point is noted where it does not move to either side, this being the equilibrium position which can be discerned after a few hours. By applying an uncharged substance on the same paper, correction can be made for the effect of electroosmosis. The equilibrium position is, of course, dependent on the intensity of evaporation and, in order to obtain the electrophoretic mobility, a reference substance of known mobility is required.

In their calculations Macheboeuf *et al.* postulate a constant evaporation as well as a constant voltage gradient. On account of the increasing buffer concentration on the paper these two assumptions are incompatible. The theoretical basis of this approach is therefore more complex than apparent from Macheboeuf's equations. The equilibrium position will thus generally vary in the course of an experiment. Although the conditions are not defined as a function of time, in the determination of mobility the deviation will cancel out due to the use of a reference substance and correct values should be obtained. It should be pointed out that to ensure a uniform voltage gradient, also in Macheboeuf's method, all readings must be performed within the central zone of uniform concentration shown in Fig. 3.

In these, as in all other previous procedures aiming at the determination of free mobilities, a constant voltage was applied, and it seems generally accepted that a reliable determination of electrophoretic mobilities can only be achieved by the maintenance of a constant voltage throughout the experiment. It must be emphasized that this is not the case. The only necessary condition is a knowledge of the voltage as a function of time. Moreover, the maintenance of a constant voltage in experiments with evaporation presents difficulties of practical as well as of theoretical nature. In spite of a constant voltage between the electrode vessels, the concentration changes along the paper result in a continuous decrease of the voltage gradient in the central part where the measurements are performed. Even if this voltage should be kept constant by a suitable regulation, very complicated functions would determine the migration of a substance. A thorough calculation with experimental verification has as yet not appeared.

In the present paper it is demonstrated that all the described difficulties can be overcome by application of a constant current during the whole experiment. This has previously been recommended in order to minimize the influence of the temperature on the migration distance [11]. The application of a constant current in the present method differs fundamentally from this, since its object is the determination of the free mobility of the migrating substance. Under these conditions it has been possible to arrive at a simple expression for the mobility, taking into account the effects of both evaporation and electroosmosis.

In addition to the effect of the buffer flow thus established, the migration velocity of a protein depends on the properties of the carrier medium. It should, however, be stressed that the equations derived above for a medium where retarding factors do not exist, are still valid when these factors come into play as far as dextran is concerned. The influence of the carrier medium as well as of the temperature increase and change in ionic strength will be evaluated in the subsequent communication [2], together with the actual determination of serum protein mobilities.

Acknowledgments. We wish to thank Dr. Tage Astrup of this Institute for his interest and stimulating discussion. This work was supported by grants from the *Teknisk-Kemisk Fond* and the *Rask-Ørsted Foundation*, to whom we are indebted.

REFERENCES

1. König, P., *Act. 3. Congr. sudam. quim.* **2** (1937) 334.
2. Waldmann-Meyer, H. and Schilling, K. *Acta Chem. Scand.* **13** (1959) 13.
3. Waldmann-Meyer, H. and Schilling, K. *Science* **124** (1956) 1028.
4. Kunkel, H. G. and Tiselius, A. *J. Gen. Physiol.* **35** (1951) 89.
5. De Wael, J. in *Ciba Found. Sympos. Paper Electrophoresis*, J. and A. Churchill Ltd., London 1956, p. 105–118.
6. Woods, E. F. and Gillespie, J. M. *Australian J. Biol. Sci.* **6** (1953) 130.
7. Macheboeuf, M., Rebeyrotte, P., Dubert, J. M. and Brunerie, M. *Bull. soc. chim. biol.* **35** (1953) 334.
8. Macheboeuf, M., Dubert, J. M. and Rebeyrotte, P. *Ibid.* **35** (1953) 346.
9. Durrum, E. L. *J. Am. Chem. Soc.* **73** (1951) 4875.
10. Durrum, E. L. *J. Colloid. Sci.* **6** (1951) 274.
11. Durrum, E. L. in *Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, Inc., New York 1958.
12. Whitehead, J.K. *Biochem. J.* **68** (1958) 653.

Received September 25, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 13—28

Determination of Free Protein Mobilities by Paper Electrophoresis with Evaporation *

II. Evaluation of Temperature and Concentration Increases, Influence of the Carrier Medium, and Measurement of Serum Protein Mobilities

HEINZ WALDMANN-MEYER and KURT SCHILLING

The Biological Institute, Carlsberg Foundation, Copenhagen, Denmark

After the determination of the joint effect of evaporation and electroosmosis by means of an uncharged substance had been established, the final equation for the calculation of free solution mobilities was obtained by introduction of correction factors for the influence of the carrier medium.

A direct quantitation of *protein adsorption* was achieved on basis of the actual electrophoretic experiment. The presence of evaporation is indispensable for this evaluation. This emphasizes the value of evaporation for accurate mobility determinations, in contrast to hitherto prevailing opinions. Differing from γ-globulin, serumalbumin was not adsorbed in the media employed.

The *path-length increase* owing to the paper structure was calculated by means of the paper/buffer conductivity ratio and found to be a paper constant. The final, uniform *salt concentration* of the migration zone was evaluated by the migration of an uncharged substance or by the voltage drop. The *temperature increase* on the strip was determined through conductivity measurements at several time intervals and appears as a function of the watt input.

42 samples of human serumalbumin and γ-globulin were run in sodium acetate and barbital buffers of 0.10 ionic strength from pH 4.0 to 8.8, and the free mobilities calculated according to the derived equation. The results were converted to their values at 1°C and compared to moving-boundary data. Absolute agreement between our values and the free electrophoresis mobilities is found.

In view of the advantages inherent to paper electrophoresis, the development of the open-strip technique into an accurate method for mobility determinations may open new perspectives for enzymological, clinical and interaction studies.

* Presented at the Meeting of the Kemisk Forening, Copenhagen, March 25th, 1958.

In spite of an immense amount of work on paper electrophoresis, very few attempts have been made to adapt this method for the estimation of protein mobilities. Presumably this is due to the prevailing opinion that any such attempt has to fail, because of the complicated effects produced by evaporation. Even when evaporation was suppressed, adsorption of the proteins to the filter paper seemed to prevent mobility determinations.

In the preceding paper [1] it was shown that the buffer flow caused by evaporation and electroosmosis can be calculated and the results experimentally confirmed. It is one of the objects of the present communication to describe how the temperature increase and the changes in the medium, inherent to the open-strip technique with free evaporation, can be evaluated and taken into account in mobility determinations.

However, the sole presence of a carrier medium produces some complications which have to be dealt with in the first instance. The migration rate of a given substance, moving on a semisolid support such as moist filter paper, would obviously differ from that found in free solution even if all other conditions could be kept identical. The rate must be lower due to the retarding effect connected with the intricate geometrical structure of such a supporting medium, and to a possible adsorption of the proteins to the carrier medium.

The results presented here demonstrate that both the geometrical factor and the adsorption can be evaluated, and that mobilities identical with those obtained in free solution — subsequently called *free mobilities* — can be calculated when corrections are introduced for these effects.

MATERIALS AND METHODS

Apparatus. As described [1].

Buffers. Sodium acetate buffers of $\Gamma/2$ 0.08 were used over the whole acid range in order to maintain a constant buffer composition. Above pH 5.3 barbital was added to 0.025 M. Experiments carried out at pH 8.6 – 8.8 were performed in Longsworth's sodium barbital buffer of the same ionic strength.

Proteins and dextran. Normal human serum albumin and γ-globulin prepared by ethanol fractionation were obtained by kindness of Dr. Albert Hansen, Statens Serum Institut, Copenhagen. 5 % (w/v) solutions in the respective buffers were prepared at 4°C and dialyzed for 24 h in the refrigerator. γ-globulin solutions were prepared once a week. The albumin was electrophoretically homogeneous, whereas a small albumin impurity was present in the γ-globulin preparation.

4 % dextran (Pharmacia, Uppsala) solutions in the corresponding buffer were prepared by gentle heating.

Procedure. As described [1], except for specimen apposition: Two transversal lines were drawn on the paper at 7 and 8 cm from the center of the strip toward the anode and the cathode, respectively. After immersion in buffer and blotting off excess fluid from the strip, the paper was placed in the apparatus. After equilibration, albumin, γ-globulin and dextran solutions were applied, each as one individual 5 μl aliquot to both of these lines, the distance between the 3 spots on a line being 2.5 cm.

For photometric evaluation of the protein migration, the paper was kept overnight in liquid petrolatum and scanned with an EEL (Harlow, Essex) Densitometer, employing a green glass filter (OGR-1). The apex of the absorption peak was taken as the end point of protein migration.

pH Measurements. The pH values were measured potentiometrically with a glass electrode, using a calomel reference electrode (Radiometer, Copenhagen), after calibration with standard phosphate. Controls were performed immediately after the run by cutting a 1×15 cm segment from the strip, which then was thoroughly eluted with boiled

distilled water, the pH measured, and compared to the same dilution of the original buffer solution. The average value, corrected for dilution, was taken as the experimental pH. Experiments showing an increase larger than 0.30 units were discarded.

Temperature. The starting and final, as well as the maximum and minimum ambient temperatures were recorded. Experiments where the maximum variation exceeded 2.5°C were discarded. The temperature on the paper was obtained by adding to the mean ambient temperature the increase (ΔT) taken from Fig. 2.

Determination of the conductivity ratio. The *electrical resistance of the moist paper* strip was found by measuring current (D.C.) and voltage after 3 h equilibration in the apparatus, at constant temperature. The *buffer content* per cm length of paper was obtained by weighing the horizontal part of the strip immediately afterwards. After drying, the difference – corrected for buffer salt content – was divided by the specific gravity and the strip length. The *conductivity* of the free solution was determined for the same temperature by means of an alternating current bridge (Danbridge, LB-1). The conductivity ratio, q, was then calculated according to eqn. II, 8.

RESULTS

A. Adsorption

Adsorption of proteins to filter paper, manifesting itself through "protein-tailing", has been considered one of the main obstacles in the determination of mobilities by paper electrophoresis. However, this problem has apparently not always been critically analysed, since it is necessary to distinguish between the effect on migration rates of reversible adsorption on one hand, and of irreversible adsorption on the other. Depending upon medium and pH, most proteins will probably undergo both processes. The "tail" left by the irreversibly adsorbed proteins does not necessarily signify a retardation of the migration rate, and Durrum's idea [2] that it might be compared to the "unrolling of a carpet" appears to give an adequate picture of the process. Provided that a sufficient amount of substance is available for migration, as it was the case in the present experiments, the "head" will reach its final position, unhindered by a possible irreversible adsorption of excess protein, and enough material will assemble at the end point as to grant the measurement of the migration distance.

On the other hand, protein migration rates would be decreased by reversible adsorption processes occurring during paper electrophoresis. Since chemical pretreatment of the paper made in order to eliminate adsorption might interfere with the protein mobilities, we have suggested the use of chromatographic R_F-values as correction factors [3]. However, a critical reappraisal of this idea showed that, although the chromatographic R_F-values possess general interest as protein-constants, the conditions in chromatography are too different from those existing in electrophoresis to allow the use of these values for correction. In ascending chromatography 19.8 μl buffer were absorbed per cm^2 paper, which is about 30 % less than the amount of fluid present during electrophoresis under our experimental conditions. Another factor which undoubtedly plays a major role is the difference between chromatographic and electrophoretic migration rates. In chromatography [3] the proteins moved at 15 cm/h, whereas the range in the electrophoretic experiments here described varied from 0.05 to 0.30 cm/h.

It was thus fundamental to evaluate the adsorption directly from the actual paper electrophoretic experiment. The following considerations demonstrate the feasibility of such a determination. The close correspondence of the "dextran shrinkage" with the ratio of initial to final voltage between the secondary electrodes in all our electrophoretic experiments [1] demonstrates that dextran is not significantly adsorbed to filter paper in the conventional buffers. This has also been substantiated by chromatographic experiments [3,4].

On this basis, a preliminary approach to the evaluation of protein adsorption was made by submitting albumin, γ-globulin and dextran to *alternating current* (50 cycles/sec.) in an electrophoresis set. Under these conditions, the migration of both protein and dextran is caused exclusively by the buffer displacement produced by evaporation. Accordingly, for an unadsorbed protein we find that the migration distances of this specimen (s_p) and of dextran (s_d) are identical:

$$s_p = s_d \qquad \text{(II,1)}$$

When adsorption occurs, the migration of the protein is delayed and a factor ($\varrho \leqq 1$) has therefore to be introduced:

$$s_p = \varrho \; s_d \qquad \text{(II,2)}$$

Thus, in alternating current experiments, the adsorption factor (ϱ) can be determined by means of one protein-dextran pair. A number of such experiments are summarized in Table 1, where it is shown that albumin and dextran migrated invariably almost equal distances, while γ-globulin was significantly adsorbed to the filter paper. A schematic presentation of an alternating current experiment is given in Fig. 1(a).

Table 1. Protein adsorption in alternating current experiments. s_p protein migration distance; s_d dextran migration distance; adsorption factor $\varrho = s_p/s_d$; σ_m standard deviation of the mean.

Buffer	Ionic strength	pH	Albumin			γ-Globulin		
			Nr. expt.	ϱ	σ_m	Nr. expt.	ϱ	σ_m
Sodium acetate	0.025—0.30	5.20	8	1.01	0.02	8	0.95	0.02
Zinc acetate	0.025—0.30	6.00	8	1.02	0.01	6	0.95	0.04

ϱ-Values corresponding to the specific experimental conditions, however, are obtained directly from the actual *electrophoretic experiment* (*i. e. direct current*), by the use of two protein dextran pairs per strip (cf. Materials and Methods). According to eqn. 13 of the preceding communication [1], which gives an expression of the mobility of an unadsorbed substance:

$$s_p - s_d = m \; V_i t \qquad \text{(I, 13)}$$

For an adsorbed protein, the equation becomes:

$$\frac{s_p}{\varrho} - s_d = m\ V_t t \qquad \text{(II,3)}$$

As eqn. II, 3 is valid irrespective of the point of application of the samples, we find that

$$\frac{s_{p1}}{\varrho} - s_{d1} = \frac{s_{p2}}{\varrho} - s_{d2} \qquad \text{(II,4)}$$

p_1d_1 and p_2d_2 indicating the two protein-dextran pairs. This relation is schematically presented in Fig. 1(b).

In consequence,

$$\varrho = \frac{s_{p1} - s_{p2}}{s_{d1} - s_{d2}} \qquad \text{(II,5)}$$

In mobility experiments described below (Fig. 3) it was seen that albumin has a mean ϱ-value of 1.025, as compared to a value of 0.903 found for γ-globulin. It is interesting to note the close correspondence of these results with those obtained in the alternating current experiments (Table 1). The scattering observed cannot be attributed to the pH, as no clear correlation between pH and ϱ is found. On the other hand, later experiments indicate that the adsorption increases parallel to the buffer concentration. The results of these investigations will be published elsewhere.

It has thus been shown how the adsorption factor can be determined both in a system based upon evaporation alone (A.C.), and in "open-strip" electrophoresis (D.C.), where evaporation, electroosmosis and protein charge contribute to the migration. The latter, direct, determination has to be preferred, as it corresponds to the specific conditions of the experiment.

In *electrophoretic systems devoid of evaporation*, however, the adsorption factor cannot be evaluated, as shown by the following considerations. Evaporation produces a liquid flow converging from both ends toward the center of the strip and diminishing in intensity as this point is approached. This flow adds to the electroosmotic flow and to the electrophoretic migration, thus giving rise to a marked asymmetry between the anodic and the cathodic side of the strip [Fig. 1(b)]. When this asymmetry is lacking, identical specimens will obviously migrate the same distances, irrespective of their starting positions on the paper [Fig. 1(c)]. In consequence, for experiments where evaporation is suppressed, it is seen that

$$s_{p1} = s_{p2} \qquad \text{and} \qquad s_{d1} = s_{d2} \qquad \text{(II,6)}$$

and, in accordance with eqn. II,5,

$$\varrho = \frac{0}{0}$$

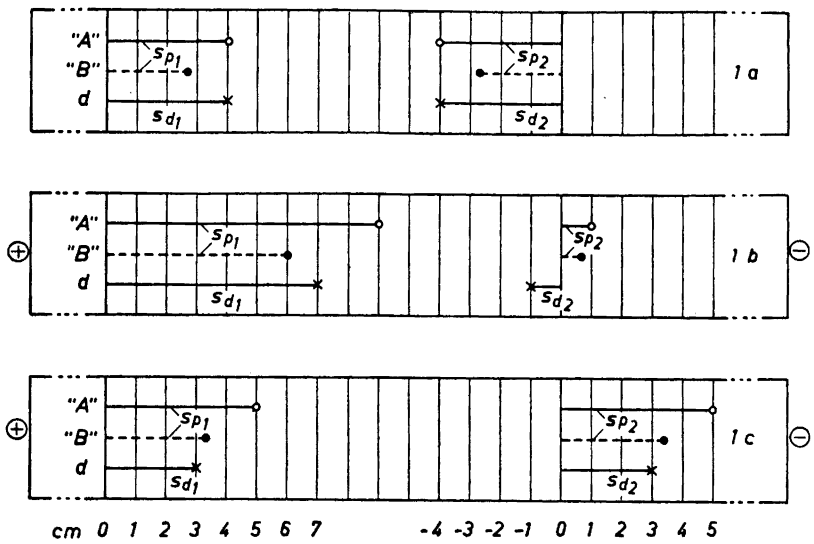


Fig. 1. Schematic illustration of migration relationships with protein adsorption. —○ Unadsorbed "A"-protein. ———● Adsorbed "B"-protein, adsorption factor $\varrho = 0.666$. —× Uncharged substance (dextran). – Assumed migration distances valid for the 3 experiments: Owing to evaporation ± 4 cm, – to electroosmosis 3 cm, and to protein charge 2 cm. (*cf.* eqn. I, 11). For simplicity, "A" and "B"-proteins are given the same charge. The points of application appear as zero. Migration to the cathode is considered positive.

1 a. Alternating current experiment. (Substances subject to evaporation only.) "A"-protein: $s_p = s_d$. "B"-protein: $s_p/\varrho = s_d$. Both sides yield the same result.
1 b. Open-strip electrophoresis. (Evaporation, electroosmosis and electrophoretic migration taking place.) "A"-protein: $s_{p_1} - s_{d_1} = s_{p_2} - s_{d_2}$. "B"-protein: $s_{p_1}/\varrho - s_{d_1} = s_{p_2}/\varrho - s_{d_2}$.
1 c. Electrophoresis devoid of evaporation. (Substances under the influence of electroosmosis and electrophoretic migration.) "A" and "B"-proteins: $s_{p_1} = s_{d_1}$. ϱ cannot be calculated.

Hence, this direct determination of the adsorption factor is not feasible in systems devoid of evaporation. From the schematic illustration in Fig. 1(c), the same conclusions can be drawn by geometrical considerations.

The result is of fundamental significance, since it is generally assumed that a possible determination of free mobilities by paper electrophoresis can be carried out only when evaporation is suppressed [2,5a]. In view of our results, it becomes clear that the presence of evaporation is even of advantage, as it allows the direct estimation of the adsorption factor. Moreover, the accuracy of the ϱ-values will increase in proportion to the migration distance due to evaporation. In order to introduce the adsorption factor into results obtained from systems devoid of evaporation, parallel alternating current experiments would have to be performed. On this ground it can be concluded that for an accurate calculation of mobilities, the open-strip method is to be preferred.

Attention should finally be drawn to the adsorption problem when protein mixtures, such as sera, are analyzed. If it is assumed that some protein is irreversibly held by the paper, the foremost specimen would saturate a number of paper centers and hereby possibly "accelerate" the fractions following behind, the ϱ-values of which thus would become higher than those of their isolated counterparts. Observations in this sense were made in our chromatographic investigations [3]. In practice, however, it is noted that the ϱ-values of albumin and γ-globulin do not differ by more than 10 % in the conventional buffers. Moreover, when — as described — two serum spots are applied, and if the order in which the fractions migrate is identical for both spots, ϱ-values different from the isolated fractions will be found, but the calculated free mobilities will be the same.

B. The influence of paper-structure

The concept of a "tortuous path" was first envisaged by Kunkel and Tiselius [6]. According to their idea, the distance covered by the migrating substance is in reality longer than that measured on the paper because of the intricate channel system proper to the filter paper texture. The observed migration distance has thus to be divided by a factor smaller than 1.0 to get the real path-length. Due to the increased path length, the measured voltage gradient has to be multiplied by the same factor in order to obtain the voltage acting on the migrating substance.

The problem is considered by McDonald *et al.*[7] under a somewhat different viewpoint in their "barrier theory". The supporting medium is supposed to act as a mechanical barrier slowing down the velocity of the migrating substance through repeated collisions. Such an effect should be more pronounced with increasing molecular weight of the migrating substance. In view of the R_F-values of approximately 1.0 found for dextran [3,4] and the correspondence between "dextran shrinkage" and the V_o/V_t ratio [1], it is apparent that the influence of the geometrical factor is the same in the case of the buffer solution as with dextran. The concept of a "tortuous path" in the sense of Kunkel and Tiselius would therefore seem more adequate than the barrier theory by McDonald *et al.*

Kunkel and Tiselius determined the real path length by comparing the conductivity of the moist paper with that of a buffer column of the same length and same amount of buffer. According to their calculations the conductivity ratio represents the ratio of the measured to the true length. This value was squared to get the correction factor for the calculation of the free mobilities. We have used the same experimental approach but applied the conductivity ratio directly to the conversion of the measured mobilities. Kunkel and Tiselius, in their paper [6], overlooked the fact that an increased path length with constant buffer content involves a corresponding reduction of the cross-sectional area, causing the resistance to increase with the square of the path length. The conductivity ratio thus gives the square of the ratio between the measured and the true length, and should be applied directly.

It is also evident that, as far as factors of identical influence, such as path-length, are concerned, the reduction in the mobility of a protein migrating on paper will be equal to that of the buffer ions and thereby to the conductivity. The free mobility, u, is thus obtained by dividing the mobility, m by the conductivity ratio, q.

$$u = \frac{m}{q} \tag{II,7}$$

The conductivity ratio q, which was used in our calculations, was determined according to the following equation:

$$q = \frac{\text{Paper conductivity}}{\text{Buffer conductivity}} = \frac{\frac{i}{V/l \cdot w}}{\varkappa} = \frac{i \cdot l}{V \cdot w \cdot \varkappa} \tag{II,8}$$

i = current employed, after equilibration of the strip (A)
l = distance between the secondary electrodes (cm)
V = voltage over distance l at current i, corrected for polarization and voltmeter resistance (V)
w = buffer per cm length of paper (ml . cm $^{-1}$)
$\varkappa$ = buffer conductivity (Ω^{-1} cm^{-1})

The value obtained from 22 experiments was 0.740 (S.D.M. $\pm$ 0.0047). Since q is equal to the square of the ratio of measured to true length, the latter is found to be 16.3 % longer than the distance measured. As observed from Table 2, q remains constant for a given brand of paper, in spite of varying buffer type, pH and ionic strength. This is in full agreement with Kunkel and Tiselius' results [6] and shows that this factor depends solely on the type of paper used. A standard error below 1 % (Table 2) indicated that small differences between different batches [5b] are without significance in the present case.

It was mentioned [1] that the "wetness" w, on which special emphasis is laid by McDonald *et al.*[7], remains constant during an experiment. That is further evidenced by the fact that the different buffer concentrations, given in Table 2, resulted in practically identical w values, and shows that no variations in q owing to wetness changes [8] occur during the experiment. Temperature increases within the observed range do not appear to influence the outcome of the q-value.

Since the different types of buffer used yielded the same q values, it can be concluded from Table 2 that reversible ion adsorption does not take place during the process. The absence of an irreversible ion adsorption was independently demonstrated by eluting a known amount if buffer from a paper strip and comparing the conductivity of the eluate with a solution of the same ion concentration as directly prepared. The difference was within the experimental error (<1 %) in the sodium and zinc buffers employed.

The q value of 0.740 (Table 2) has been applied to all subsequent mobility calculations in the following way. According to eqn. II,3:

$$m = \frac{\frac{s_p}{\varrho} - s_d}{V_t t} \tag{II,3}$$

Table 2. Conductivity ratio, q, calculated according to eqn. II,8. Muncktell paper No. 20/150 employed.

No.	Buffer	$\Gamma/2$	pH	q
1	Sodium acetate	0.051	5.25	0.732
2	id. id.	0.051	5.25	0.747
3	id. id.	0.080	4.05	0.734
4	id. id.	0.080	4.44	0.730
5	id. id.	0.080	4.90	0.723
6	id. id.	0.080	5.32	0.761
7	id. id.	0.150	5.37	0.730
8	id. id.	0.150	5.37	0.730
9	id. id.	0.300	5.32	0.718
10	id. id.	0.300	5.32	0.730
11	id. id. + 0.02 M barbital	0.080	5.87	0.734
12	id. id.	0.082	6.80	0.773
13	id. id.	0.083	6.20	0.726
14	Phosphate	0.050	7.60	0.723
15	id.	0.100	7.60	0.709
16	Sodium barbital	0.075	8.45	0.797
17	id. id.	0.080	8.80	0.752
18	id. id. + 0.06 M sodium chloride	0.150	8.45	0.783
19	id. id.	0.200	8.55	0.747
20	id. id.	0.250	8.55	0.752
21	id. id.	0.350	8.50	0.723
22	id. id.	0.400	8.50	0.722
			MEAN	0.740
			σ_m	0.005

By introducing the conductivity quotient according to eqn. II,7, it is found that

$$u^{T°C} = \frac{m}{q} = \frac{\frac{s_p}{\varrho} - s_d}{q \cdot V_t t} \qquad \text{(II,9)}$$

$u^{T°C}$ = free mobility at experimental temperature T°C ($cm^2V^{-1}sec^{-1}$)
s_p = protein migration distance measured (cm)
s_d = dextran migration distance measured (cm)
ϱ = adsorption factor (eqn. II,5)
q = conductivity ratio (eqn. II,8)
V_t = voltage/cm at the conclusion of the experiment, corrected for polarization and voltmeter resistance ($V \cdot cm^{-1}$)
t = duration of the experiment (sec)

Eqn. II,9 is the final formula employed in the calculation of free mobilities.

The correction factors for adsorption and increased path length may also be introduced into the equation I, 11 of the previous communication [1], resulting in

$$u = \frac{1}{q}\left(\frac{s}{\varrho V_i t} - e - afi\right) \qquad \text{(II, 10)}$$

From this equation it would be possible to determine free mobilities on paper without the use of an uncharged reference substance provided that the constants e, f, and ϱ had been determined beforehand. Because of the obvious uncertainty of such a procedure, the use of dextran cannot be dispensed with, and the present method has to be preferred.

C. Temperature determination

Since the use of thermocouples during an experiment would produce considerable technical difficulties, the determination of the actual temperature on the strip indicated the convenience of an evaluation of the temperature increase (ΔT) as function of the watt input.

It has been shown in the preceding communication [1] that in experiments with constant current the reciprocal voltage plotted against time gives a straight line (Fig. 6 in Ref.[1]). By extrapolation to the ordinate an expression of the initial conductivity at the temperature prevailing during the experiment is obtained ($1/V'$). The difference between $1/V'$ and $1/V_0$ is directly proportional to the conductivity increase caused by the temperature rise.

The viscosity of the buffer solution used is nearly identical with that of pure water, and the temperature function of the latter was therefore employed to convert the conductivity increase to temperature. This conversion is performed as follows: The reciprocal viscosity of water corresponding to the mean ambient temperature during such an experiment, is taken from a table. This value is increased by the percentage of conductivity increase found as described above, and the corresponding temperature read from the table. ΔT is then obtained by subtracting the mean ambient temperature from this value.

In Fig. 2 the temperature increase found in 16 buffers of widely varying ionic strengths and different composition by plotting the reciprocal voltage against time, is correlated with the initial watt input ($V_0^{(15cm)} \times$ mA per strip). In spite of some scattering, it appears justified to accept a ΔT-value of 3°C per $W_0^{(15cm)}$/strip.

In the experiments which laid the basis for Fig. 2 no substances were applied to the strips after equilibration, as it was the case in the mobility determinations. Since the equilibrium undoubtedly will be disturbed by the opening of the covering plates, as required with our apparatus, the voltage before and after protein and dextran application was measured. It was seen that the difference between the two values is of no significance at all, as even 5 min opening, together with the application of six 5 μl-aliquots, does not produce a voltage decrease greater than 1 %, which in the present series would mean a maximum error of 5×10^{-3} °C.

The $\Delta T/W_0$ correlation will obviously vary with the apparatus used and is the only function here described which will have to be determined at each laboratory.

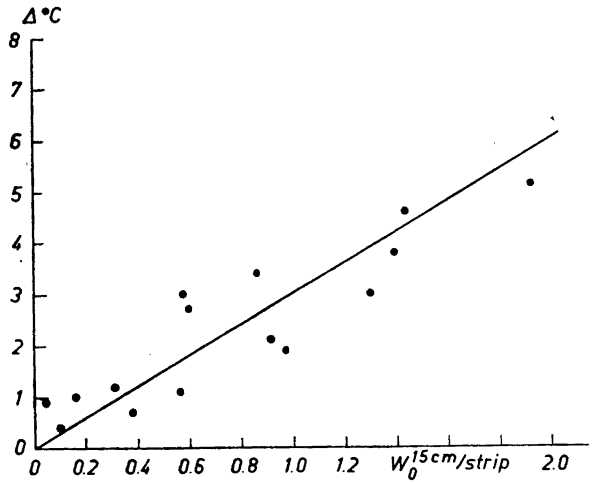


Fig. 2. The temperature increase as function of the initial watt input. $W^{15\ cm}$: Watt per 15 cm strip length (120 cm^2). Sodium acetate, barbital and zinc acetate buffers, ranging from $\Gamma/2 = 0.025$ to 0.30, used. Experimental points obtained by extrapolation procedure (see p. 22).

When highly concentrated or viscous buffers are employed, the conductivity should be measured as function of the temperature, and the resulting curve used as reference for exact temperature conversions. In the ordinary concentration range, however, the difference would be negligible.

From the described experiments it has been seen that the temperature increase occurs within the first hour, and it appears therefore justified to add ΔT, taken from the $\Delta T/W_0$ plot, directly to the mean ambient temperature. The resulting value is then used for the conversion of the mobilities to their values at 1°C. The viscosity ratio was used to this end. This is in accordance with Tiselius' procedure [9] and it has been shown by McDonald *et al.*[7] that the free mobility as function of the temperature is, in fact, proportional to the reciprocal viscosity. Deviations from this relation, which have been mentioned for certain inorganic ions [10], do apparently not apply to serum proteins. We used 1°C for comparison, since, although not always clearly stated, most free electrophoresis data are obtained at a temperature of about 1°C within the cell. A variation of 1°C in this range changes the mobility value by about 2 %.

D. The concentration increase and its effects

Open-strip electrophoresis suffers from the characteristic inherent to all procedures with evaporation, that the salt concentration on the strip increases during the run. The final ionic strength can easily be calculated by multiplying the initial concentration by the "dextran shrinkage" (distance between the starting positions divided by the distance between the final positions). The mean ionic strength ($\Gamma/2_m$) is then considered valid for the whole experiment.

In our mobility determinations an initial ionic strength of $\Gamma/2 = 0.080$ to 0.084 was used, which increased to about 0.12, thus resulting in a mean concentration of $\Gamma/2_m = 0.102$ (S.D. = ± 0.0036).

The error involved by taking the average ionic strength is rather insignificant, since the mobility changes only slightly with the ionic strength in monovalent ion buffers [11]. According to our own determinations, the mobilities of both serum albumin and γ-globulin in sodium acetate buffer of $\Gamma/2_m = 0.08$ differ by about 5—7 % from the values found in the same buffer of $\Gamma/2_m = 0.12$, the change becoming less pronounced in higher concentrations. These determinations were made at pH 4.75 and 5.65. It is evident that the average concentration ($\Gamma/2_m$) would differ even much less from the real value.

pH changes measured on the strip could not be caused by a concentration increase of the magnitude observed. Since the buffer capacity was sufficient and electrolysis products were prevented from diffusing into the paper, the pH variations were produced by the evaporation of volatile buffer components. Routine controls were therefore especially required in sodium acetate buffers. No pH gradients can be supposed to occur within the uniform concentration zone.

E. The mobilities of human serum albumin and γ-globulin from pH 4.0 to 8.8. Determination and calculation

The crucial test for the validity of the considerations set forth above is the determination of a number of mobilities and their comparison with results obtained by free electrophoresis.

As shown in the preceding chapters, the final eqn. II,9 for the computation of mobilities has been derived from eqn. 13 in Ref.[1] by gradual introduction of correction factors for adsorption and paper structure. The migration distance of the protein is divided by the corresponding ϱ-value, the dextran migration then subtracted, and the difference divided by the conductivity ratio, final voltage and time.

The temperature conversion to 1°C is then performed as described above.

It will be noted that two protein-dextran pairs were applied per strip in order to evaluate the adsorption factor ϱ. Once this factor has been calculated, it is seen from eqn. II,4 that either pair can be used for the mobility calculation according to eqn. II,9.

The mobilities of human serum albumin and γ-globulin were determined over a pH range from 4.0 to 8.8, and the results calculated according to eqn. II,9.

In Fig. 3 the mobilities are presented as function of the pH, together with previous findings by other authors working with free electrophoresis (moving boundary). Values obtained from media other than those employed in this investigation were disregarded.

A full accordance between free and paper electrophoretic results is observed. Isoelectric points of pH 4.68 for albumin and of pH 6.83 for the γ-globulin preparation were obtained by our method, the scattering of the experimental points being less than to be expected from a corresponding study employing free electrophoresis.

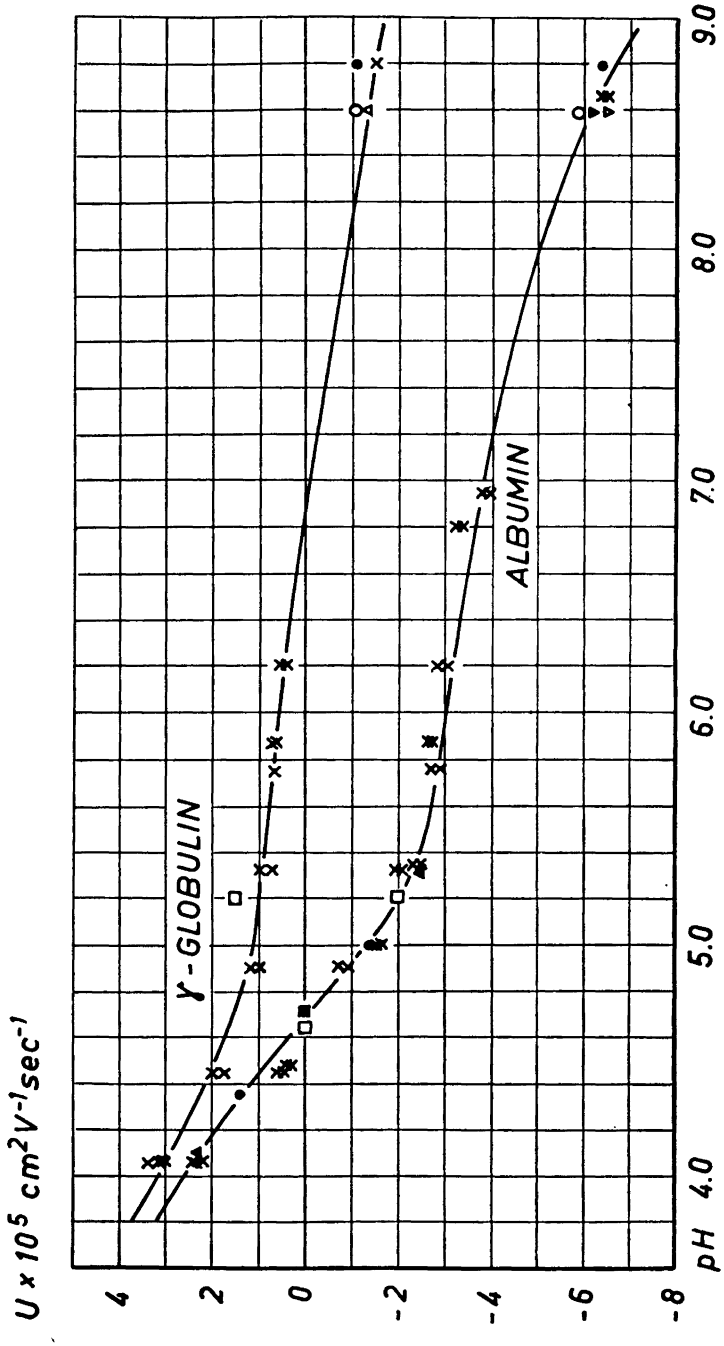


Fig. 3. The electrophoretic mobilities of human serumalbumin and γ-globulin at 1°C in 0.10 ionic strength buffers. pH 4.0—5.5 sodium acetate; pH 5.5—7.0 sodium acetate + barbital; pH 8.6—8.8 barbital.

× *RESULTS OBTAINED BY THE PRESENT PAPER ELECTROPHORESES.*

Moving boundary electrophoresis:
● Kunkel and Tiselius [6]
◀ ■ ▼ Schilling [20], Baldwin [19], Pigman *et al.* [22]
□ Stenhagen [18]
○ Dole and Braun [21]
▽ Woods and Gillespie [12]
△ own determination

The excellent agreement of the paper electrophoretic results with those obtained in free solution establishes the value of the present procedure.

DISCUSSION

In view of the reported results, previous work by other authors on the determination of free mobilities by means of paper electrophoresis is discussed first.

Procedures working without evaporation were described by Kunkel and Tiselius [6] and McDonald *et al.*[7] In experiments by Woods and Gillespie [12] a correction was introduced. The only method working with uninhibited evaporation has been published by Macheboeuf *et al.*[13,14] who even take advantage of this factor.

In the first group mentioned, the sole complications are the electroosmotic flow and the influence of the supporting medium. In the fundamental study of Kunkel and Tiselius [6] — the only publication giving a comparison with moving boundary data for more than one pH value — the uncharged dextran was used as indicator of the electroosmotic flow. Correct mobilities for serumalbumin were obtained at three pH values, in spite of the fact that the square of the conductivity ratio was erroneously applied as tortuous path factor. It might be presumed that this was compensated by a retarding of the protein migration owing to the silicone employed in Kunkel and Tiselius' closed-strip system. No correction for adsorption effects was introduced. In this specific case, however, this does not influence the final mobility values, since — as shown above — serumalbumin remains unadsorbed in the conventional media.

McDonald *et al.*[7] describe several approaches to the attainment of free mobilities, all of them obligatorily working at low temperatures. In a method working with slight evaporation the mobility of serumalbumin at pH 8.6 was obtained by extrapolation of mobility *vs.* time to zero time on the basis of about 50 experiments, whereas γ-globulin could not be estimated because of its low mobility. In a "direct method" requiring 24—36 h per determination, the maximum voltage applicable is 0.5 V/cm. Apparently, no account is taken of the electroosmosis nor of the "barrier effect", so strongly emphasized by the authors. Since it is furthermore shown that a change in the type of paper may change the results by a factor of about 2.0, the agreement with free mobility values appears rather fortuitous. In a third approach, where ionic strengths of 0.0125 may not be exceeded, a reference substance of known mobility is required to correct for the effects of electroosmosis and paper interaction. In an empirical equation various constants are determined using results obtained with the reference protein. When the migration velocity of an unknown substance is then introduced into this equation, it is claimed to yield the free mobility. The different interaction with the paper is estimated separately in conductivity measurements which require a considerable amount of the substance tested. The procedure presents appreciable technical difficulties, and the results reported for γ-globulin, showing 20 % deviation from the free mobility value after determining the parameters by means of albumin, cannot be considered to establish the validity of the proposed equation.

According to McDonald [5c] mobilities may be defined as specific for a given support. This was actually done in a later publication [15], and means — in

our view — a virtual abandonment of the attempts to determine free mobilities by paper electrophoresis, since the mobility is only significant when considered as a physical constant independent of the carrier employed.

Mobility determinations in the presence of evaporation, reported by Woods and Gillespie [12], contain an empirical correction for evaporation effects. It should be noted that the comparison with free mobilities is based on an erroneous temperature correction, since a 2 % change of the mobility value per °C, as derived from the viscosity, is only valid when converting from 25°C to 0°C, and not in the opposite sense. Their value would thus be 27.2 % lower than that found by free electrophoresis. The authors do not consider any corrections for the paper structure. If this is done, the discrepancy would be reduced.

Important investigations have been carried out by Macheboeuf *et al.* [13,14]. The procedure called "electrorheophoresis" has been discussed in the preceding communication. An example of its practical application has been published by Meulemans [23].

Both in Macheboeuf *et al.*'s method and in McDonald *et al.*'s third approach, proteins of known mobility are used as reference substances, in order to correct for buffer flow and carrier medium effects. However, the fact is neglected that, as shown above, the adsorption depends on the specific protein, and some doubts concerning results thus obtained must be expressed.

In this connection, it should be pointed out that the use of dextran, too, involves a series of problems which remain to be solved. Our calculations are based on the assumption that no interaction between the dextran indicator and neither the carrier nor the buffer takes place. While this holds true in the present experiments, conditions may be conceived where dextran is adsorbed or acquires a significant charge. In experiments aiming beyond the conventional media it may therefore be necessary to gain a previous knowledge of the electrochemical behavior of dextran under these conditions. An approach to this problem is at present being worked out and will be published. It does not appear probable that dextran can be easily replaced by a more appropriate indicator, but a final solution might possibly be found by resorting to labelled molecules.

It is evident that paper electrophoresis shares with the moving boundary method certain problems, inherent to the migration of colloidal particles in the electric field, which await further elucidation. Preliminary observations indicate that the mobility of albumin, at a given pH, decreases with increasing protein concentration, which is in agreement with Svensson's results in moving-boundary electrophoresis [16]. Nevertheless, extrapolation to zero protein concentration is not usual in routine mobility determinations and the protein concentration is simply stated together with the other parameters.

One of the most significant advantages of paper electrophoresis being the minute quantity of specimen required, the present results open new ways for studies on the electrochemical properties of enzymes and pathological samples only available in scanty amounts.

An interesting application of the new procedure lies in the field of ion interaction studies. As stressed by Steinberg and Mihalyi [17], some of the subfractions observed in the moving-boundary method may be artifacts produced by

incomplete ion binding resulting from the high protein/free ion ratio. In paper electrophoresis, on the other hand, a considerable and constant excess of the free ion assures the displacement of the equilibrium toward protein-ion binding. By computation of the changes produced in the mobility through addition of increasing ion concentrations to the buffer, binding constants and charge of the different fractions can be evaluated. When working with ions whose activity coefficients change appreciably with the concentration increase caused by evaporation, corrections would have to be introduced. The open-strip technique offers the possibility of further implementing such studies by the introduction of labelled ions.

On this ground it is hoped that the present results, aside from providing an exact micromethod for mobility determinations, may contribute to the attainment of new insights into protein binding and structure.

Acknowledgments. The authors express their gratefulness to Dr. Tage Astrup of this Institute for his most valuable advice and constant encouragement. The skillful technical assistance of Miss Jytte Walter is acknowledged. Thanks are due to Miss Mary A. Bloomstrom for aid with the manuscript. We are indebted to the *Rask-Ørsted Foundation* and the *Teknisk-Kemisk Fond* for grants supporting this investigation.

REFERENCES

1. Schilling, K. and Waldmann-Meyer, H. *Acta Chem. Scand.* **13** (1959) 1.
2. Durrum, E. L. in *Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, Inc., New York 1958.
3. Waldmann-Meyer, H. and Schilling, K. *Science* **124** (1956) 1028.
4. Wood, S. E. and Strain, H. H. *Anal. Chem.* **26** (1954) 1869.
5. *CIBA Symposium on Paper Electrophoresis*, J. A. Churchill, London 1956 : *a*) p. 100, *b*) p. 115, *c*) p. 116.
6. Kunkel, H. G. and Tiselius, A. *J. Gen. Physiol.* **35** (1951) 89.
7. McDonald, H. J., Lappe, R. J., Marbach, E. P., Spitzer, R. H. and Urbin, M. C. *Ionography: Electrophoresis in Stabilized Media*, Year Book Publ., Chicago 1955.
8. Crawford, R. and Edward, J. T. *Anal. Chem.* **29** (1957) 1543.
9. Tiselius, A. *Biochem. J.* **31** (1937) 1472.
10. Edsall, J. T. and Wyman, J., in *Biophysical Chemistry*, Vol. I, p. 399, Academic Press, New York 1958.
11. Smith, E. R. B. *J. Biol. Chem.* **113** (1936) 473.
12. Woods, E. F. and Gillespie, J. M. *Australian J. Biol. Sci.* **6** (1953) 130.
13. Macheboeuf, M., Rebeyrotte, P., Dubert, J. M. and Brunerie, M. *Bull. soc. chim. biol.* **35** (1953) 334.
14. Macheboeuf, M., Dubert, J. M. and Rebeyrotte, P. *Ibid.* **35** (1953) 346.
15. Bermes, Jr., E. W. and McDonald, H. J. *Biochim. et Biophys. Acta* **20** (1956) 416.
16. Svensson, H. *Arkiv Kemi, Mineral. Geol.* **22A** (1946) Nr. 10.
17. Steinberg, D. and Mihalyi, E. *Ann. Rev. Biochem.* **26** (1957) 383.
18. Stenhagen, E. *Biochem. J.* **32** (1938) 714.
19. Baldwin, R. L., Laughton, P. M. and Alberty, R. A. *J. Phys. & Colloid Chem.* **55** (1951) 111.
20. Schilling, K. *Acta Chem. Scand.* **11** (1957) 1103.
21. Dole, V. P. and Braun, E. *J. Clin. Invest.* **23** (1944) 708.
22. Pigman, W., Patton, F. M. and Platt, D. *Arch. Biochem. Biophys.* **69** (1957) 334.
23. Meulemans, O. *Maandschr. v. Kindergeneesk.* **23** (1955) 488.

Received September 25, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 29—34

N-Substituted Alkyltriaminosilanes

II. On the Reaction of N-Substituted *n*-Propyltriaminosilanes with Amines

LEWI TANSJÖ

Department of Organic Chemistry, University of Lund, Lund, Sweden

As a part of an investigation on aminosilanes the reaction between N-substituted *n*-propyltriaminosilanes and some amines was studied. These reactions are apparently catalyzed by hydrogen ion and observations were made which indicate influence by the steric properties of the reagents.

The preparation of *n*-propyltri-(*n*-amylamino)-silane, *n*-propyltri-(*n*-hexylamino)-silane and *n*-propyltri-(benzylamino)-silane is described.

In Part I[1] of this work the author described the reaction of *n*-propyltrichlorosilane with various primary and secondary amines to give N-substituted *n*-propyltriaminosilanes. The great reactivity of the Si—N bonds in these compounds has now been used for the preparation of some compounds of this type by an exchange reaction according to the formula

$$n\text{-}C_3H_7Si(NHR')_3 + 3R''NH_2 \rightarrow n\text{-}C_3H_7Si(NHR'')_3 + 3R'NH_2 \qquad (A)$$

The method has earlier been extensively used for the preparation of N-substituted trialkylaminosilanes, dialkyldiaminosilanes and diphenyldiaminosilanes [2-8].

As it can be assumed that reaction (A) is reversible, it is desirable — in order to obtain good yields — to employ an excess of $R''NH_2$ and to choose R′ so that liberated $R'NH_2$ can be easily removed from the reaction mixture. Of the compounds described in Part I, therefore, only *n*-propyltri-(methylamino)-silane and *n*-propyltri-(ethylamino)-silane were used. They reacted on heating with an excess of *n*-butylamine, *n*-amylamine, *n*-hexylamine, benzylamine, *cyclo*hexylamine and aniline to give the following compounds

I n-$C_3H_7Si(NHC_4H_9$-$n)_3$
II n-$C_3H_7Si(NHC_5H_{11}$-$n)_3$
III n-$C_3H_7Si(NHC_6H_{13}$-$n)_3$
IV n-$C_3H_7Si(NHCH_2C_6H_5)_3$
V n-$C_3H_7Si(NHC_6H_{11})_3$
VI n-$C_3H_7Si(NHC_6H_5)_3$

II—IV have not earlier been reported in the literature. Their physical constants and analyses are given in Table 1. I, V and VI were earlier prepared in Part I of this work.

Table 1. Physical constants and analyses of n-$C_3H_7Si(NHR')_3$

No.	M	B.p. °C	Pressure mm Hg	n_D^{20}	d^{20}_4	MR_D	% Si		Equiv.wt.	
							found	calc.	found	calc.
II	329.64	164–65	3	1.4503	0.8516	104.08	8.6	8.5	110.2	109.9
III	371.72	182–83	2	1.4527	0.8507	118.04	7.6	7.6	123.6	123.9
IV	389.60	216–18	1	1.5665	1.0503	121.09	7.2	7.2	131.5	129.9

n-Propyltri-(methylamino)-silane and n-propyltri-(ethylamino)-silane both failed to react with N-methylaniline, which may be ascribed to the steric requirement of the bulky groups in this amine. That reaction (A) is influenced by steric effects is also supported by the fact that *cyclo*hexylamine which reacted with n-propyltri-(ethylamino)-silane failed to react with n-propyltri-(*iso*propylamino)-silane.

Addition of a proton donator to the reaction mixture considerably accelerated reaction (A). Thus with a small amount of ammonium chloride or ammonium sulfate present, there was in all reactions a pronounced increase in the rate of liberation of $R'NH_2$. In the presence of one of these proton donators *cyclo*hexylamine reacted readily even with n-propyltri(*iso*-propylamino)-silane. With still more bulky groups around the silicon atom, however, as in n-propyltri-(*tert.*-butylamino)-silane, the reaction with *cyclo*hexylamine seemed to be quite impeded even in the presence of these accelerators. With a small amount of ammonium sulfate added, even N-methylaniline liberated some ethylamine from n-propyltri-(ethylamino)-silane, but complete reaction was not obtained even on prolonged heating of the reaction mixture. In general ammonium sulfate was found to be more convenient as proton donator than amine hydrochlorides which during the course of the reactions partly sublimed into the reflux condensor.

The accelerating effect of protons on the reactions of aminosilanes with amines has been reported in the literature prior to this work. Thus Langer, Conell and Wender [8] recently demonstrated a pronounced catalytic effect by hydrogen or ammonium ion on the reaction between hexamethyldisilazane and primary amines, and Speier, Zimmerman and Webster [9] recognized this catalysis in the preparation of allylaminotrimethylsilane from hexamethyldisilazane and allylamine.

In the presence of a proton donator the initial step in reaction (A) seems to be the formation of an ion, $Si—\overset{+}{N}H_2R'$, which on the following nucleophilic attack from $R''NH_2$ splits off $R'NH_2$. The fact that aminosilanes do not give hydrochlorides with hydrogen chloride but are spontaneously converted to chlorosilanes by this reagent demonstrates clearly the great reactivity of the $Si—\overset{+}{N}$ bond.

n-Propyltri-(methylamino)-silane and *n*-propyltri-(ethylamino)-silane could both be refluxed for several hours without any liberation of methylamine or ethylamine, which indicates that the rate of the intermolecular condensation reaction, the first step of which would be

$$2n\text{-}C_3H_7Si(NHR')_3 \rightarrow n\text{-}C_3H_6Si(NHR')_2 - NR' - Si(NHR')_2 - C_3H_7\text{-}n + R'NH_2$$

is very low. Thus it is evident that such a reaction will not accompany reaction (A) to any great extent. As can be expected, however, also the condensation reaction is catalyzed by hydrogen ion. This will be further described in a later part of this work.

Reaction (A) can be considered as the sum of three consecutive competitive reactions:

$$n\text{-}C_3H_7Si(NHR')_3 + R''NH_2 \xrightarrow{k_1} n\text{-}C_3H_7Si(NHR')_2(NHR'') + R'NH_2$$

$$n\text{-}C_3H_7Si(NHR')_2(NHR'') + R''NH_2 \xrightarrow{k_2} n\text{-}C_3H_7Si(NHR')(NHR'')_2 + R'NH_2$$

$$n\text{-}C_3H_7Si(NHR')(NHR'')_2 + R''NH_2 \xrightarrow{k_3} n\text{-}C_3H_7Si(NHR'')_3 + R'NH_2$$

Therefore, if the reaction is performed with a mole ratio $R''NH_2$: $n\text{-}C_3H_7(NHR')_3$ less than 3:1, a mixture of four compounds will result. The relative amounts of these compounds will depend on the ratio $k_1:k_2:k_3$ between the rate constants of the competitive reactions above. This was demonstrated in the case of *n*-hexylamine and *n*-propyltri-(ethylamino)-silane, which were mixed in the mole ratio 1:1 and heated in the presence of some ammonium chloride giving a mixture containing all the expected compounds.

The conceivable redistribution between two aminosilanes of the type $n\text{-}C_3H_7Si(NHR')_3$ with different R'-groups was studied in the case of *n*-propyltri-(ethylamino)-silane and *n*-propyltri-(*n*-amylamino)-silane. These compounds could be refluxed together for several hours without any noteworthy change. In the presence of some ammonium sulfate, however, heating to reflux temperature for a few hours caused redistribution resulting in a mixture of the expected four compounds.

EXPERIMENTAL

The method of the determination of the physical data of the compounds and the analyses have been previously described [1]. The preparation of *n*-propyltri-(*tert.*-butylamino)-silane will be described in the next part of this work.

n-*Propyltri-(*n-*butylamino)-silane (I).* A mixture of 3.2 g (0.02 mole) of *n*-propyltri-(methylamino)-silane and 5.9 g (0.08 mole) of *n*-butylamine was heated to reflux temperature for one hour in the presence of 0.1 g of ammonium chloride. Distillation of the reaction mixture gave 4.0 g (70 %) of I, b.p. 159—161°/12 mm, n_D^{20} = 1.4472, d^{20} = 0.8525, equiv. wt. 96.0 (calc. 95.9).

n-*Propyltri-(*n-*amylamino)-silane (II).* A mixture of 3.2 g (0.02 mole) of *n*-propyltri-(methylamino)-silane and 8.7 g (0.1 mole) of *n*-amylamine was heated in a flask equipped with thermometer and reflux condensor. Liberated methylamine was titrated with standard hydrochloric acid using methyl red as the indicator. Sixty five per cent of the expected amount of methylamine escaped from the reaction vessel during a period of 1.5 h, and during that time the reflux temperature rose from 108 to 122°. After cooling about 0.1 g of methylamine hydrochloride was added to the reaction mixture. On heating methylamine evolution began below 100° and the expected amount of it was liberated within 15 min. Distillation of the reaction mixture gave 4.9 g (74 %) of II.

In another experiment a mixture of 2.0 g (0.01 mole) of *n*-propyltri-(ethylamino)-silane and 4.4 g (0.05 mole) of *n*-amylamine was heated to reflux temperature for one hour. Only about 30 % of the calculated amount of ethylamine were liberated. On addition of 10 mg of ammonium sulfate to the reaction mixture, however, the rest of the ethylamine disappeared within 30 min.

n-*Propyltri-(*n-*hexylamino)-silane (III).* 3.1 g (0.015 mole) of *n*-propyltri-(ethylamino)-silane and 7.6 g (0.075 mole) of *n*-hexylamine were heated at about 125° for one hour. The liberated amount of ethylamine during that period corresponded to only 20 % of the expected amount. After addition of 50 mg of ammonium sulfate to the reaction mixture gas evolution began at about 100° and the rest of the ethylamine disappeared within 25 min. Distillation of the reaction mixture gave 4.2 g (75 %) of III.

n-*Propyltri-(benzylamino)-silane (IV).* A mixture of 2.0 g (0.01 mole) of *n*-propyltri-(ethylamino)-silane, 4.3 g (0.04 mole) of benzylamine and 20 mg of ammonium sulfate gave upon heating to reflux temperature for 45 min the expected amount of ethylamine. On distillation of the reaction mixture 3.0 g (77 %) of IV were obtained.

n-*Propyltri-(*cyclo*hexylamino)-silane (V).* 7.9 g (0.08 mole) of *cyclo*hexylamine liberated on heating to reflux temperature for one hour about 30 % of the expected amount of ethylamine from 4.1 g (0.02 mole) of *n*-propyltri-(ethylamino)-silane. On addition of 50 mg of ammonium sulfate to the reaction mixture complete reaction was obtained after reflux for one hour. Distillation gave 6.1 g (83 %) of V, b.p. 192—93°/3 mm, n_D^{20} = 1.4918, d^{20} = 0.9479, equiv. wt. 121.6 (calc. 121.9).

n-*Propyltrianilinosilane (VI).* 3.2 g (0.02 mole) of *n*-propyltri-(methylamino)-silane and 7.4 g (0.08 mole) of aniline were heated to reflux temperature for one hour. About 50 % of the expected amount of methylamine were evolved. On addition of 50 mg of ammonium sulfate the rest of the methylamine evolved in 20 min on heating to reflux temperature. Distillation gave 5.4 g (78 %) of VI, b.p. 220°/2 mm, equiv. wt. 115.3 (calc. 115.8).

Reaction with N-methylaniline. A mixture of *n*-propyltri-(methylamino)-silane and N-methylaniline did not react after 6 h of reflux. In another experiment 3.1 g (0.015 mole) of *n*-propyltri-(ethylamino)-silane and 8.0 g (0.075 mole) of N-methylaniline were heated to reflux temperature for 8 h without reaction. On addition of 50 mg of ammonium sulfate to the reaction mixture 50 % of the amount of the ethylamine, calculated for complete reaction, were liberated on heating to reflux temperature for 6 h. On fractionation of the mixture most of the N-methylaniline was recovered. Between 160 and 200° at 10 mm Hg 2.0 g of a substance was obtained which contained N-methylaniline and may have been a mixture of *n*-propyl-(N-methylanilino)-di-(ethylamino)-silane and *n*-propyldi-(N-methylanilino)-(ethylamino)-silane. It is probable that the evolution of ethylamine was partly due to an intermolecular condensation of *n*-propyltri-(ethylamino)-silane.

Reaction of cyclo*hexylamine with* n-*propyltri-(*iso*propylamino)-silane and* n-*propyltri-(*tert.-*butylamino)-silane.* No *iso*propylamine distilled from the reaction mixture when

7.9 g (0.08 mole) of *cyclo*hexylamine and 4.9 g (0.02 mole) of *n*-propyltri-(*iso*propylamino)-silane were heated to 125° for 4 h. On addition of 50 mg of ammonium sulfate to the reaction mixture more than 90 % of the calculated amount of *iso*propylamine distilled over on heating to 115° for one hour. From *n*-propyltri-(*tert.*-butylamino)-silane, however, under the same conditions no liberation of *tert.*-butylamine occurred on prolonged heating to 125°, even in the presence of some ammonium sulfate.

n-*Propyltri-(ethylamino)-silane and* n-*hexylamine.* 30.5 g (0.15 mole) of *n*-propyltri-(ethylamino)-silane and 15.2 g (0.15 mole) of *n*-hexylamine were heated for 2 h to reflux temperature in the presence of about 0.1 g (0.002 mole) of ammonium chloride. The calculated amount of ethylamine (0.15 mole) was liberated, most of this during the first 45 min. The precipitate was filtered from the reaction mixture. On distillation at 11 mm Hg the following fractions were obtained:

A:	90— 95°	7.2 g	equiv.wt. = 68.1
B:	95—135	1.9 g	» = 82.7
C:	135—137	11.1 g	» = 86.3
D:	137—180	3.1 g	» = 89.7
E:	180—182	9.3 g	» = 105.3
F:	182—200	1.8 g	» = 111.3
G:	dist. residue	2.2 g	» = 179

The following compounds, E_i = equiv.wt., can be expected to be present in the reaction mixture:

n-$C_3H_7Si(NHC_2H_5)_3$	E_1 = 67.8
n-$C_3H_7Si(NHC_2H_5)_2(NHC_6H_{13}$-$n)$	E_2 = 86.5
n-$C_3H_7Si(NHC_2H_5)(NHC_6H_{13}$-$n)_2$	E_3 = 105.2
n-$C_3H_7Si(NHC_6H_{13}$-$n)_3$	E_4 = 123.9

The found equivalent weights of the fractions seem to indicate that:

A = E_1
B = E_1 (0.3 g) and E_2 (1.6 g)
C = E_2
D = E_2 (2.5 g) and E_3 (0.6 g)
E = E_3
F = E_3 (1.1 g) and E_4 (0.7 g)

If it is further assumed that G is a mixture of E_4 (1.5 g) and products of total hydrolysis (0.7 g) the following compounds seem to have been formed:

7.2 + 0.3	= 7.5 g of	E_1 = 0.037 mole
11.1 + 1.6 + 2.5	= 15.2 g »	E_2 = 0.059 »
9.3 + 0.6 + 1.1	= 11.0 g »	E_3 = 0.035 »
and about	2 g »	E_4 = 0.005 »
		Sum: 0.136 mole

If k_1:k_2:k_3 is 3:2:1, *i. e.*, if the difference in steric and polar properties of the species are neglected, it can be shown [10] that E_1:E_2:E_3:E_4 would be 8:12:6:1. For 0.15 mole of starting material this makes E_1 = 0.044; E_2 = 0.067; E_3 = 0.033 and E_4 = 0.006 mole.

Fraction C, which was assumed to be E_2, had n_D^{20} = 1.4455; d^{20} = 0.8532; MR_D = 81.02; E, which was assumed to be E_3, had n_D^{20} = 1.4499; d^{20} = 0.8521; MR_D = 99.51.

Redistribution. 11.8 g (0.058 mole) of *n*-propyltri-(ethylamino)-silane and 15.3 g (0.041 mole) of *n*-propyltri-(*n*-amylamino)-silane were heated to reflux temperature for 3 h. On distillation most of the starting materials were recovered. Less than one g of substance was obtained between 100—200° at 12 mm Hg, so it is evident that redistribution had occurred only to a very small extent. In another experiment, however, 8.1 g (0.04 mole) and 13.2 g (0.04 mole), respectively of the compounds were heated to reflux

temperature for 3 h in the presence of 20 mg of ammonium sulfate. Now distillation of the reaction mixture gave: 92—127°: 1.0 g, E = 72.3; 127—136°: 2.4 g, E = 82.2; 136—162°: 2.7 g, E = 86.5; 162—170°: 4.3 g, E = 95.0; 170—185°: 2.8 g, E = 98.2; 185—205°: 2.4 g, E = 100.1, and distillation residue 3.0 g, so it is evident that under these conditions redistribution had occurred. Some ethylamine was liberated during the course of reaction, which seems to indicate that the reaction was accompanied by an intermolecular condensation reaction.

The author is indebted to Professor E. Larsson for his kind interest in this work.

REFERENCES

1. Tansjö, L. *Acta Chem. Scand.* **11** (1957) 1613.
2. Larsson, E. *Svensk Kem. Tidskr.* **61** (1949) 59.
3. Larsson, E. and Smith, B. *Acta Chem. Scand.* 3 (1949) 487.
4. Larsson, E. *Acta Chem. Scand.* **4** (1950) 45.
5. Mjörne, O. *Svensk Kem. Tidskr.* **62** (1950) 120.
6. Larsson, E. and Mårin, R. *Acta Chem. Scand.* **5** (1951) 1173.
7. Larsson, E. and Bjellerup, L. *J. Am. Chem. Soc.* **75** (1953) 995.
8. Langer, S. H., Conell, S. and Wender, I. *J. Org. Chem.* **23** (1958) 50.
9. Speier, J. L., Zimmerman, R. and Webster, J. *J. Am. Chem. Soc.* **78** (1956) 2278.
10. Fuoss, R. M. *J. Am. Chem. Soc.* **65** (1943) 2406.

Received September 30, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 35—39

N-Substituted Alkyltriaminosilanes

III. On the Preparation of Some Sterically-Hindered Compounds

LEWI TANSJÖ

Department of Organic Chemistry, University of Lund, Lund, Sweden

n-Propyltribromosilane and *n*-propyltriiodosilane were shown to react more readily than *n*-propyltrichlorosilane with *tert.*-butylamine and diethylamine.

Some N-substituted alkyltriaminosilanes which could not be prepared by the direct treatment of halosilanes with the bulky amines in question could be obtained from the corresponding amine magnesium halogenides.

The preparation of fifteen compounds which have not earlier been reported is described.

Part 1. In a previous paper [1] it was shown that only two chlorine atoms are replaced when *n*-propyltrichlorosilane reacts with *tert.*-butylamine, diethylamine and N-methylaniline. This was ascribed to the steric hindrance exerted by the bulky groups in these amines, for the remaining chlorine atom could readily be exchanged by ammonia and amines with less bulky groups, such as ethylamine and even *iso*propylamine.

Thus the reactivity of the Si—Cl bond is not sufficient to give the N-substituted *n*-propyltriaminosilanes with the amines above even on prolonged reflux. In order to obtain these triaminosilanes, two other methods have been tried, both giving the desired result.

A. From the fact that in general the reactivity of halosilanes increases in the order F, Cl, Br, I, it could be expected that *n*-propyltribromosilane and *n*-propyltriiodosilane would give a better yield of the triaminosilanes than *n*-propyltrichlorosilane does in their reaction with the amines in question. This assumption was verified by the fact that *n*-propyltriiodosilane reacted completely with *tert.*-butylamine and *n*-propyltribromosilane gave a good yield of *n*-propyltri-(diethylamino)-silane. N-Methylaniline, however, replaced only two bromine atoms in *n*-propyltribromosilane; the second one not readily.

Previously [1] it has also been reported that di-*iso*propylamine only exchanged one chlorine atom in *n*-propyltrichlorosilane. Now it was shown that even in *n*-propyltribromosilane only one halogen atom was replaced by di-*iso*propylamine, illustrating the great steric requirement of this amine.

B. Breederveld and Waterman [2-3] used *tert.*-butylamine magnesium bromide and diethylamine magnesium bromide for the preparation of tetra-*tert.*-butylaminosilane and the corresponding diethylamine compound, and this method was successful also in this work. Thus *n*-propyltri-(*tert.*-butylamino)-silane was obtained in good yield by heating a mixture of *n*-propyldi-(*tert.*-butylamino)-chlorosilane and *tert.*-butylamine magnesium bromide at about 160°C for several hours, and the corresponding diethylamine compound was obtained in an analogous way from diethylamine magnesium bromide. *n*-Propyltri-(N-methylanilino)-silane was prepared directly from *n*-propyltrichlorosilane and N-methylaniline magnesium iodide. Prolonged heating of a mixture of *n*-propyl-(di-*iso*propylamino)-dichlorosilane and di-*iso*propylamine magnesium bromide seems to make possible the exchange of two chlorine atoms in *n*-propyltrichlorosilane, but no pure compound was isolated in this case.

Part 2. Whereas *n*-propyltrichlorosilane gives N-substituted *n*-propyldiaminochlorosilanes with *tert.*-butylamine and diethylamine and practically no triaminosilanes, silicon tetrachloride reacts with these amines to give the N-substituted triaminosilanes in good yield [4-5]. It must be concluded, therefore, that the reactivity of the compounds $(t\text{-}C_4H_9NH)_2SiClX$ and $[(C_2H_5)_2N]_2SiClX$ is much greater for X = Cl than for X = n-C_3H_7 in the reaction

$$(t\text{-}C_4H_9NH)_2SiClX + 2t\text{-}C_4H_9NH_2 \longrightarrow (t\text{-}C_4H_9NH)_3SiX + t\text{-}C_4H_9NH_3Cl \quad (1)$$

and the analogous reaction for diethylamine. The influence on the reactivity for some different X in the reaction above has now been qualitatively investigated in the following way. Compounds of the type $(t\text{-}C_4H_9NH)_2SiClX$ and $[(C_2H_5)_2N]_2SiClX$ were prepared with X = n-C_3H_7, C_2H_5, CH_3, H and Cl, and

Table 1. $(t-C_4H_9NH)_2SiClX + t-C_4H_9NH_2$.

X	Hydrochloride g	Per cent reaction
$n-C_3H_7$	<0.2	<2
C_2H_5	1.4	13
CH_3	5.3	48
H	10.4	95
Cl	10.1	92

Table 2. $[(C_2H_5)_2N]_2SiClX + (C_2H_5)_2NH$

X	Hydrochloride g	Per cent reaction
$n-C_3H_7$	<0.2	<2
C_2H_5	0.4	4
CH_3	1.4	13
H	9.8	89
Cl	10.0	91

these were refluxed with an excess of *tert.*-butylamine and diethylamine, respectively, in benzene solution. The amount of hydrochloride precipitated after ten hours was taken as a measure of the per cent reaction. From the results presented in Tables 1 and 2, it is seen that the reactivity increases in the order n-C_3H_7, C_2H_5, CH_3, H and that X = Cl gives a greater reactivity than any alkyl group. It also appears that *tert.*-butylamine reacts more readily than diethyl amine, probably due to the difference in steric requirement between these amines.

From the compounds, prepared in part 1 and 2 the following have not been described before (the Arabic figures refer to the numbering in the experimental part):

I	(1)	n-$C_3H_7Si(NHC_4H_9$-$t)_3$
II	(2)	n-$C_3H_7Si[N(C_2H_5)_2]_3$
III	(4)	n-$C_3H_7Si[N(CH_3)(C_6H_5)]_3$
IV	(9)	$C_2H_5Si(NHC_4H_9$-$t)_3$
V	(9)	$C_2H_5Si[N(C_2H_5)_2]_3$
VI	(9)	$CH_3Si(NHC_4H_9$-$t)_3$
VII	(9)	$CH_3Si[N(C_2H_5)_2]_3$
VIII	(7)	$C_2H_5Si(NHC_4H_9$-$t)_2Cl$
IX	(7)	$C_2H_5Si[N(C_2H_5)_2]_2Cl$
X	(7)	$CH_3Si(NHC_4H_9$-$t)_2Cl$
XI	(7)	$CH_3Si[N(C_2H_5)_2]_2Cl$
XII	(7)	$HSi(NHC_4H_9$-$t)_2Cl$
XIII	(7)	$HSi[N(C_2H_5)_2]_2Cl$
XIV	(3)	n-$C_3H_7Si[N(CH_3)(C_6H_5)]_2Br$
XV	(5)	n-$C_3H_7Si[N(C_3H_7$-$i)_2]Br_2$

Their physical constants and analyses are given in Table 3.

EXPERIMENTAL

The method of the determination of the physical data of the compounds and the analyses have been previously described [1] and also the general performance of the syntheses. The preparation of n-propyltribromosilane and n-propyltriiodosilane will be described in a later paper.

1. n-*Propyltri-(*tert.-*butylamino)-silane (I). A.* 18.1 g (0.04 mole) of n-propyltriiodosilane in 50 ml of benzene were added dropwise to 21.9 g (0.3 mole) of *tert.*-butylamine in 75 ml of the same solvent, and then the mixture was refluxed for 10 h. Filtration left 24.1 g (calc. for the replacement of three halogen atoms 24.1 g) of *tert.*-butylamine hydroiodide, and fractionation of the filtrate gave 9.8 g (85 %) of I with the data given in Table 3.

B. 12.0 g (0.11 mole) of ethylbromide in 25 ml of ether were added dropwise to 2.7 g (0.11 mole) of magnesium in 15 ml of ether. After the addition the mixture was stirred for 15 min. Thereafter 8.8 g (0.12 mole) of *tert.*-butylamine in 20 ml of ether were slowly added, and the mixture was gently refluxed for 15 min. To the suspension of *tert.*-butylamine magnesium bromide formed, 25.1 g (0.1 mole) of n-propyldi(*tert.*-butyl-

Table 3. Physical constants and analyses of the compounds I–XV.

No.	M	B.p. °C	Pressure mm Hg	n_D^{20}	d^{20}	MR_D	% Si		Equiv.wt.	
							found	calc.	found	calc.
I	287.56	119–20	11	1.4427	0.8452	90.14	9.8	9.8	96.7	95.9
II	287.56	136–37	10	1.4599	0.8752	89.97	9.9	9.8	96.1	95.9
III	389.60	227–29	2–3				7.2	7.2	130.0	129.9
IV	273.53	115–16	14	1.4414	0.8459	85.46	10.2	10.3	92.6	91.2
V	273.53	133–34	14	1.4595	0.8773	85.32	10.3	10.3	92.3	91.2
VI	259.51	96–97	12	1.4370	0.8413	80.81	10.9	10.8	86.5	86.5
VII	259.51	115–16	12	1.4515	0.8672	80.65	10.9	10.8	87.0	86.5
VIII	236.86	95–96	12	1.4465	0.9280	68.13	11.8	11.9	236.3	236.9
IX	236.86	100–01	12	1.4517	0.9395	67.97	11.8	11.9	238.0	236.9
X	222.84	81–82	12	1.4419	0.9259	63.68	12.6	12.6	222.4	222.8
XI	222.84	84–85	12	1.4453	0.9343	63.52	12.6	12.6	223.7	222.8
XII	208.81	74–75	12	1.4379	0.9261	59.18	13.6	13.5	210.2	208.8
XIII	208.81	77–78	11	1.4433	0.9354	59.20	13.6	13.5	209.9	208.8
XIV	363.38	205–06	10	1.5770	1.2126	99.31	7.6	7.7	358.6	363.4
XV	331.19	114–15	10				8.6	8.5	335.1	331.2

amino)-chlorosilane were added. The ether was distilled off, and then the mixture was heated for 14 h at 125°C. On distillation 15 g of a liquid were obtained between 114° and 116°C at 10 mm Hg with a neutralization equivalent of 106 (calc. for I 95.9) and n_D^{20} = 1.4437; d^{20} = 0.8572. To complete the reaction this product was again heated with a small quantity of *tert.*-butylamine magnesium bromide for 14 h at 160°C. Distillation now gave 12.0 g (42 %) of a compound freed from chlorine and with the same data as the compound obtained in A.

2. n-*Propyltri-(diethylamino)-silane (II).* *A.* 9.3 g (0.03 mole) of *n*-propyltribromosilane in 40 ml of benzene were added to 16.1 g (0.22 mole) of diethylamine in 75 ml of benzene. The mixture was refluxed for 4 h. Filtration left 12.3 g diethylamine hydrobromide on the filter (calc. for complete reaction 13.9 g). 10 g of diethylamine were added to the filtrate, and the mixture was then refluxed overnight. Filtration now left 1.5 g of hydrobromide. Fractionation gave 5.0 g (58 %) of II with the data given in Table 3.

B. 25.1 g (0.1 mole) of *n*-propyldi-(diethylamino)-chlorosilane were added to diethylamine magnesium bromide obtained in the same way as the corresponding *tert.*-butylamine compound. After evaporation of the ether the mixture was heated for 4 h at 130°C. Distillation at 9 mm Hg gave three fractions: 110–120°C, 4 g, n_D^{20} = 1.4560; 120–127°C, 4 g, n_D^{20} = 1.4575; 127–135°C, 10 g, n_D^{20} = 1.4590. After a new treatment with about 0.05 mole of diethylamine magnesium bromide for 10 h at 160°C, 9.1 g (32 %) of II and 6 g of a first fraction with E = 103 were obtained.

3. n-*Propyldi-(N-methylanilino)-bromosilane (XIV).* 9.3 g (0.03 mole) of *n*-propyltribromosilane in 40 ml of benzene were added to 22.5 g (0.21 mole) of N-methylaniline in 50 ml of benzene. After the addition some hydrobromide had precipitated, but on warming this was quantitatively dissolved. The solution was refluxed overnight, and the hydrobromide precipitated after cooling was filtered off. The benzene and the excess of N-methylaniline were distilled off. On fractionation of the residue, 3.3 g (30 %) of XIV were obtained. 4.5 g of a product distilled between 160° and 180°C at 10 mm Hg and presumably contained *n*-propyl-(N-methylanilino)-dibromosilane. Only a small residue was left in the distillation flask, showing that *n*-propyltri-(N-methylanilino)-silane cannot be obtained in this way.

4. n-*Propyltri-(N-methylanilino)-silane (III).* 8.9 g (0.05 mole) of *n*-propyltrichlorosilane in 25 ml of ether were slowly added to N-methylaniline magnesium iodide obtained from 3.9 g (0.16 mole) of magnesium, 22.7 g (0.16 mole) of methyliodide and 21.4 g (0.2 mole) of N-methylaniline. After refluxing for 30 min, the ether was distilled off and

the residual mixture was heated for 6 h at 130°C. Fractionation gave 7.0 g (36 %) of III as a very viscous syrup and a first fraction of about 6 g between 160° and 200°C at 2—3 mm Hg.

5. n-*Propyl-(di-*iso*propylamino)-dibromosilane (XV).* 31.1 g (0.1 mole) of *n*-propyltribromosilane in 100 ml of benzene were added dropwise to 50.6 g (0.5 mole) of di-*iso*propylamine in 100 ml of the same solvent. The mixture was then refluxed for 4 h. Filtration left 18.9 g (calc. for the replacement of one bromine atom 18.2 g) of di-*iso*-propylamine hydrobromide on the filter. Further refluxing of the filtrate for 30 h gave only 0.3 g more hydrobromide, so it is seen that the diaminosilane cannot be obtained in reasonable yield by this method. The yield of XV was 22.5 g (68 %). It is immediately hydrolyzed in contact with moist air and was never quite freed from hydrobromide.

6. 24.2 g (0.1 mole) of *n*-propyl-(di-*iso*propylamino)-dichlorosilane were heated for 22 h at 150°C with di-*iso*propylamine magnesium bromide obtained from 2.7 g (0.11 mole) of magnesium, 12.0 g (0.11 mole) of ethylbromide and 12.1 g (0.12 mole) of di-*iso*propyl-amine. On fractionation of the mixture at 9 mm Hg most of the dichlorosilane was recovered, but at 120—135°C there were obtained 5.1 g of a product which consumed acid to an extent corresponding to about 75 % *n*-propyldi-(di-*iso*propylamino)-chloro-silane and 25 % of the dichlorosilane. No further attempts were made to isolate a pure compound from this fraction.

7. The compounds VIII—XIII used for the investigation in part 2 were all prepared by addition dropwise of 59 g (0.8 mole) of *tert.*-butylamine or diethylamine in 100 ml of ether to 0.2 mole of the appropriate trihalosilane in 100 ml of the same solvent and refluxing for 2 h. The amine hydrochlorides precipitated (43.8 ± 0.5 g; calc. 43.8 g) were filtered off. The yields of diamino compounds ranged from 55 to 70 %.

The preparation of *n*-propyldi-(*tert.*-butylamino)-chlorosilane and the corresponding diethylamine compound, which were also used in part 2, has been earlier described [1]. Di-(*tert.*-butylamino)-dichlorosilane and di-(diethylamino)-dichlorosilane have been prepared by Breederveld and Waterman [4-5]. Here they were prepared in the same way as VIII—XIII, and the data of the compounds obtained were in good agreement with those given in Refs.[4-5].

8. The experiments in part 2 were performed by refluxing a mixture of 0.10 mole of the compounds $(t\text{-BuNH})_2SiClX$ or $[(C_2H_5)_2N]_2SiClX$ with 21.9 g (0.3 mole) of *tert.*-butyl-amine respectively diethylamine in 100 ml of benzene for 10 h. After cooling, the precipitated amine hydrochloride was separated from the reaction mixture, carefully washed with benzene and dried. The appropriate filtrates were used for the syntheses below.

9. As is seen from Tables 1 and 2 the direct treatment of ethyltrichlorosilane or methyltrichlorosilane with *tert.*-butylamine or diethylamine will not give the compounds IV—VII in good yields. These compounds were more easily obtained according to method B in part 1:

Ethyltri-(tert.-*butylamino)-silane (IV).* After the benzene had been distilled off from the filtrate, mentioned in 8, the residue was added to 0.1 mole of *tert.*-butylamine magnesium bromide and the mixture was heated for 12 h at 160°C. On the following fractionation 10.2 g (37 %) of IV were obtained.

Ethyltri-(diethylamino)-silane (V) (9.8 g; 36 %), *methyltri-*(tert.-*butylamino)-silane* (VI) (10.5 g; 40 %) and *methyltri-(diethylamino)-silane* (VII) (9.6 g; 37 %) were obtained in exactly the same way as IV.

The author is indebted to Professor E. Larsson for his kind interest in this work.

REFERENCES

1. Tansjö, L. *Acta Chem. Scand.* **11** (1957) 1613.
2. Breederveld, H. and Waterman, H. I. *Research London* **6** (1953) s. 48.
3. Breederveld, H. and Waterman, H. I. *Research London* **6** (1953) s. 1.
4. Breederveld, H. and Waterman, H. I. *Research London* **6** (1953) s. 43.
5. Breederveld, H. and Waterman, H. I. *Research London* **5** (1952) 537.

Received September 30, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 40—49

Studies on Succinic Acids

I. Preparation of α,α′-Dialkyl- and Tetraalkyl-Substituted Succinic Acids by Kolbe Electrolysis

LENNART EBERSON

Department of Organic Chemistry, Chemical Institute, University of Lund, Sweden

This paper is the first one in a series dealing with problems concerning the stereochemistry of alkyl-substituted succinic acids. The preparation of α,α′-dimethyl-, α,α′-diethyl-, α,α′-di*iso*propyl-, α,α′-di-(*tert.*-butyl)-, α,α′-di*neo*pentyl-, α,α′-di*cyclo*hexyl-, α,α′-dimethyl-α,α′-diethyl-, tetraethyl-, α,α′-diethyl-α,α′-di*iso*propyl-, α,α,α′,α′-bis-(ethylene)-, α,α,α′,α′-bis-(trimethylene)-, and α,α,α′,α′-bis-(tetramethylene)-succinic acids by Kolbe electrolysis of the corresponding malonic monoesters in methanolic solution and subsequent hydrolysis of the coupled products is described. Treatment with concentrated sulphuric acid at moderate temperatures has been found to be an effective and rapid hydrolysis method for the sterically hindered succinic esters. The melting points of the acids are found to be dependent on the method of determination, generally being appreciably higher when determined with a Kofler apparatus than with the conventional capillary method in an electrically heated block.

The Kolbe electrolysis method has frequently been used for the preparation of α,α′-dialkyl- and tetraalkyl-substituted succinic acids from half esters of mono- and dialkylmalonic acids [1–6]. Most of these electrolyses have been carried out in aqueous solution and a reinvestigation with methanol as solvent seemed desirable, as the conditions for obtaining optimum yields are less critical in this medium [7]. Moreover, the electrolysis of malonic esters containing alkyl groups of more varying type should give a better understanding of the scope and limitations of the reaction.

The electrolyses were carried out in methanolic solution at a smooth platinum anode with a current density of about 0.5 A/cm^2. The pH of the solution was below 7 and the temperature was kept below 35° by external cooling. The results of the present and earlier investigations are summarized in Table 1.

Table 1. Yield of coupled products.

Substituents in the malonic half ester	Yield (%) of succinate in the present investigation [a]	Yield (%) of succinate and electrolysis medium in earlier investigations
CH_3, H	34	40[b]; water [1]
C_2H_5, H	42	46[b]; water [1]
iso-C_3H_7, H	67	
tert.-C_4H_9, H	85 [c]	
neo-C_5H_{11}, H	52	
$(CH_3)_3SiCH_2$, H		40; methanol [2]
cyclo-C_6H_{11}, H	63	17; water [2] 33[b]; water [1]
CH_3, CH_3		21; methanol-pyridine [4] 25; methanol [4]
CH_3, C_2H_5	28 [c]	
C_2H_5, C_2H_5	32	12[b]; water [1] not stated; water [5]
C_2H_5, *iso*-C_3H_7	22	
n-C_3H_7, *n*-C_3H_7		not stated; water [6]
iso-C_3H_7, *iso*-C_3H_7	0	
	12 [c]	
	22 [c]	
	17 [c]	

[a] Based on unrecovered malonic diester. [b] Product of unspecified purity. [c] *Trans*-esterification occurred.

The complication that *trans*-esterification occurred with some of the esters investigated was not serious, as no difficulties in the purification of the coupled products were introduced by this reaction, especially as the preparation of the acids was the main purpose.

HYDROLYSIS OF THE SUCCINIC ESTERS

Diethyl α,α'-dimethyl- and α,α'-diethylsuccinate were hydrolyzed by boiling with concentrated hydrochloric acid and 50 % sulphuric acid, respectively, and separated into diastereoisomers by fractionated crystallization from water. Diethyl α,α'-di*iso*propylsuccinate on hydrolysis with 78 % sulphuric acid exclusively gave the anhydride of the low-melting form of α,α-di*iso*-propylsuccinic acid. Hydrolysis by warming the ester with concentrated sulphuric acid for a few minutes gave a mixture of the high-melting acid and the anhydride of the low-melting acid. The product from the electrolysis of monoethyl *tert.*-butylmalonate was first purified by distillation *in vacuo* and then separated into two solid diethyl α,α'-di-(*tert.*-butyl)-succinates, m.p. 85—87°

and 36—40°, and a liquid dimethyl ester. The ester with m.p. 85—87° on treatment with concentrated sulphuric acid at 80° for 5 min gave a mixture of high-melting α,α′-di-(*tert.*-butyl)-succinic acid and the anhydride of the low-melting diastereoisomer which was converted into the corresponding acid by boiling with 20 % potassium hydroxide. The ester with m.p. 36—40° on treatment with concentrated sulphuric acid gave the anhydride of the low-melting form, while the liquid dimethyl ester gave a mixture of high-melting acid and anhydride of the low-melting acid.

Diethyl α,α′-di*neo*pentylsuccinate and its silicon analogue, diethyl α,α′-bis-(trimethylsilylmethyl)-succinate [2], were readily hydrolyzed by boiling with ethanolic potassium hydroxide, and the acid mixtures obtained were separated into diastereoisomers by fractionated crystallization from suitable solvents.

Murray and Taylor [3] separated the solid diethyl esters of α,α′-di*cyclo*hexyl-succinic acid and found that they were difficult to hydrolyze in alkaline media, and instead used heating with a mixture of concentrated sulphuric acid, acetic acid, and water. However, warming with concentrated sulphuric acid for a few minutes again was found to effect hydrolysis. The high-melting ester gave the high-melting acid, whereas the low-melting one gave the anhydride of the low-melting acid. No interconversion of the diastereoisomers was observed, which was the case under the vigorous conditions used by Murray and Taylor.

The α,α′-dimethyl-α,α′-diethyl-, tetraethyl-and α,α′-diethyl-α,α′-di*iso*propylsuccinates all were very resistant to hydrolysis in alkaline media, but they were easily converted into the corresponding anhydrides by concentrated sulphuric acid at 70—80°. The anhydrides on boiling with strong potassium hydroxide solution and subsequent acidification gave the acids. The α,α,α′,α′-bis-(ethylene)- and α,α,α′,α′-bis-(trimethylene)-succinates were hydrolyzed by boiling with 10 % ethanolic potassium hydroxide. The α,α,α′,α′-bis-(tetramethylene)-succinate required treatment with concentrated sulphuric acid for effecting hydrolysis and then gave α,α,α′,α′-bis-(tetramethylene)-succinic anhydride, m.p. 62—63°. The anhydride readily dissolved in warm 20 % potassium hydroxide solution and on acidification the corresponding acid was obtained.

DETERMINATION OF THE MELTING POINTS

It is well known that an alkyl-substituted succinic acid decomposes into the corresponding cyclic anhydride and water on heating near its melting point. This phenomenon lowers the melting point and makes it dependent on the rate and time of heating and the method of determination. This is clearly shown in Table 2, which gives melting points for the above acids plus tetramethylsuccinic acid under varying conditions. The first column refers to determination with the Kofler apparatus, the heating block being pre-warmed to about 30° below the melting point before introduction of the sample, and then heated at a rate of about 2°/min. The second and third columns give melting points by the conventional capillary method with open and sealed capillaries, respectively, under the same heating conditions as above. The sealed capillaries were wholly immersed into the electrically heated block to

Table 2. Melting points of alkyl-substituted succinic acids.

Succinic acid	Melting point, °C, Kofler apparatus	Melting point, °C, open capillary	Melting point, °C, sealed capillary	Melting point, °C, from the literature	Reference
Racem-α,α′-dimethyl-	121—122.5	120—122.5	119.5—122	123—127	8
Meso-α,α′-dimethyl-	197—201	195—197	192 —195	209	8
Racem-α,α′-diethyl-	130—134	132—133	129.5—130.5	131—133	9
Meso-α,α′-diethyl-	204—206	192.5—194	187 —191	194—195	9
Racem-α,α′-di*iso*propyl-	193—197	166.5—169	163 —164	171	10
Meso-α,α′-di*iso*propyl-	228—235	221.5—223	214 —216	226—228	10
Racem-α,α′-di-(*tert.*-butyl)-	159—162	151—152	145 —146.5		
Meso-α,α′-di-(*tert.*-butyl)-	294—303	310—312	294.5—297.5		
Racem-α,α′-di*neo*pentyl-	182—184	169—171.5	165 —171		
Meso-α,α′-di*neo*pentyl-	274—275	270—271	266 —269		
Racem-α,α′-bis-(trimethylsilylmethyl)-[2]	164—166	160—162	158 —162		
Meso-α,α′-bis-(trimethylsilylmethyl)-[2]	245—246	229—232	225 —229		
Racem-α,α′-di*cyclo*hexyl-	151—154	143—146	139 —143.5	147	3
Meso-α,α′-di*cyclo*hexyl-	236—238	219—222	218 —221.5	225	3
Tetramethyl [a]-	sublimes	176—178	180 —183	192	11
α,α′-dimethyl-α,α′-diethyl-	157—160	142—144	140 —141	147—148	12
Tetraethyl-	140—143	128—129	125.5—126.5	150—151	12
α,α′-diethyl-α,α′-di*iso*propyl-	123—129	122—125	117.5—122		
α,α,α′,α′-bis-(ethylene)-	sublimes	sublimes	256 —275		
α,α,α′,α′-bis-(trimethylene)-	201—203	182—185	184 —186		
α,α,α′,α′-bis-(tetramethylene)-	202—204	167—174	170 —174	157.7—158.5	13

[a] Tetramethylsuccinic acid was prepared from *iso*butyric acid and potassium persulphate according to Fichter and Heer [14].

prevent the decomposition products from distilling to the cold parts of the capillary. The last column gives melting points from the literature, the most recent values having been selected all of which have probably been determined by the capillary method.

The rather large discrepancy between the Kofler and capillary method is readily explained by the fact that the decomposition products can escape almost freely from the unchanged acid in the Kofler apparatus, while this is more difficult in an open capillary and impossible in a sealed one. Even the Kofler method may give melting points differing by as much as 15° for different samples from the same bulk of a substance, dependent on the rate of heating, especially for the tetra-alkylated acids. The values in Table 2 may therefore be too low, and it is possible that other investigators will find still higher melting points. However, the melting point determined in a sealed capillary (which is not a real melting point but a mixed melting point between acid and anhydride under the pressure of acid, anhydride, and water in the vapour phase) was found to be comparatively insensitive to the heating conditions and thus is of value for identification purposes.

Tetraethyl and α,α′-diethyl-α,α′-di*iso*propylsuccinic acids in the solid phase slowly pass into their anhydrides on standing even at room temperature, while the low-melting forms of α,α′-di*iso*propyl-, α,α′-di-(*tert.*-butyl)-, and α,α′-di*cyclo*hexylsuccinic acids, and α,α′-dimethyl-α,α′-diethyl- and α,α,α′,α′-bis-(tetramethylene)-succinic acids tend to lose water during recrystallization.

CONFIGURATIONS OF THE DIASTEREOISOMERS

The low-melting forms of α,α′-dimethyl- and α,α′-diethylsuccinic acid have previously been shown to be *racemic* by resolution with appropriate alkaloids [15,16]. In this paper it is shown by means of partial resolution with brucine that the low-melting forms of α,α′-di*iso*propyl- and α,α′-di-(*tert.*-butyl)-succinic acid possess *racemic* configurations; knowledge of the configuration is necessary for the discussion in coming papers of this series. By analogy in melting points, solubility, and especially the behaviour on anhydrization by heat the low-melting forms of α,α′-di*neo*pentyl- and α,α′-bis-(trimethylsilylmethyl)-succinic acid [2] are tentatively ascribed the *racemic* configuration, as also Murray and Taylor [3] have done in the case of α,α′-di*cyclo*hexylsuccinic acid.

Stefl [17] has stated that an α,α′-dimethyl-α,α′-diethylsuccinic acid with m.p. 173° prepared by him was partially resoluble into optically active components by means of brucine. However, the structure of this acid is questionable, as Stefl has shown it to undergo conversion into an anhydride, claimed to be of opposite configuration, which requires the assumption of breaking and recombination of C—C bonds in good yields if actually α,α′-dimethyl-α,α′-diethylsuccinic acid should be involved. It has been shown that optically active compounds which have no possibility to enolize at the asymmetric center, cannot be racemized even under very vigorous conditions [18]. The present author has not been able to reproduce this conversion with a sample of the acid prepared by the Kolbe method. No resolution experiments have been attempted, and as only one isomer has been obtained any conclusions regarding the configuration cannot be drawn. The same applies to α,α′-diethyl-α,α′-di*iso*propylsuccinic acid.

EXPERIMENTAL

Preparation of the succinic esters

Preparation of the monoethyl malonates. The malonic diester, 0.5 mole, was dissolved in 200 ml of absolute ethanol and a solution of 0.5 mole of potassium hydroxide in 300 ml of absolute ethanol added with good agitation during 5 min. The mixture was allowed to stand over night, whereupon the ethanol was distilled off with gradual addition of water. Unreacted diester was removed by extraction with ether, and the aqueous layer was acidified with concentrated hydrochloric acid. The monoester was extracted with ether and the ether solution thoroughly washed with water. Benzene (about one third of the volume of the solution) was added and finally the solvents were distilled off under reduced pressure at 40–50° to avoid decomposition. By this procedure dry crude monoester was obtained which was sufficiently pure to be directly used in the electrolysis experiments.

Electrolysis of the monoethyl malonates. The electrolysis of a substituted malonic monoester was carried out according to the following procedure: Dry crude monoester, prepared from 0.5 mole of diester according to the above method, was dissolved in 150–200 ml of methanol together with 0.5–1 g of sodium. The mixture was placed in a cell consisting of a 400 ml beaker fitted with a water cooling jacket, a mechanical stirrer, a platinum spiral anode (surface area 3.0 cm^2), a platinum net cathode (total surface area *ca.* 50 cm^2), and a thermometer reaching into the liquid. A current of 1.5–2.0 A was passed through the solution until it became neutral. Rapid agitation and passing a rapid stream of tap water through the cooling jacket was necessary to keep the temperature of the electrolyte below 35°.

Table 3. Physical constants and analyses of substituted succinic esters.

Substituents in the succinate	B.p., °C	Pressure, mm Hg	M. P., °C	n_D^{20}	d_4^{20}	r_D found	r_D calc.	% C found	% C calc.	% H found	% H calc.
α,α'-dimethyl	98— 99	11		1.4223	0.9988	0.2546	0.2557	59.3	59.4	8.9	9.0
α,α'-diethyl	113—116	11		1.4273	0.9757	0.2633	0.2650	62.6	62.6	9.5	9.6
α,α'-di*iso*propyl	125—127	11		1.4350	0.9650	0.2704	0.2722	64.8	65.1	10.1	10.1
α,α'-di-(*tert.*-butyl) [a]			85— 87					67.4	67.1	10.5	10.6
α,α'-di-(*tert.*-butyl) [a]			36—40					66.9	67.1	10.5	10.6
α,α'-di-(*tert.*-butyl) [a]	111—112	2.5		1.4455	0.9774	0.2726	0.2722 [b]	64.8	65.1 [b]	10.1	10.1 [b]
α,α'-di*neo*pentyl	117—118	2		1.4374	0.9317	0.2818	0.2828	68.7	68.7	10.8	10.9
α,α'-di*cyclo*hexyl [c]			119—121								
α,α'-di*cyclo*hexyl [c]			59 —62								
α,α'-dimethyl-α,α'-diethyl	128—132	11		1.4443	1.0004	0.2658	0.2650 [b]	63.4	62.6 [b]	9.7	9.6 [b]
Tetraethyl	122—124	1		1.4595	1.0004	0.2736	0.2777	66.9	67.1	10.6	10.6
α,α'-diethyl-α,α'-di*iso*propyl	132—135	1.5		1.4607	0.9809	0.2796	0.2828	68.3	68.7	10.8	10.9
α,α,α',α'-bis-(ethylene)	105—112	5		1.4528	1.0814	0.2498	0.2464 [b]	60.8	60.6 [b]	7.7	7.1 [b]
α,α,α',α'-bis-(trimethylene)	108—113	2		1.4603	1.0673	0.2568	0.2545 [b]	63.7	63.7 [b]	8.4	8.1 [b]
α,α,α',α'-bis-(tetramethylene)	159—167	11		1.4730	1.0706	0.2621	0.2589 [b]	66.9	66.1 [b]	9.0	8.7 [b]

[a] The separation of these esters is described below.
[b] Calculated for dimethyl ester.
[c] The crude product was separated into two forms according to Murray and Taylor [3]. They found m.p.'s 120° and 60°, respectively.

The reaction product was isolated by dilution of the cell contents with water, distilling off the methanol on a water-bath, and taking up the organic layer in ether. The ether solution was washed with 5 % aqueous sodium carbonate and water and finally dried with anhydrous magnesium sulphate. The ether was distilled off and the residue fractionated *in vacuo*. In most cases also low-boiling material, probably consisting of disproportionation products from intermediate radicals [7], was obtained, but it was not further investigated.

Yields of coupled products are given in Table 1, and physical constants and analyses in Table 3.

*Separation of the esters of α,α'-di-(*tert.*-butyl)-succinic acid.* The crude product, isolated as above, was first purified by distillation, b. p. 102—110°/2 mm, whereby a semi-solid crystal mass was obtained. It was dissolved in boiling 90 % methanol and the solution allowed to cool slowly. The crystals were collected, m. p. 83—85°, m. p. after another recrystallization from the same solvent 85—87° (yield 31 %). The mother liquor was freed from solvent and vacuum-distilled, b. p. 109—112°/2.5 mm. This product was liquid but gradually deposited crystals on standing at 0° for one month, after which time the crystals were filtered on a sintered glassfunnel and sucked as dry as possible, m. p. 33—38°. They were dissolved in petroleum ether, the solution cooled to —80° and the solid rapidly filtered, m. p. 36—40° (yield 15 %).

The mother liquor from the filtration of the crystals with m. p. 33—38° was then very carefully fractionated in an efficient column and a fraction with b. p. 111—112°/2.5 mm was collected, according to analyses consisting of nearly pure dimethyl α,α'-di-(*tert.*-butyl)-succinate (yield 39 %).

Hydrolysis of the succinic esters

Melting points of the acids, determined as described above, are given in Table 2. Other melting points in the experimental part were determined with the Kofler apparatus unless otherwise stated. The neutralization equivalent weight of an acid was determined by potentiometric titration in 50 % ethanol (by weight) with standard alkali in the same solvent and was calculated from the most distinct break in the titration curve. The behaviour of these acids on titration will be discussed in a coming paper of this series.

α,α'-Dimethylsuccinic acid. Diethyl α,α'-dimethylsuccinate, 10.0 g, was hydrolyzed by boiling with concentrated hydrochloric acid for 8 h. After cooling the crystal mass was filtered off and the mother liquor extracted with ether. The ether was distilled off and the remaining solid combined with the first crystal crop dissolved in hot water. *Meso*-α,α'-dimethylsuccinic acid (4.1 g) crystallized out and was further purified by two recrystallizations from water.

The mother liquor was evaporated to dryness and the residue recrystallized four times from concentrated hydrochloric acid to give almost pure *racem*-α,α'-dimethylsuccinic acid (2.0 g).

α,α'-Diethylsuccinic acid. The diethyl ester was hydrolyzed by boiling with 50 % sulphuric acid over night and the acid mixture separated into *meso* and *racemic* forms according to Schotte and Rosenberg [9].

α,α'-Diisopropylsuccinic acid. A. Diethyl α,α'-di*iso*propylsuccinate, 10.0 g, was boiled for 20 min with a mixture of concentrated sulphuric acid, 40 ml, and 20 ml of water. During this procedure the mixture became dark, probably due to oxidation. After cooling, it was diluted with water and the organic layer taken up in ether. The ether was distilled off and the residual oil (crude anhydride) dissolved in warm 10 % aqueous potassium hydroxide and finally the acid liberated by acidification with concentrated hydrochloric acid. Crude *racem*-α,α'-di*iso*propylsuccinic acid (7.8 g) was obtained, which was purified by recrystallization from ethyl acetate. It was necessary to keep the heating time as short as possible to avoid too much anhydrization.

B. Diethyl α,α'-di*iso*propylsuccinate, 10.0 g, was dissolved in 20 ml of concentrated sulphuric acid and warmed on a water-bath for 10 min. After cooling, the mixture was diluted with water and the organic components taken up in ether. The ether solution was extracted twice with 10 % sodium carbonate solution, which then on acidification gave crude *meso*-α,α'-di*iso*propylsuccinic acid (2.7 g). This acid was purified by recrystallization from hot glacial acetic acid.

The ether solution with the same treatment as above gave *racem*-α,α'-di*iso*propylsuccinic acid.

Partial resolution of low-melting α,α'-diisopropylsuccinic acid. Low-melting α,α'-di*iso*propylsuccinic acid, 4.04 g, and brucine, 7.88 g, were dissolved in 250 ml of hot water and the solution filtered warm. After standing for four days at room temperature the crystals formed were filtered off (6.3 g) and recrystallized twice from hot water. The acid was liberated by treatment with dilute hydrochloric acid, dried and recrystallized from ethyl acetate. It showed $[\alpha]_D^{20} = +1.5°$ in absolute ethanolic solution. The rotation was small but significant, as the rotation of the alkaloid is of opposite sign.

α,α'-Di-(tert.-*butyl*)-*succinic acid.* High-melting diethyl α,α'-di-(*tert.*-butyl)-succinate, 5.0 g, was warmed with 10 ml of concentrated sulphuric acid at about 80° for 5 min. The solution was diluted with water and after cooling the crystal mass was filtered off, washed with water and finally dried. It was treated with 25 ml of boiling ethyl acetate, which left the *meso*-α,α'-di-(*tert.*-butyl)-succinic acid undissolved. The acid (1.2 g) was filtered from the hot solution and then recrystallized from boiling glacial acetic acid. (Found: C 62.6; H 9.6; equiv. wt. 116.1. Calc. for $C_{12}H_{22}O_4$: C 62.6; H 9.6; equiv. wt. 115.2.)

The mother liquor was evaporated to dryness leaving behind *racem*-α,α'-di-(*tert.*-butyl)- succinic anhydride (2.5 g), a small sample of which was recrystallized from 80 % aqueous acetone, m. p. 114—115°. (Found: C 67.9; H 9.5. Calc. for $C_{12}H_{20}O_3$: C 67.9; H 9.5.)

Crude anhydride, 2.0 g, was boiled with 20 % aqueous potassium hydroxide until it had completely dissolved, which required about 2 h. Acidification yielded an oil from which the aqueous layer was decanted and replaced by water. The mixture was heated to 80—90° whereby the oil quickly solidified and could be filtered off. It was recrystal-

lized from ethyl acetate or benzene. Care must be exercised in recrystallizing this compound, since prolonged boiling resulted in partial anhydrization. (Found: C 62.8; H 9.6; equiv. wt. 231.0. Calc. for $C_{12}H_{22}O_4$: C 62.6; H 9.6; equiv. wt. 230.3.)

The liquid dimethyl ester on the same treatment gave both *meso* acid and *racemic* anhydride, whereas the low-melting diethyl ester only gave *racemic* anhydride.

Partial resolution of low-melting α,α'-di-(tert.-butyl)-succinic acid. Low-melting α,α'-di-(*tert.*-butyl)-succinic acid, 8.3 g, and brucine, 14.2 g, were dissolved in 200 ml of boiling water, filtered, and allowed to cool slowly. The salt (11.1 g) was collected and recrystallized once from water. The acid was liberated by treatment with dilute hydrochloric acid and showed after recrystallization from benzene $[\alpha]_D^{20} = +6.1°$ in absolute ethanolic solution. In spite of further recrystallizations of the brucine salt higher rotations could not be achieved.

α,α'-Dineopentylsuccinic acid. Diethyl α,α'-di-*neo*pentylsuccinate, 10.0 g, was boiled with excess of 10 % ethanolic potassium hydroxide over night. The ethanol was distilled off with gradual addition of water, and on acidification a solid was obtained, which was filtered off and dried. The acid mixture was dissolved in boiling ethyl acetate, filtered, and allowed to cool slowly. *Meso-α,α'-dineo*pentylsuccinic acid (3.7 g) crystallized out. It was recrystallized once from ethyl acetate. (Found: C 64.9; H 10.1; equiv. wt. 129.5. Calc. for $C_{14}H_{26}O_4$: C 65.1; H 10.1; equiv. wt. 129.2.)

The mother liquor from the above filtration was freed from ethyl acetate and the residue boiled with 15 ml of acetic anhydride for 3 h. The acetic anhydride together with acetic acid formed was distilled off *in vacuo* and the remaining oil dissolved in warm 10 % aqueous potassium hydroxide. On acidification the *racemic* form (3.1 g) was obtained, which was recrystallized from benzene. Before analysis it had to be dried at 105° for 2 h. (Found: C 65.3; H 10.2; equiv. wt. 130.0. Calc. for $C_{14}H_{26}O_4$: C 65.1; H 10.1; equiv. wt. 129.2.)

On anhydrization by heat the two isomers behaved analogously with the two forms of α,α'-bis-(trimethylsilylmethyl)-succinic acid [2], the high-melting form giving the anhydride of the low-melting acid and the low-melting form its own anhydride.

α,α'-Bis-(trimethylsilylmethyl)-succinic acid. The two forms of these acids have been prepared previously [2].

α,α'-Dicyclohexylsuccinic acid. High-melting diethyl α,α'-di-*cyclo*hexylsuccinate, 5.0 g, was heated on a boiling water-bath with 10 ml of concentrated sulphuric acid for 4 min. A longer heating time caused isomerization. Cooling and dilution with water precipitated a solid which was filtered, dried and recrystallized from glacial acetic acid to give 3.1 g of pure *meso-α,α'-dicyclo*hexylsuccinic acid.

Low-melting diethyl ester, 5.0 g, was heated on a boiling water-bath with 10 ml of concentrated sulphuric acid for 10 min. After cooling and dilution with water an oil was obtained which was taken up in ether, the ether distilled off, and the residue dissolved in boiling 10 % aqueous potassium hydroxide. Acidification gave an oil which did not solidify when trying the usual methods for effecting crystallization. However, boiling with water for a few minutes caused the oil to crystallize. The acid was collected, dried and recrystallized from benzene to give 2.8 g of *racem-α,α'-dicyclo*hexylsuccinic acid. Care was required during this procedure, as prolonged boiling in benzene resulted in anhydrization.

α,α'-Dimethyl-α,α'-diethylsuccinic acid. Dimethyl α,α'-dimethyl-α,α'-diethylsuccinate, 10.0 g, was heated on a boiling water-bath with concentrated sulphuric acid for 15 min. Cooling and dilution with water yielded an oil which was taken up in ether. The ether was distilled off and the residue boiled with 10 % aqueous potassium hydroxide until it had dissolved, which required about 45 min. Acidification gave 7.1 g of α,α'-dimethyl-α,α'-diethylsuccinic acid, which was recrystallized from ethyl acetate. (Found: C 59.2; H 9.0; equiv. wt. 203.1. Calc. for $C_{10}H_{18}O_4$: C 59.4; H 9.0; equiv. wt. 202.2.)

Stefl [17] described one liquid and one solid form of α,α'-dimethyl-α,α'-diethylsuccinic anhydride and also found that the liquid form dissolved on boiling with aqueous potassium hydroxide. On acidification an acid with m. p. 173° was obtained. If the liquid anhydride was dissolved in 40 % aqueous potassium hydroxide and the solution kept at reflux temperature for three days it was stated that the solid anhydride was obtained directly on acidification of the reaction mixture. No acid corresponding to this anhydride could be obtained. However, the present author was not able to reproduce this conver-

sion with α,α′-dimethyl-α,α′-diethylsuccinic anhydride prepared by the Kolbe method. Only α,α′-dimethyl-α,α′-diethylsuccinic acid with m. p. 155—159° was recovered in 85 % yield.

Tetraethylsuccinic acid. Diethyl tetraethylsuccinate on treatment with concentrated sulphuric acid as described in the foregoing preparation gave tetraethylsuccinic anhydride, m. p. 86—87°, in almost quantitative yield. The anhydride was converted into the acid by boiling with 40 % aqueous potassium hydroxide for 4 h. The yield of crude acid was 95 % and it was recrystallized from ethyl acetate, though with considerable loss due to anhydrization. After standing for about two years the solid acid had transformed into anhydride to an extent of about 30 %.

α,α′-Diethyl-α,α′-diisopropylsuccinic acid. Diethyl α,α′-diethyl-α,α′-di*iso*propylsuccinate, 10.0 g, was heated on a boiling water-bath with 20 ml of concentrated sulphuric acid for 5 min. Dilution with water produced an oil which was taken up in ether. The ether solution was washed with water and dried with anhydrous magnesium sulphate. The ether was distilled off and the anhydride distilled *in vacuo*, the main fraction (5.1 g) being collected at 140—143°/3 mm. The oil soon solidified and was recrystallized from petroleum ether at −80°, m. p. 44—50°. (Found: C 69.8; H 10.0. Calc. for $C_{14}H_{24}O_3$: C 70.0; H 10.1.)

α,α′-Diethyl-α,α′-di*iso*propylsuccinic anhydride, 2.0 g, was boiled with 20 % ethanolic potassium hydroxide for 2 h. The solution was evaporated to dryness and the residue treated with ice-cold dilute hydrochloric acid. The solid (1.8 g) was filtered, washed with water and rapidly dried in a vacuum-desiccator. A small sample was recrystallized from ethyl acetate as rapidly as possible and then analyzed immediately. (Found: C 65.5; H 10.2; equiv. wt. 261. Calc. for $C_{14}H_{26}O_4$: C 65.1; H 10.1; equiv. wt. 258.4).

The solid acid spontaneously lost water on standing. After one month the m. p. (in a sealed capillary) was 80—105°, the depression being due to the presence of anhydride.

α,α,α′,α′-Bis-(ethylene)-succinic acid. Dimethyl α,α,α′,α′-bis-(ethylene)-succinate, 5.0 g, was boiled with 10 % ethanolic potassium hydroxide over night, the ethanol distilled off and the residue acidified with dilute hydrochloric acid. The solid was collected and recrystallized from 50 % acetic acid to give pure α,α,α′,α′-bis-(ethylene)-succinic acid (3.1 g). (Found: C 56.3; H 5.8; equiv. wt. 84.5. Calc. for $C_8H_{10}O_4$: C 56.5; H 5.9; equiv. wt. 85.1.)

α,α,α′,α′-Bis-(trimethylene)-succinic acid. Dimethyl α,α,α′,α′-bis-(trimethylene)-succinate, 5.0 g, was boiled with 10 % ethanolic potassium hydroxide over night, the ethanol distilled off and the residue acidified with dilute hydrochloric acid. The solid was filtered and recrystallized from 50 % acetic acid to yield pure α,α,α′,α′-bis-(trimethylene)-succinic acid (2.3 g). (Found: C 60.5; H 7.2; equiv. wt. 198.0. Calc. for $C_{10}H_{14}O_4$: C 60.6; H 7.1; equiv. wt. 198.2.)

α,α,α′,α′-Bis-(tetramethylene)-succinic acid. Dimethyl α,α,α′,α′-bis-(tetramethylene)-succinate, 5.0 g, was heated on a boiling water-bath together with 10 ml of concentrated sulphuric acid for 5 min. On dilution with water an oil separated which solidified upon cooling. The solid (3.6 g) was filtered and a small sample recrystallized from light petroleum, m. p. 65—66°. According to analyses it consisted of α,α,α′,α′-bis-(tetramethylene)-succinic anhydride. (Found: C 69.4; H 7.8; Calc. for $C_{12}H_{16}O_3$: C 69.2; H 7.8.)

Crude anhydride, 3.1 g, was dissolved in 20 % aqueous potassium hydroxide by boiling for 15 min. Acidification yielded a solid which was recrystallized from 90 % acetic acid, though with loss due to anhydrization. According to analyses it was α,α,α′,α′-bis-(tetramethylene)-succinic acid. (Found: C 63.7; H 8.1; equiv. wt. 225.3. Calc. for $C_{12}H_{18}O_4$: C 63.7; H 8.0; equiv. wt. 226.3.)

Overberger *et al.*[13] found m. p. 63—64° for the anhydride.

The author wishes to express his sincere gratitude to Professor Erik Larsson for his kind interest in this work. A grant from *Doktor P. Håkanssons Stiftelse* is gratefully acknowledged.

REFERENCES

1. Crum Brown, A. and Walker, J. *Ann.* **274** (1893) 41.
2. Eberson, L. *Acta Chem. Scand.* **10** (1956) 629.
3. Murray, A. K. and Taylor, T. W. J. *J. Chem. Soc.* **1937**, 1450.

4. Alberti, C. *Gazz. Chim. Ital.* **86** (1956) 1195.
5. Walker, J. and Walker, A. P. *J. Chem. Soc.* **87** (1905) 961.
6. Crichton, J. *J. Chem. Soc.* **89** (1906) 929.
7. Weedon, B. C. L. *Quart. Revs. (London)* **6** (1952) 380.
8. Linstead, R. P. and Whalley, M. *J. Chem. Soc.* **1954**, 3722.
9. Schotte, L. and Rosenberg, A. *Arkiv Kemi* **8** (1956) 551.
10. Bone, W. A. and Sprankling, C. H. G. *J. Chem. Soc.* **77** (1900) 654.
11. Ansell, M. F., Hickinbottom, W. J. and Holton, P. G. *J. Chem. Soc.* **1955**, 349.
12. Dox, A. W. *J. Am. Chem. Soc.* **47** (1925) 1471.
13. Overberger, C. G., Biletch, H., Huang, P. T. and Blatter, H. M. *J. Org. Chem.* **20** (1955) 1717.
14. Fichter, F. and Heer, J. *Helv. Chim. Acta* **18** (1935) 1276.
15. Berner, E. and Leonardsen, R. *Ann.* **538** (1939) 1.
16. Wren, H. and Haller, J. W. E. *J. Chem. Soc.* **1937**, 230.
17. Stefl, A. *Über Bernsteinsäure und ihre Homologen* (Diss.) München 1914.
18. Hückel, W. *Theoretische Grundlagen der Organischen Chemie*, Akademische Verlagsgesellschaft, Leipzig 1952.

Received October 1, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 50—59

On the Complex Formation of Nickel with Dimethylglyoxime

DAVID DYRSSEN, FRANC KRAŠOVEC and LARS GUNNAR SILLÉN

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The distribution of $^{63}Ni^{2+}$ at 25°C between chloroform and 0.1 M perchlorate solution has been studied as a function of the concentration of H^+ and of dimethylglyoxime (HA) in the aqueous phase. The data may be completely explained with the equilibria; $NiA_2 \rightleftharpoons NiA_2$ (in $CHCl_3$), $\log \lambda_2 = 2.51 \pm 0.10$; $Ni^{2+} + 2HA \rightleftharpoons NiA_2$(in $CHCl_3$) + $2H^+$, $\log K = -1.17 \pm 0.10$. Previous solubility [1] and emf [2] data have been recalculated.

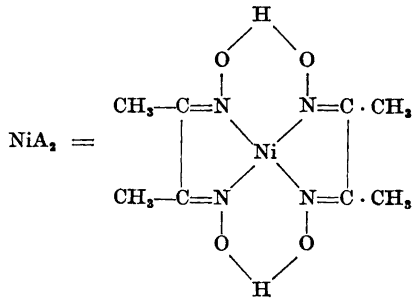
The present distribution data give no evidence for the presence of significant amounts of NiA^+. By curve-fitting it is concluded that $\log K_1 \leq \log K_2 - 1.2$; the same conclusion is drawn from the solubility data [1]. By using the value for K_a(HA) calculated from Ref[1], β_2 is obtained as 17.24 ± 0.07 (at $I = 0.10$) from the distribution data, and 17.23 ± 0.05 from the solubility data.

Charles and Freiser's emf data [2] would indicate $\log K_1 \sim 10.55$, $\log K_2 \sim 11.15$ in their medium, 50 % dioxane.

Dimethylglyoxime (HA) is a well-known reagent for nickel. The complex NiA_2 has a very low solubility in water but may be extracted, presumably as NiA_2 molecules, with organic solvents such as chloroform.

The crystal structure of NiA_2 (s) has been studied by Godycki and Rundle[3] and its infrared spectrum by Rundle and Parasol [4]. The molecule is planar. There are two very strong, probably symmetrical, hydrogen bonds between the two A groups in each molecule, and metal-metal bonds between neighboring NiA_2 molecules. From a discussion of the properties of NiA_2 and a number of related compounds, Sharpe and Wakefield [5] conclude, as was already suggested by Rundle [3,4], that the metal-metal bonds are the main factor in making NiA_2 insoluble.

It might be of interest to know to what extent the intermediate complex NiA^+ is formed in aqueous solution. Christopherson and Sandell [1] could explain their solubility data assuming only Ni^{2+} and NiA_2 in the aqueous phase; however, we shall see that their experiments were perhaps not ideally designed for a search for NiA^+. On the other hand, Charles and Freiser [2],



from pH measurements (presumably in 50 % dioxane-water), give complex formation constants K_1 and K_2 for A^-—Ni^{2+}, indicating $K_1 \sim 4K_2$. They point out, quite correctly, that a more accurate calculation of their data would give a lower K_1 and a higher K_2.

To study the importance of NiA^+, we have applied the distribution method (for surveys, see Rydberg [6], Dyrssen [7]). In this case, we have measured how the distribution ratio, q, for Ni between chloroform and an aqueous solution varies with the concentrations [HA] and $[H^+] = h$, in the aqueous solution.

GENERAL EQUATIONS

There is no evidence that the aqueous solutions with which we are concerned should contain Ni^{2+} and A^- in any species other than Ni^{2+}, NiA^+, NiA_2, A^-, and HA. With the usual definitions of the acidity constant, K_a, and the complex formation constants, $\beta_1 = K_1$ and $\beta_2 = K_1K_2$ we have:

$$[A^-] = K_a[HA]h^{-1}\,;\ [NiA^+] = \beta_1[Ni^{2+}][A^-]\,;\ [NiA_2] = \beta_2[Ni^{2+}][A^-]^2 \quad (1)$$

Our equations will be transformed to use h and [HA] as variables; in general, [HA] is more accurately known than $[A^-]$, since $[HA] \gg [A^-]$ in the solutions. We shall find it convenient to introduce an auxiliary variable, v, and a constant parameter, p:

$$v = [A^-]^{-1}\beta_2^{-\frac{1}{2}} = h[HA]^{-1}K_a^{-1}\beta_2^{-\frac{1}{2}}\,;\ p = \beta_1\beta_2^{-\frac{1}{2}} = \sqrt{K_1/K_2} \quad (2)$$

The paramter, p, indicates the tendency towards formation of NiA^+. In Dyrssen and Sillén's »two-parameter» notation [8], $\beta_1 = 10^{a+b}$, $\beta_2 = 10^{2a}$ and $p = 10^b$. If the distribution of A over the Ni atoms were statistical, one would have $p = 2$.

From (1) and (2) we may easily derive

$$[Ni^{2+}] = [NiA_2]h^2[HA]^{-2}\beta_2^{-1}K_a^{-2} = [NiA_2]v^2$$
$$[NiA^+] = [NiA_2]\,h\,[HA]^{-1}\beta_1\beta_2^{-1}K_a^{-1} = [NiA_2]pv$$

The total amount of Ni in the aqueous solution is thus

$$[NiA_2] + [NiA^+] + [Ni^{2+}] = [NiA_2]\,(1 + pv + v^2) \tag{3}$$

Solubility data. In Christopherson and Sandell's experiments [1], solutions of ionic strength 0.05 M were brought to equilibrium with NiA_2 (s) and HA (s) at 25°C. At equilibrium with these solids we have

$$[HA] = s_1 = \text{constant};\ [NiA_2] = s_2 = \text{constant}$$

Thus the total solubility of NiA_2 in the solution is

$$S = [NiA_2] + [NiA^+] + [Ni^{2+}] = [NiA_2]\,(1 + pv + v^2) = s_2\,(1 + pv + v^2) \tag{4a}$$

$$v = hs_1^{-1}\,K_a^{-1}\beta_2^{-\frac{1}{2}} = h \cdot \text{constant} \tag{4b}$$

To find the unknown constants we may plot the experimental data in a diagram log S (log h) and move it along a family of standardized curves $Y\ (X)$

$$Y = \log\,(1 + pv + v^2);\ X = \log v \tag{5}$$

which may easily be constructed (Sillén [9]). From the curve that gives the best fit, one may obtain the parameter, p. From the positions of the coordinate axes at the best fit one obtains from (4) and (5).

$$\log S - Y = \log s_2;\ \log h - X = \log s_1 + \log K_a + \tfrac{1}{2}\log \beta_2 \tag{6}$$

Distribution data. When an aqueous solution with Ni^{2+} and HA is equilibrated with chloroform, the chloroform takes up NiA_2 and HA; the experiments have given no reason to assume any other species in the organic phase. Denoting by λ_2 the real equilibrium constant for the distribution of NiA_2 and by q the total distribution ratio for Ni, we have

$$[NiA_2]_{org} = \lambda_2\,[NiA_2] = q([NiA_2] + [NiA^+] + [Ni^{2+}]) = q[NiA_2]\,(1 + pv + v^2) \tag{7}$$

This gives immediately

$$q^{-1} = \lambda_2^{-1}\,(1 + pv + v^2) \tag{8}$$

Since $v = h\,[HA]^{-1}$. constant, (2), we may make a plot of $(-\log q)$ as a function of $\log (h\,[HA]^{-1})$ and compare it with the same family (5) of normalized curves as before, Y (X). From the position at the best fit we obtain

$$-\log q - Y = -\log \lambda_2;\ \log (h[HA]^{-1}) - X = \log K_a + \tfrac{1}{2}\log \beta_2 \tag{9}$$

Emf data. Charles and Freiser [2] made pH titrations in 50 % dioxane and calculated log $[A^-]$ at the two points where $\bar{n}$, the average number of A bound per Ni, is 0.5 and 1.5. We have the equation

$$\bar{n} = \frac{2\,[NiA_2] + [NiA^+]}{[NiA_2] + [NiA^+] + [Ni^{2+}]} = \frac{2 + pv}{1 + pv + v^2} \tag{10}$$

From (10) we may derive the values for v where $\bar{n} = 0.5$ and 1.5:

$$v_{0.5} = (\sqrt{12 + p^2} + p)/2 \; ; \; v_{1.5} = (\sqrt{12 + p^2} - p)/6$$

$$v_{0.5}v_{1.5} = 1 \; ; \; v_{0.5}/v_{1.5} = (\sqrt{12 + p^2} + p)^2/4$$

Inserting the definition (2) of v we find

$$\log [A^-]_{0.5} + \log [A^-]_{1.5} = -\log \beta_2$$

$$\log [A^-]_{1.5} - \log [A^-]_{0.5} = 2 \log (\sqrt{12 + p^2} + p) - \log 4 \qquad (11)$$

CHRISTOPHERSON AND SANDELL'S SOLUBILITY DATA

Christopherson and Sandell [1] determined the solubility at 25°C of HA(s) in dilute $NaHCO_3$-Na_2CO_3 buffers, or NaOH, of ionic strength 0.05(NaCl), and of NiA_2(s) in KCl-HCl mixtures of ionic strength 0.05, when saturated with HA(s), and also in some other solutions of pH between 5.6 and 8.9 and the same ionic strength, all saturated with HA(s). They gave the »pH» of the solution,

$$\text{»pH»} = -\log h - \log f$$

where f is the activity factor of the H^+ ion. In the following we shall give the equilibrium constants as the concentration quotients at the corresponding ionic strength. As long as f remains constant (constant ionic strength), many of the conclusions are independent of any uncertainty that may exist in the f assumed.

For the solubility of HA, Christopherson and Sandell found a linear plot, solubility *versus* $(fh)^{-1}$, with the equation

$$\text{solubility} = 10^{-2.268} (1 + 10^{-10.585} (fh)^{-1})$$

Now it may easily be seen that the solubility is

$$[HA] + [A^-] = s_1 (1 + K_a h^{-1})$$

whence we deduce

$$s_1 = 10^{-2.268} \; ; \; K_a = f^{-1} \cdot 10^{-10.585} \; (I = 0.05(\text{NaCl}))$$

The solubility, S, of NiA_2 is plotted in Fig. 1 as log S (log (fh)). Over these experimental points is placed the family of curves, $Y(X)$, in the position of best fit. The full-drawn curve is the one calculated for $p = 0$, and the dotted one for $p = 1$. The broken lines are the asymptotes. It is readily seen that most of the experimental points are in parts where the curves differ very little on varying the value for p. Only one point is in the region with maximum influence of p, and thus of the intermediate complex NiA^+, and this point

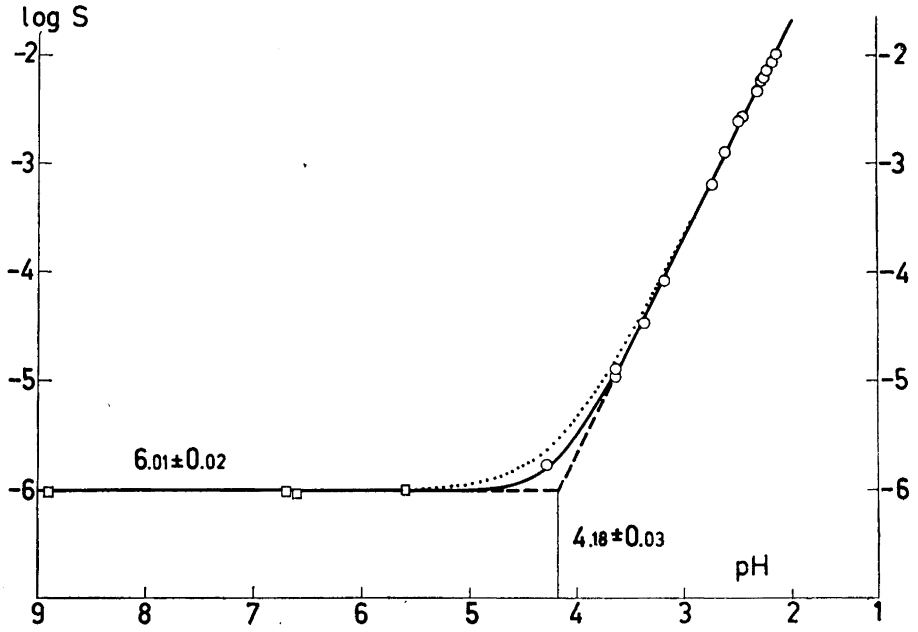


Fig. 1. Solubility of NiA_2 (25°C, $I = 0.05$, saturated with HA(s)) according to Christopherson and Sandell [1]). Experimental data: $\log S$ *versus* — "pH" = $\log h + \log f$. "Normalized curves" $Y(X)$, eqn. (5), in position of best fit, for $p = 0$ (full-drawn) and $p = 1$ (dotted). The broken lines are the asymptotes.

agrees well with $p \sim 0$. The data hence give no certain evidence for the existence of NiA^+. Assuming $p = 0$, we obtain at the best fit (see eqn. 6):

$$\log S - Y = -6.01 \pm 0.02 = \log s_2$$

$$\log (fh) - X = -4.18 \pm 0.03 = \log s_1 + \log K_a + \tfrac{1}{2} \log \beta_2 + \log f$$

Combining this with the above values for s_1 and K_a we obtain

$$\log s_2 = -6.01 \pm 0.02; \log \beta_2 = 17.35 \pm 0.04 \ (I = 0.05)$$

It may be noted that the value for β_2 is independent of any uncertainty in f; so is the conclusion that $p \sim 0$.

We might have obtained the same result and the following equation for S by using for the two constants average values from the data near to each of the asymptotes

$$S = 10^{-6.013} + 10^{+2.340} (fh)^2 = [NiA_2] + [Ni^{2+}]$$

Applying the activity factors given by Kielland [10] for the ions, and by Christopherson and Sandell [1] for the uncharged species, we may calculate, from their data, the following equilibrium constants for the ionic strengths 0, 0.05, and 0.10:

		$I = 0$	$I = 0.05$	$I = 0.10$
$HA(s) \rightleftharpoons HA$	$\log s_1 =$	− 2.26	− 2.27	− 2.28
$HA \rightleftharpoons H^+ + A^-$	$\log K_a =$	−10.67	−10.52	−10.46
$NiA_2(s) \rightleftharpoons NiA_2$	$\log s_2 =$	− 6.00	− 6.01	− 6.03
$Ni^{2+} + 2A^- \rightleftharpoons NiA_2$	$\log \beta_2 =$	17.83	17.35	17.23

Reference[1] seems to have used the activity factors for 0.025 M instead of 0.05 M, which is easily done since Kielland's table gives f as a function of $2I$.

For distribution between chloroform and water, Christopherson and Sandell [1] moreover give

$$HA \rightleftharpoons HA(\text{in } CHCl_3) \qquad \log K_d = -1.08$$
$$NiA_2 \rightleftharpoons NiA_2(\text{in } CHCl_3) \qquad \log \lambda_2 = 2.61$$

In water, saturated with $CHCl_3$, the equilibrium constants may differs slightly from those in aqueous solution free from $CHCl_3$. For instance, in the presence of $CHCl_3$, $\log s_2 = -5.92$.

DISTRIBUTION EXPERIMENTS

Experimental. The experiments were made in a room thermostated at 25°C. All the reagents used were of analytical grade, and the chloroform was made alcohol-free by washing it with water. The ionic strength was kept constant at 0.1 M using $HClO_4$, $NaClO_4$, and NaOH. In some experiments, small amounts of acetate ($\leqq 0.01$ M) were added to buffer the aqueous phase.

The distribution ratio, q, was measured using ^{63}Ni (half-life 80 years, 0.063 MeV β). The two phases had equal volumes, 15 ml. HA and Ni^{2+} were originally present in the aqueous phase, at concentrations $[HA]_{total} = 0.001$ to 0.0032, and $[Ni]_{tot} = 1.30 \times 10^{-4}$M. After equilibration and separation of the phases, 1 ml of the chloroform phase was taken out, evaporated in a cup of stainless steel, and counted in a Tracerlab Flow Proportional Counter SC-16. The aqueous phase could not be evaporated directly, since it contained $NaClO_4$; to 5 ml aqueous phase, ammonia was added to pH $\sim$ 9, and the nickel was extracted with 5 ml of chloroform, 1 ml of which was then evaporated, and the Ni counted as before.

In the aqueous phase, $[H^+] = h$ was measured with a glass electrode and a Radiometer pHM3i valve potentiometer, standardized against 0.01 M $HClO_4$ + 0.09 M $NaClO_4$. To obtain the real [HA] in the aqueous phase, from the $[HA]_{tot}$ added originally, it was necessary to introduce corrections for $[HA]_{org}$ and in some parts of the experimental range also for $[A^-]$ and $[NiA_2]_{org}$. The amounts of NiA_2 and NiA^+ in the aqueous phase were always negligible in comparison to $[HA]_{tot}$. We easily obtain

$$[HA] = ([HA]_{tot} - 2\,[Ni]_{tot}\, q(1+q)^{-1})\,(1 + K_d + K_a h^{-1})^{-1}.$$

The values for K_d and K_a were taken from Christopherson and Sandell's data [1]. K_d is small, only 1/12, and any slight error in it will mean only a constant shift along the log (h[HA]$^{-1}$) axis. The correction for K_a is negligible for most points, and the exceptions are not in the critical part of the curve.

The data ([HA]$_{tot}$, log h, log q) are given in Table 1; Fig 2 gives (log q) as a function of (log h — log [HA]). As in Fig 1, the calculated curves for $Y(X)$ with $p = 0$ (full-drawn) and $p = 1$ (dotted), and the asymptotes (broken) are given in the position of best fit. The spread is seen to be greater than for the solubility measurements, which is due to the difficulty of measuring dry samples with the very weak α-emitter ^{63}Ni (0.063 MeV). On the other hand, a much larger number of experimental points has been placed in the critical region from which the value of p can be deduced. As seen from Fig. 2, the data agree, within the experimental error, with the curve for $p = 0$, and thus give no evidence for the existence of NiA^+ in appreciable amounts.

From the position at the best fit we find, using points close to the asymptotes

$$q^{-1} = 10^{-2.51} + 10^{1.17}\,(h/[HA])^2$$

We may also read directly (see eqn. 9)

$$-\log q - Y = -\log \lambda_2 = -2.51 \pm 0.10$$

$$(\log h - \log [HA]) - X = \log (h/[HA]v) = \log K_a + \tfrac{1}{2} \log \beta_2 = -1.84 \pm 0.07$$

which gives, for 25°C and $I = 0.1$, the following equilibrium constants:

$$NiA_2 \rightleftharpoons NiA_2 \text{ (in } CHCl_3) \quad ; \log\lambda_2 = 2.51 \pm 0.10$$

$$Ni^{2+} + 2HA \rightleftharpoons NiA_2 \text{ (in } CHCl_3) + 2H^+ \quad ; \log K = \log\lambda_2 + 2 \log K_a + \log \beta_2 = -1.17 \pm 0.10$$

Inserting the value log $K_a = -10.46$, calculated from the solubility data [1], we obtain

$$Ni^{2+} + 2A^- \rightleftharpoons NiA_2 \; ; \log\beta_2 = 17.24 \pm 0.07$$

which is seen to agree very well with the estimate from the solubility data [1], 17.23.

The crossed and solid symbols in Fig. 2 denote experiments where up to 0.01 M acetate was added for buffering. A comparison with the open symbols indicates that the acetate had no perceptible influence, which is reasonable in view of the equilibrium constants given by Fronæus [11] (log $K_1 \sim 0.7$, log $\beta_2 \sim 1.25$ for Ac^--Ni^{2+} in 1 M $NaClO_4$ at 20°C).

Estimates of p and K_1

We have seen from Figs. 1 and 2 that both the solubility and the distribution data, when plotted and compared with »normalized curves», $Y(X)$, give good agreement with the curve for $p = 0$. A deviation of p from the value 0

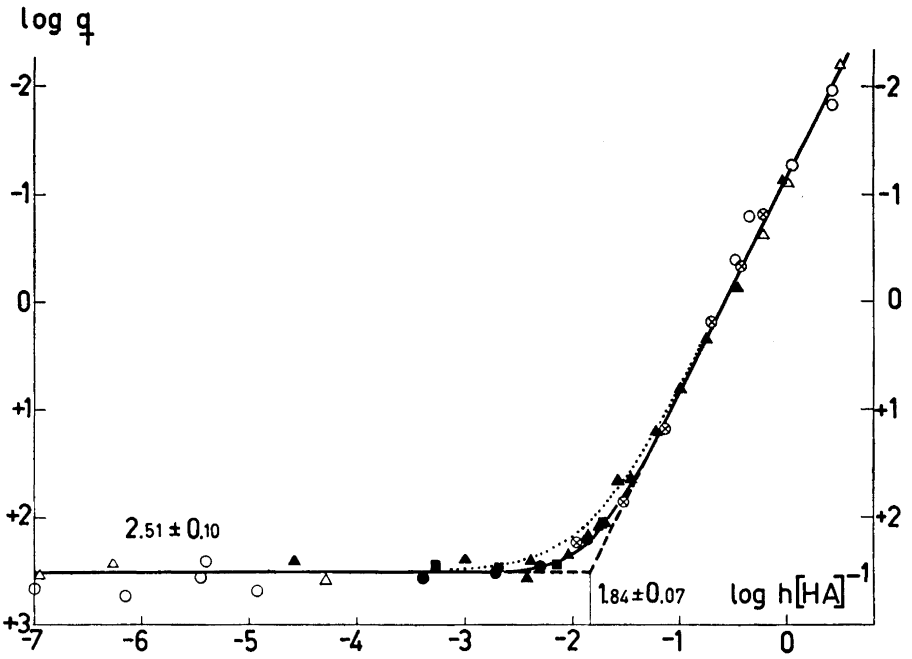


Fig. 2. Distribution data (present work). Experimental points: (— log q), distribution ratio, *versus* (log h — log [HA]). "Normalized curves" $Y(X)$ in position of best fit, for $p = 0$ (full-drawn) and $p = 1$ (dotted). The broken lines are the asymptotes.

Table 1. Distribution of Ni^{2+} between chloroform and 0.1 M perchlorate solutions at 25°C. Initial concentrations in the aqueous phase $[Ni]_{tot} = 1.30 \times 10^{-4}$ M, $[HA]_{tot} =$ 0.0032 (○ ⊗ ●), 0.002 (■) or 0.001 (△ ▲) M. Acetate concentrations: 0 (○, △), 0.004 (⊗), or 0.010 (●, ■, ▲) M.

log q	— log h	log q	— log h	log q	— log h
0.0032 M HA, ○		0.0032 M HA, ●		0.001 M HA, ▲	
— 1.962	2.12	+ 2.214	4.44	— 1.066	3.09
— 1.830	2.12	+ 2.464	4.88	— 0.130	3.55
— 1.255	2.49	+ 2.521	5.30	+ 0.339	3.88
— 0.793	2.89	+ 2.560	5.96	+ 0.812	4.15
— 0.388	3.03	0.002 M HA, ■		+ 1.200	4.38
+ 2.675	7.50	+ 2.049	4.52	+ 1.636	4.62
+ 2.409	7.97	+ 2.438	4.95	+ 1.660	4.75
+ 2.555	8.02	+ 2.467	5.48	+ 2.042	4.87
+ 2.727	8.74	+ 2.447	6.07	+ 2.078	4.92
+ 2.658	9.81	0.001 M HA, △		+ 2.162	5.03
0.0032 M HA, ⊗		— 2.206	2.45	+ 2.358	5.23
— 0.806	2.75	— 1.095	3.03	+ 2.397	5.56
— 0.328	2.97	— 0.626	3.29	+ 2.564	5.59
+ 0.194	3.24	+ 2.566	7.46	+ 2.383	6.17
+ 1.182	3.70	+ 2.432	9.47	+ 2.391	7.75
+ 1.864	4.10	+ 2.531	10.32		
+ 2.244	4.54				

would cause the largest deviation in the curve at the abcissa corresponding to $X = 0$ (intersection point of the asymptotes). Here, $Y = \log (2 + p)$. as seen from eqn. (5). Fron the data it may be estimated that the deviation from the curve with $p = 0$, denoted by Δ by Dyrssen and Sillén [8], is probably less than 0.05:

$$\Delta = \log (2 + p) - \log 2 \leq 0.05$$

thus $p \leq 0.25$, or $\log K_1 \leq \log K_2 - 1.2$

From Charles and Freiser's data [2] for 50 % dioxane (25° to 39.6°C) we obtain, using eqns. (11)

$$\log\beta_2 = -\log([A^-]_{0.5}\,[A^-]_{1.5}) = 21.7 \pm 0.1$$

$$\log ([A^-]_{1.5}/[A^-]_{0.5}) = 2 \log (\sqrt{12 + p^2} + p) - \log 4 = 0.60 \pm 0.02$$

thus $p = 0.5 \pm 0.1$, $\log K_1 \sim 10.55$, $\log K_2 \sim 11.15$ (in 50 % dioxane)

Charles and Freiser have also measured the complex formation between Ni^{2+} and the O-monomethyl ether of dimethylglyoxime, HA_{Me} in 50 % dioxane at 25°C. Applying the same eqns. (11) to their single set of $[A^-_{Me}]$ values, we obtain

$$\text{for } A^-_{Me}\text{—}Ni^{2+}\text{: } \beta_2 = 12.07\,;\ 2 \log (\sqrt{12 + p^2} + p) - \log 4 = 0.69$$

thus $p \sim 0.85$, $\log K_1 = 5.97$, $\log K_2 = 6.10$

Accordingly we would have for A^-_{Me}—Ni^{2+}, $K_1 \sim 10^{6.0}$ and $K_2 \sim 10^{6.1}$. Even in this case, the second complex would be favored since p is less than the »statistical» value, 2. This is remarkable, since with A^-_{Me} the formation of the second complex cannot be favored by hydrogen bonds as with A^-. The great difference makes one suspect for a moment that there might be in the aqueous phase a complex where Ni is bound to the doubly charged ion of HA; we might formally write this complex as $NiH_{-1}A$, and it could have no counterpart with HA_{Me}. For such a complex we would have

$$[NiH_{-1}A] = K\,[NiA_2]\,[HA]^{-1}$$

In the solubility experiments, with [HA] = constant, considerable amounts of such a complex might have passed as »NiA_2». In the distribution experiments, however, it would make q shift with [HA], whereas really q is found to be a function of $h/[HA]$ only, so this complex does not seem to be important.

On the other hand, one might disbelieve completely the p obtained from Charles and Freiser's data but still put some confidence in their β_2. Estimating that K_1 for A^-_{Me} is $\sim 10^6$ and that K_1 for A^- has the same order of magnitude, we would have K_2 for A^- about 10^{16}, and $p \sim 10^{-5}$, in 50 % dioxane. This is, however, a very uncertain estimate.

Returning to our first treatment: if Charles and Freiser's data [2] are taken at their face value, the first complex of the methyl ether, A^-_{Me}, would be much

less stable than that of A^-. One possible reason is a steric hindrance, or strain in the chelate ring caused by the CH_3 group. It would be interesting to know the crystal structure of $Ni(A_{Me})_2$, and also to have more accurate equilibrium data on the A_{Me}—Ni^{2+} complexes.

The only conclusion that the present data allow us to draw about p for A^-—Ni^{2+} in aqueous solution is that it is certainly less than 1, and probably less than 0.25. At any rate, p is thus less than would be expected from a statistical distribution of A^- over Ni^{2+} ions: 2.0 at $I = 0$, 1.65 at $I = 0.05$, and 1.55 at $I = 0.10$, corrected with Kielland's activity coefficients [10].

Finally, we should like to remark that the quite good agreement of the experimental data with the calculated curve over a wide range of concentrations has added to our confidence in the distribution method as a way of studying equilibria. If there had been any serious systematic errors, they would have been likely to show up in this very simple system which contains only two adjustable parameters, λ_2 and the product $K_a^2\beta_2$.

Acknowledgements. The present investigation has been supported by *Atomkommittén (Swedish Atomic Energy Commission)*, and by a grant from the *Institut Josef Stefan*, Ljubljana, Yugoslavia.

REFERENCES

1. Christopherson, H. and Sandell, E. B. *Anal. Chim. Acta* **10** (1954) 1.
2. Charles, R. G. and Freiser, H. *Anal. Chim. Acta* **11** (1954) 101.
3. Godycki, L. E. and Rundle, R. E. *Acta Cryst.* **6** (1953) 487.
4. Rundle, R. E. and Parasol, M. *J. Chem. Phys.* **20** (1952) 1487.
5. Sharpe, A. G. and Wakefield, D. B. *J. Chem. Soc.* **1957** 281.
6. Rydberg, J. *Svensk Kem. Tidskr.* **67** (1955) 499.
7. Dyrssen, D. *Svensk Kem. Tidskr.* **68** (1956) 212.
8. Dyrssen, D. and Sillén, L. G. *Acta Chem. Scand.* **7** (1953) 663.
9. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 186.
10. Kielland, J. *J. Am. Chem. Soc.* **59** (1937) 1675.
11. Fronæus, S. *Acta Chem. Scand.* **6** (1952) 1200.

Received August 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 60—74

The Effect of Some Pro-oxidants and Antioxidants on the Stability of Natural Vitamin A

KARL BÄCK*

Division of Food Chemistry, Royal Institute of Technology, Stockholm, Sweden

A study was made on the influence of some pro-oxidants (metal ions) and antioxidants on the stability of vitamin A in fish liver oils and vitamin A concentrates from fish liver oils. The destruction of vitamin A and the change in peroxide number were determined in oil samples stored at 34.5°C.

Under the conditions used, the effects of the pro-oxidants and antioxidants were small. Iron and copper had the greatest pro-oxidative effect of the metal ions tested. Among the antioxidants tested, NN′-diphenyl-*p*-phenylene diamine was the best stabilizer.

Fish liver oils are the best natural sources of the vitamins A and D and are extensively used to supply these essential compounds. When the oils turn rancid, their vitamin contents are destroyed. Rancidity in fish liver oils is mainly due to atmospheric oxidation. As vitamin D has been found to be more stable to oxidation than vitamin A, most of the work in this field has been devoted to studies of the stability of vitamin A.

In this investigation, the catalytic effect of various concentrations of the metal ions Al^{3+}, Cu^{2+}, Fe^{3+}, Ni^{2+}, Sn^{4+} and Zn^{2+} on the destruction of vitamin A was studied. The pro-oxidative effect observed with the metal ions was weak compared to those reported by other investigators.

The stabilizing effect of the following antioxidants and synergists was also investigated: ascorbic acid, ascorbyl palmitate, citraconic acid, citric acid, BHA,** BHT, lecithin, NN′-diphenyl-*p*-phenylene diamine, NDGA, tiodiphenylamine and some commercial antioxidant mixtures. NN′-diphenyl-*p*-phenylene diamine was the best stabilizer and on the whole the effects of the antioxidants were comparatively small.

* Present address, Blå Band Produkter AB, Halmstad, Sweden.

** The abbreviations BHA, BHT and NDGA denote butylated hydroxy anisole, butylated hydroxy toluene and nordihydroguaiaretic acid respectively.

EXPERIMENTAL

The properties of the vitamin oils used in these experiments are summarized in Table 1. Investigations were made on fish liver oils and on vitamin A concentrates obtained by molecular distillation of low potency fish liver oils. In the oils A, C, F and M the contents of the trace metals Cu, Mn, Ni and Fe were estimated. The amount of iron was in the range 0.1—0.5 p.p.m. and the amounts of the other metals were in the range 0.00—0.05 p.p.m. After the oils had been percolated through an ion exchange resin column, the amounts of Cu, Mn and Ni were in the range 0.00—0.01 p.p.m. and the amount of iron was less than 0.1 p.p.m.

Analysis

Vitamin A analysis. Vitamin A was determined using a method which closely resembles that given by Dowler and Laughland [1].

Adsorbent. Commercial bone meal was extracted in a Soxhlet apparatus with hexane and then with acetone in order to remove fatty material soluble in these solvents. The bone meal was dried in an oven at 110°C and the fraction which passed a 100 mesh but was retained by a 200 mesh sieve was used. To remove the finest particles the sieving on the 200 mesh sieve was made with the bone meal suspended in water. After this treatment, the adsorbent was again dried at 110°C.

Procedure. The chromatographic tube used had a length of 120 mm and an internal diameter of 8 mm. At the lower end it had a constriction. A plug of glass wool was placed in the tube and the bone meal adsorbent poured into the tube in the form of a suspension in hexane. After settling the height of the adsorbent column was 60 mm. Without further arrangements the rate of flow of the solvent through this column was 40—60 drops per min.

A 2 ml solution of oil in hexane which contained approximately 100 IU of vitamin A per ml was placed on the top of the column. The vitamin A esters were eluted by three successive portions of 2, 5, and 10 ml of hexane. As no vitamin A alcohol could be detected in any of the batches of oil used in these experiments, no measures were taken to elute this substance in the routine analyses.

The vitamin A in the eluate was determined by measuring the absorbancies at 312, 325 and 335 mμ in a Beckman spectrophotometer model DU. A corrected value for the absorbancy at 325 mμ was calculated by using the formula

$$A_{corr} = 7\ A_{325} - 3.045\ A_{312} - 3.955\ A_{335}.$$

Using this corrected value, the $E^{1\%}_{1\,cm}$-value was calculated and the vitamin A contents were determined by using a conversion factor of 1 900. This method has certain limitations especially when the oil is oxidized. Components initially present in the oil or developing during its oxidation and having a relatively high absorbancy in the interval 290—325 mμ are not quantitatively removed by the simple purification on the bone meal column. On the other hand, it is not possible to use a method involving saponification when the oil has a high content of peroxides.

Peroxide value. The peroxide value was determined essentially as suggested by Wheeler [2]. Samples (1 g) of oil were dissolved in 25 ml of a mixture of 60 % glacial acetic acid and 40 % chloroform in 200 ml conical flasks. A saturated solution of potassium iodide (0.5 ml) was added, and the flask gently rotated for 1 min. Distilled water (50 ml) was then added and the liberated iodine titrated immediately with 0.02 N sodium thiosulphate. Starch was used as an indicator. The results are expressed in millimoles of peroxide per kg of oil.

Estimation of trace metals. The metals Cu, Fe, Mn and Ni were detected and semi-quantitatively estimated by a method given by Täufel [3]. Oil (20 g) was weighed in a thoroughly cleaned quartz crucible with a diameter of about 50 mm and a height of about 50 mm. The crucible was placed on an electric heating plate and when the oil had reached its ignition temperature, the vapours were ignited. The energy input to the electric plate was regulated to keep the flame of the burning oil as small as possible.

Table 1. Properties of the different oils tested

Oil	Vitamin A contents IU/g × 10^3	Initial peroxide value	Time for 50 % destruction of vitamin A, hours	Peroxide value in the oil when 50 % of the vitamin A initially present is destroyed
A	92.6	22.1	440	80
B	94.2	2.8	578	41
C	44.4	1.9	965	17
D	84.3	2.2	276	52
E	94.4	7.4	212	67
F	45.7	9.6	276	81
G	84.0	1.8	216	71
H	81.0	5.3	209	57
J	97.7	2.9	750	38
K	101.7	2.2	632	34
L	81.8	3.1	247	55
M	38.0	0.5	205	48
N	77.4		272	58
O	72.4	2.3	264	70
P	39.8	0.5	204	59

The 20 g of oil were usually burnt to a coke-like residue in about 1—2 h. The crucible was then placed in an electric oven, where the combustion was completed at 500°C in 3—4 h.

The ash was dissolved in 0.5 ml of 6 N HCl. The resulting solution was evaporated to dryness by placing the quartz crucible in a slanting position on a sand bath in order to keep the resulting dry chloride in as small an area as possible. The dry chloride was dissolved in 0.2 ml of 6 N HCl. Sheets of Whatman No. 4 filter paper (230 × 420 mm) were washed to remove metal ions which might be present, by passing a solvent system of *n*-butanol (83 %) and 6 N HCl (17 %) through them by using a descending technique. The paper sheets were dried in a stream of warm air.

The hydrochloric acid solution of ash from the oil was placed in two spots on the starting line of two of the above mentioned washed paper sheets. The total amount of the solution was equally divided between the two spots. On each sheet four more spots with known amounts of metal chlorides were placed for comparison. On one sheet the chlorides of copper and nickel were placed and on the other the chlorides of iron and manganese. The paper sheets were chromatographed with the above mentioned solvent system for 5—7 h. During this time the solvent front had moved 200 — 300 mm. The paper sheets were dried in a stream of warm air. The sheet with the control spots of copper and nickel was sprayed with a solution of rubeanic acid (0.05 %) in 99 % ethanol and then treated with ammonia vapour. Copper gave a grey to green spot with an R_F-value of 0.1—0.2 and nickel a violet spot with an R_F-value of 0.02—0.08. Amounts of 0.05 μg of copper and nickel were easily detected. The sheet with the control spots of iron and manganese was sprayed near the starting line (to $R_F = 0.3$) with a water solution of sodium cyanide (15 %) in order to bind the nickel ion in a complex that would not interfere with the determination of manganese. This metal was detected as a brown spot (R_F-value 0.05—0.15) after spraying the upper part of the sheet with a solution of formaldoxim according to Hecht and Gottlieb [4*]. The sheet was subsequently treated with ammonia vapour. The method readily permits the detection of 0.05 μg of manganese.

* The formaldoxim solution according to Hecht and Gottlieb is prepared by dissolving hydroxylamine hydrochloride (19.95 g) and paraformaldehyde (10 g) in distilled water (100 ml).

Iron present in amounts of approximately 5 μg could easily be seen as a yellow spot. Even 1 μg quantities were easily detected if the sheets were sprayed with a methanolic solution of ammonium rhodanide (5 %) and subsequently treated with hydrochloric acid vapours. The R_F-value of iron was 0.5—0.7.

Recovery experiments in which Cu, Mn and Ni were added to the oil in amounts of 0.1 p.p.m. showed that complete recovery was obtained with Mn and Ni, whereas a negligible loss of Cu occurred during the analytical process.

Procedure

The testing of the oil was performed according to a method used by Simons *et al.*[5] Oil (2 ml) was carefully measured into several beakers, 23 mm in diameter and 27 mm in height, which had been thoroughly cleaned just prior to use. Care was taken to avoid smearing the oil on the walls of the beakers. The samples were placed in a constant temperature oven at 34.5 ± 0.5°C. At recorded intervals, three (in some experiments two) beakers of each sample of oil were removed and individual determinations of the peroxide values and vitamin A contents were immediately performed.

Metallic ions were removed from the oils in the following way: A chromatographic tube, approximately 400 mm in length and 22 mm in diameter, was used. At its lower end there was a glass filter plate and a stopcock. The tube was filled with a slurry of an ion exchange resin (Amberlite IR 100, analytical quality) in distilled water to give a bed approximately 320 mm deep. The level of the liquid was not allowed at any time to fall below that of the resin. Percolation of 5 % HCl (1 800 ml) through the column over a period of at least 15 min ensured that the resin was in the hydrogen form. The column was washed with successive portions of distilled water (10 ml) until the effluent had a pH of about 4. The column was then prepared for the oil by percolating 100 ml of 2-propanol through it in order to remove water from the resin, followed by 100 ml of a mixture of 85 % 2-propanol and 15 % benzene.

Fifty ml of oil was mixed with 15 ml of benzene and 35 ml of 2-propanol and the mixture percolated through the column. Fifty ml of 30 % benzene in 2-propanol and then pure 2-propanol were added until all the oil had passed through the column. The solvents were removed from the oil-2-propanol-benzene mixture by evaporation under vacuum.

Metal ions were usually incorporated in the oil samples as chlorides dissolved in absolute ethanol. These solutions were added to the oil to give a final concentration of 2 % of the total amount of oil. In two experiments the ions of copper and iron were incorporated in the oils as stearates dissolved in paraffin oil. The solutions were added to give a final concentration of 2 % of the vitamin A oil. Antioxidants were added as solutions in propylene glycol or ground-nut oil and these solutions were always added to give a concentration of 2 % of the total amount of the vitamin A oil. Citric acid was added as a 30 % solution in absolute ethanol. As citric acid was usually added to a total concentration of 0.01 %, the amount of ethanol added in this way seldom exceeded 0.05 %.

RESULTS

The experiments are summarized in Tables 2—20.

In the tables the term "relative stability" means ratio of the time for 50 % destruction of vitamin A in oil containing metal ion or antioxidant, to the time for the same distruction of vitamin A in the same oil containing no metal ion or antioxidant.

Figs. 1—4 are typical diagrams showing peroxide value relative to time, percentage destruction of vitamin A relative to time, and percentage destruction of vitamin A relative to peroxide value.

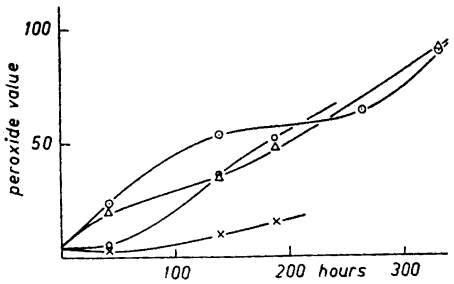


Fig. 1. Increase in peroxide value in oil from which metal ions had been removed and to which various amounts of Cu had been added (as $CuCl_2$). The samples were kept in the dark and exposed to air at 34.5°C for different lengths of time.

⊙ Oil, (metal ions removed)
△ » » » » + 1 ppm Cu
○ » » » » + 10 ppm Cu
× » » » » + 100 ppm Cu

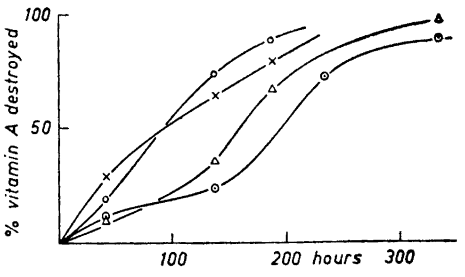


Fig. 2. Destruction of vitamin A in oil from which metal ions had been removed and to which various amounts of Cu had been added (as $CuCl_2$). The samples were kept in the dark and exposed to air at 34.5°C for different lengths of time.

⊙ Oil, (metal ions removed)
△ » » » » + 1 ppm Cu
○ » » » » + 10 ppm Cu
× » » » » + 100 ppm Cu

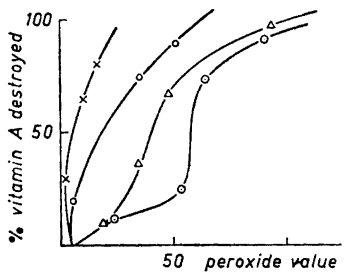


Fig. 3. Destruction of vitamin A relative to peroxide value in oil from which metal ions had been removed and to which various amounts of Cu had been added (as $CuCl_2$). The samples were kept in the dark and exposed to air at 34.5°C for different lengths of time.

⊙ Oil (metal ions removed)
△ » » » » + 1 ppm Cu
○ » » » » + 10 ppm Cu
× » » » » + 100 ppm Cu

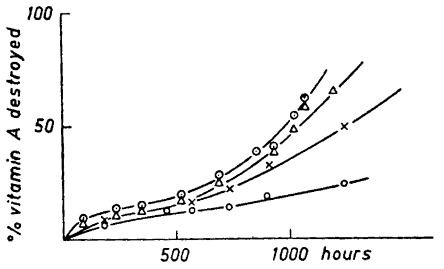


Fig. 4. Destruction of vitamin A in oil with various antioxidants added and with no addition of antioxidant. The samples were kept in the dark and exposed to air at 34.5°C for different lengths of time.

⊙ No antioxidant
△ 0.01 % citric acid
○ 0.01 % DPPD
× 0.01 % citric acid + 0.1 % BHT + 0.02 % lecithin

Not only did the metal ions increase the destruction rate of vitamin A, they also decreased the peroxide value for the oil at a certain percentage of vitamin A destruction. When the metal ions were added as stearates, as in the two experiments with iron and copper, their catalytic effect was not so pronounced as when they were added as chlorides.

Table 2. Effect of $Al^{3}+$

Oil	Concentration of $Al^{3}+$ p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
A	0	440	80	1
A	10	268	61	0.61
A	100	216	30	0.48
B	0	578	41	1
B	1	526	35	0.91
B	10	380	22	0.66
B	100	298	13	0.52
C	0	965	17	1
C	100	379	12	0.38

Aluminium ions markedly increased the rate of vitamin A destruction and this effect was noticeable even at concentrations of 1 p.p.m. (Table 2).

At concentrations of 1 p.p.m. and 10 p.p.m., copper was the strongest accelerator of the destruction of vitamin A of all the metals tested. The addition of 10 p.p.m. and 100 p.p.m. of copper ions caused the same rate of destruction, but the peroxide value for the same percentage of vitamin A destruction was much lower in the oil with 100 p.p.m. of copper (Table 3).

Table 3. Effect of $Cu^{2}+$.

Oil	Concentration of $Cu^{2}+$ p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
P	0	204	59	1
P	1	173	43	0.85
P	10	96	27	0.47
P	100	96	5	0.47
E	0	212	67	1
E	1 *	219	66	1.03
E	10 *	127	50	0.60
F	0	276	81	1
F	10	89	23	0.28
F	100	89	11	0.28
C	0	965	17	1
C	100	145	5	0.15

* The copper ions were added as stearates dissolved in paraffin oil.

Table 4. Effect of Fe^{3+}

Oil	Concentration of Fe^{3+} p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
P	0	204	59	1
P	1	238	60	1.17
P	10	144	43	0.71
P	100	44	23	0.22
G	0	216	71	1
G	6*	195	65	0.90
G	60*	122	54	0.56
F	0	276	81	1
F	10	126	46	0.46
C	0	965	17	1
C	100	87	8	0.09

* The ferric ions were added as stearates dissolved in paraffin oil.

Ferric ions at a concentration of 100 p.p.m. caused more rapid destruction of vitamin A than any concentration of the other metals tested. Iron did not accelerate the breakdown of vitamin A at 1 p.p.m. but increased its detruction at 6 p.p.m. The decrease in peroxide value at 50 % destruction of vitamin A was less pronounced when iron was added to the oil than when copper was added (Table 4).

Nickel ions had a moderately accelerating effect on the destruction of vitamin A. The decrease in peroxide value for 50 % destruction of vitamin A with nickel ions was less than for ions of copper and iron. It was confusing to notice that in oil C, which was found to be extremely sensitive to the

Table 5. Effect of Ni^{2+}

Oil	Concentration of Ni^{2+} p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
H	0	209	57	1
H	1	191	57	0.91
H	10	138	54	0.66
H	100	76	38	0.36
C	0	965	17	1
C	100	610	14	0.63

Table 6. Effect of Sn^{4+}

Oil	Concentration of Sn^{4+} p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
B	0	578	41	1
B	1	546	38	0.95
B	10	526	40	0.91
B	100	386	28	0.66
C	0	965	17	1
C	100	385	9	0.40

presence of all the other metal ions tested, the effect of nickel was comparatively small (Table 5).

The pro-oxidative effects of tin and zinc were weak. The increase in vitamin A destruction and the decrease in peroxide value were hardly detectable when these metal ions were added at concentrations of 1 p.p.m. At 10 p.p.m. these metals had a noticeable effect (Tables 6 and 7).

Table 8 shows that citric acid definitely stabilized vitamin A. In the range 0.1—0.01 %, the effect was practically independent of the concentration. Citric acid was effective in "trapping" copper ions which is shown in Table 12. Ascorbic acid had good antioxidant properties. The addition of 0.01 % of ascorbic acid to one of the tested oils increased the time for 50 % destruction of vitamin A by about 20 %. Ascorbyl palmitate had no effect (Tables 9 and 10).

Citraconic acid is considered to be a powerful antioxidant for margarine [6]. In these experiments, however, citraconic acid had no antioxidant properties

Table 7. Effect of Zn^{2+}

Oil	Concentration of Zn^{+2} p.p.m.	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
A	0	440	80	1
A	100	268	41	0.61
B	0	578	41	1
B	1	532	38	0.92
B	10	552	39	0.96
B	100	476	23	0.82
C	0	965	17	1
C	100	490	13	0.51

Table 8. Effect of citric acid on the stability of vitamin A oil alone and together with other antioxidants

Oil	Citric acid %	Other antioxidants	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
N	0	—	272	58	1
N	0.01	—	309	60	1.14
M	0	—	205	59	1
M	0.01	—	215	63	1.05
M	0	Lecithin 0.1 %	268	53	1.30
M	0.01	Lecithin 0.1 %	271	48	1.32
K	0	—	632	34	1
K	0.05	—	695	33	1.10
K	0.1	—	695	33	1.10
K	0	Ascorbyl palm. 0.05 %	620	36	0.98
K	0.05	Ascorbyl palm. 0.05 %	680	34	1.08
C	0	—	965	17	1
C	0.01	—	1 064	16	1.10

(Tables 11 and 12). Table 12 shows the effects of citric, ascorbic and citraconic acids in an oil in which 10 p.p.m. of copper was incorporated.

Lecithin was found to be a very good antioxidant for stabilizing vitamin A. In the concentration range 0.01—0.1 % the stabilizing effect increased approximately linearly with the concentration. The effect of lecithin was added to the effect of phenolic antioxidants present but, as could be expected, the combined effect of citric acid and lecithin was less than the sum of the effects for each of them (Table 13).

DPPD (NN'-diphenyl-*p*-phenylene diamine) was the most powerful stabilizer of vitamin A of all the antioxidants tested. In the range 0.001—0.01 %,

Table 9. Effect of ascorbic acid on the stability of vitamin A oil alone and together with citric acid

Oil	Ascorbic acid %	Citric acid %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
O	0	0	264	70	1
O	0.01	0	321	73	1.22
L	0	0	247	55	1
L	0.01	0.01	285	54	1.15

Table 10. Effect of ascorbyl palmitate on the stability of vitamin A oil alone and together with citric acid

Oil	Ascorbyl palmitate %	Citric acid %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initally present is destroyed	Relative stability
K	0	0	632	34	1
K	0.01	0	613	36	0.97
K	0.05	0	620	36	0.98
K	0.05	0.05	680	34	1.08
J	0	0	750	38	1
J	0.05	0.05	790	37	1.05

Table 11. Effect of citraconic acid on the stability of vitamin A oil alone and together with citric acid

Oil	Citraconic acid %	Citric acid %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initally present is destroyed	Relative stability
O	0	0	264	70	1
O	0.01	0	254	73	0.99
L	0	0	247	55	1
L	0.1	0.01	268	48	1.09

Table 12. Effect of some acids together with Cu^{2+}

Oil	Antioxidant	Cu^{2+} p.p.m. as stearate in paraffin oil	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
O	—	—	264	70	1
O	Ascorbic acid 0.01 %	—	321	73	1.18
O	» » 0.01 %	10	100	34	0.38
O	Citric acid 0.01 %	—	288	68	1.09
O	» » 0.01 %	10	211	72	0.80
O	Citracon. acid 0.01 %	—	254	73	0.96
O	» » 0.01 %	10	70	17	0.27

Table 13. Effect of different concentrations of lecithin on the stability of vitamin A oil alone and together with other antioxidants

Oil	Lecithin %	Other antioxidant	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destoryed	Relative stability
N	0	—	272	58	1
N	0.1	Citric acid 0.01 %	355	65	1.30
P	0	—	204	59	1
P	0.01	—	241	58	1.18
P	0.03	—	244	58	1.20
P	0.1	—	268	53	1.31
P	0.1	Citric acid 0.01 %	271	48	1.33
M	0	—	205	48	1
M	0.1	—	254	50	1.24
M	0.1	BHA 0.02 %	275	55	1.34
M	0.1	BHA 0.02 %	288	54	1.40
M	0.1	Tiodiph.amine 0.01 %	236	46	1.15
M	0.1	DPPD 0.01 %	370	50	1.80

its effect increased approximately linearly with the logarithm of the concentration (Table 14).

The results from the experiments made with BHA and BHT are summarized in Tables 15, 16, and 17. Table 15 shows that BHA and a mixture of equal amounts of BHA and BHT were inferior to BHT in their ability to stabilize vitamin A.

Table 14. Effect of DPPD on the stability of vitamin A oil alone and together with lecithin

Oil	DPPD %	Lecithin %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
C	0	0	965	17	1
C	0.01	0	1 875	16	1.94
C	0.01	0	2 000	18	2.07
M	0	0	205	48	1
M	0.001	0	222	No test	1.08
M	0.003	0	262	» »	1.28
M	0.01	0	340	38	1.66
M	0.01	0	314	48	1.53
M	0.01	0.1	370	50	1.80
M	0	0.1	254	50	1.24

Table 15. Comparison between BHA and BHT

Oil	Antioxidant mixture	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
M	Lecithin 0.1 % BHA 0.02 % Citric acid 0.01 %	329	49	1.34
M	Lecithin 0.1 % BHA 0.01 % BHT 0.01 % Citric acid 0.01 %	375	49	1.55
M	Lecithin 0.1 % BHT 0.02 % Citric acid 0.01 %	428	50	1.77

In this experiment the temperature in the constant temperature oven fell to room temperature and remained at that level for 24 h. The figures are valid only when compared with each other.

Some experiments were made with propyl gallate, NDGA and tiodiphenylamine. The results are summarized in Tables 18 and 19. NDGA and propyl gallate had distinctly antioxidative effects but tiodiphenylamine had no effect. The latter substance even seemed to be antagonistic to lecithin.

Table 16. Effect of BHT on the stability of vitamin A oil together with lecithin and/or citric acid

Oil	BHT %	Lecithin %	Citric acid %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
M	0	0	0	205	48	1
M	0	0.1	0	254	50	1.24
M	0.02	0.1	0.	288	63	1.40
M	0.02	0.1	0.01	296	65	1.44
M	0.02	0.1	0.01	295	55	1.44
M	0.02	0.1	0.01	362	46	1.77
C	0	0	0	965	17	1
C	0	0	0.01	1 064	16	1.10
C	0.02	0.1	0.01	1 255	17	1.30
N	0	0	0	272	58	1
N	0	0	0.01	314	60	1.14
N	0.02	0	0.01	337	64	1.24
N	0	0.1	0.01	355	65	1.30
N	0.02	0.1	0.01	375	66	1.38

Table 17. Effect of BHA on the stability of vitamin A oil together with lecithin

Oil	BHA %	Lecithin %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
M	0	0	205	48	1
M	0	0.1	254	50	1.24
M	0.02	0.1	275	55	1.34
M	0.02	0.1	288	54	1.40

Table 18. Effect of propyl gallate and NDGA on the stability of vitamin A oil together with citric acid

Oil	Antioxidant	Citric acid	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
L	—	0	247	55	1
L	—	0.01	268	55	1.08
L	Propyl gallate 0.01 %	0.01	294	62	1.19
L	NDGA 0.01 %	0.01	300	56	1.21

Table 19. Effect of tiodiphenyl amine on the stability of vitamin A oil alone and together with lecithin

Oil	Tiodiphenyl amine %	Lecithin %	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
M	0	0	205	48	1
M	0.01	0	202	43	0.99
M	0	0.1	254	50	1.24
M	0.01	0.1	236	46	1.15

Some commercial antioxidant mixtures were also tested. The results from these experiments are summarized in Table 20. A mixture containing BHT, BHA, propyl gallate and lecithin citrate gave the best results. When added at a concentration of 0.05 %, as recommended by the manufacturer, this mixture increased the time for 50 % destruction of vitamin A by 50 %.

Table 20. Effect of some commercial antioxidant mixtures on the stability of vitamin A oil

Oil	Trade name	Conc. = maximum recommended by manufacturer	Time for 50 % destruction of vitamin A, hours	Peroxide number in the oil when 50 % of the vitamin A initially present is destroyed	Relative stability
J	—	—	750	38	1
J	Tenox II	0.05	803	37	1.07
M	—	—	205	48	1
M	Tenox II	0.05	267	51	1.30
J	—	—	750	38	1
J	"Keep It" *Green*	0.05	770	37	1.03
J	"Keep It" *Blue*	0.05	722	35	0.96
M	—	—	205	48	1
M	G4	0.05	270	55	1.32
M	G15	0.05	315	61	1.54
M	G15	0.05	300	46	1.46
M	Sustane F	0.02	263	52	1.28

DISCUSSION

The effect of metal ions on the oxidation of various fats and oils has been studied by Lea [7], Morris *et al.*[8], and Evans *et al.*[9] and others. Lea studied the accelerating effect of metal ions at room temperature in the presence of an aqueous phase while the other investigators used an A.O.M. stability test. Lea found that 1 p.p.m. of Fe and 0.1 p.p.m. of Cu greatly enhanced the oxidation of herring oil. Morris *et al.* found that the same amounts of these metals decreased the stability of lard by 50 %. Evans *et al.* found that 0.3 p.p.m. of Fe greatly accelerated the oxidation of soya-bean oil.

Under the conditions of this investigation the effect of trace metals is less pronounced. To double the rate of destruction of vitamin A, 10 p.p.m. of copper or iron has to be added to the oil when stored in the dark at 34.5°C. Aluminium or nickel must be present at a concentration of about 100 p.p.m. in order to accelerate the destruction to the same extent. As much as 100 p.p.m. of tin or zinc do not usually increase the rate of vitamin A destruction by 100 %. Metal ions seem to have at least two different effects on the oxidation of vitamin A in fish liver oil. One is a direct enhancement of the oxidation of vitamin A. The other is an acceleration of the breaking down of the peroxides formed. This second reaction seems to decrease the rate of oxidation of vitamin A *via* peroxides.

The effects of various antioxidants on the stability of vitamin A have previously been studied by Buxton [10,11], Caspe and Hadjopoules [12], Kring [13], Swain and Idler [14], Kamath and Magar [15], Spruyt [16], and Wodsack [17] and others. Different methods have been tried for testing and determining the vitamin A and peroxide values.

In this investigation, citric acid was found to act as a good stabilizer of vitamin A. It is not considered to be a true antioxidant [6,9], but is effective as an inactivator of trace metals and as a synergist for true antioxidants. As the fish liver oils tested contained detectable amounts of trace metals and as fish liver oils have been shown to contain antioxidants [10], it is understandable that citric acid should have some stabilizing effect.

Antioxidative properties have been reported for ascorbyl palmitate [17] and for citraconic acid [6]. In the experiments with fish liver oils reported here, however, no antioxidative effect could be detected.

Lecithin has a comparatively good stabilizing effect on vitamin A in fish liver oil. This effect is, however, only slightly increased by the addition of citric acid. The phosphorous acid group of lecithin is itself able to inactivate trace metals and therefore no additional effect of citric acid can be expected.

Acknowledgements. The author wishes to express his thanks to Professor H. Lundin, Head of the Division of Food Chemistry, for his valuable advise in the preparation of this paper. Thanks are due to Miss M. Ahrens, Mr. E. Kivisäkk, and Mr. E. T. Okine for technical assistance. The financial support of Kjellbergs Successors AB is greatfully acknowledged.

REFERENCES

1. Dowler, M. W. and Laughland, D. H. *Anal. Chem.* **24** (1952) 1047.
2. Wheeler, D. H. *Oil & Soap* **10** (1922) 89.
3. Täufel, K. and Romminger, K. *Fette Seifen Anstrichmittel* **58** (1956) 104.
4. Hecht, F. and Gottlieb, A. *Mikrochemie ver. Mikrochem. Acta* **35** (1950) 337.
5. Simons, E. J., Buxton, L. O. and Colman, H. B. *Ind. Eng. Chem.* **32** (1940) 706.
6. Hennecke, H. *Fette u. Seifen* **53** (1951) 636.
7. Lea, C. H. *J. Soc. Chem. Ind. London* (1936) 293.
8. Morris, S. G., Myers, J. S., Kip, M. L. and Riemenschneider, R. W. *J. Am. Oil Chemists' Soc.* **27** (1950) 105.
9. Evans, C. D., Cooney, P. M., Moser, H. A. and Schwab, A. W. *J. Am. Oil Chemists' Soc.* **30** (1953) 143.
10. Buxton, L. O. *Ind. Eng. Chem.* **39** (1947) 225.
11. Buxton, L. O. *Ibid* **39** (1947) 1171.
12. Caspe, S. and Hadjopoulos, L. G. *Am. J. Pharm.* **110** (1938) 533.
13. Kring, P. *Dansk Tidskr. Farm.* **24** (1950) 211.
14. Swain, L. A. and Idler, D. R. *Progr. Rep. Pacific Coast Stations* **103** (1955) 11.
15. Kamath, G. G. and Magar, N. G. *J. Indian Chem. Soc.* **30** (1953) 335.
16. Spruyt, J. P. *J. Am. Oil Chemists' Soc.* **32** (1955) 197.
17. Wodsack, W. *Fette u. Seifen* **53** (1951) 73.

Received September 27, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 75—96

Zur Kenntnis der Carbonylgruppen im Lignin. I

ERICH ADLER und JOSEF MARTON

Institutionen för organisk kemi, Chalmers Tekniska Högskola, Göteborg, Schweden

Die Reduktion verschiedener Aldehyde und Ketone der Guajacylpropanreihe mittels Natriumborhydrid in alkalischer Lösung wurde spektrophotometrisch untersucht und die Veränderungen des Absorptionsspektrums der Lösungen beim Übergang der Carbonylverbindungen in die entsprechenden Alkohole wurden in Form der Borhydridreduktions-$\Delta\varepsilon$-Kurven ($\Delta\varepsilon_r$-Kurven) dargestellt.

Die $\Delta\varepsilon_r$-Kurven für Björkman-Lignin und dessen durch Behandlung mit Diazomethan erzeugtes Derivat wurden, ausgehend von den $\Delta\varepsilon_r$-Kurven der Modellsubstanzen, in qualitativer und quantitativer Hinsicht analysiert. Als Ergebnis wurden folgende Werte für die Anzahl von carbonylhaltigen Einheiten per OCH_3-Gruppe im Björkman-Lignin gefunden: <0,01 phenolische Coniferylaldehydeinheiten (Typ I), 0,03 verätherte Coniferylaldehydeinheiten (Typ II), 0,005—0,01 phenolische 4-Ketoguajakolgruppen (Typ III) und 0,05—0,06 verätherte 4-Ketoguajakolgruppen (Typ IV). Neben diesen konjugierten Carbonylgruppen (insgesamt 0,09—0,11/OCH_3) dürften etwa ebensoviele isolierte Ketogruppen (in der β-Stellung der Propanseitenkette) vorkommen.

Durch Oximierung von Björkman-Lignin (= "Milled wood lignin", M.W.L.)[1,2] aus Fichte mit Hydroxylaminhydrochlorid und potentiometrische Titrierung der freigelegten Salzsäure wurde gefunden, dass dieses Lignin auf etwa jede fünfte Guajacylpropaneinheit eine Carbonylgruppe enthält (0,18—0,21 CO/OCH_3)[3,4]. Auch wenn die Feinmahlung des Holzes vor der Extraktion des Lignins in Stickstoffatmosphäre vorgenommen wurde [2], liessen sich die Carbonylgruppen nachweisen, woraus hervorgehen dürfte, dass die lezteren tatsächlich dem nativen Lignin angehören und nicht erst während des Isolierungsprozesses (oxydativ) gebildet werden.

Für die nähere Kenntnis der Biosynthese und der Struktur des Lignins wäre es von Bedeutung, Aufschluss über die Lokalisierung und Verteilung der Carbonylgruppen im Ligninmolekül zu gewinnen. Qualitative Hinweise liefert das Infrarotspektrum. Im IR-Spektrum des Björkman-Lignins (Fig. 1) zeichnen sich zwei für Carbonyle typische Bandengruppen ab. Die längerwellige (1 690—1 660 cm^{-1}, Schwerpunkt bei 1 675 cm^{-1}) kann die konjugierten Aldehydgruppen der phenolischen and verätherten Coniferylaldehydeinheiten (I, II) sowie Arylketone (III, IV) umfassen. Die kürzerwellige und schwächere

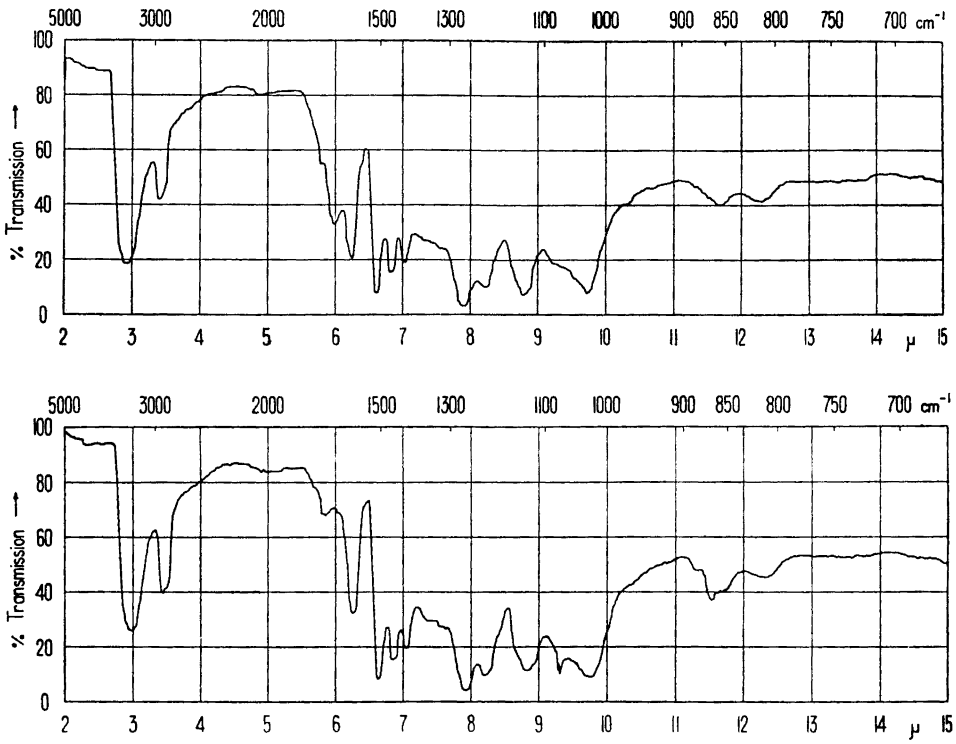
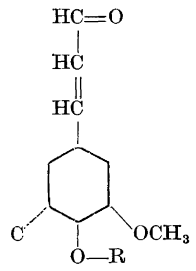


Fig. 1. Oben: IR-Spektrum von Björkman-Lignin L II *(Picea abies). Unten:* IR-Spektrum eines mit $NaBH_4$ reduzierten Björkman-Lignins.

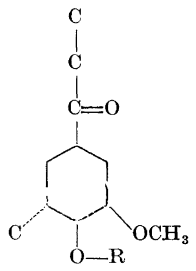
Absorption (1 725—1 710 cm^{-1}) lässt isolierte, d. h. in der Mitte der Propanseitenkette stehende Ketogruppen (V, VI) erkennen. Nichtkonjugierte Aldehydgruppen, die bei 1 740—1 720 cm^{-1} absorbieren sollten, können nicht streng ausgeschlossen werden. War die Feinmahlung des Holzes unter Stickstoff ausgeführt worden, so zeigte daraus isoliertes Björkman-Lignin keine merkbare Absorption für nichtkonjugierte Aldehydgruppen. Eine Zusammenstellung der C=O-Streckungsfrequenzen für eine Reihe von Modellsubstanzen findet sich in Tab. 1. (S. 86).

Aus Tab. 1 ist zu entnehmen, dass die für Arylketone normalerweise bei 1 700—1 680 cm^{-1} liegende Carbonylbande in einigen Fällen nach niedrigerer Frequenz verschoben ist. Dies gilt besonders für die Oxyketone XI, XX und XXa, mit Frequenzen von 1 668—1 665 cm^{-1}, und für die Diketone XIV und XV (1 665—1 660 cm^{-1}). Die Banden solcher α-Ketone würden also mit denen der Coniferylaldehydgruppen zusammenfallen.

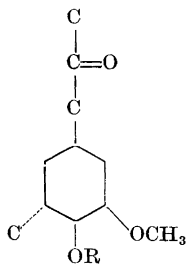
Im Spektrum eines mit Natriumborhydrid reduzierten Björkman-Lignins [5] (Fig. 1) fehlt die längerwellige Bandengruppe. Die Intensität der kürzerwelligen Bandengruppe ist herabgesetzt; die restliche Absorption (bei 1 725—1 700 cm^{-1}) ist vielleicht auf Spuren von Carboxylgruppen zurückzuführen.



I : R = H
II : R = C-Atom einer Seitenkette



III : R = H
IV : R = C-Atom einer Seitenkette



V : R = H
VI : R = C-Atom einer Seitenkette

Für den qualitativen und quantitativen Nachweis der im IR-Spektrum erkennbaren ungesättigten Aldehydgruppen vom Coniferylaldehydtyp (I und II) stehen mehrere schon bekannte Methoden zur Verfügung. Björkman-Lignin zeigt die Wiesner'sche Reaktion (Rotfärbung mit Phloroglucin-Salzsäure) sowie Gelbfärbung mit aromatischen Aminen, z. B. *p*-Aminobenzoesäure, Reaktionen, die für Gruppen vom Typus I und II charakteristisch sind [6,7]. Der kolorimetrische Vergleich der durch *p*-Aminobenzoesäure hervorgerufenen Färbung des Björkman-Lignins (λ_{max} in 60 %-iger Essigsäure bei 445 mμ) mit den entsprechenden Farbstoffen aus Coniferylaldehyd oder dessen Äthern deutet darauf hin, dass auf etwa 35 Guajacylpropaneinheiten im Lignin eine Coniferylaldehydgruppe (I + II) trifft (etwa 0,03 Gruppen I + II per OCH_3)[4]. Derselbe Wert wurde für Brauns-Lignin erhalten, und Aulin-Erdtman und Hegbom [8] geben auf Grund von Messungen der spektralen Veränderungen bei Ionisierung, Sulfonierung und alkalischer Desulfonierung ($\Delta\varepsilon$-Methoden) von Brauns-Lignin *(Picea abies)* die Werte für I und II zu 0,01 bzw. 0,02 per OCH_3 an.

Die Aldehydgruppen in Einheiten vom Coniferylaldehydtyp (I und II) machen somit nur einen kleinen Teil des Gesamtcarbonyls aus. Auch der Anteil von 4-Ketoguajakolgruppen (mit freiem Phenolhydroxyl) (Typ III) scheint sehr begrenzt zu sein. Solche Gruppierungen verursachen beim Übergang von neutraler zu alkalischer Lösung eine Extinktionserhöhung im Gebiet um 350 mμ, und aus den Ionisierungs-$\Delta\varepsilon$-Werten für diesen Bereich hat Aulin-Erdtman [8] auf das Vorkommen von 0,02—0,03 Gruppen III per OCH_3 (in Brauns-Lignin aus *Picea abies*) geschlossen. Björkman-Lignin gibt bei 350 mμ ungefähr dieselben Ionisierungs-$\Delta\varepsilon$-Werte [2]. Aulin-Erdtman [8] hat schliesslich auch versucht, die Menge von verätherten 4-Ketoguajakolgruppen (Typ IV) zu schätzen und gibt für Brauns-Lignine aus *Picea mariana* und *Tsuga heterophylla* Werte von 0,02 bzw. 0—0,01 Gruppen IV per OCH_3 an.

Wir haben nun die Frage der differenzierten Bestimmung der verschiedenen Typen von Carbonylgruppen, besonders im Björkman-Lignin, mit Hilfe einer neuen Methode angegriffen. Die Methode schliesst sich an Beobachtungen an, die kürzlich von D. C. C. Smith [9] mitgeteilt wurden. Smith fand, dass *p*-Oxyaryl-aldehyde und -ketone sowie Coniferylaldehyd von Natriumborhydrid in 0,01 N NaOH langsam reduziert werden, während die Reduktion

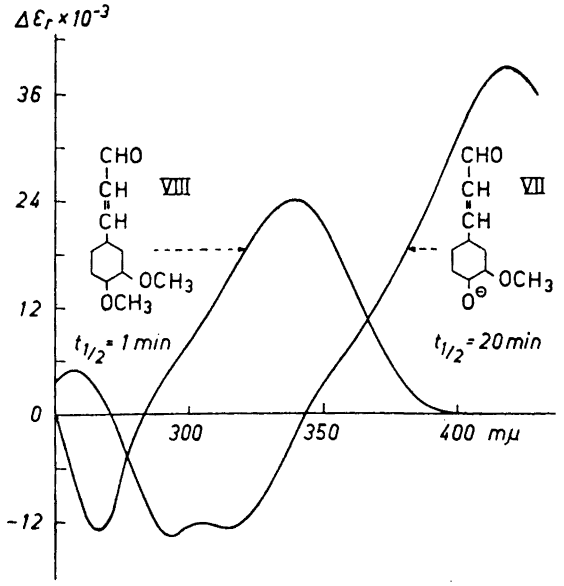


Fig. 2. $\Delta\varepsilon_r$-Kurven für Coniferylaldehyd (VII) und Methylconiferylaldehyd (VIII) nach 100 min Reduktionsdauer (Versuchsbedingungen, siehe Experimenteller Teil).

entsprechender Carbonylverbindungen mit verätherten Phenolhydroxyl rasch verläuft. Reduktion von vier verschiedenen Brauns-Ligninen verursachte einen Abfall der Extinktion der Lösungen bei 350 mμ; eine rasch verlaufende Anfangsphase wurde der Reduktion von verätherten Coniferylaldehydeinheiten (Typ II), eine folgende langsame Phase der Reduktion von 4-Ketoguajakolgruppen (III) zugeschrieben.

Es war nun zu erwarten, dass ein genauerer Vergleich der spektralen Veränderungen bei der Borhydridreduktion von Lignin bzw. von geeigneten Modellsubstanzen in alkalischer Lösung eine quantitative Bestimmung der carbonylhaltigen Gruppierungen I—IV erlauben würde, da diese als konjugierte Chromophore in spezifischer Weise zur Ultraviolettabsorption des Ligninmoleküls beitragen. Eine direkte Erfassung der Gruppierungen V und VI mit nicht-konjugierter Ketogruppe erschien weniger aussichtsreich. Wie weiter unten gezeigt wird, gaben jedoch auch Modellsubstanzen vom Typ V und VI bei der Borhydridreduktion spektrale Änderungen, die bei der Deutung der mit Lignin erhaltenen Befunde berücksichtigt werden mussten.

Dem Vergleich zwischen Lignin und Modellverbindungen wurden die Differenzkurven zugrundelegt, die durch Subtraktion der ε-Werte der reduzierten Lösung von den ε-Werten der (alkalischen) Lösung ohne Borhydrid konstruiert wurden. (Die so erhaltenen "Borhydridreduktions-$\Delta\varepsilon$-Kurven" werden im folgenden kurz als "$\Delta\varepsilon_r$-Kurven" bezeichnet.) Durch diese Anwendung der von Aulin-Erdtman [10] eingeführten $\Delta\varepsilon$-Methode auf die Borhydridreduktion des Lignins werden die konjugierten Carbonylsysteme (I—IV) von übrigen Chromophoren gesondert sichtbar und der Auswertung zugänglich gemacht.

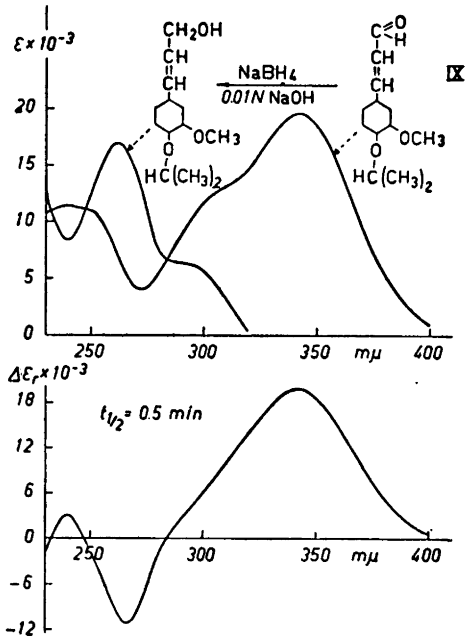


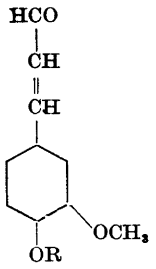
Fig. 3. Oben: Absorptionskurven von *Iso*propylconiferylaldehyd (IX) und dessen Borhydridreduktionsprodukt (50 min Reduktionsdauer). *Unten:* $\Delta\varepsilon_r$-Kurve.

Borhydridreduktion von Modell-Carbonylverbindungen

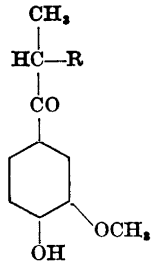
Coniferylaldehyd (VII) — als Vertreter des Strukturtyps I — zeigt in alkalischer Lösung eine intensive Absorption mit einem Maximum bei 418 mμ (ε_{max} = 39 000) (vgl. auch [11]). Bei Zusatz von überschüssigem Natriumborhydrid verschwand das langwellige Absorptionsband und es bildeten sich neue Maxima bei 290 und 315 mμ aus, die dem Übergang in Coniferylalkohol (vgl.[12]) entsprechen. Die Absorptionsänderung war nach 100 Min. fast beendet, und die Halbwertzeit ($t_{½}$) für den Extinktionsabfall bei 420 mμ war 20 Min. Subtraktion der Extinktionswerte der reduzierten Lösung von den Ausgangswerten lieferte die in Fig. 2 angegebene $\Delta\varepsilon_r$-Kurve.

In Tab. 1 sind die langwelligen Maxima der $\Delta\varepsilon_r$-Kurven für die untersuchten Modelle zusammengestellt. Dieselbe Tabelle gibt auch Aufschluss über die Geschwindigkeit der Borhydridreduktion (Halbwertzeiten), ermittelt aus dem zeitlichen Verlauf des Extinktionsabfalls im Absorptionsmaximum.

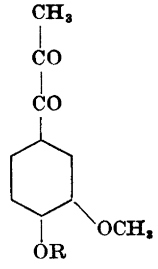
Coniferylaldehyd-methyläther (VIII) und der entsprechende *Iso*propyläther (IX) — als Vertreter des Strukturtyps II — haben im Gegensatz zu dem ionisierbaren Coniferylaldehyd in alkalischer Lösung dasselbe Absorptionsspektrum wie in neutraler Lösung ($\lambda_{max.}$ 340 bzw. 342 mμ) und die sehr rasch verlaufende $NaBH_4$-Reduktion liefert das Spektrum der entsprechenden ver-



VII : R = H
VIII : R = CH_3
IX : R = $CH(CH_3)_2$

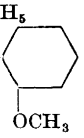


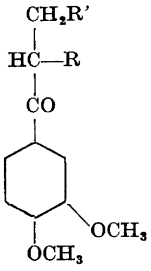
X : R = H
XI : R = OH
XII : R = OC_2H_5



XIV : R = H
XV : R = CH_3

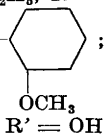
XIII[13] : R =





XVI : R = R' = H
XVII : R = OH; R' = H
XVIII : R = OC_2H_5; R' = H

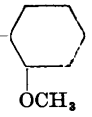
XIX[13] : R =



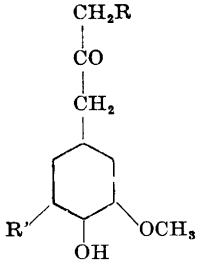
; R' = H

XX : R = H; R' = OH

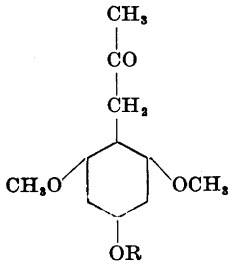
XXI : R =



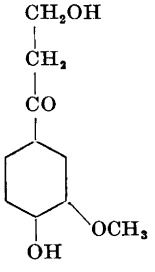
; R' = OH



XXII : R = R' = H
XXIII : OCH_3 statt OH; R = R' = H
XXIV : R = H, R' = OCH_3
XXV : R = OH; R' = H



XXVI : R = H
XXVII : R = CH_3



XX a

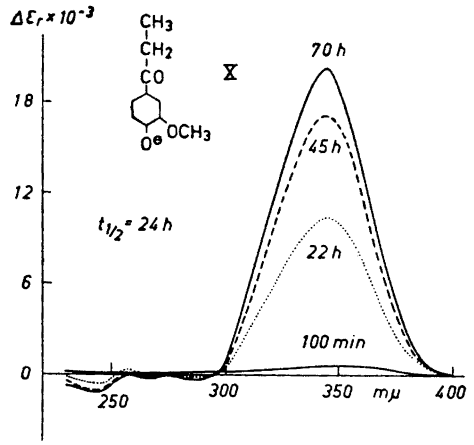


Fig. 4. $\Delta\varepsilon_r$-Kurven für 4-Propioguajakon (X) nach verschiedenen Reduktionszeiten.

ätherten Coniferylalkohole. Die $\Delta\varepsilon_r$-Kurve für VIII ist ebenfalls in Fig. 2 wiedergegeben; für IX sind in Fig. 3 als Beispiel für die Konstruktion der $\Delta\varepsilon_r$-Kurven sowohl die Absorptionskurven vor und nach der Reduktion sowie die Differenzkurve dargestellt.

Als Modelle für den phenolischen 4-Ketoguajakoltyp (III) wurden die Verbindungen X—XIII und als Sonderfall das Diketon XIV untersucht. Die Hauptmaxima der Absorptionsspektren für die ionisierten Phenolketone X—XIII (in 0,01 N NaOH) liegen zwischen 345 und 356 mμ, und da die ent-

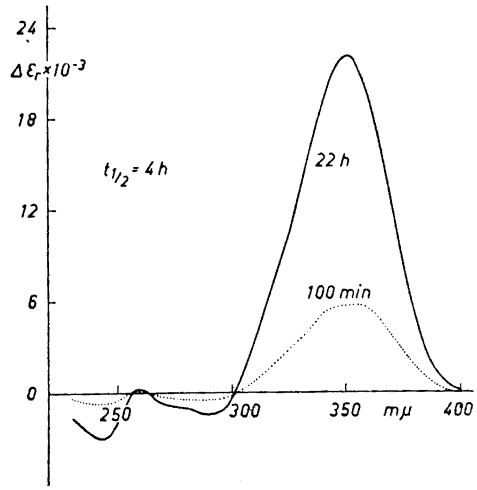


Fig. 5. $\Delta\varepsilon_r$-Kurven für α-Oxy-propioguajakon (XI) nach verschiedenen Reduktionszeiten.

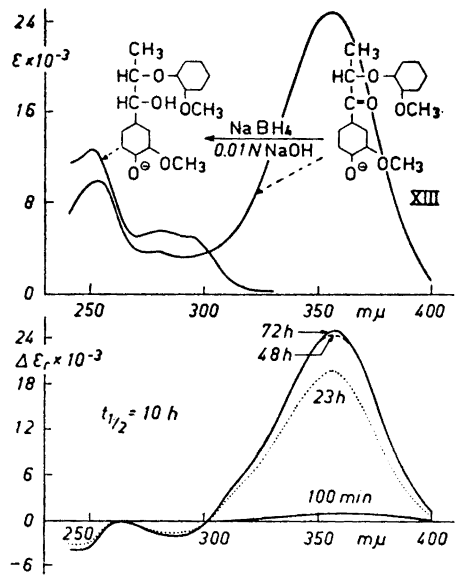


Fig. 6. Oben: Absorptionskurven von α-Guajakoxy-propioguajakon (XIII) und dessen Borhydridreduktionsprodukt (72 h Reduktionsdauer). *Unten:* $\Delta\varepsilon_r$-Kurven nach verschiedenen Reduktionszeiten.

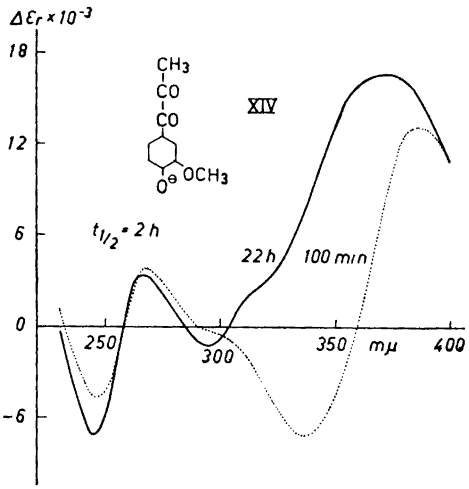


Fig. 7. $\Delta\varepsilon_r$-Kurven für Vanilloylmethylketon (XIV) nach verschiedenen Reduktionszeiten.

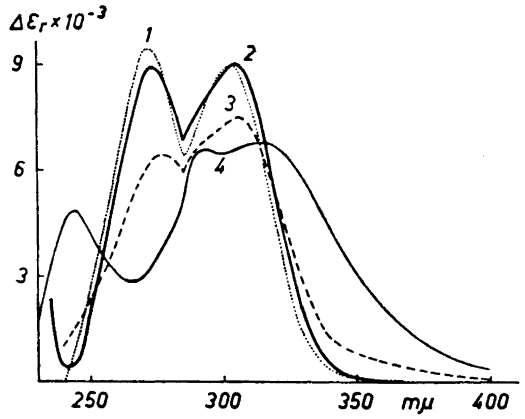


Fig. 8. $\Delta\varepsilon_r$-Kurven für *1)* 4-Propioveratron (XVI); *2)* β-Oxy-propioveratron (XX); *3)* α-Oxy-propioveratron (XVII); *4)* Veratroylmethylketon (XV), nach 100 min Reduktionsdauer.

sprechenden Phenolalkohole auch in alkalischer Lösung bei diesen Wellenlängen nicht mehr absorbieren, erhalten die $\Delta\varepsilon_r$-Kurven die Maxima und die Intensitäten der langwelligen Banden der ionisierten Phenolketone. Die Reduktion der Phenolketone verläuft sehr langsam. Nach 100 Min. Reduktionsdauer in 0,01 N NaOH war bei X und XIII der Extinktionsabfall nur sehr geringfügig, und erst nach etwa 70 Stunden hatte die Reaktion ihr Ende erreicht. Vanilloylmethylketon (XIV) unterliegt einer stufenweisen Reduktion, indem zunächst die β-ständige Carbonylgruppe sehr rasch reduziert wird, wobei sich das Ketol XI bildet, das langsam weiterreagiert.

Die $\Delta\varepsilon_r$-Kurven für die Ketone X, XI, XIII und XIV sind in den Fig. 4—7 wiedergegeben. (Langwellige Maxima der $\Delta\varepsilon_r$-Kurven und Halbwertzeiten für X—XIV, vgl. Tab. 1.)

Die Substanzgruppe XVI—XXI umfasst Vertreter des verätherten 4-Ketoguajakol-Typs (IV), zu dem auch das verätherte Diketon XV zu rechnen ist. Die $\Delta\varepsilon_r$-Kurven für Propioveratron (XVI), α-Oxy-propioveratron (XVII), β-Oxy-propioveratron (XX) und Veratroylmethylketon (XV) sind in Fig. 8 dargestellt. Für α-Guajakoxy-β-oxy-propioveratron (XXI) sind in Fig. 9 teils die Absorptionskurven der alkalischen Lösung vor und nach der Reduktion, teils die daraus konstruierte $\Delta\varepsilon_r$-Kurve wiedergegeben. (Vgl. auch Tab. 1.) Noch stärker ausgeprägt als in der Gruppe der Coniferylaldehyde verläuft bei den α-Ketonen die Reduktion relativ sehr viel rascher, wenn die phenolische Hydroxylgruppe verätherт ist (Tab. 1).

Überraschenderweise unterschied sich die Absorption der alkalischen Lösung des Ketols XXI (Fig. 9), obwohl es keine freie Phenolhydroxylgruppe enthält, von derjenigen seiner neutralen Lösung. In 0,1 N NaOH (in 50 proz. Äthanol) waren die Extinktionswerte (ε), verglichen mit der neutralen Lösung, bei 247 mμ um einen Betrag von 950, bei 340 mμ um 900 erhöht, während sie bei 280 mμ um 400, bei 310 mμ um 200 vermindert waren. Die entsprechenden Extinktionsänderungen in 1 N NaOH waren +3 000,

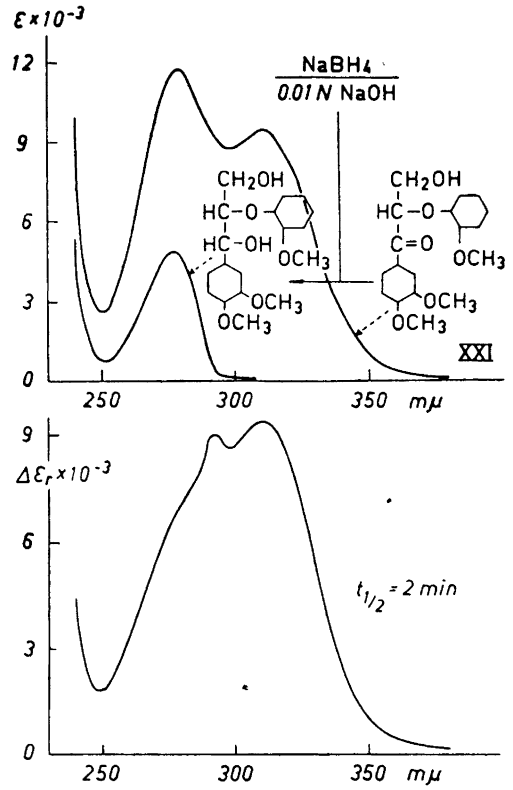


Fig. 9. Oben: Absorptionskurven von α-Guajakoxy-β-oxy-propioveratron (XXI) und dessen Borhydridreduktionsprodukt (50 min Reduktionsdauer). *Unten:* $\Delta\varepsilon_r$-Kurve.

+1 800, −1 300 und −440. Diese Alkaliverschiebung erinnert an die bei der Ionisierung von konjugierten Phenolen auftretende — jedoch sind die $\Delta\varepsilon$-Werte dort viel höher (vgl.[10]) —, und es lag daher der Verdacht nahe, dass das verwendete Präparat Spuren des entsprechenden Phenols als Verunreinigung enthielt. Die Alkaliverschiebung des Spektrums blieb jedoch nach Reinigung der Substanz (Ausschütteln ihrer ätherischen Lösung mit verdünnter Lauge) erhalten. Bei einigen anderen verätherten α-Ketonen, z. B. α-Oxypropioveratron (XVII), und weniger ausgeprägt bei den Ketonen XVIII und XIX, wurde ein ähnlicher Effekt beobachtet. Möglicherweise handelt es sich hier um eine Enolisierung.

Die Substanzen XXII, XXIV, XXV und XXVI [14] wurden als Modelle für die β-Ketongruppierung mit phenolischer Hydroxylgruppe (Strukturtyp V) untersucht. Obwohl bei diesen Verbindungen die Reduktion des Ketons zum sekundären Alkohol nicht mit der Aufhebung einer Konjugation verknüpft ist, bedingt sie doch eine gewisse Änderung des Absorptionsspektrums. Die β-Ketogruppe steht über das α-Kohlenstoffatom mit dem aromatischen Kern in Beziehung (vgl.[15]). In neutraler Lösung ist ihr Einfluss allerdings so gering,

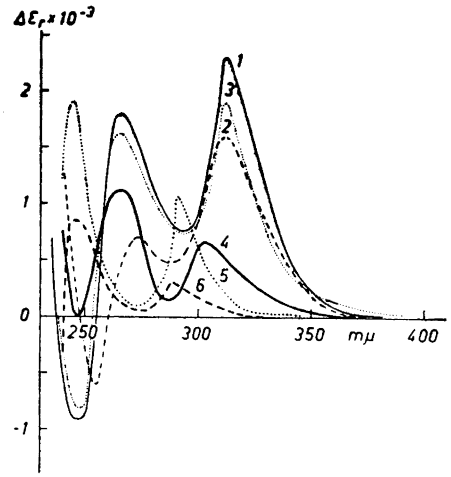


Fig. 10. $\Delta\varepsilon_r$-Kurven für *1)* Guajacylaceton (XXII); *2)* Syringylaceton (XXIV); *3)* ω-Oxyguajacylaceton (XXV); *4)* 4-Oxy-2,6-dimethoxyphenylaceton (XXVI); *5)* Veratrylaceton (XXIII); *6)* 2,4,6-Trimethoxyphenylaceton (XXVII), nach 100 min Reduktionsdauer.

dass die Absorption der β-Ketone derjenigen von unkonjugierten Guajacylpropanen weitgehend ähnlich ist. In alkalischer Lösung dürfte jedoch infolge der erhöhten Polarisation der Ketogruppe deren Einfluss auf die chromophoren Eigenschaften der Gesamtstruktur deutlicher werden; auch an eine schwache Enolisierung, die dem Auftreten einer *Iso*eugenolstruktur entspräche, könnte man denken. Die Abwesenheit solcher Effekte im entsprechenden sekundären Alkohol dürfte die Absorptionsänderung erklären. Die $\Delta\varepsilon_r$-Kurven von Guajacylaceton (XXII), Syringylaceton (XXIV) und ω-Oxyguajacylaceton (XXV) sind einander sehr ähnlich (Fig. 10); ihre Intensitäten sind aber ungefähr zehnmal kleiner als die der phenolischen α-Ketone (Typ III). Das Phloroglucinderivat XXVI schliesst sich den übrigen Acetonverbindungen gut an. (Siehe auch Tab. 1.)

ω-Oxyguajacylaceton (XXV), das im allgemeinen als "β-Oxyconiferylalkohol" (vgl. z. B.[16]), d. h. als die Enolform von XXV, bezeichnet wird, zeigt im IR-Spektrum (in fester Phase) eine normale C=O-Bande (1 718 cm^{-1}) und in neutraler Lösung (95 % Äthylalkohol) das UV-Spektrum einer unkonjugierten Guajakolverbindung, besitzt also unter solchen Bedingungen die Ketonstruktur (XXV). In alkalischer Lösung wird es ziemlich rasch oxydativ verändert, was sich in einer fortschreitenden Erhöhung der Extinktion im Bereich um 350 mμ zu erkennen gibt. Die Bestimmung der Absorptionskurven des Ketols XXV — sowie der übrigen phenolischen β-Ketone — erforderte deshalb besondere Vorsichtsmassnahmen (N_2-Atmosphäre).

Veratrylaceton (XXIII) und 2,4,6-Trimethoxyphenylaceton (XXVII)[14] dienten schliesslich als Modelle für die verätherte β-Ketongruppierung (Typ VI). Der Unterschied zwischen der Absorption dieser Ketone und derjenigen der entsprechenden Alkohole ist, besonders im Bereich höherer Wellenlänge, sehr geringfügig (Fig. 10, Tab. 1).

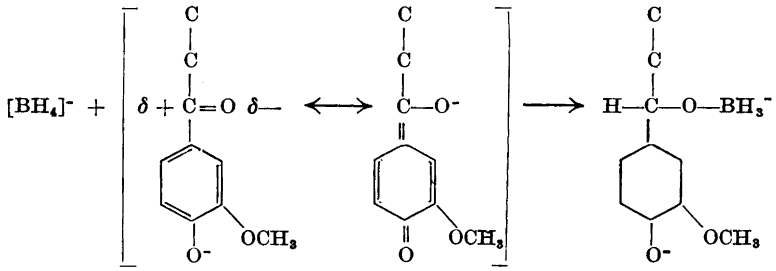
Tabelle 1. Modellsubstanzen: Frequenzen der C=O-Streckungsschwingungen, langwellige Maxima der Borhydridreduktions-$\Delta\varepsilon$-Kurven und Halbwertzeiten der Borhydridreduktion.

Substanz	C=O Streckungsschwingung im IR-Spektrum [a] cm^{-1}	$\Delta\varepsilon_r$-Maximum nach Reduktionsdauer [b] von	λ in mμ	$\Delta\varepsilon_r$	Halbwertzeit ($t_{1/2}$) für die Borhydridreduktion [b] min	Halbwertzeit ($t_{1/2}$) für die Borhydridreduktion [b] h
Typ I:						
Coniferylaldehyd (VII)	1 668	100 min [c]	418	39 000	20	
Typ II:						
Methylconiferylaldehyd (VIII)	1 670	100 »	340	24 000	1	
*Iso*propylconiferylaldehyd (IX)	1 677	100 »	342	19 800	0,5	
Typ III:						
4-Propioguajakon (X)	1 675	100 »	358	700		
		22 h	345	10 500		
		45 h	345	17 200		24
		70 h	345	20 400		
α-Oxypropioguajakon (XI)	1 668	100 min	355	5 800		
		22 h	352	22 200		4
α-Äthoxypropioguajakon (XII)	1 692	100 min	354	1 300		
		22 h	354	12 000		18
		70 h	354	20 200		
α-Guajakoxypropioguajakon (XIII)	1 684	100 min	365	1 200		
		22 h	356	19 300		10
		45 h	356	24 300		
		70 h	356	24 600		
β-Oxypropioguajakon (XXa)	1 666					
Vanilloylmethylketon (XIV)	1 710 / 1 662	100 min	388	13 000		
		22 h	372	16 600		2
Typ IV:						
4-Propioveratron (XVI)	1 681	100 min	303	8 900	18	
α-Oxypropioveratron (XVII)	1 680—1 690	100 »	307	7 500	2,5	
α-Äthoxypropioveratron (XVIII)	1 689	100 »	308	8 900	8	
α-Guajakoxypropioveratron (XIX)	1 692	100 »	309	9 500	3	
β-Oxypropioveratron (XX)	1 665	100 »	305	9 000	13	
α-Guajakoxy-β-oxypropioveratron (XXI)	1 690	100 »	310	9 400	2	
Veratroylmethylketon (XV)	1 710 / 1 665—1 660	100 »	314	6 750	2,5	

Typ V:						
Guajacylaceton (XXII)	1 715	100	min	312	2 300	8
Syringylaceton (XXIV)	1 705	100	»	312	1 600	8
ω-Oxy-guajacylaceton (XXV)	1 718	100	»	312	1 900	1
4-Oxy-2,6-dimethoxy-phenylaceton (XXVI)		100	»	303	660	ca. 50
Typ VI:						
Veratrylaceton (XXIII)	1 715	100	min	290	1 060	2,5
2,4,6-Trimethoxyphenyl-aceton (XXVII)		100	»	288	300	ca. 45

a Feste Substanzen wurden in KBr-Tabletten, Öle zwischen NaCl-Platten gemessen.
b Versuchsbedingungen, siehe Experimenteller Teil.
c Wenn andere Zeitangaben fehlen, war die Reduktion nach 100 min vollständig.

Die Abhängigkeit der Reduktionsgeschwindigkeit von der Struktur der Carbonylverbindung (vgl. Tab. 1) ist vereinbar mit der Annahme eines nucleophilen Angriffs durch das $[BH_4]^-$-Ion oder das $H:^-$-Ion auf das Kohlenstoffatom der Carbonylgruppe; die Reduktion verläuft umso schneller, je stärker positiv dieses C-Atom ist. Bei den sehr langsam reagierenden phenolischen α-Ketonen (Typ III) ist durch die Resonanz des Phenolatketon-anions der positive Charakter des Carbonyl-C-Atoms stark abgeschwächt; bei den entsprechenden Äthern ist die Resonanz zwischen dem substituierten Kern und der Ketogruppe weniger ausgeprägt und sie reagieren deshalb rascher.



Ähnliches gilt für Coniferylaldehyd (Typ I) bzw. dessen Äther (Typ II).

Elektronenanziehende Substituenten an dem der Carbonylgruppe benachbarten C-Atom wirken positivierend und erhöhen die Reduktionsgeschwindigkeit sowohl in der Reihe der phenolischen wie der verätherten 4-Ketoguajakole (OH > Guajacyl > OC_2H_5); ein OH-Substituent am ω-C-Atom wirkt schwächer beschleunigend (vgl. XX resp. XVII mit XVI). Andrerseits setzen elektronenabstossende (negativierende) Substituenten an der Carbonylgruppe die Reduktionsgeschwindigkeit herab, wie aus folgenden Beispielen hervorgeht:

	Halbwertzeit in 0,01 N NaOH
Vanillin	127 Min.
Propioguajakon (X)	24 Std.
Veratrumaldehyd	2 Min.
Propioveratron (XVI)	18 Min.

Erhöhung der Alkalikonzentration im Reaktionsgemisch der Borhydridreduktion (von der in der vorliegenden Arbeit angewandten 0,01 N NaOH auf 0,1 N NaOH) wirkte stark verlangsamend sowohl auf die Reduktion der phenolischen wie der verätherten

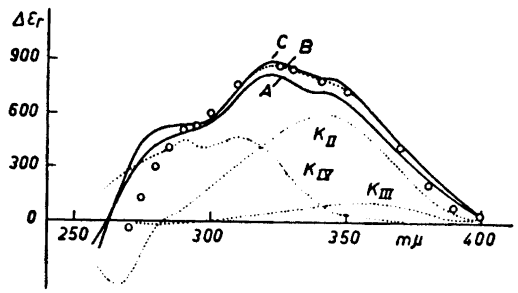


Fig. 11. $\Delta\varepsilon_r$-Kurven von Björkman-Lignin L I (vgl. Text), nach 50 min (A), 22 h (B) und 46 h (C) Reduktionsdauer, sowie berechnete $\Delta\varepsilon_r$-Kurven für 0,03 Mol/l *Iso*propyl-coniferylaldehyd IX (K_{II}), 0,005 Mol/l α-Guajakoxy-propioguajakon XIII (K_{III}) und 0,05 Mol/l α-Guajakoxy-β-oxy-propioguajakon XXI (K_{IV}). Addition der $\Delta\varepsilon_r$-Werte von K_{II}, K_{III} und K_{IV} ergibt die mit O bezeichneten Werte. Versuchsbedingungen, siehe Experimenteller Teil.

α-Ketone. Die in Tab. 1 angegebenen Halbwertzeiten wurden mindestens auf das 2—4-fache erhöht. (Propioguajakon X lässt sich durch mehrtägige Einwirkung von Borhydrid in 0,01 N NaOH präparativ sehr gut in Guajacyl-äthyl-carbinol überführen; in 0,1 N NaOH dagegen ist die Methode praktisch kaum mehr anwendbar.) Auch dieser Effekt scheint sich in die oben dargestellten Zusammenhänge einzufügen; bezüglich des Alkalieinflusses auf die Reduktion der verätherten α-Ketone sei auch auf die durch steigende Alkalikonzentration hervorgerufene successive Änderung der UV-Absorption des Ketons XXI hingewiesen (Enolisierung? vgl. S. 83—84).

Borhydridreduktion von Björkman-Lignin

Die Borhydridreduktion wurde an zwei unbehandelten Präparaten aus *Picea abies*, L I (14,84 % OCH_3) und L II (15,23 % OCH_3), sowie an einem mit Diazomethan methylierten [17] Präparat, DL III (19,41 % OCH_3), spektrophotometrisch verfolgt. Präparat L I entstammte einem Holzmehl, dessen Feinmahlung in der Schwingmühle unter Stickstoff vorgenommen worden war [2].

Die Einwirkung von Borhydrid auf das in 0,01 N NaOH (Lösungsmittel: Monomethylglykol-Äthanol-Wasser 4:6:5) gelöste Björkman-Lignin L I verursachte einen raschen Abfall der Extinktion im Bereich von 270—400 mμ. Nach 50 Min. Reduktionsdauer hatte die $\Delta\varepsilon_r$-Kurve das in Fig. 11, Kurve A, angegebene Aussehen. Die Reduktion verlief danach so langsam weiter, dass eine nach 100 Min. ausgemessene Kurve sich von Kurve A kaum unterscheiden liess. Nach 22 Stunden dagegen waren die $\Delta\varepsilon_r$-Werte deutlich erhöht (Kurve B), und nach 46 Stunden (Kurve C) war die Reaktion offenbar zum Stillstand gekommen.

Auf Grund der Modellversuche sollte Kurve A die (rasch verlaufende) Reduktion von verätherten Coniferylaldehydgruppen (Typ II) sowie von verätherten 4-Ketoguajakolgruppen (Typ IV) wiederspiegeln. Die Schulter bei etwa 342 mμ stimmt mit den Maxima der $\Delta\varepsilon_r$-Kurven der verätherten Coniferylaldehydmodelle (Fig. 2, 3) überein. Das Maximum bei 320—322 mμ ist

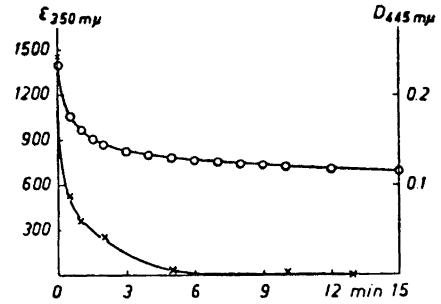


Fig. 12. Borhydridreduktion von Coniferylaldehydgruppen in Björkman-Lignin L II: Abfall der Extinktion bei 350 mμ (Kurve O und linke Ordinate). Farbreaktion mit *p*-Aminobenzoesäure, gemessen bei 445 mμ (Kurve × und rechte Ordinate). Versuchsbedingungen, siehe Exp. Teil.

gegenüber dem für das $\Delta\varepsilon_r$-Maximum der Ketone vom Typ IV erwarteten (305—310 mμ, vgl. Fig. 8, 9) nach Rot verschoben; wie weiter unten gezeigt wird, kann dies auf Überlagerung der $\Delta\varepsilon_r$-Kurven von Typ II und Typ IV zurückgeführt werden. Nach 50—100 Min. langer Reduktion müssten auch bereits phenolische Coniferylaldehydgruppen (Typ I) in der $\Delta\varepsilon_r$-Kurve sichtbar werden. Sie würden verhältnismässig hohe $\Delta\varepsilon_r$-Werte am langwelligen Ende der Kurve verursachen. Da aber die $\Delta\varepsilon_r$-Werte in diesem Gebiete sehr niedrig sind, können Coniferylaldehydgruppen mit freiem Phenolhydroxyl nur in Spuren im Präparat L I vorhanden sein. Aus demselben Grunde kann auch die Anwesenheit von Vanilloylmethylketongruppierungen (XIV) (vgl. Fig. 7) ausgeschlossen werden.

Der geringe Extinktionsabfall während der langsamen Reduktionsphase (Erhöhung der $\Delta\varepsilon_r$-Werte von A nach B und C), besonders im Bereich um 350 mμ, kann nach den Modellversuchen auf die Reduktion phenolischer 4-Ketoguajakolgruppen (III) zurückgeführt werden.

Die oben vorgenommene Zuordnung des langwelligen Teils der $\Delta\varepsilon_r$-Kurve A zur Reduktion von (verätherten) Coniferylaldehydeinheiten konnte auf folgende Weise gestützt werden. Für eine Lösung von Björkman-Lignin in 0,01 N NaOH wurde teils der Extinktionswert bei 350 mμ gemessen, teils wurde in einer mit Essigsäure angesäuerten Probe der Lösung die Intensität der durch Zusatz von *p*-Aminobenzoesäure hervorgerufenen Gelbfärbung bestimmt. Die alkalische Lösung wurde hierauf mit $NaBH_4$ versetzt und es wurde teils der Abfall der Extinktion bei 350 mμ, teils, in abpipettierten Proben, die *p*-Aminobenzoesäurereaktion zeitlich verfolgt. Hierbei zeigte sich, dass die Intensität der Farbreaktion parallel mit dem Abfall der Extinktion abnimmt und Null wird, wenn die rasche Phase des Extinktionsabfalls im Reduktionsgemisch abgeschlossen ist (Fig. 12).

Durch Vergleich der $\Delta\varepsilon_r$-Werte der Kurven A—C (Fig. 11) mit den $\Delta\varepsilon_r$-Kurven der Modellsubstanzen lässt sich nun auch eine mengenmässige Schätzung der verschiedenen Carbonyltypen in dem untersuchten Ligninpräparat vornehmen.

Wie bereits erwähnt, kann man vom Vorkommen merklicher Mengen von Coniferylaldehydgruppen mit freiem Phenolhydroxyl (Typ I) im Präparat L I

absehen. Kurve A kann man sich aus den $\Delta\varepsilon_r$-Werten für die zwei rasch reagierenden Carbonyltypen, nämlich verätherte Coniferylaldehydgruppen (Typ II) und verätherte 4-Ketoguajakole (Typ IV) zusammengesetzt denken. Als Vergleichssubstanzen für diese Gruppierungen wählten wir den *Iso*propylconiferylaldehyd (IX) und das α-Guajakoxy-β-oxypropioveratron (XXI). Substanz IX besitzt ein $\Delta\varepsilon_r$-Maximum von 19 800 bei 342 mμ (Fig. 3 und Tab. 1); das $\Delta\varepsilon_r$-Maximum von Substanz XXI ist 9 400 bei 310 mμ. (Fig. 9 und Tab. 1.) Bei 310 mμ hat Substanz IX $\Delta\varepsilon_r = 10\,500$, während Substanz XXI bei 342 mμ ein $\Delta\varepsilon_r$ von 2 020 besitzt. Kurve A (Fig. 11) hat bei 310 mμ den $\Delta\varepsilon_r$-Wert 750, bei 342 mμ den $\Delta\varepsilon_r$-Wert 720. Bezeichnet man die Anzahl verätherter Coniferylaldehydgruppen (II) in Lignin (in Mol per OCH_3) mit x, die der verätherten 4-Ketoguajakolgruppen (IV) mit y, so gilt:

$$\text{bei } 310 \text{ m}\mu\text{:} \quad 10\,500\ x + 9\,400\ y = 750,$$
$$\text{bei } 342 \text{ m}\mu\text{:} \quad 19\,800\ x + 2\,020\ y = 720.$$

Hieraus ergibt sich $x = 0{,}031$ Gruppen II per OCH_3, und $y = 0{,}046$ Gruppen IV per OCH_3 in Präparat L I.

Die Anzahl phenolischer 4-Ketoguajakolgruppierungen (Typ III) lässt sich direkt aus der Grösse des langsamen Extinktionsabfalls ermitteln. Bei 356 mμ z. B. ist die Differenz zwischen den $\Delta\varepsilon_r$-Werten von Kurve A und Kurve C 110; wählt man als Modell das α-Guajakoxy-propioguajakon (XIII) mit $\Delta\varepsilon_r = 24\,600$ im Maximum (356 mμ), so ergibt sich ein Wert von 110/24 600 = 0,005 Gruppen III per OCH_3.

Um die Zuverlässigkeit der voranstehend beschriebenen quantitativen Auswertung zu prüfen, wurden $\Delta\varepsilon_r$-Kurven für die ermittelten Anteile der 3 Komponenten gesondert konstruiert, indem man die $\Delta\varepsilon_r$-Werte der Modelle IX (für Typ II), XIII (für Typ III) und XXI (für Typ IV) mit 0,03, 0,005 bzw. 0,05 multiplizierte. Die so erhaltenen $\Delta\varepsilon_r$-Kurven der einzelnen Komponenten sind in Fig. 11 als K_{II}, K_{III} und K_{IV} bezeichnet. Addition der $\Delta\varepsilon_r$-Werte dieser drei Kurven bei verschiedenen Wellenlängen ergab die mit o bezeichneten Werte. Diese fallen im Bereich zwischen 290 und 370 mμ mit der experimentell erhaltenen Kurve C zusammen. Die geringfügige Abweichung oberhalb 370 mμ dürfte auf dem Vorkommen sehr kleiner Mengen phenolischer Coniferylaldehydgruppen, die hier vernachlässigt wurden, beruhen. Die mangelnde Übereinstimmung im Bereich unterhalb 290 mμ kann durch die Wahl des Vergleichsmodells XXI bedingt sein; andere Modelle, wie Propioveratron (XVI) und β-Oxy-propioveratron (XX), haben in diesem Bereich höhere $\Delta\varepsilon_r$-Werte. Möglicherweise macht sich hier auch der Einfluss von nicht-konjugierten (β)-Ketonen geltend (s. unten).

Die $\Delta\varepsilon_r$-Kurven für das Björkman-Lignin-präparat L II (Fig. 13) zeigen etwas höhere $\Delta\varepsilon_r$-Werte als die Kurven für L I. Da Präparat L II aus einem Holzmehl stammte, dessen Schwingmahlung in gewöhnlicher Weise in Luft vorgenommen worden war, während bei L I die Mahlung unter Stickstoff erfolgte, deuten die erhöhten $\Delta\varepsilon_r$-Werte auf eine oxydative Neubildung von konjugierten Carbonylgruppen hin. Einen Hinweis hierauf hatte bereits der Vergleich der Ionisierungs-$\Delta\varepsilon$-Kurven geliefert [2]. Die Hydroxylaminmethode gab einen kleinen aber eindeutigen Unterschied im Gesamt-Carbonylgehalt (0,18 CO/OCH_3 in L I; 0,21 CO/OCH_3 in L II), und wie die Auswertung der $\Delta\varepsilon_r$-

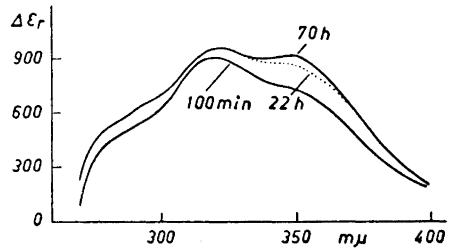


Fig. 13. $\Delta\varepsilon_r$-Kurven von Björkman-Lignin L II.

Kurven zeigt, hält sich auch die Neubildung von konjugiertem Carbonyl innerhalb bescheidener Grenzen.

Am langwelligen Ende der $\Delta\varepsilon_r$-Kurven für L II (Fig. 13) sind die $\Delta\varepsilon_r$-Werte deutlich höher als bei L I (Fig. 11). Würde man den bei 400 mμ gefundenen Wert (180) auf phenolische Coniferylaldehydeinheiten zurückführen, so würde sich, da Coniferylaldehyd bei 400 mμ $\Delta\varepsilon_r = 32\,000$ besitzt, ein Gehalt von $180/32\,000 = 0{,}006$ phenolischen Coniferylaldehydgruppen per OCH_3 ergeben.

Die weitere Analyse der $\Delta\varepsilon_r$-Kurven für L II lieferte folgende Werte: 0,03 verätherte Coniferylaldehydgruppen (Typ II), 0,01 phenolische 4-Ketoguajakolgruppen (Typ III) (gegenüber 0,005 bei L I) und 0,05—0,06 verätherte 4-Ketoguajakolgruppen (Typ IV) per Methoxyl (Summe: 0,09—0,10 konjugierte Carbonylgruppen per OCH_3; unter Einbeziehung der oben diskutierten phenolischen Coniferylaldehydgruppen würde sich diese Summe auf 0,10—0,11 konjugiertes CO/OCH_3 erhöhen).

Die bei der Borhydridreduktion des mit Diazomethan methylierten Björkman-Lignins (DL III) erhaltenen Resultate stehen in guter Übereinstimmung mit den oben beschriebenen. Methylierung mit Diazomethan beeinflusst die in Frage stehenden konjugierten Carbonyleinheiten in verschiedener Weise. Nach

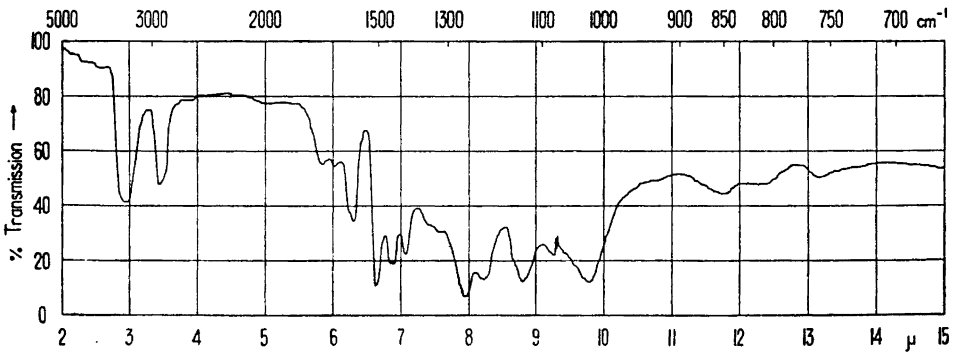


Fig. 14. IR-Spektrum eines mit CH_2N_2 methylierten Björkman-Lignins (Präparat DL III).

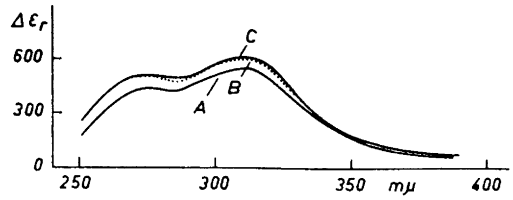


Fig. 15. $\Delta\varepsilon_r$-Kurven eines mit CH_2N_2 methylierten Björkman-Lignins (DL III) nach 100 min (A), 22 h (B) und 46 h (C) Reduktionsdauer.

Kratzl und Wittmann [18] lagert sich Diazomethan an die Äthylenbindung der Coniferylaldehydseitenketten unter Bildung eines Pyrazolinrings an. Hierbei wird die Konjugation der Aldehydgruppe mit dem aromatischen Kern aufgehoben; die typischen Farbreaktionen werden nicht mehr gegeben. Ferner werden phenolische Ketongruppierungen (III und V) durch Methylierung des Phenolhydroxyls in verätherte (IV und VI) übergeführt.

Im IR-Spektrum von DL III (Fig. 14) ist die langwellige Carbonylbande infolge der Umsetzung der Coniferylaldehydgruppen mit Diazomethan viel schwächer als im Spektrum des unbehandelten Lignins (vgl. Fig. 1, oben). Die vorhandene Absorption um 1 665 cm^{-1} kann mit dem Vorkommen von Arylketongruppierungen mit 1,3-Ketolstruktur, wie z. B. beim β-Oxypropioveratron (XX), erklärt werden (vgl. S. 76 und Tab. 1). Auch Spuren von Coniferylaldehydgruppen, welche der Anlagerung von Diazomethan entgangen sein mögen, könnten zu dieser Absorption beitragen. Die Absorptionsbande für isolierte Carbonylgruppen liegt im Bereich von 1 725 — 1 705 cm^{-1}.

Die Effekte der Diazomethanbehandlung auf die carbonyltragenden Einheiten des Lignins zeichnen sich auch in der Borhydridreduktions-$\Delta\varepsilon$-Kurve des Präparats DL III (Fig. 15) deutlich ab. Die für die nicht methylierten Präparate (L I, L II) charakteristische Schulter mit relativ hohen $\Delta\varepsilon_r$-Werten bei 340—350 mμ fehlt in der Kurve für DL III infolge der Absättigung der Coniferylaldehydseitenketten durch die Anlagerung von Diazomethan. Die nach 100 Min. erhaltenen $\Delta\varepsilon_r$-Werte um 350 mμ erhöhen sich nicht bei verlängerter Reduktionsdauer, da nun die Phenolhydroxyle der ursprünglichen 4-Ketoguajakole vom Typ III methyliert sind.

Das bei den unmethylierten Präparaten bei 322—325 mμ liegende Maximum ist im Falle des methylierten Lignins DL III nach etwa 310 mμ gerückt, d. h. in den für das $\Delta\varepsilon_r$-Maximum der verätherten 4-Ketoguajakole (Typ IV) charakteristischen Bereich. Diese Verschiebung des Maximums ebenso wie die Erniedrigung der $\Delta\varepsilon_r$-Werte ist ohne weiteres durch das Wegfallen der Überlagerung mit den $\Delta\varepsilon_r$-Werten der verätherten Coniferylaldehydgruppen zu verstehen (vgl. Fig. 11). Für die geringfügige Erhöhung der $\Delta\varepsilon_r$-Werte im kürzerwelligen Gebiet bei verlängerter Reaktionsdauer gibt es gegenwärtig keine auf Modellversuche zu stützende Erklärung.

Der quantitative Vergleich der $\Delta\varepsilon_r$-Werte um 310 mμ für das methylierte Lignin DL III mit den entsprechenden Werten für das Modell XXI gibt an,

dass DL III 0,06—0,07 verätherte 4-Ketoguajakolgruppen (Typ IV) per OCH_3 enthält. Dieser Wert stimmt überein mit der Summe der Anzahl von verätherten (0,05—0,06) und phenolischen (0,01) 4-Ketoguajakolgruppen im nicht-methylierten Lignin.

Wir haben bei der Analyse der $\Delta\varepsilon_r$-Kurven der untersuchten Ligninpräparate bisher den eventuellen Beitrag durch β-Ketongruppierungen (Typ V und VI) unberücksichtigt gelassen. Wie oben gezeigt (S. 84—85, Fig. 10), geben phenolische β-Ketone, wie Guajacylaceton (XXII) und ω-Oxy-guajacylaceton (XXV), $\Delta\varepsilon_r$-Kurven mit Maximum bei 312 mμ, d. h. im selben Bereich wie die verätherten 4-Ketoguajakole, z. B. das Modell XXI (310 mμ). Wollte man das Hauptmaximum der $\Delta\varepsilon_r$-Kurven der unmethylierten Lignine (L I und L II) anstatt auf verätherte 4-Ketoguajakole (Typ IV) ausschliesslich auf die Anwesenheit von phenolischen β-Ketonen (Typ V) zurückführen, so würde dies bedeuten, dass die Ligninpräparate statt 0,05 Gruppen vom Typ IV etwa 0,25 Gruppen vom Typ V per OCH_3 enthalten. (Die $\Delta\varepsilon_r$-Werte der phenolischen β-Ketonmodelle XXII und XXV, 2 300 bzw. 1 900 bei λ_{max} = 312 mμ, sind etwa fünfmal kleiner als die Maximum-Werte der verätherten 4-Ketoguajakolmodelle, s. Tab. 1.) Es kann jedoch ausgeschlossen werden, dass phenolische β-Ketone nennenswert zum $\Delta\varepsilon_r$ im Maximum der Ligninkurven beitragen. Bei der Methylierung von Lignin mit Diazomethan würden nämlich die phenolischen β-Ketongruppen (Typ V) in verätherte (Typ VI) übergehen, und die letzteren sollten im Bereich von 310—312 mμ nur noch kaum messbare $\Delta\varepsilon_r$-Werte geben (vgl. z. B. Veratrylaceton, XXIII, Fig. 10). In Wirklichkeit bleibt aber, wie oben gezeigt, der $\Delta\varepsilon_r$-Wert bei 310 mμ (nach Abzug des Beitrags durch verätherte Coniferylaldehydeinheiten, s. Fig. 11) beim Übergang von Lignin zu methyliertem Lignin erhalten.

Es wurde gefunden, dass ω-Oxy-guajacylaceton (XXV) mit Diazomethan unter Verschwinden der Carbonylfunktion reagiert (IR-Spektrum). Wären phenolische β-Ketone von der Struktur XXV in grösserer Menge im Lignin vorhanden, so könnte man also auch aus dem eben genannten Grunde erwarten, dass beim diazomethan-methylierten Lignin der $\Delta\varepsilon_r$-Wert bei 310—312 mμ deutlich niedriger wäre als beim unbehandelten Lignin, was jedoch nicht der Fall ist.

Es mag schliesslich noch erwähnt werden, dass Brauns-Lignin *(Picea abies)* und diazomethan-methyliertes Brauns-Lignin bei der Borhydridreduktion ähnliche $\Delta\varepsilon_r$-Kurven lieferten wie die untersuchten Björkman-Ligninpräparate. Entsprechend dem höheren Totalgehalt an phenolischen Einheiten [5] sind auch die phenolischen 4-Ketoguajakolgruppen im Brauns-Lignin etwas reichlicher vertreten. Neben 0,03 verätherten Coniferylaldehydeinheiten (Typ II) und 0,04—0,05 verätherten 4-Ketoguajakoleinheiten (Typ IV) wurden 0,015—0,02 phenolische 4-Ketoguajakolgruppen (Typ III) per OCH_3 gefunden. Aulin-Erdtman [8] fand mit anderen $\Delta\varepsilon$-Methoden — neben 0,01 phenolischen Coniferylaldehydgruppen (Typ I) — 0,02 Gruppen von Typ II und 0,02—0,03 Gruppen vom Typ III.

Zusammenfassung und Diskussion

Unsere mit der Borhydridreduktionsmethode für Björkman-Lignin erhaltenen Resultate können folgendermassen zusammengefasst werden.

Das Lignin enthält per OCH_3 folgende Mengen mit dem aromatischen Kern konjugierte Carbonylgruppen:

a) in phenolischen Einheiten
<0,01 Aldehydgruppen in Coniferylaldehydeinheiten (I),
0,005—0,01 α-ständige Ketogruppen (III);
b) in nicht-phenolischen Einheiten
0,03 Aldehydgruppen in verätherten Coniferylaldehydeinheiten (II),
0,05—0,06 α-ständige Ketogruppen (IV).

Insgesamt sind damit 0,09—0,11 (konjugierte) Carbonylgruppen per OCH_3 lokalisiert. Dies ist ungefähr die Hälfte der Gesamtmenge von Carbonylgruppen (0,18—0,21 CO/OCH_3), bestimmt mittels der Hydroxylaminmethode [3,4]. Da β-ständige Carbonylgruppen in phenolischen Einheiten (Typ V) nicht in fassbarer Menge vorzukommen scheinen (s. S. 93) und da nicht-konjugierte Aldehydgruppen auf Grund des IR-Spektrums wenig wahrscheinlich sind, sollte die fehlende Hälfte der Carbonylgruppen der Hauptsache nach aus β-ständigen Ketogruppen in nicht-phenolischen Einheiten (Typ VI) bestehen. Auf sie spricht die $\Delta\varepsilon_r$-Methode nicht an (s. S. 85 und 93).

Gierer und Söderberg [19,20] haben neuerdings die gasvolumetrische Messung des Borhydridverbrauchs nach Lindberg [21] auf die Bestimmung des Gesamtcarbonylgehaltes von Lignin angewandt und kamen für Björkman-Lignin *(Picea abies)* zu Werten von 0,41—0,45 CO/OCH_3. Diese Werte sind etwas mehr als doppelt so hoch wie die von uns und auch von Gierer und Söderberg mittels der Hydroxylaminhydrochlorid-Methode gefundenen. Zur Erklärung der Diskrepanz zwischen den beiden Methoden weisen Gierer und Söderberg darauf hin, dass gewisse Modell-Carbonylverbindungen nur sehr langsam oximiert werden. Besonders langsam reagierte das sowohl in β- wie in γ-Stellung substituierte α-Keton XXI ("sterische Hinderung").

Auch im hiesigen Institut war, im Zusammenhang mit der Bestimmung des Gesamtcarbonyls in Lignin, die Geschwindigkeit der Oximierung mehrerer der auf S. 80 angegebenen Modellsubstanzen geprüft worden [3]. Unter den angewandten Bedingungen, die den von Gierer und Söderberg [20] angegebenen ähnlich waren, waren die am langsamsten reagierenden Verbindungen (gewisse α-Ketone) erst nach 300 Stunden voll oximiert; beim Vanilloylmethylketon (XIV), reagierte das erste Carbonyl ziemlich rasch, das zweite jedoch erst im Laufe von etwa 600 Stunden. Die von Gierer und Söderberg [19,20] gefundene Ausnahmestellung des α-Ketons XXI wurde nachträglich bestätigt; es war nach 500 Stunden erst zu 60 % oximiert. Für die Ligninpräparate wählten wir eine Oximierungsdauer von 500 Stunden (die Anwesenheit nennenswerter Mengen von Vanilloylmethylketongruppierungen konnte aus spektrochemischen Gründen ausgeschlossen werden).

Da nun mit Hilfe der in der vorliegenden Arbeit beschriebenen spektrophotometrischen Borhydridmethode festgestellt wurde, dass die Gesamtmenge von α-Carbonylgruppen (in phenolischen und verätherten Einheiten, III und IV) nicht mehr als 0,06—0,07/OCH_3 beträgt, kann ausgesagt werden, dass solche Gruppen nicht oder jedenfalls nur zu einem kleinen Teil für die Differenz zwischen den mit der Hydroxylaminmethode und der gasvolumetrischen Borhydridmethode erhaltenen Werten für das Gesamtcarbonyl verantwortlich gemacht werden können. Coniferylaldehydgruppen werden mit Hydroxylamin

sehr rasch umgesetzt, und ihre Menge (0,03—0,04/OCH_3) ist ausserdem durch verschiedene Methoden übereinstimmend festgelegt.

Will man daher annehmen, dass die Differenz zwischen den mit der Hydroxylaminmethode und den mit der gasvolumetrischen Borhydridmethode erzielten Werten darauf beruht, dass gewisse Typen von Carbonylgruppen durch die erstgenannte Methode nicht erfasst werden, so hätte man diese nicht-oximierbaren Carbonyle in der Reihe der β-Ketone zu suchen. Die Menge solcher Carbonyle sollte dann 0,20—0,25/OCH_3 betragen. Modellsubstanzen mit β-ständiger, nicht oximierbarer (aber durch Borhydrid reduzierbarer) Ketogruppe sind bisher nicht angetroffen worden; es wäre von Interesse, die Oximierbarkeit von Derivaten des ω-Oxyguajacylacetons (XXV), die am ω-Hydroxyl veräthert und eventuell auch am α-C-Atom substituiert sind, zu untersuchen. Auch an irgendwie "maskierte" Carbonylgruppen, die zwar Borhydrid verbrauchen aber nicht mit Hydroxylamin reagieren, wäre vielleicht zu denken.

Herrn Dr. J. C. Pew, Madison, Wisconsin, U. S. A., danken wir für die Überlassung von Coniferylaldehyd-*iso*propyläther, Herrn Dr. J. A. F. Gardner, Vancouver, Canada, für eine Probe von ω-Oxy-guajacyl-aceton. Herrn Docent A. Björkman, Säffle, Schweden, verdanken wir das Ligninpräparat L I.

Bei der Ausführung der Versuche erfreuten wir uns der Mitarbeit von Frau Terezia Marton.

Wir danken *Statens Tekniska Forskningsråd* für die Gewährung eines Forschungsbeitrags.

EXPERIMENTELLES

$\Delta\varepsilon_r$-Kurven der Modellsubstanzen. Das UV-Spektrum der Substanzen wurde gemessen in einer Lösung, die durch Mischen von 2 ml einer 0,1—0,4 mM Lösung der Substanz in Äthanol mit 1 ml 0,03 N wässriger Natronlauge bereitet wurde. In einem zweiten Reaktionsgemisch wurde anstelle der Natronlauge 1 ml einer 50 mM oder 100 mM Lösung von $NaBH_4$ in 0,03 N NaOH verwendet und das Absorptionsspektrum nach verschiedenen Zeiten gemessen. Die Stabilität des Spektrums der alkalischen Lösung ohne $NaBH_4$ wurde ebenfalls nach verschiedenen Zeiten geprüft. Als Vergleichslösungen dienten die entsprechenden Gemische ohne Substanz.

$\Delta\varepsilon_r$-Kurven der Ligninpräparate. Als Lösungsmittel für die Ligninpräparate diente ein Gemisch von 40 Volumteilen Monomethylglykol und 60 Volumteilen Äthanol. Die Konzentration der Lösungen war 0,1 g/l (für L I und L II) bzw. 0,2 g/l (für DL III). Die Messlösungen wurden bereitet aus 2 ml der Ligninlösung und 1 ml 0,03 N NaOH bzw. 1 ml einer 50 mM, im Falle von DL III einer 100 mM Lösung von $NaBH_4$ in 0,03 N NaOH. Die Extinktion ε ist auf die Konzentration an OCH_3 bezogen.

Die *p-Aminobenzoesäurereaktion* auf Coniferylaldehydgruppen (vgl. S. 89 und Fig. 12) wurde folgendermassen ausgeführt: Von der mit Borhydrid versetzten alkalischen Lösung des Lignins (Zusammensetzung wie voranstehend, jedoch Dioxan als Lösungsmittel) wurden nach verschiedenen Zeiten Proben von 1 ml abpipettiert und sofort mit 1 ml Eisessig vermischt. Die erhaltene Lösung wurde mit 2 ml *p*-Aminobenzoesäurereagens (1,65 g *p*-Aminobenzoesäure, 60 ml Eisessig und 25 ml 4 N HCl, mit Wasser auf 100 ml verdünnt) versetzt. Die optische Dichte der Lösung bei 445 mμ wurde gegen eine Vergleichslösung ohne Lignin gemessen. Der *D*-Wert für die Zeit Null wurde mit einer Ligninlösung ohne Borhydridzusatz bestimmt.

Die Messungen der Ultraviolettabsorption wurden mit einem Beckman DU-Gerät ausgeführt.

Zur Aufnahme der Infrarotspektren (KBr-Methode) diente ein Perkin-Elmer Instrument, Modell 21. Von den Modellsubstanzen wurden 1—1,5 mg, von den Ligninpräparaten 1,8 mg per 500 mg KBr verwendet.

LITERATUR

1. Björkman, A. *Svensk Papperstidn.* **59** (1956) 477.
2. Björkman, A. und Person, B. *Svensk Papperstidn.* **60** (1957) 158.
3. Adler, E. und Walldén, I. *Unveröffentlicht.*
4. Adler, E. und Gierer, J. in Treiber, E., Die Chemie der Pflanzenzellwand, Springer-Verlag, Berlin 1957, S. 446.
5. Adler, E., Hernestam, S. und Walldén, I. *Svensk Papperstidn.* **61** (1958) 641.
6. Adler, E., Björkqvist, K. J. und Häggroth, S. *Acta Chem. Scand.* **2** (1948) 93; Adler, E. und Ellmer, L. *ibid.* **2** (1948) 839.
7. Kratzl, K. *Monatsh.* **76** (1948) 173.
8. Aulin-Erdtman, G. und Hegbom, L. *Svensk Papperstidn.* **61** (1958) 187.
9. Smith, D. C. C. *Nature* **176** (1955) 927.
10. Aulin-Erdtman, G. *Svensk Kem. Tidskr.* **70** (1958) 145 (Übersicht über vorangehende Arbeiten).
11. Aulin-Erdtman, G. *Svensk Papperstidn.* **56** (1953) 91.
12. Aulin-Erdtman, G. und Hegbom, L. *Svensk Papperstidn.* **60** (1957) 671.
13. Adler, E. und Delin, S. *Unveröffentlicht.*
14. Magnusson, R. *Acta Chem. Scand.* **12** (1958) 791.
15. Ramart-Lucas, P. *Bull. Soc. Chim.* **51** (1932) 289, 965; **10** (1943) 13.
16. Fisher, H. E. und Hibbert, H. *J. Am. Chem. Soc.* **69** (1947) 1208.
17. Björkman, A. und Person, B. *Svensk Papperstidn.* **60** (1957) 285.
18. Kratzl, K. und Wittmann, E. *Monatsh.* **85** (1954) 7.
19. Gierer, J. *Privatmitteilung.*
20. Gierer, J. und Söderberg, S. *Acta Chem. Scand.* **13** (1959) 127.
21. Lindberg, B. und Misiorny, A. *Svensk Papperstidn.* **55** (1952) 13; Lindberg, B. und Theander, O. *ibid.* **57** (1954) 83.

Eingegangen am 30. August 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 97—108

On the Design and Efficiency of Isothermal Reaction Calorimeters

S. SUNNER and I. WADSÖ

Thermochemistry Laboratory, University of Lund, Lund, Sweden

A number of isothermal reaction calorimeters have been designed, built and tested. From an initial, very simple Dewar-type calorimeter a system was developed that showed an accuracy of 0.1 % and an equilibration time less than 2 minutes. To check the accuracy of the electrical calibrations, the heat of solution of potassium chloride was measured. The results obtained were compared with those of previous investigators.

In principle, changes in enthalpy accompanying a chemical reaction can always be calculated from heat of formation data, given that these are available for all the participating substances. In most cases this is not so — even the determination of a single heat of combustion value can be a laborious and very time-consuming task. Besides, in some reactions one (or more) of the participants may be too unstable for investigation by the combustion method [1]. A few years ago we therefore began to develop suitable apparatus for reaction calorimetry.

The variety in types of chemical reactions makes it impossible to design a more or less universally useful reaction calorimeter (in the same sense as a bomb calorimeter can be constructed). We therefore restricted the methodological work to calorimetric equipment suited to reactions obeying the following limiting conditions:

1. The reactions should be reasonably fast, the duration of the reaction period not exceeding one hour.
2. The total amount of heat evolved (or absorbed) during the reaction should be 50 cal. or less.

Further, the following conditions should be emphasized:

3. The size of the calorimeter should be *ca.* 100 ml.
4. The calorimeter should be simple in design and easy to operate, and
5. The accuracy achieved should be 0.1 % or 0.05 cal. whichever is larger.

Having these conditions in mind it was felt that a calorimeter of the "constant-temperature environment" (isothermal) type should be adopted. Furthermore,

there being no systematic treatise on reaction calorimeters available, it was decided to start by assembling a very simple Dewar-type calorimeter, and from the experience gained with this, to develop a more elaborate apparatus.

Altogether five different calorimeters have been built and tested, and the present paper reports briefly on the experience gained with these.

CALORIMETERS IN THE DEVELOPMENT STAGE

The essential features of design of the calorimeters are shown in Fig. 1. The second earliest type (A) consisted of a silvered Dewar vessel with an inner thin-walled glass-shield (e) and an inner socket standard joint *. All connections were made through the cone-joint: the thermistor (a) (in the beginning a Beckmann thermometer was used); the calibration element (b) and two short glass tubes carrying the stirrer (c) and the ampoule holder (d). A rigid mounting was achieved by use of sheets of cork interspaced by cork crumbs as insulating materials. The inner end of the standard cone-joint was protected against absorption of moisture from the calorimeter liquid by a paraffin wax-poly*iso*butene coating.

The temperature was measured by use of a thermistor (Stantel F 2311/300) in a conventional Wheatstone bridge arrangement. The thermistor was sealed into a glass tube; only the small thermistor bead extruded into the calorimeter liquid. The stirrer hung freely through the glass tube into the calorimeter liquid and was mounted directly on the shaft of a 600 rpm synchronous motor. At the start of an experiment, the Dewar-vessel was completely submerged in a thermostat, the temperature of which was constant to within 0.002°C. After equilibration the measurement was performed in the usual way by observing the temperature during the initial, reaction and final periods. The reaction was started by breaking a sealed glass ampoule on the bottom of the Dewar.

The heat equivalent of the calorimeter was determined by passing a known current through the known calibration resistance for a known length of time.

Although this common type is very easy to build and operate, it suffers from several weaknesses. The reproducibility was not very high, $\pm$ 0.5 %. The reasons for this are several. The mounting of the stirrer was not rigid enough to ensure an even heat of stirring. The ampoule, which was positioned very near the stirrer, broke irregularly causing a sudden change in the heat of stirring. The most serious disadvantage, however, was that the time for reaching thermal equilibrium was very long mainly due to the slow equilibration. This calorimeter has been used for several measurements [2,3].

In order to overcome these difficulties a new calorimeter was built (Fig. 1). This consisted of a steel can (B) which could be used together with one of three different surrounding systems — a solid copper block (B 1), a double walled glass flask (B 2) or a single-walled glass flask (B 3).

The calorimeter can was made out of high grade stainless steel and consisted of a thin-walled cylinder to which a lid and a bottom had been soldered. A

* The first calorimeter built consisted of an ordinary Dewar vessel, volume 150 ml, fitted with a heavy rubber stopper.

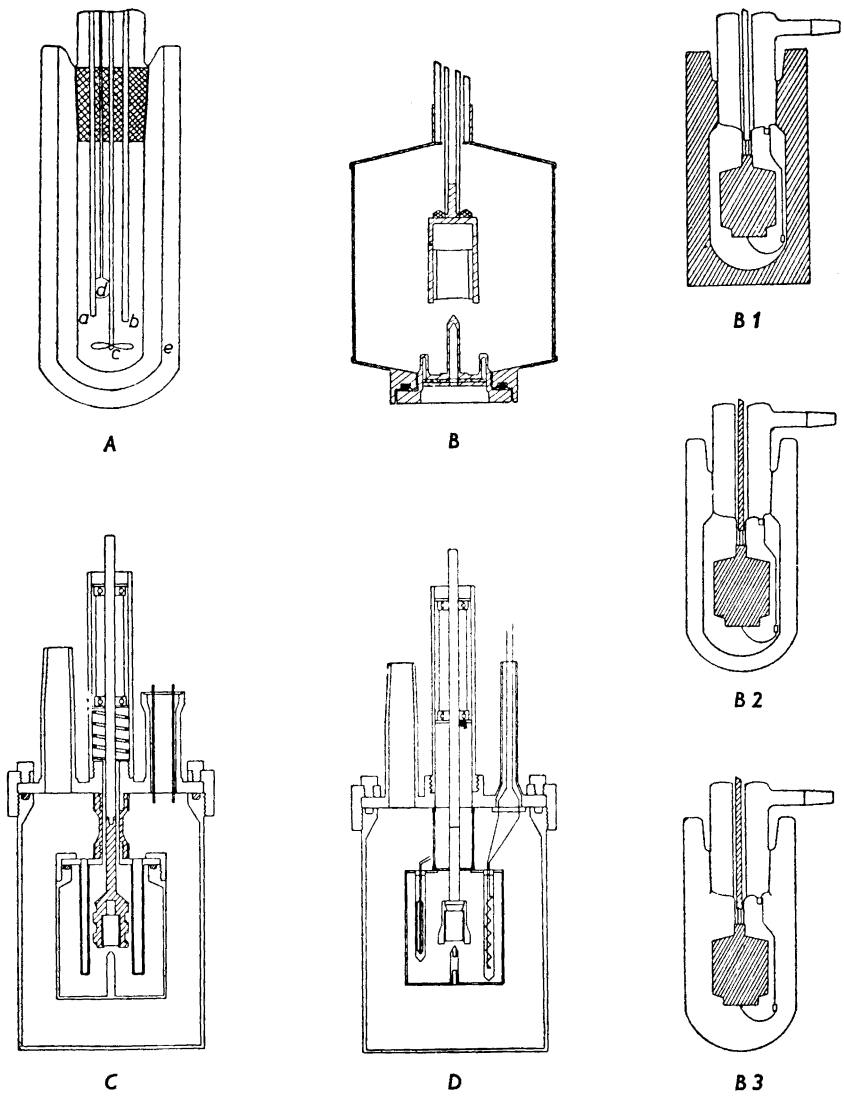


Fig. 1. Principle design of the calorimeters.

plug, machined from one piece of steel and having two wells was screwed into the bottom, and a high-vacuum tight seal was achieved by use of an O-ring gasket. The central well served to house the thermistor and the concentric well contained the calibration heater. This consisted of a 2 mm strip of thin mica on which a 0.15 mm manganin wire was wound (resistance 15 Ω). The strip was inserted into the bottom of the well and insulated from the walls by

use of mica. The lower part of the well was closed by a PVC ribbon; further insulation between the heater and the outside of the plug was effected by a PVC disk kept in place by a steel plate.

The calorimeter can was sealed to a tube extruding from a hollow glass stopper. Through the stopper, the space between the calorimeter can and the surrounding flask could be evacuated. The electrical connections were carried from the plug in the calorimeter can to four terminals placed on a strip of phosphor-bronze, and then passed through the stopper to the outside of the calorimeter.

The stirrer was made of steel and shaped like a three-armed clutch grasping the ampoule and provided with small wings for the stirring. It was sealed with sulphur to a short glass tube, the upper end of which was sealed to a steel rod. The stirrer and shaft were precisely centered during the sealing operation. The steel rod was mounted in two ball bearings outside the hollow stopper and could be moved up and down during a run.

At the start of an experiment, the calorimeter was held in an upside down position. The stirrer shaft was moved so that the ampoule holder projected from the can. The ampoule was inserted and the stirrer was moved back to give a leak-proof seal between the lid of the can and the ampoule holder by means of a small rubber gasket. The calorimeter can was filled with 90 ml of solution, the plug was screwed tight and the can was inserted into the jacket, which was afterwards submerged in the thermostat.

This calorimeter can together with the surrounding system (B 1) fulfilled the given conditions reasonably well and was used for several measurements [4-8]. It was, however, not convenient for reactions with a heavy second reaction component (bromine, or certain solids) as the stirring did not affect the liquid in the ditch between the thermistor and the calibration element wells. Further, the stainless steel was corroded by bromine or showed catalytic activity in the reaction $SO_2 + Br_2$ or I_2. Finally, with certain calorimeter liquids, *e. g.* amines, the rubber-gasket did not always give a leak-proof seal due to swelling or dissolution processes.

We therefore built a third calorimeter (Fig. 1, C), of a rather common design (*e. g.* Ref.[9]). This was machined from stainless steel and consisted of a body, a lid with tubes for the thermistor and the calibration element and a screw-nut ring. The high-vacuum seal was made with an O-ring gasket. All inner parts were coated with several layers of teflon, of total thickness 0.12 mm. The stirrer was made from a teflon-rod. The calorimeter can hung from the lid of the surrounding chromium-plated brass can in a tube of teflon.

This calorimeter was tested and immediately rejected because the equilibration was very slow, particularly if the calorimeter can was not completely or almost completely filled by liquid.

THE FINAL CALORIMETER

Finally, a calorimeter (Fig. 1, D), was built according to the main principles of the design of calorimeter B.

A thin-walled (0.5 mm) open can machined from one piece of brass was silver soldered to a brass lid. The bottom had a central rod to support an

ampoule-breaking pin. The lid was provided with two thin-walled tubes, and had a central, wide opening surrounded by two concentric thin brass-rings. After being chromium-plated, all interior parts of the can were coated with teflon as described above.

The calorimeter can hung from the lid of a surrounding brass can in a thin-walled (0.7 mm) glass tube which was sealed to the metal parts by Araldite applied to groves between the brass-rings. Thus, a rigid mounting as well as a high-vacuum tight seal were attained. In order to minimize the air gap between the stirring rod and the walls of the opening in the calorimeter can, a snugly fitting teflon coated brass gasket was introduced into the glass tube. The only free space between the gas phase in the calorimeter and the surrounding air was thus reduced to a 0.5 mm air gap around the stirring shaft.

The combined ampoule holder and stirrer was made of teflon-coated stainless steel and fastened to a teflon rod, the upper end of which was screwed on to the steel spindle of the stirring device. The whole stirrer was exactly centered and could be easily unscrewed from the lid of the outer brass can.

The thermistor was introduced into one of the tubes in the calorimeter can. The calibration element was placed in the second tube and both tubes were vacuum-tightly sealed with Araldite. The electrical connections were passed through the outer can by metal-glass seals soldered to the lid of the can.

The interspace was evacuated through a B19 standard cone, using a mechanical rotary pump and a three stage glass mercury pump attached to a liquid air trap. The evacuation was very rapid and a vacuum better than 10^{-4} mm Hg was reached within a few minutes after the start of the pumping.

This calorimeter was very easy and rapid to operate, one experimental cycle being completed within *ca.* 1 h.

Technique of mixing

The reaction period was started by breaking a glass ampoule and mixing the liquid or solid contents with the calorimeter liquid. In order to achieve rapid mixing a modification has been developed of a well-established procedure [9–12], in which the stirrer performs the breaking of the bulb.

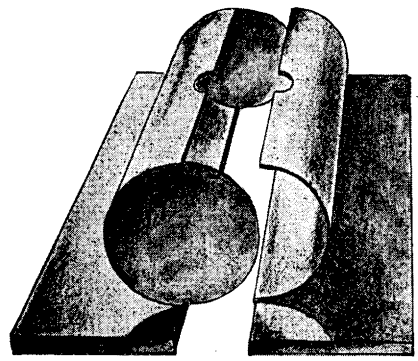


Fig. 2. Ampoule mould.

The ampoules were blown in a mould (Fig. 2) from selected thin-walled glass tubing. The method has two advantages: the ampoules are of uniform and predetermined size, and the end walls are very thin, facilitating the breaking and minimizing possible heat effects. For liquid samples, the ampoule shaft was drawn to a fine capillary as near the bulb as possible and for solid samples, the shaft was only slightly narrowed. When the sample had been introduced, the bulb was placed in a small copper block which was cooled by dry ice and the shaft was sealed very close to the bulb wall with a micro flame. The ampule was inserted centrally in the stirrer which during rotation gave the liquid an upward motion. By lowering the stirrer, a steel or platinum needle was forced to break through the end walls of the ampoule and a rapid mixing was achieved, the contents of the bulb being thrown out into the calorimeter liquid.

Heat effects arrising from bulb breaking

Many authors have pointed out that the breaking of a bulb may give rise to one or more small heat effects [11-13]. Principally such a heat effect can be traced back to one or several of the following causes:

1. The heat of stirring is dependent on the environmental situation — other conditions being equal. If the bulb is situated close to the stirrer the heat generated by friction between the liquid and the unbroken bulb might be significantly different from that generated between the liquid and the remaining parts of the broken bulb. This effect is the only one which is dependent on the speed of stirring and can therefore easily be detected by observing the time-temperature curves before and after breaking an empty bulb [12]. By varying the speed of stirring the highest rpm value can be found at which the change in the heat of stirring is insignificant.

It is of course desirable to make the breaking of the bulbs as uniform as possible. This is difficult when using the type of bulb which is very common and which we used in our first apparatus. However, a moulded bulb tends to break very uniformly. In our first calorimeter we found a varying and sometimes quite appreciable heat effect of this kind. It was not detectable in calorimeters B and D.

2. The heat of rupture of the bulb depends on the mechanical properties of the glass: wall thickness, internal strain *etc.* With rather large and rigid ampoules, the heat of rupture may be considerable [11].

In our case, in which the volume of the bulb is only *ca.* 1 ml and the end walls break through for a pressure of about 10 g this effect is negligible.

3. The heat of wetting of the inner surface of the glass bulb is usually a negligible quantity. Only in microcalorimetric measurements of, *e. g.*, heats of adsorption is it necessary to take this effect into consideration [13].

4. Heat effects due to a change of state of one or two of the phases in the calorimeter may or may not be significant depending on the experimental conditions. Bartell and Suggitt [11] have pointed out that the breaking of an evacuated bulb is followed by an evaporation. In the same way, the rupture of a bulb partly filled by a volatile liquid is accompanied by a condensation process.

5. The vaporized reactant in a partly filled ampoule may partly escape the surrounding liquid when the bulb is broken.

The magnitude of both effects 4 and 5 are easily estimated from vapor pressure data and the volume of the gaseous phase in the bulb. With 1 ml bulb they are usually negligible.

Time of equilibration

In high precision calorimeters it is of great importance to minimize the time of equilibration as much as possible. The method of comparative measurements is not rigorously applicable as the heat cannot be generated at the same rate in the calibration experiment as in the actual measurement. Evidently, a simultaenous measurement of the kinetics and exothermicity of a particular reaction also requires a small time lag of the apparatus compared to the total time of reaction.

The time of equilibration was experimentally determined for the different calorimeters by studying the heat transfer constant, K, as a function of time in a calibration experiment. K is defined as

$$K = \frac{| g_f - g_i}{| \Theta_i - \Theta_f}$$

where

g_i is the rate of temperature change during the initial period,
g_f is the rate of temperature change during the final period,
Θ_i is the mean temperature during the initial period and
Θ_f is the mean temperature during the final period.

By chosing different reaction times in one and the same experiment, different values of K were obtained and plotted against the time elapsing from the moment when the calibration current was shut off until the middle of the final period. In such a way the curves in Fig. 3 were obtained.

The following conclusions can be drawn from the results: Any construction which creates an indetermined boundary of the calorimeter system towards the surroundings leads to a slow equilibration. A stopper which is in reasonably good contact both with the calorimeter system as well as with the surroundings and which contains insulating materials (in order to slow down the heat transfer) introduces an appreciable time lag. Also, a radiation shield with a not negligible heat capacity compared to that of the calorimeter increases the time of equilibration. The three-parted calorimeter C was slow, due to the low heat conductivity of steel together with the fact that the lid and the screw-nut ring represent rather heavy masses far removed from the liquid phase. When the amount of liquid in the calorimeter was increased from 90 to 110 ml (the vessel was then almost full), the time of equilibration dropped from 40 to 15—20 min. Both calorimeter B with single glass wall (B 3) or copper block (B 1) as well as calorimeter D reached equilibrium rapidly. Indeed, D equilibrated within 2 min, as is best seen from the curve in Fig. 4.

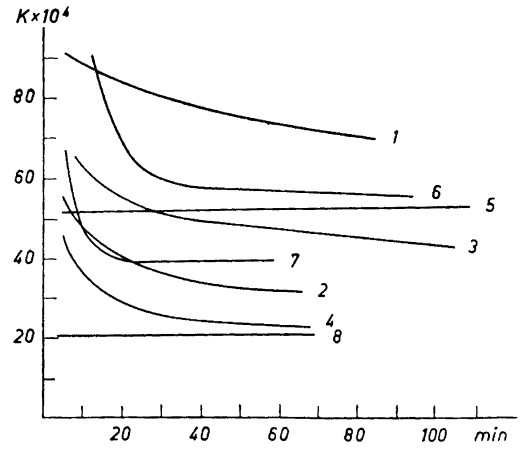


Fig. 3. *K*-values *versus* time. At time zero the input of *ca.* 50 cal of electrical energy was completed.

1) Ordinary Dewar, fitted with a heavy rubber stopper. 100 ml of water.
2) Calorimeter A. 75 ml of water.
3) Calorimeter B 2. 90 ml of water, 10^{-1} mm Hg.
4) Calorimeter B 2. 90 ml of water, 10^{-4} mm Hg.
5) Calorimeter B 1 and B 3. 90 ml of water, 10^{-1} mm Hg.
6) Calorimeter C. 90 ml of water, 10^{-1} mm Hg.
7) Calorimeter C. 110 ml of water, 10^{-1} mm Hg.
8) Calorimeter D. 100 ml of water, 10^{-4} mm Hg.

Temperature measuring system

The temperature was measured by a thermistor (Stantel F 2311/300) in a conventional D.C. bridge circuit [14]. Instead of using a high current sensitivity galvanometer together with scale and lamp outfit we have used a spotlight galvanometer (Cambridge Spot Galvanometer, 41159/2, sensitivity 0.03 μA/scale division for calorimeters A—C; Sullivan T 2001/D, sensitivity 0.005 μA/scale division for calorimeter D) together with a 40 × microscope. The sensitivity corresponds to a temperature change of the thermistor of less than 0.0001°C. The galvanometer was placed on a heavy iron plate resting on rubber knobs directly on a table. The mounting is simple and sturdy and the galvanometer is reasonably insensitive to vibrational disturbances.

The uncertainty in the determination of a temperature difference is estimated to $\pm$ 0.0002°C.

Electrical calibration system

The calibration resistance was wound from 0.15 mm lacquered and silk spun manganin wire on a thin-walled glass tube, coated with an epoxy resin and inserted into a glass or metal tube, which was then carefully sealed. The resis-

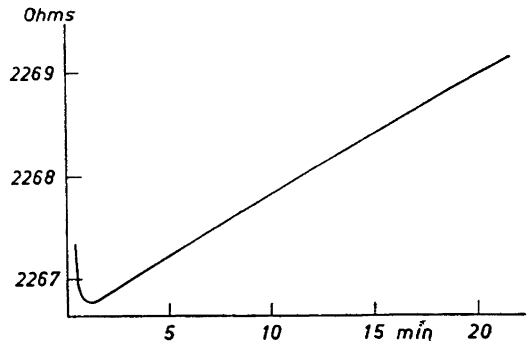


Fig. 4. Time of equilibration for calorimeter D, with water as calorimetric liquid. At time zero the input of *ca.* 50 cal of electrical energy was completed.

tance of the heater was measured between the leads to the heater at the midpoints between the calorimeter can and the outer jacket. The resistance was about 15 Ω (calorimeter B) and 65 Ω (calorimeter D). By separate experiments it was found that the resistance did not change within 0.001 Ω during a run.

The calibration was performed by the substitution method. The current from a 12 V storage battery was passed through a dummy heater, of the same resistance as the calibration resistance. When the current became constant, as found from the potential drop over a standard resistor in series with the dummy heater, the calibration was started by switching over to the calibration heater for a fixed time. The current was measured as above at regular intervals by use of a potentiometer (Leeds — Northrup Student Potentiometer, calorimeters A—C, and Rubicon High Precision Type B Potentiometer, calorimeter D).

The time during which the current was passed through the heater was chosen to equal the time of an actual experimental determination, if possible, the shortest time being *ca.* 240 sec. and the longest *ca.* 1 000 sec. The current was chosen to produce a temperature increase as near to that in an actual run as possible. The total input of electrical energy was usually kept at *ca.* 200 J. The uncertainty of the electrical calibration as calculated from the errors in the determinations of voltage, resistance and time was ±0.15 % for calorimeters A—C, and ± 0.05 % for calorimeter D.

All heat quantities are given in thermochemical calories, defined by 1 calorie = 4.1840 abs. joules.

Heat of solution measurements of potassium chloride

Mishchenko and Kaganovich [15] suggested in 1949 the solution of potassium chloride in water be used as a standard process to calibrate reaction calori-

meters. The authors critically examined the relevant literature and concluded that the best value for the integral heat of solution according to the equation

$$KCl\ (c) + 200\ H_2O\ (l) \rightarrow KCl,\ 200\ H_2O$$

is $\Delta H_{25°} = 4\,194 \pm 3$ cal.mole^{-1}.

In order to check the reliability of our calorimeters we therefore made heat of solution measurements with calorimeters B 1 and D.

A sample of pure KCl (Merck's pro analysi) was pulverized, carefully dried at 105°C for 2 h and stored in vacuum over phosphorus pentoxide. About 800 mg were weighed out, corresponding to a molar ratio KCl to H_2O of *ca.* 1 to 450. With calorimeter B 1 two sets of calibration experiments were performed (Table 1), one with pure salt and pure water (I) and the second with potassium chloride solution (II). The first set together with the dissolution experiments (Table 2) gave ΔH(soln) at 24.73°C and the second set gave ΔH(soln) at 25.17°C. In calculating the results in Table 2 the average value from Table 1 was used and the heat of solution values therefore refer to 24.95°C.

In Table 1, the first column gives the corrected logarithmic resistance ratio value (which is proportional to the temperature increase), the second column the input of electrical energy in calories and the third column the value of the heat capacity in calories per unit of $\log(R_i/R_f)$. In Table 2, the first column gives the amount of KCl (vacuum weight), the second the corrected temperature increase as above, the third column gives the heat absorbed, the forth

Table 1. Calibration experiments. Calorimeter B 1.

	$10^4 \times \log(R_i/R_f)$	q, cal.	$\varepsilon \times 10^{-2}$
I	64.085	44.046	68.73
	64.450	44.349	68.81
	64.442	44.265	68.70
	64.667	44.340	68.57
	62.114	42.699	68.74
			Mean 68.71 ± 0.03
II	64.621	44.265	68.50
	64.715	44.298	68.45
	63.680	43.614	68.49
	61.123	41.817	68.41
	63.533	43.539	68.53
			Mean 68.48 ± 0.03

Table 2. Heat of solution measurements. Calorimeter B 1.

mg KCl	$10^4 \times \log(R_i/R_f)$	q, cal.	ΔH(soln) cal.mole^{-1}	$\Delta H°$(soln) cal.mole^{-1}
816.73	66.785	45.811	4 182	4 180
829.40	67.975	46.627	4 192	4 190
770.28	63.006	43.219	4 183	4 182
766.78	62.586	42.931	4 174	4 173
645.63	52.880	36.273	4 189	4 190
				Mean 4 183 ± 3

Table 3. Calibration experiments. Calorimeter D.

$10^4 \times \log (R_i/R_f)$	q, cal.	$\varepsilon \times 10^{-4}$
52.148	47.12	90.36
53.097	48.01	90.41
54.733	49.47	90.38
53.535	48.30	90.22
54.259	48.95	90.22
		Mean 90.32 ± 0.05

Table 4. Heat of solution measurements. Calorimeter D.

mg KCl	$10^4 \times \log (R_i/R_f)$	q, cal.	ΔH(soln) cal.mole^{-1}	$\Delta H°$(soln) cal.mole^{-1}
891.78	55.416	50.05	4 184	4 187
871.10	54.041	48.81	4 177	4 180
923.33	57.299	51.69	4 173	4 177
919.06	56.974	51.46	4 174	4 179
889.80	55.378	50.02	4 191	4 184
947.72	58.901	53.20	4 185	4 189
				Mean 4 183 ± 2

column gives the uncorrected heat of solution in cal.mole^{-1} and the last column gives the heat of solution value corrected to KCl, 450 H_2O and 25.00°C. The magnitudes of the corrections were obtained from the literature [16,17].

The same series of experiments were performed with calorimeter D, although only one set of calibration experiments was taken. The results are shown in Tables 3 and 4.

The agreement shows that systematic errors are either absent or equal in the two calorimetric systems. However, the difference between our value and that recommended by Mishchenko and Kaganovich, corrected to KCl, 450 H_2O, 4 196 ± 3 cal.mole^{-1}, is more than twice the sum of the standard deviations and therefore seems to be significant [18].

The recent careful investigation by Coops *et al.*[12] gives a heat of solution value of 4 126 cal.mole^{-1} for KCl, 5 170 H_2O. Corrected [16] to KCl, 450 H_2O this value becomes 4 165 cal.mole^{-1}.

From a comparison of the results given, it is obvious that systematic errors are present in one or more of the determinations. With calorimeters of an expected accuracy of *ca.* 0.1 %, the agreement between values obtained in different laboratories for a standard reaction should definitely be better. For the following reasons we question if the solution of KCl in water is really suitable for calibration purposes:

1. ΔH(soln)/ΔT at room temperature is too high (36 cal.mole^{-1} per centigrade).

2. On a molar basis, the heat of solution of KCl is inconveniently low. It might therefore easily be influenced by impurities, crystal shape and size, and dissolved gases in the water phase.

3. When used in connection with a measured reaction having a large temperature change, a rather large amount of KCl must be used. The con-

centration of the KCl solution will be high and the differential heat of solution for concentrated solutions is high. Thus not only has the temperature to be known with an accuracy of *ca.* 0.005°C but also the concentration must be very well defined in each experiment.

Acknowledgements. This investigation has been supported by grants from *Statens Naturvetenskapliga Forskningsråd, Statens Tekniska Forskningsråd* and *Knut och Alice Wallenbergs Stiftelse.*

REFERENCES

1. Sunner, S. *Nature* **176** (1955) 217.
2. Sunner, S. and Wadsö, I. *Acta Chem. Scand.* **13** (1959). *In press.*
3. Sunner, S. and Wadsö, I. *Trans. Faraday Soc.* **53** (1957) 455.
4. Wadsö, I. *Acta Chem. Scand.* **11** (1957) 1745.
5. Sunner, S. *Acta Chem. Scand.* **11** (1957) 1757.
6. Bjellerup, L., Sunner, S. and Wadsö, I. *Acta Chem. Scand.* **11** (1957) 1761.
7. Wadsö, I. *Acta Chem. Scand.* **12** (1958) 630.
8. Wadsö, I. *Acta Chem. Scand.* **12** (1958) 635.
9. Hutchinson, E. and Manchester, K. E. *Rev. Sci. Instr.* **26** (1955) 364.
10. Westrum, E. F., Jr. and Eyring, L. *J. Am. Chem. Soc.* **74** (1952) 2045.
11. Bartell, F. E. and Suggitt, R. M. *J. Phys. Chem.* **58** (1954) 36.
12. Coops, J., Balk, A. N. and Tolk, N. W. *Rec. trav. chim.* **75** (1956) 75.
13. LaPorte, F. *Ann. Phys.* [12] **5** (1950) 5.
14. Pritchard, H. O. and Skinner, H. A. *J. Chem. Soc.* (1950) 272.
15. Mishchenko, K. P. and Kaganovich, Ya.Ya. *Zhur. Priklad. Khim.* **22** (1949) 1078; *Chem. Abstracts* **44** (1950) 921a.
16. Lange, E. and Monheim, J. *Z. physik. Chem.* **A 150** (1930) 349.
17. Popoff M. M., Chomjakoff, K. G., Feodossjeff, N. N. and Schirokich, P. K. *Z. physik. Chem.* **A 167** (1933) 29.
18. Rossini, F. D. and Deming, W. E. *J. Wash. Acad. Sci.* **24** (1939) 416.

Received September 24, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 109—114

The Crystal Structure of Ru_7B_3

BERTIL ARONSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The crystal structure of Ru_7B_3 has been determined from single crystal data. There are two formula units in the unit cell, which Åselius [14] has determined to be hexagonal with $a = 7.467$ Å and $c = 4.713$ Å. The space group is $P6_3mc$. The ruthenium atoms are situated in two six-fold positions 6 (*c*) with $x_I = 0.4563$, $z_I = 0.318$, and $x_{II} = 0.1219$, $z_{II} = 0.000$ and one two-fold position 2(*b*) with $z_{III} = 0.818$. The structure of Ru_7B_3 is very closely related to that of the trigonal chromium carbide Cr_7C_3.

Very little is known about the borides of the platinum metals. Buddery and Welch [1] have shown that a number of such borides exists and are easily prepared by direct combination of the elements. These authors have given the approximate compositions of many borides of the platinum metals and, in some cases, also have established the unit cell dimensions. Later Mooney and Welch[2] determined the crystal structure of Rh_2B from single crystal data. Rh_2B seems to be the only noble metal boride with known crystal structure and, as Geller [3] points out, it is isomorphous with Rh_2Ge[3], δ-Ni_2Si[4] and Co_2Si[5].

Since a knowledge of the platinum metal borides is very essential for a better understanding of the transition metal borides, X-ray investigations in this group of compounds have been started at this institute. The borides of ruthenium were supposed to be those most related to the well-known molybdenum and iron borides and were therefore chosen as the first object of investigation. In an investigation of the Ru-B system, Åselius [14], confirming the results of Buddery and Welch, has found that the most metal-rich intermediate phase in this system has the approximate composition Ru_2B. He was able to index the powder photograph, chosing a hexagonal unit cell with $a = 7.467$ Å, $c = 4.713$ Å and $c/a = 0.631$. The density was found to be 10.7 g/cm^3, showing that there are 14 ruthenium atoms in the unit cell and indicating that the formula should be written Ru_7B_3 rather than Ru_2B. Åselius [14] has also found the isomorphous phase Rh_7B_3 with $a = 7.47$ Å, $c = 4.78$ Å and $c/a = 0.640$.

The crystal structure of Ru_7B_3 has been determined with single crystals kindly donated by Åselius. The relation of this structure to that of the trigonal chromium carbide [6], Cr_7C_3, and to other transition metal borides will be discussed.

EXPERIMENTAL

Some small single crystals were picked out of an arc melted alloy. By use of Mo$K\alpha$-radiation, Weissenberg photographs around the a- and c-axes were taken of two crystals which were found to be free from satellites. The multiple film technique (with thin iron foils between the films) was used and the intensities were estimated visually. With the exception of a few low angle reflexions from the larger crystal (rotated around the a-axis), no serious influence from absorption or extinction could be detected. Log I_o/I_c decreased linearly with $\sin^2\Theta/\lambda^2$, and for both crystals the temperature constant B was found to be 0.42 Å^2. The $\varrho_o(xy)$ and $\varrho_c(xy)$ electron density projections were computed on the Hägg-Laurent [7] machine and with Beevers-Lipson strips. Twenty eight nonequivalent $hki0$ reflexions were used in these calculations.

DETERMINATION AND DESCRIPTION OF THE STRUCTURE

The Weissenberg photographs showed that the crystals were hexagonal. There was no evidence that the symmetry should be lower. The systematic extinctions of $hh\bar{2}\bar{h}l$ reflexions for l odd gave the possible space groups $P6_3/mmc$, $P\bar{6}2c$ and $P6_3mc$. The approximate positions of the 14 ruthenium atoms in the unit cell were deduced from the following two conditions:

(1). The ruthenium atoms cannot all have the z-parameters 0, 1/4, 1/2 and 3/4. This is a consequence of the relation $|F|_{0008} < |F|_{0002} < |F|_{0004} < |F|_{0006}$ which was found from the intensities of the $000l$-reflexions.

(2). No Ru-Ru contacts are shorter than 2.5 Å. This seems a reasonable assumption since the shortest Ru-Ru distance in pure ruthenium is 2.65 Å. In particular, this condition establishes that two ruthenium atoms cannot have the same x- and y-parameters but different z-parameters because such a situation would imply Ru-Ru contacts shorter than 2.36 Å ($c/2 = 2.357$ Å).

These two conditions eliminate all the positions of space group $P6_3/mmc$. Condition (2) rules out 12(k), 12(i), 6(g), 4(f), 4(e), 2(b) and 2(a). Condition (1) rules out any combination of 12(j), 6(h) and the remaining two-fold positions. In the same way any combination of the positions of space group $P\bar{6}2c$ violates one or both of the above conditions. Only the space group $P6_3mc$ then remains. Of the positions in this space group condition (2) eliminates 12(d) and 2(a). The only alternative remaining is to put the 14 ruthenium atoms into two six-fold positions 6(c) and one two-fold 2(b) position. An evaluation of the threedimensional Patterson function in the section $P(xy0)$ and along $P(00z)$ showed that no Ru-Ru contacts shorter than 2.7 Å exist in the basal plane or in the direction of the c-axis. This supports the correctness of condition (2). In addition, the Patterson function gave approximate x-parameters of the ruthenium atoms. Approximate z-parameters could be obtained from space considerations.

The x-parameters were refined from successive $\varrho_o(xy)$ and $\varrho_c(xy)$ syntheses until the final "backshift"-corrections were less than $\pm$ 0.001. The final x-parameters (Table 1) are estimated to be reliable to within $\pm$ 0.002. No

Table 1. Final atomic parameters of Ru_7B_3. Space group $P6_3mc$. (Estimated accuracy of x-parameters $\pm$ 0.002, of z-parameters $\pm$ 0.003.)

6 Ru_I in 6(*c*)	$x_I = 0.456_3$	$z_I = 0.318$
6 Ru_{II} in 6(*c*)	$x_{II} = 0.121_9$	$z_{II} = 0.000$
2 Ru_{III} in 2(*c*)	$x_{III} = 1/3$	$z_{III} = 0.818$
6 B in 6(*c*)	$x_B = 0.18_7$	$z_B = 0.58_0$

significant difference in the scattering parameters (number of electrons) of the different ruthenium atoms was revealed in the Fourier projections. The z-parameter of the Ru_{II} atom (Table 1) was chosen to be zero. The z-parameter of the Ru_I atom was obtained by comparison between calculated and observed $|F|$ values of $3n0\bar{3}nl$ reflexions ($l = 5, 7, 9$), the contribution of the Ru_{III} atom to these reflexions being zero. In the same way the z-parameter of the latter was obtained from inspection of $20\bar{2}l$ reflexions ($l = 5, 7, 9$) since the contribution of the Ru_I atom to the intensities of these reflexions was found to be very small. A variation of $\pm$ 0.001 in the z-parameters changed the $|F|_{calc}$ values by 1—5 %, and the final z-parameters are estimated to be reliable to within $\pm$ 0.003. With the final atomic parameters given in Table 1, the reliability index of the observed 28 nonequivalent $hki0$ reflexions was found to be 8.2 % while that of the observed 64 nonequivalent $h0\bar{h}l$ reflexions was 11.8 %.

As seen from Fig. 1 the ruthenium skeleton of Ru_7B_3 is fairly densely packed and is built up mainly of metal tetrahedra, some of which are outlined in the figure. Besides the tetrahedra there are metal octahedra with their centres in 0, 0, 1/4 and 0, 0, 3/4 and sharing faces in the c-direction. Between

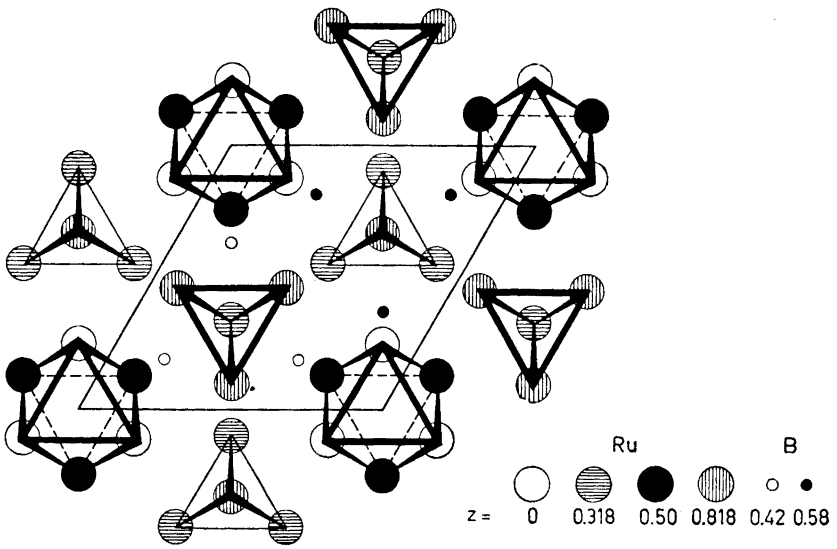


Fig. 1. The structure of Ru_7B_3 as seen along the hexagonal axis.

the octahedra and the tetrahedra there are two types of 'holes' whose centres in the mirror planes correspond to positions 6(*c*). The larger 'hole' has a roughly trigonal prismatic environment and the parameters of its centre are $x = 0.20$; $z = 0.58$. The smaller hole is more irregular and the parameters of its 'centre' are $x \sim 0.20$; $z \sim 0.42$. In addition to these two types of holes only the octahedral holes of the metal skeleton can possibly accomodate the boron atoms. (The tetrahedral interstices are much too small for a boron atom.)

The difference ($F_o - F_c$) synthesis (only the contribution from the ruthenium atoms was included in the F_c terms) was carried out in the *xy*-projection. No maximum was found in the origin, indicating that there are no boron atoms in the octahedral holes. The highest maximum of the difference synthesis (amounting to 5 % of the ruthenium maxima of the F_o synthesis) had the coordinates $x \sim 0.17$; $y \sim 0.83$, which indicates that one of the remaining 'holes' did contain boron atoms. An attempt has been made to obtain some experimental evidence showing which of the two holes is actually occupied. Since the x_B-parameter is about 0.20, the $50\bar{5}l$ ($l = 2, 4$) are the $h0\bar{h}l$ reflexions that are most likely to give some information about the *z*-parameter of the boron atom. Fortunately, these two reflexions are fairly weak and therefore a considerable influence of the boron atoms on their intensities can be anticipated. The $|F_o - F_c|/|F_o|$ values for the $50\bar{5}2$ and $50\bar{5}4$ reflexions are given below. The value of $|F_c|$ has been computed for the two alternative boron positions and also for the situation when the contribution from boron is neglected.

$|F_o - F_c|/|F_o|$ values for the $50\bar{5}2$ and $50\bar{5}4$ reflexions

	neglecting boron	with 6 boron atoms in 6(*c*) and $x_B = 0.20$, $z_B = 0.58$; $x_B = 0.20$, $z_B = 0.42$	
$50\bar{5}2$	0.21	0.11	0.31
$50\bar{5}4$	0.16	0.11	0.27

As seen from the table, a z_B value of 0.58 is strongly preferred over the value of 0.42. In fact, with $x_B = 0.20$ the only z_B parameters which will improve the agreement between observed and calculated structure factors for the above reflexions are $z_B \sim 0.1$ and $z_B \sim 0.6$, and the first of these is impossible since it would give a Ru-B distance of about 1 Å.

Thus, there is considerable experimental evidence that the boron atoms are situated in the largest hole of the ruthenium skeleton, corresponding to a position 6(*c*) with $x_B \sim 0.20$ and $z_B \sim 0.58$. Since the experimental material does not permit an accurate determination of the boron positions, the coordinates of the centre of the hole have been chosen as boron parameters. Aside from the experimental evidence, it is very unlikely from space considerations that boron atoms can occupy the 'smaller' holes of the metal skeleton since this would involve Ru-B distances shorter than 2.0 Å.

The final atomic parameters are given in Table 1. The interatomic distances (< 3.5 Å) are given in Table 2. A projection of the structure along the *c*-axis is shown in Fig. 1.

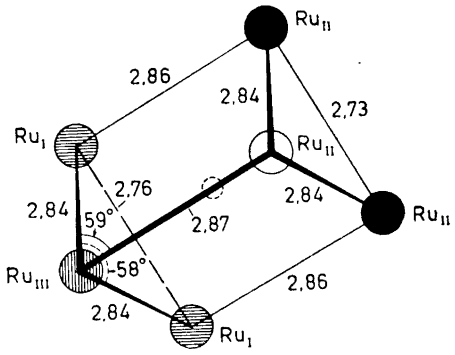


Fig. 2. The environment of the boron atoms in Ru_7B_3 as seen along the hexagonal axis.

DISCUSSION

The Ru-Ru contacts (Table 2) are on the average only 0.10 Å longer in Ru_7B_3 than in ruthenium where the average interatomic distance is 2.68 Å. There are also contacts shorter than those in pure ruthenium. The Ru_I atoms form zig-zag rows in the *c*-direction with an intermetallic distance of 2.61 Å. The boron atom has its six closest neighbours at an average distance of 2.16 Å. (With $r_{Ru} = 1.34$ Å, r_B will be 0.82 Å.) This value is rather small and is in accordance with the Me-B distances found in MoB (B-6 Mo 2.23 Å) [8] and in the recently discovered borides of the cementite (Fe_3C) type [9], *e.g.* in Ni_3B (B-6 Ni 2.04 Å). In these two phases the boron atoms have a trigonal prismatic environment as in Ru_7B_3. In Mo_2B[8] and Rh_2B[2] where the boron atoms have more than six close neighbours, the Me-B distances are considerably larger. In Mo_2B the boron atoms have eight metal neighbours at 2.37 Å; in Rh_2B nine at distances between 2.38 Å and 2.63 Å with an average value of 2.50 Å.

The most remarkable feature of the structure of Ru_7B_3 is its close similarity to that of the trigonal chromium carbide [6], Cr_7C_3. The formula and the unit cell of this carbide were determined by Westgren and Phragmén [10], who found the unit cell to be hexagonal with $a = 13{,}87$ kX, $c = 4.53$ kX and $2c/a = 0.654$. Later Westgren *et al.*[11] found a small deviation from the hexagonal symmetry and concluded that Cr_7C_3 was indeed trigonal. In a more recent paper on this

Table 2. Interatomic distances (in Å) in Ru_7B_3. (Estimated accuracy ± 0.03 Å.)

		Average
Ru_I	— 11 Ru 2.61(2); 2.72; 2.76(2); 2.81(2); 2.84(2); 2.86(2).	2.77 Å.
	— 4 B 2.15(2); 2.66(2).	
Ru_{II}	— 11 Ru 2.73(2); 2.81(2); 2.84(4); 2.86(2); 2.87.	2.82 Å.
	— 4 B 2.15; 2.16(2); 2.86.	
Ru_{III}	— 12 Ru 2.72(3); 2.84(6); 2.87(3).	2.78 Å.
	— 3 B 2.20(3).	
B	— 9 Ru 2.15; 2.15(2); 2.16(2); 2.20; 2.66(2); 2.86.	2.16 Å.
	— 2 B 3.22(2).	(six first)

phase, Westgren [6] gave its approximate crystal structure. As *hki*0 reflexions appeared only when h was even and k was even, a projection on the basal plane could be divided into four identical parts. The atomic arrangements projected on such a part were determined by Westgren. Using the space group $P6_3mc$ he found 6 Cr_I in 6(c) with $x_I = 0.455$, 6 Cr_{II} in 6(c) with $x_{II} = 0.125$ and 2 Cr in 2(b). The chromium parameters of Westgren agree within 0.001 and 0.003 with those reported here for the ruthenium atoms of Ru_7B_3. Westgren did not try to determine the z-parameters but he gave a description of the complete structure using the hexagonal space group $P6_3mc$. In particular, he pointed out the trigonal prismatic environment of the carbon atoms. It is evident from Westgren's proposed structure that the near environment of the atoms in Cr_7C_3 and Ru_7B_3 is practically the same. The structure of Cr_7C_3 can be described as a slightly distorted superstructure of Ru_7B_3, making the unit cell four times larger and causing the symmetry to decrease to trigonal.

Ru_7B_3 is another example of a boride with close similarities to the 'complicated' carbides of chromium, manganese and iron. Other recently discovered examples are Co_3B and Ni_3B[9] which crystallize in the cementite (Fe_3C) structure. It has also been found that boron and carbon to a large extent can substitute for each other in the 'complicated' carbide structures, *e.g.* in $Fe_3(C, B)$[12] and in $Fe_{23}(C, B)_6$[13]. It does not seem unlikely that further studies on the platinum metal borides will reveal additional similarities between borides and carbides.

Acknowledgements. The research described in this paper was sponsored by the *European Commission of Air Research and Development Command* under Contract No. AF 61(052)—40. The financial support from this authority is gratefully acknowledged.

The author wishes to thank professor G. Hägg for his kind interest. Many thanks are also due to Dr S. Rundqvist for valuable suggestions and to Mr J. Åselius who has permitted me to quote unpublished results.

REFERENCES

1. Buddery, J. H. and Welch, A. J. E. *Nature* **167** (1951) 362.
2. Mooney, R. W. and Welch, A. J. E. *Acta Cryst.* **7** (1954) 49.
3. Geller, S. *Ibid.* **8** (1955) 15.
4. Toman, K. *Ibid.* **5** (1952) 329.
5. Geller, S. *Ibid.* **8** (1955) 83.
6. Westgren, A. *Jernkontorets Ann.* **119** (1935) 231.
7. Hägg, G. and Laurent, T. *J. Sci. Instr.* **23** (1956) 155.
8. Kiessling, R. *Acta Chem. Scand.* **1** (1947) 893.
9. Rundqvist, S. *Ibid.* **12** (1958) 658.
10. Westgren, A. and Phragmén, G. *Kgl. Svenska Vetenskapsakad. Handl.* **III:2** (1926) No. 5.
11. Westgren, A., Phragmén, G. and Negresco, T. *J. Iron Steel Inst.* **117** (1928) 386.
12. Nicholson, M. E. *J. Metals* **9** (1957) 1.
13. Carrol, K. G., Darken, L. S., Filer, E. W. and Zweel, L. *Nature* **174** (1954) 978.
14. Åselius, J. *Private communication.*

Received October 6, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 115—119

Chemistry of Arylboric Acids IX. Reaction of Arylboric and Alkylboric Acids with Mercuric Halides *

KURT TORSSELL

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

The reaction between mercuric salts and arylboric acids is an electrophilic displacement reaction. Electron attracting substituents hamper the boron-mercury exchange whereas electron releasing substituents facilitate the reaction. Most arylboric acids react readily in aqueous solution; unreactive arylboric acids are rapidly converted in excellent yield to the corresponding mercury compound in glacial acetic acid medium with perchloric acid as catalyst. Alkylboric acids are converted in acetic acid medium to alkylmercuric salts without any catalyst.

According to Michaelis [1] phenylboric acid is converted to phenylmercuric chloride by aqueous mercuric chloride:

$$C_6H_5B(OH)_2 + HgCl_2 \longrightarrow C_6H_5HgCl + HCl + H_3BO_3$$

The mercuration reaction has been proved to proceed quantitatively and has been used for analytical purposes [2,3]. Diarylboric acids,[4,5] triphenylboron [2] and lithium tetraphenylboron [2] react in the same manner:

$$(C_6H_5)_2BOH + 2HgCl_2 \longrightarrow 2C_6H_5HgCl + 2HCl + H_3BO_3$$
$$(C_6H_5)_3B + 3HgCl_2 \longrightarrow 3C_6H_5HgCl + 3HCl + H_3BO_3$$
$$(C_6H_5)_4BLi + 4HgCl_2 \longrightarrow 4C_6H_5HgCl + 3HCl + H_3BO_3 + LiCl$$

From lithium bis-(*o,o'*-diphenyl)-boron Wittig and Herwig [6] obtained *o,o'*-bis(chloromercuri) diphenyl in a yield of 51 %. Alkylboric acids react slowly [7] or not at all [8] with mercuric halides.

In alkaline solution diphenylmercury is obtained [7] and according to Nesmejanow *et al.*[9] asymmetrical diarylmercurials can be prepared by reacting arylmercuric halides with arylboric acid containing a different arylgroup:

$$ArHgX + Ar'B(OH)_2 \xrightarrow{NaOH} ArHgAr' + H_3BO_3$$

* Part VIII. *Acta Chem. Scand.* 12 (1958) 1373.

Table 1. Conversion of arylboric acids into arylmercuric halides by treatment with mercuric halides in aqueous solution. PhBA = Phenylboric acid.

	Arylboric Acid	Arylmercuric halide		Yield, crude product, %	M.p., °C	M. p. Lit., °C	Analysis			
		ArHgX	Mol. wt.				Calc C %	Calc H %	Found C %	Found H %
1	PhBA	PhHgCl	313.2	74	257—8	258 [a]	—	—	—	—
2	PhBA	PhHgBr	357.6	42	282—3	280 [b]	—	—	—	—
3	4-OCH_3-PhBA	4-OCH_3-PhHgCl	343.2	93	249—50	239 [c]	24.50	2.06	24.38	1.97
4	4-OCH_3-PhBA	4-OCH_3-PhHgBr	387.7	62	255—6	243—4 [d]	21.69	1.82	22.13	1.74
5	α-NaphthylBA	α-NaphthylHgCl	363.3	95	190—1	191 [a]	—	—	—	—
6	α-NaphthylBA	α-NaphthylHgBr	407.7	50	203—4	202 [b]	—	—	—	—
7	2-CH_2OH-PhBA	2-CH_2OH-PhHgCl	343.2	59	135—6	—	24.50	2.06	24.16	1.83
8	2-CHO-PhBA	2-CHO-PhHgCl	341.2	88	176—7	—	24.64	1.48	24.81	1.46
9	2-CH_3-3.5diNO_2-PhBA	2-CH_3-3.5diNO_2-PhHgCl	417.2	32 [e]	253—4,d.	—	20.15	1.21	19.88	1.00
10	3-COOH-PhBA	3-COOH-PhHgCl	357.2	—	268—9,d.	—	23.53	1.41	23.82	1.63
11	2-NO_2-4-NH_2-PhBA	2-NO_2-4-NH_2-PhHgCl	373.2	88	255—6,d.	—	19.31	1.35	19.69	1.16
12	3-NO_2-5-NH_2-PhBA	3-NO_2-5-NH_2-PhHgCl	373.2	40	273—5,d.	—	19.31	1.35	19.78	1.26
13	2-NO_2-4-$COOCH_3$-PhBA	2-NO_2-4-$COOCH_3$-PhHgCl	416.2	0	—	—	—	—	—	—
14	3-NO_2-5-$COOCH_3$-PhBA	3-NO_2-5-$COOCH_3$-PhHgCl	416.2	trace	—	—	—	—	—	—
15	*iso*-Butylboric acid	*iso*-butyl-HgBr	337.6	0	—	—	—	—	—	—
16	2-NO_2-4-$COOCH_3$-PhBa [f]	2-No_2-4-$COOCH_3$-PhHgCl	416.2	97	172—3	—	23.09	1.45	23.00	1.40
17	3-NO_2-5-$COOCH_3$-PhBA [f]	3-NO_2-5-$COOCH_3$-PhHgCl	416.2	92	247—9	—	23.09	1.45	23.17	1.53
18	*iso*-Butylboric acid [g]	*iso*-butyl-HgBr	337.6	50	54—5	55.5 [h]	—	—	—	—
19	*n*-Octylboric acid [g]	*n*-octyl-HgCl	349.3	81	115—6	115—5.5 [i]	—	—	—	—

[a]. Nesmejanov, A. N. *Ber.* **62** (1929) 1011. [b]. Slotta, K. H. and Jakobi, K. R. *J. prakt. Chem.* (2) **120** (1929)249. [c]. Michaelis, A. and Rabinerson, F. *Ber.* **23** (1890) 2342. [d]. Challenger, F. and Miller, S. A. *J. Chem. Socc.* **1938** 894. [e]. 56% of the starting material recovered. [f]. The reaction was carried out in glacial acetic acid with perchloric acid as catalyst. [g]. The reaction was carried out in glacial acetic acid without catalyst. [h]. Marvel, C. S., Ganerke, C. G. and Hill, E. L. *J. Am. Chem. Soc.* **47** (1925) 3009. [i]. Whitmore, F. C. and Bernstein, H. *Ibid.* **60** (1938) 2626.

Johnson *et al.*[10,11] have used the reaction between arylboric acids and mercuric chloride for the structural determination of nitroderivatives obtained by nitration of arylboric acids. The corresponding mercury derivatives were formed in good yields.

In a series of papers, Torssell *et al.*[12–15] have shown that a number of reactions and rearrangements can be performed with arylboric acids, even under drastic conditions, without splitting off the dihydroxyboron group (borono group). The availability of new arylboric acids and their apparent ready conversion to mercury compounds by simple treatment with an aqueous mercury halide might therefore render this mercuration reaction of value as a preparative method.

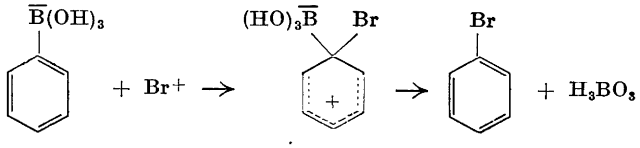
There seems to be one limitation. Arylboric acids containing many electron attracting substituents *e.g.* $-NO_2$, -COOR, react very slowly or not at all in aqueous solution. The unreactivity of certain compounds can, however, be easily overcome by using glacial acetic acid as medium and perchloric acid as catalyst. Klapproth and Westheimer [16] had found previously that the rate of mercuration of aromatic compounds increases with the acid concentration.

When, *e.g.*, 2-nitro-4-carbomethoxy-phenylboric acid, which is unaffected by aqueous mercuric chloride, is reacted with the mercuric halide in glacial acetic acid to which a small amount of perchloric acid is added, the corresponding mercuric organic compound is formed practically instantaneously in good yield. (Table 1, numbers 16 and 17.)

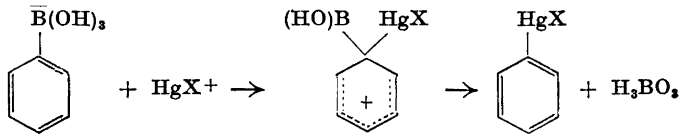
Mercuration of the aromatic nucleus is recognized as an electrophilic attack by positively charged mercuric ions [16]. Substituents increasing the electron density of the nucleus therefore facilitate the reaction, whereas substituents withdrawing electrons from the nucleus have the opposite effect.

The mercury-boron exchange seems to follow the same scheme. If mercuric chloride is added to a cold aqueous phenylboric acid solution, the mercury compound will be formed and precipitate immediately whereas a *m*-nitrophenylboric acid solution must be heated for a short time before precipitation occurs. From Table 1 it can be seen that 2-nitro-4-carbomethoxyphenylboric acid does not react and 3-nitro-5-carbomethoxyphenylboric acid gives only traces of the mercury compound. When 2-methyl-3,5-dinitrophenylboric acid is reacted with mercuric chloride for 20 min, only a part of the boron compound yields the mercury derivative and another part can be recovered as unreacted material. The ring carbon atom, to which the boron atom is attached, is electron deficient in these compounds. The mononitro [10] and monocarboxy derivatives [4], however, give the corresponding mercury compounds in good yield, which is also the case with all the other compounds tested in the present study.

Kuivila and Hendrickson [17] have shown that the bromolysis of arylboric acids is an electrophilic substitution:



The mercuration can be formulated in the same way:



The unreactivity of alkylboric acids towards mercuric salts has been reconfirmed. When *iso*-butylboric acid, for example, is heated with mercuric chloride in aqueous solution for 30 min no mercury organic compound can be detected. Part of the *iso*-butylboric acid decomposes into boric acid and butanol during the heating, a reaction that also takes place when no mercuric salt is present.

However, the corresponding alkylmercuric halide is formed in good yields when the reaction is carried out in acetic acid medium. No catalyst is necessary. Prolonged heating or presence of perchloric acid causes formation of yellow colored products difficultly soluble in ordinary solvents (M.p. > 260°).

EXPERIMENTAL

General procedure for converting an arylboric acid into the corresponding arylmercuric halide compound. The arylboric acid is dissolved in hot water. For compounds difficultly soluble in water a mixture of ethyl alcohol and water, 1:1, is employed. A sufficient amount of solvent is thereby used to ensure that no arylboric acid precipitates when the aqueous mercuric chloride is added. Mercuric chloride (25 % excess) is dissolved in warm water and added to the arylboric acid solution. Usually the arylmercuric halide precipitates after a few minutes or less. In order to ensure complete conversion, the mixture is boiled for *ca.* 15 min and left standing overnight in a refrigerator. The precipitate is filtered, washed with water and dried.

Conversion of less reactive arylboric acids into arylmercuric halide in glacial acetic acid medium with perchloric acid as catalyst. 3-Nitro-5-carbomethoxy-phenylboric acid, 0.1 g, is dissolved in 3 ml of glacial acetic acid at 80—90°; mercuric acetate, 0.16 g, is added and then 60 % perchloric acid, 0.05 ml. After 2 min at this temperature, the solution is poured into 15 ml of water containing potassium chloride, 0.2 g. A white precipitate of 3-nitro-5-carbomethoxyphenylmercuric chloride is formed immediately. Yield 97 %, m. p. 247—249°, from ethyl alcohol.

Conversion of alkylboric acids into alkylmercuric halides. n-Octylboric acid,[18] 0.10 g, is dissolved in 2 ml of cold glacial acetic acid. Mercuric acetate, 0.26 g, is added and the solution is warmed to 90—100° for 4—5 min whereafter it is poured into 12 ml water containing sodium chloride, 0.1 g. *n*-Octylmercuric chloride precipitates immediately, 0.18 g or 81 %, m. p. 115—116°.

iso-Butylmercuric bromide is prepared in the same way. Yield 50 %, m. p. 54—5°.

This work was supported by funds from *Beskowska Fonden* which are gratefully acknowledged.

REFERENCES

1. Michaelis, A. and Becker, P. *Ber.* **15** (1882) 180.
2. Wittig, G., Keicher, G., Rückert, A. and Raff, P. *Ann.* **563** (1949) 110.
3. Flaschka, H., Amin, A. M. and Holasek, A. *Z. anal. Chem.* **138** (1953) 241.
4. König, W. and Scharrnbeck, W. *J. prakt. chem.* (2) **128** (1930) 153.
5. Torssell, K. *Acta Chem. Scand.* **9** (1955) 239.

6. Wittig, G. and Herwig, W. *Ber.* **88** (1955) 962.
7. Challenger, F. and Richards, O. V. *J. Chem. Soc.* **1934** 405.
8. Snyder, H. R., Kuck, J. A. and Johnson, J. R. *J. Am. Chem. Soc.* **60** (1938) 105.
9. Freidlina, R. Ch., Nesmejanov, A. N. and Kozeschkow, K. A. *Ber.* **68** (1935) 565.
10. Seaman, W. and Johnson, J. R. *J. Am. Chem. Soc.* **53** (1931) 711.
11. Bean, F. R. and Johnson, J. R. *Ibid.* **54** (1932) 4415.
12. Torssell, K. *Arkiv Kemi* **10** (1957) 473.
13. Torssell, K., Meyer, H. and Zacharias, B. *Ibid.* **10** (1957) 497.
14. Torssell, K. *Ibid.* **10** (1957) 507.
15. Torssell, K. *Ibid.* **10** (1957) 513.
16. Klapproth, W. J. and Westheimer, F. H. *J. Am. Chem. Soc.* **72** (1950) 4461.
17. Kuivila, H. G. and Hendrickson, A. R. *J. Am. Chem. Soc.* **74** (1952) 5068.
18. Torssell, K. and Larsson, N. V. *Acta Chem. Scand.* **11** (1957) 704.

Received September 29, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 120—126

The Kinetics of Ox Liver Arylsulphatase A

S. O. ANDERSEN

Universitetets Fysisk-Kemiske Institut, Copenhagen, Denmark

The hydrolysis of 2-hydroxy-5-nitrophenyl sulphate catalyzed by ox liver arylsulphatase A was investigated by continued automatic titration of the liberated acid. It has been possible to keep the concentration of the substrate constant during an experiment, and the liberated sulphate has been precipitated as $BaSO_4$. It is shown that steady state is only reached after the course of more than one hour. A reaction mechanism is proposed involving slow, reversible inactivation of the enzyme.

Of the three arylsulphatases present in mammalian liver, arylsulphatase A has been found to exhibit anomalous kinetics when hydrolysing 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) [1–3]. The investigations reported in this paper have been performed with another technique than in the above mentioned investigations, in the hope that it would be possible to clarify some of the problems.

EXPERIMENTAL

Dipotassium nitrocatechol sulphate free from nitropyrogallol disulphate was prepared according to the method of Roy [4] and Dodgson and Spencer [5]. The equivalent weight determined by complete enzymic hydrolysis in a pH-state was found to be 347 (theoretical value for the dihydrate 347.3). A stock solution of nitrocatechol sulphate being 0.03 M and having pH 5.0 was prepared and stored in the refrigerator until use.

The enzyme was prepared from ox liver according to the method of Roy [2], with a few modifications. The acetone dried liver powder was extracted for 4 h with 0.1 M KCl at room temperature. After having performed the two fractionations with acetone the enzyme extract was heated at 65°C for 5 min and then centrifuged. The sulphatase activity was not affected by this procedure. The clear colourless supernatant was used without further purifications in the experiments described in this paper. It showed an enzyme activity towards nitrocatechol sulphate at about 10 000 units/mg N (one unit is defined as the amount of enzyme which liberates 1 μg of nitrocatechol from a 0.003 M nitrocatechol sulphate solution at pH 5.0 and 37°C in one hour [2]).

A diluted enzyme preparation was incubated at 20°C and pH 5.0. At suitable intervals samples were taken for determination of the enzyme activity. It was found that under these conditions the enzyme was stable for at least 48 h.

The hydrolysis of the nitrocatechol sulphate was followed by automatic titration of the liberated sulphuric acid in a pH-state. All experiments were performed at pH 5.0 and 20.0°C. A suitable amount of the enzyme preparation was diluted with so much 0.1 M

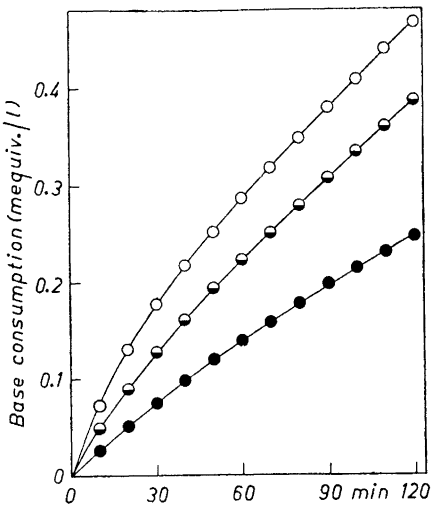


Fig. 1. Effect of addition of potassium sulphate on the enzymic hydrolysis of nitrocatechol sulphate. O—O—O: without sulphate, ◒—◒—◒: 0.3 mM K_2SO_4, ●—●—●: 1.5 mM K_2SO_4.

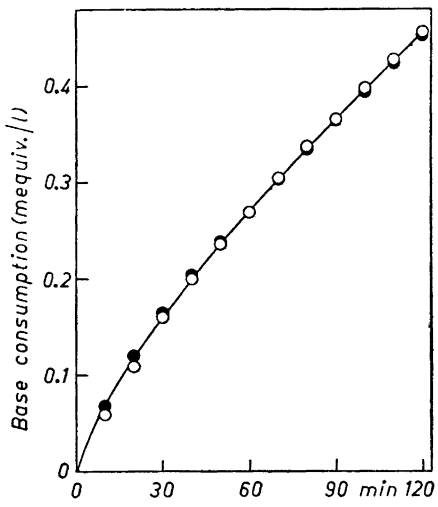


Fig. 2. Effect of addition of nitrocatechol on the enzymic hydrolysis of nitrocatechol sulphate. O—O—O: without nitrocatechol, ●—●—●: 3 mM nitrocatechol.

KCl that a final volume of 30 ml was obtained, and the pH was adjusted to 5.0. The reaction was started by adding the desired amount of substrate solution. The amount of base necessary to keep the pH constant was added automatically from a syringe, and corresponding values of time and amount of base added were read. The small dilution of the reaction mixture caused by this addition of base has not been considered in the calculations as it only amounts to a few per cent.

Roy[6] and Dodgson and Spencer[1] has shown that the reaction is influenced by sulphate ions. To overcome this complication the equivalent amount of $BaCl_2$ was added to the base in the syringe, so that the liberated sulphate would be precipitated as $BaSO_4$. It was found necessary to use exactly the equivalent amount of $BaCl_2$, as barium ions in excess inhibit the enzymic reaction. Before an experiment was started, a small amount of $BaSO_4$ was added to the reaction mixture to avoid supersaturation.

In most of the experiments reported the substrate concentration was kept constant. This was done by titrating with a mixture of equivalent amounts of base and substrate. The substrate is sufficiently stable in alkaline solution at room temperature to make such a procedure possible.

RESULTS

Experiments with added sulphate or nitrocatechol show that sulphate in a concentration of 0.0003 M inhibits the reaction (Fig. 1), and that nitrocatechol in a concentration of 0.003 M has very little influence on the reaction velocity (Fig. 2).

Experiments, where the substrate concentration was kept constant and where the liberated sulphate was precipitated as $BaSO_4$, showed that the reaction velocity decreased to about half the initial velocity during the first hour

Table 1. Measured (ξ_{exp}) and calculated (ξ_{calc}) values of the amount of substrate split at the time t. ξ_{exp} and ξ_{calc} are expressed in 10^{-6} M. E_{rel} is the relative enzyme concentration.

t	$E_{rel} = 1$		$E_{rel} = 1.7$		$E_{rel} = 3$		$E_{rel} = 4$		$E_{rel} = 6$	
min	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}
10	17	16	26	27	44	46	61	62	92	91
20	29	28	48	48	84	84	113	113	167	165
30	38	39	65	67	116	117	158	157	228	228
40	52	49	84	83	145	145	195	195	288	284
50	61	58	98	99	171	171	231	230	338	335
60	67	66	112	112	197	195	263	263	379	382
70	75	73	125	126	220	218	293	294	424	426
80	82	81	139	138	241	240	325	324	466	469
90	89	88	150	151	261	262	351	352	507	511
100	96	95	162	163	283	283	381	382	550	552
110	101	102	175	175	303	303	409	410	591	592
120	108	109	187	187	324	324	436	437	633	633

of the experiment, after which the velocity was constant for at least seven hours.

Incubation of the diluted enzyme for one hour at 20°C and pH 5.0 before starting the experiment has no inluence on the reaction progress. This indicates that the substrate is among the factors involved in the initial decrease of the reaction velocity.

Increasing the substrate concentration in an experiment when the reaction velocity had been constant for some time, showed that the velocity increased suddenly and then decreased slowly again for about one hour, when it again became constant at a higher level than before.

Experiments were performed at five different enzyme concentrations, the substrate concentrations being kept constant at 0.002 M nitrocatechol sulphate. The liberated sulphate was precipitated as $BaSO_4$. It was found that

Table 2. The absolute and relative values of the parameters A, B and C used to calculate the values of ξ_{calc} in Table 1.

E_{rel}	A 10^{-6} mole × l^{-1} min^{-1}	B 10^{-6} mole × l^{-1}	C min^{-1}	A_{rel}	B_{rel}	C_{rel}
1	0.684	27.4	0.038	1	1	1
1.7	1.170	46.8	0.038	1.71	1.71	1
3	2.03	81.9	0.038	2.97	2.99	1
4	2.74	109.5	0.038	4.01	4.00	1
6	3.94	162.0	0.038	5.76	5.91	1

Table 3. Measured (ξ_{exp}) and calculated (ξ_{calc}) values of the amount of substrate split at the time t. ξ_{exp} and ξ_{calc} are expressed in 10^{-6} M. a is the substrate concentration.

t min	a = 0.25 mM		a = 0.50 mM		a = 1.00 mM		a = 2.00 mM		a = 3.00 mM	
	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}
10	16	15	23	23	40	40	55	55	63	62
20	31	29	44	44	74	74	102	100	110	109
30	44	43	63	63	105	105	139	138	148	148
40	54	55	81	81	132	132	172	171	182	181
50	65	67	98	98	157	156	201	201	209	211
60	77	78	114	114	180	180	228	228	236	238
70	90	90	130	130	202	202	254	255	264	265
80	101	101	145	145	223	223	278	280	290	291
90	112	111	160	159	243	243	304	304	316	316
100	122	121	175	174	264	264	328	328	340	341
110	132	132	189	188	283	284	353	352	367	366
120	141	142	204	203	303	304	378	376	392	390

under these circumstances the progress of the reaction could be described by the expression:

$$\xi = At + B(1 - e^{-Ct})$$

where ξ is the amount of acid liberated at the time t, and A, B and C are parameters, the values of which were determined to give the best description of the reaction progress. The results are given in Tables 1 and 2. It is seen that A and B are proportional to, and C independent of the enzyme concentration.

Experiments were performed at five different substrate concentrations, and with the same enzyme concentration. The substrate concentrations were kept constant in the individual experiments, and the liberated sulphate was precipitated as $BaSO_4$. In these experiments too the reaction progress could be described by the expression:

$$\xi = At + B(1 - e^{-Ct})$$

as seen in Table 3. The values of the parameters A, B and C are given in Table 4.

Table 4. The values of the parameters A, B and C used to calculate the values of ξ_{calc} in Table 3.

a mM	A	B	C
	10^{-6} mole × l^{-1} × min^{-1}	10^{-6} mole × l^{-1}	min^{-1}
0.25	0.972	27.8	0.022
0.50	1.37	40.5	0.026
1.00	1.91	76.0	0.032
2.00	2.29	101.2	0.038
3.00	2.43	98.7	0.048

REACTION MECHANISM

To explain the results presented in this paper, it has been assumed that the active form of the enzyme can be transformed into an inactive form. This transformation proceeds slowly to an equilibrium, and the substrate seems to be necessary for this transformation.

The suggested reaction scheme can be represented in the following way:

$$\begin{aligned} X_1 + A &\rightleftharpoons X_2 && (\pm 1)\\ X_2 &\rightarrow X_1 + P && (+ 2)\\ X_2 &\rightleftharpoons X_3 && (\pm 3) \end{aligned}$$

where X_1, X_2 and X_3 are different forms of the enzyme, A is the substrate and P is the reaction products.

The two first reactions are the usual Henri-Michaelis scheme, and reaction ($\pm$ 3) is the slow, reversible inactivation of the enzyme here postulated.

If we assume steady state at any moment with respect to the two first reactions, we have

$$x_1 = (K_m/a)x_2 \tag{4}$$

where K_m is $(k_2 + k_{-1})/k_1$. Small letters are used to denote concentrations, and k_i is the velocity constant for reaction i.

Further we have for the total enzyme concentration

$$E = x_1 + x_2 + x_3 \tag{5}$$

and that

$$\mathrm{d}x_3/\mathrm{d}t = k_3x_2 - k_{-3}x_3 \tag{6}$$

From (4) and (5) we get

$$x_3 = E - (K_m/a + 1)x_2$$

and this together with (6) gives, if a is kept constant,

$$-(K_m/a + 1)\mathrm{d}x_2/\mathrm{d}t = (k_3 + k_{-3}(K_m/a + 1))x_2 - k_{-3}E \tag{7}$$

This equation can be solved with respect to x_2, and if we assume that the concentration of x_3 is zero at the beginning of the experiment, we get

$$x_2 = c_1 + c_2\mathrm{e}^{-\lambda t} \tag{8}$$

where $c_1 = k_{-3}E/(k_3 + k_{-3}(K_m/a + 1))$

and $c_2 = k_3E/(k_3 + k_{-3}(K_m/a + 1))(K_m/a + 1)$

and $\lambda = (k_3 + k_{-3}(K_m/a + 1))/(K_m/a + 1)$

The velocity of the reaction is

$$v = \mathrm{d}\xi/\mathrm{d}t = k_2x_2$$

hence

$$\xi/k_2 = c_1t + \frac{c_2}{\lambda}(1 - \mathrm{e}^{-\lambda t}) \tag{9}$$

This expression is of the same form as that determined from the results of the experiments:

$$\xi = At + B(1 - \mathrm{e}^{-Ct})$$

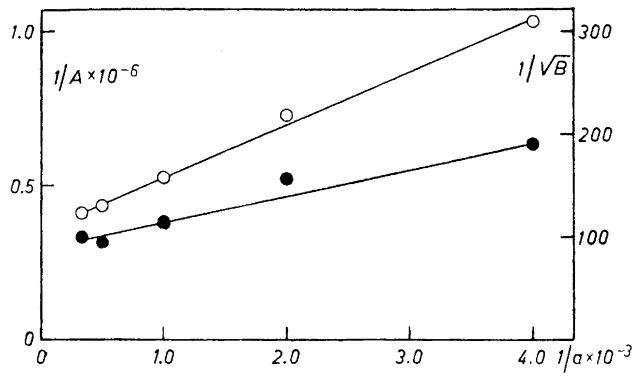


Fig. 3. The calculated values of 1/A and $1/\sqrt{B}$ plotted against the reciprocal substrate concentration.

In is seen that c_1 and c_2/λ are proportional to and λ is independent of the enzyme concentration, which is in accordance with the experiments. Moreover it can be found that there is a rectilinear relationship between $1/c_1$ and $1/a$ and between $\sqrt{\lambda/c_2}$ and $1/a$. In Fig. 3 the experimentally determined values of 1/A and $1/\sqrt{B}$ are plotted against $1/a$.

DISCUSSION

The experiments reported in this paper show that even when the substrate concentration is kept constant, and the liberated sulphate is precipitated as $BaSO_4$, the reaction velocity only reaches a constant value more than one hour after the beginning of the experiment. As it has been shown that the liberated nitrocatechol has only little influence on the reaction velocity, the cause of this phenomenon must be sought in the enzyme. A reaction mechanism has been proposed and shown to fit the experiments. The special feature of this reaction mechanism is a slow reversible inactivation of the enzyme, in which

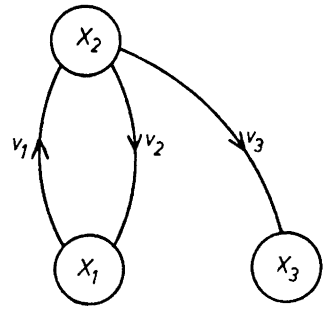


Fig. 4.

the substrate is involved. The reaction scheme can be expressed in the notation proposed by Christiansen [7,8] as in Fig. 4, where the circles symbolize the different enzyme forms.

It must be noted that there are some differences between the results obtained in other laboratories and those here reported. Roy [3] has found that free nitrocatechol causes an overall increase in the reaction velocity, and both Roy [3] and Dodgson and Spencer [1] report that there can be observed an increase in velocity after the initial decrease in the reaction velocity. Both groups have performed there experiments at 37°C and used an acetate buffer as medium, and they have followed the reaction spectrophotometrically. In this work 0.1 M KCl has been used as medium at 20°C, and the reaction was followed by titrating the liberated acid. Before more experiments have been performed it will not be possible to decide whether the cause of the discrepancies has to be sought in the different techniques used.

Acknowledgement. This work has been carried out with financial support from the *Carlsberg foundation* which is gratefully acknowledged.

REFERENCES

1. Dodgson, K. S. and Spencer, B. *Biochem. J.* **62** (1956) 30 p.
2. Roy, A. B. *Biochem. J.* **55** (1953) 653.
3. Roy, A. B. *Experientia* **13** (1957) 32.
4. Roy, A. B. *Biochem. J.* **53** (1953) 12.
5. Dodgson, K. S. and Spencer, B. *Biochim. et Biophys. Acta* **21** (1956) 175.
6. Roy, A. B. *Biochem. J.* **59** (1955) 8.
7. Christiansen, J. A. *Acta Chem. Scand.* **3** (1949) 493.
8. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.

Received September 27, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 127—137

Über die Carbonylgruppen des Lignins

JOSEF GIERER und SIGURD SÖDERBERG

Schwedisches Holzforschungsinstitut, Holzchemische Abteilung, Stockholm, Schweden

Carbonyl group estimations, by a volumetric method involving the use of borohydride, indicate that in lignins isolated under mild conditions (Brauns "native lignin" and Björkmans "milled wood lignin") there are 0.41—0.48 CO groups/OCH_3 group. This contrasts with other determinations, in which hydroxylamine hydrochloride was used, where values of 0.12—0.19 were obtained.

The cause of this discrepancy has been sought in the present work. Similar types of determinations to those above have been carried out on compounds related to lignin and containing carbonyl groups. A study of the reaction of those compounds with hydroxylamine hydrochloride showed that the rate of reaction is highly dependent on the position of the carbonyl group in the side-chain and on the structure of the side-chain. Under the experimental conditions normally employed the use of hydroxylamine hydrochloride, therefore, resulted in values, which in many cases were too low, whereas the use of borohydride gave values near to the theoretical.

The possibilities to correlate the difference between the two carbonyl determinations with the position of the carbonyl groups in the side-chains and to reconcile the new findings with other experimental evidence are briefly discussed.

Seit dem Nachweis von Carbonylgruppen in Lignosulfonsäuren durch Klason [1] ist wiederholt wersucht worden, diese Gruppen in verschiedenen Ligninpräparaten auch quantitativ zu bestimmen. Die erhaltenen Werte schwankten innerhalb weiter Grenzen je nach Wahl des Untersuchungsmaterials und der Bestimmungsmethode [2]. Die Kenntnis des genauen Carbonylgehaltes ist jedoch von erheblicher strukturchemischer Bedeutung. Ausserdem zeigten vorläufige Versuche [3], in Übereinstimmung mit bereits früher gemachten Annahmen [4], dass Carbonylgruppen eine wichtige Funktion bei der Sulfitierung des Lignins ausüben dürften. Eine zuverlässige Bestimmungsmethode für diese Gruppen sollte daher auch das Studium des Verlaufes dieser technisch wichtigen Delignifizierungsreaktion wesentlich erleichtern.

Von den bekannten, analytisch auswertbaren Carbonylreaktionen (Zusammenfassung siehe [5]) wurde in letzter Zeit vor allem die Umsetzung mit Hydroxylamin-Chlorhydrat zur quantitativen Bestimmung der CO-Gruppen in unter schonenden Bedingungen isolierten Ligninpräparaten und in Ligno-

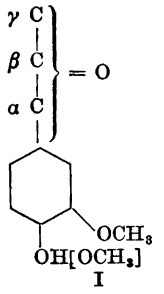
Tabelle 1. Mit Hilfe der Borhydrid-Methode ermittelte Carbonylgehalte verschiedener Ligninpräparate.

Ligninpräparat	Carbonylgehalt CO/OCH_3
Brauns' "native lignin"	0,48
Brauns' "native lignin", 24 Stunden bei Zimmertemperatur mit 0,5 %iger methanolischer Salzsäure methyliert [6,21]	0,45
Björkman-Lignin *	0,44, 0,43, 0,41
Björkman-Lignin, 2 Tage in der Schwingkugelmühle gemahlen	0,45
Björkman-Lignin, mit Natriumborhydrid reduziert und isoliert [21]	0,023
Björkman-Lignin, 24 Stunden bei Zimmertemperatur mit 0,1 N Natronlauge behandelt und wiederisoliert	0,48
Björkman-Lignin, 3 Tage lang bei Zimmertemperatur und pH 4,0 oximiert und wiederisoliert	0,21
Björkman-Lignin, 5 Stunden unter Rückflusskochung bei pH 4,0 oximiert und wiederisoliert	0,29
Björkman-Lignin, 24 Stunden bei Zimmertemperatur mit 0,5 %iger methanolischer Salzsäure methyliert [6,21]	0,40
Dehydrierungspolymerisat, hergestellt nach dem Zulaufverfahren [11]	0,33
Salzsäurelignin (Rheinau)	0,38

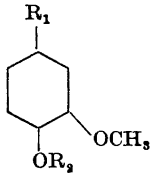
* verschiedene nach dem gleichen Verfahren hergestellte Präparate

sulfonsäuren herangezogen. Der bei der Oximbildung freiwerdende Chlorwasserstoff wurde potentiometrisch mit Natronlauge titriert, wobei sich in den Zeit - Umsatzkurven eine rasch und eine langsam verlaufende Phase abzeichnete. Die erstgenannte wurde der tatsächlichen Oximierungsreaktion, die letztgenannte hingegen einer oxydativen Zersetzung des überschüssigen Hydroxylamin-Chlorhydrates zugeschrieben [6]. Der Carbonylgehalt ergab sich daher durch Extrapolation auf die Zeit Null. In Brauns' "native lignin" [6] wurden auf diese Weise 0,12 CO/OCH_3, in Björkman-Lignin [7] 0,19 CO/OCH_3 und in einer Lignosulfonsäure [8] 0,10 CO/OCH_3 gefunden.

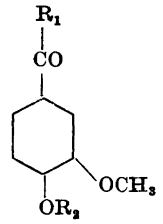
Vor kurzem teilten *Lindberg und Mitarbeiter* [9,10] eine neue Methode zur Bestimmung von Carbonylgruppen in Kohlenhydraten mit, die auf einer quantitativen Auswertung der Reduktion mit überschüssigem Borhydrid beruht. Der Verbrauch an Wasserstoff wird hierbei nach Ablauf einer bestimmten Reaktionszeit durch Zersetzung des Borhydridüberschusses (mit H_2SO_4) gegen eine entsprechende Blindprobe als Differenz ermittelt.



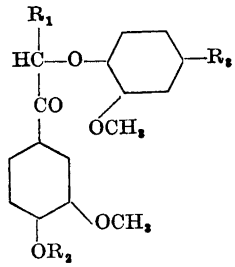
I



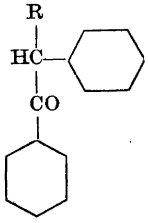
II R_1 = CH=CH–CHO, R_2 = H
III R_1 = CH=CH–CHO, R_2 = CH_3
IV R_1 = CH_2–CO–CH_3, R_2 = OC–CH_3
V R_1 = CH_2–CO–CH_3, R_2 = CH_3



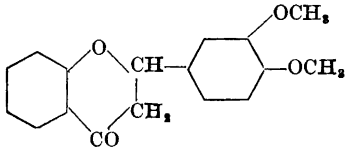
VI R_1 = H, R_2 = H
VII R_1 = H, R_2 = CH_3
VIII R_1 = CH_3, R_2 = H
IX R_1 = C_2H_5, R_2 = H
X R_1 = C_2H_5, R_2 = CH_3
XI R_1 = CHOH-CH_3, R_2 = H
XII R_1 = CO-CH_3, R_2 = H



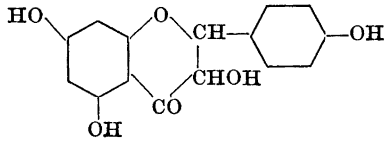
XIII R_1 = H, R_2 = CH_3, R_3 = H
XIV R_1 = CH_3, R_2 = H, R_3 = H
XV R_1 = CH_3, R_2 = CH_3, R_3 = H
XVI R_1 = CH_2OH, R_2 = CH_3, R_3 = H
XVII R_1 = CH_2OH, R_2 = CH_3, R_3 = CHO



XVIII R = H
XIX R = CH_2OH



XX



XXI

Bei Anwendung dieser Methode auf schonend isolierte Ligninpräparate (aus Fichtenholz) wurden nun Carbonylgehalte gefunden, die, wie Tabelle 1 zeigt, durchwegs viel höher als die entsprechenden mit Hilfe der Hydroxylamin-Methode bestimmten Werte (siehe oben) liegen. Ein ähnlicher Unterschied im Ergebnis der beiden Bestimmungsmethoden konnte auch bei einem enzymatisch nach dem Zulaufverfahren [11] aus Coniferylalkohol synthetisierten

"Dehydrierungspolymerisat" (DHP) * beobachtet werden. Mit Hilfe der Hydroxylamin-Methode wurden nur 0,07 CO/OCH_3, mit Hilfe der Borhydrid-Methode hingegen 0,33 CO/OCH_3 gefunden (Tab. 1). Um sicherzustellen, dass der hohe Wasserstoffverbrauch tatsächlich auf eine Reduktion und nicht etwa auf eine Adsorption an dem bei Säurezusatz wieder teilweise ausfallenden Lignin (siehe experimenteller Teil) zurückzuführen ist, wurde die Bestimmungsmethode auch an einem mit Natriumborhydrid vorreduzierten und wiederisolierten Ligninpräparat ausgeführt. Der hierbei beobachtete Wasserstoffverbrauch war sehr gering (siehe Tab. 1) und dürfte durch eine partielle Reoxydation des vorreduzierten Lignins während und nach seiner Isolierung bedingt sein.

Für die auffallende Diskrepanz zwischen den Ergebnissen der beiden Carbonylbestimmungsmethoden müssen daher chemische Faktoren verantwortlich sein. Es muss angenommen werden, dass entweder die Umsetzung mit Hydroxylamin-Chlorhydrat nicht alle Carbonyle des Lignins erfasst oder dass die Borhydrid-Methode auch auf andere Gruppen (als Carbonylgruppen) anspricht. Um zwischen diesen Alternativen zu entscheiden, wurden die beiden Methoden an geeigneten Modellsubstanzen näher untersucht.

HYDROXYLAMIN-METHODE

Die Umsetzung der Modellsubstanzen und der Ligninpräparate mit Hydroxylamin-Chlorhydrat wurde mit Hilfe einer verbesserten Apparatur untersucht. Der Laugenzusatz wurde automatisch geregelt und der Luftsauerstoff von einem Stickstoffstrom durch das thermokonstant gehaltene Reaktionsgefäss verdrängt. Dadurch konnte die Zersetzung des überschüssigen Hydroxylamin-Chlorhydrates sehr vermindert werden (Blindversuch).

Selbst bei Anwendung dieser apparativen Ausrüstung zeigten jedoch die Zeit-Umsatzkurven im Falle der Ligninpräparate nach einer rasch verlaufenden Initialphase einen langsamen sich über Tage erstreckenden Anstieg, der wesentlich steiler als der des entsprechenden Blindversuches (Versuch nur mit Hydroxylamin-Chlorhydrat) war (siehe Abb. 1). Dies deutet darauf hin, dass der langsam freigelegte Chlorwasserstoff seine Entstehung nicht nur einer spontanen Zersetzung des Reagenzes, sondern teilweise auch einer Umsetzung des letztgenannten mit sehr träge reagierenden Carbonylgruppen verdankt.

Ein Studium der Oximierungsreaktion mit ligninverwandten Verbindungen u. a. vom Typus der 3-Methoxy-4-hydroxy- und der 3,4-Dimethoxy-phenylpropanone bzw.-propanale (I) ergab, dass β- und γ-ständige Carbonylgruppen (II—V) — wie auch aromatische Aldehyde, z. B. VI und VII — überaus rasch (siehe Abb. 2), α-ständige Carbonylgruppen (aromatische Ketone, VIII—XIX) hingegen langsam reagieren (Abb. 4). Die Reaktionsgeschwindigkeit der α-ständigen Carbonylgruppen ist stark abhängig von der Substitution am β-ständigen Kohlenstoffatom der Seitenkette und nimmt von Verbindungen mit einer der Ketogruppe benachbarten Methylengruppe (VIII, IX, X, XIII, XVIII, XX) über solche mit einer Hydroxymethylengruppe (z. B. XI) zu

* Für die freundliche Überlassung dieses Präparates danken wir Herrn Dr. G. Billek, Wien.

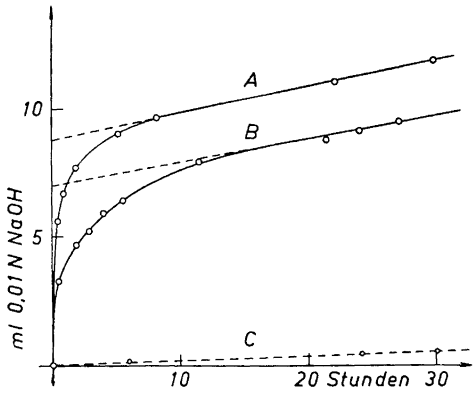


Abb. 1. Zeitlicher Verlauf der Oximierung von A) Björkman-Lignin (100 mg), B) Brauns' "native lignin" (100 mg), C) Blindversuch.

solchen mit einer Aroxymethylengruppierung (XIV—XVI) oder mit einer Arylmethylengruppierung (z. B. XIX) hin ab (siehe Abb. 4). Besonders langsam reagieren α-Carbonylgruppen in Einheiten, die ausser einer β-ständigen Aroxyl- oder Arylgruppe auch eine γ-ständige Hydroxylgruppe enthalten (z. B. XVI bzw. XIX). Derartige Substitutionstypen an den β- und γ-ständigen Kohlenstoffatomen der Seitenketten werden bekanntlich aufgrund biosynthetischer Modellversuche [11–14], sowie aufgrund von Untersuchungen über den Abbau von Ligninpräparaten durch Äthanolyse [15–18] und "Acidolyse" [19] als dominierend angesehen.

Hingegen ist offenbar die Oximierungsgeschwindigkeit unabhängig davon, ob die zur Seitenkette p-ständige phenolische Hydroxylgruppe frei oder veräthert (bzw. verestert) ist (vgl. II mit III, IV mit V und VI mit VII, Abb. 2, sowie IX mit X und XIV mit XV, Abb. 4).

Die grossen Unterschiede in der Oximierungsgeschwindigkeit verschiedener Carbonyle zeigen sich auch deutlich bei Substanzen, welche 2 Carbonylgruppen verschiedener Reaktionsfähigkeit im gleichen Molekül enthalten, wie z. B. bei den Verbindungen XII und XVII. Aufgrund der oben erwähnten Modellversuche konnte erwartet werden, dass die β-ständige Ketogruppe in XII (vgl. mit IV und mit V), sowie die Aldehydgruppe in XVII (vgl. mit VII) sehr rasch, die α-ständigen Ketogruppen in beiden Substanzen (vgl. mit XI bzw. mit XVI) hingegen nur langsam oximierbar sind. Der Verlauf der Zeit — Umsatzkurven stimmt hiermit überein. In beiden Fällen werden während der raschen Reaktionsphase nur ca 50 % des Totalcarbonyls erfasst (Abb. 3). Dass im Diketon XII tatsächlich die β-ständige Ketogruppe rasch und die α-ständige langsam oximiert wird, konnte auf folgende Weise gezeigt werden: Nach Ablauf der raschen Reaktionsphase wurde das partiell oximierte Produkt (Monoxim) isoliert (Schmp. 162—163°) und mit Natriumborhydrid reduziert. Das Reduktionsprodukt gab bei Kupplung mit Chinononochlorimid in schwach alkalischer Lösung eine intensive Farbreaktion, woraus auf das Vorkommen einer freien (nicht oximierten) α-ständigen Ketogruppe im ursprünglichen Monoxim geschlossen werden darf [20,21].

Die Carbonylgruppe der Substanz XX, welche einem Flavanonring angehört, ist mit ungefähr der gleichen Geschwindigkeit wie gewöhnliche α-Carbonyle mit benachbarter Methylengruppe (z. B. IX und X) oximierbar, die der Substanz XXI hingegen reagiert — vermutlich bedingt durch die Anwesenheit der beiden benachbarten Hydroxylgruppen — extrem langsam (Abb. 4).

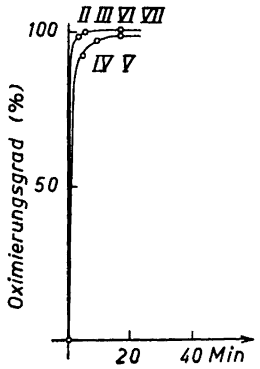


Abb. 2. Zeitlicher Verlauf der Oximierung von β- und von γ-Carbonylverbindungen des 3-Methoxy-4-hydroxy- und des 3,4-Dimethoxyphenylpropantypus (I), sowie von Vanillin (VI) und von Veratrumaldehyd (VII).

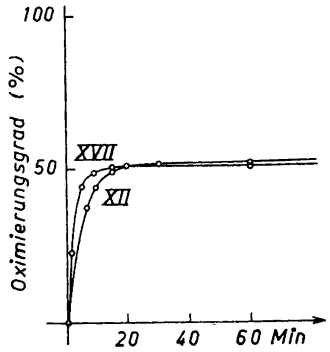


Abb. 3. Zeitlicher Verlauf der Oximierung zweier Dicarbonylverbindungen (XII und XVII) mit Carbonylgruppen verschiedener Reaktionsfähigkeit.

Wird die Oximierung anstatt bei Zimmertemperatur unter Rückflusskochung (5 St.) ausgeführt, so setzen sich auch die nur sehr träge reagierenden α-Carbonyle (z. B. in den Substanzen XII, XIV—XVII und XIX) zum grössten Teil um (Tab. 2).

Brauns "native lignin" und Björkman-Lignin ergaben unter diesen Reaktionsbedingungen höhere Carbonylwerte (0,3 bzw. 0,25 CO/OCH_3) als bei Zimmertemperatur (0,12 bzw. 0,19 CO/OCH_3). Die mit Hilfe der Borhydrid-Methode gefundenen hohen Carbonylwerte (0,48 bzw. 0,44 CO/OCH_3, siehe Tab. 1) konnten demnach durch Erhöhung der Oximierungstemperatur nicht erreicht werden.

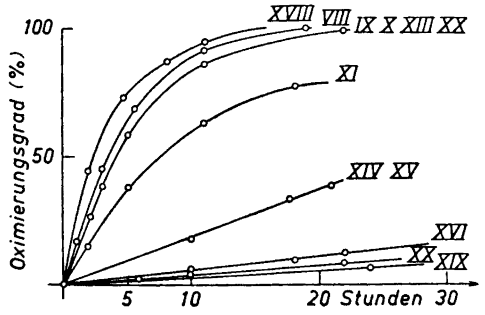


Abb. 4. Zeitlicher Verlauf der Oximierung von α-Ketoverbindungen des 3-Methoxy-4-hydroxy- und des 3,4-Dimethoxy-phenylpropantypus (I).

Tabelle 2. Vergleich der Oximierungsgrade (in Prozenten des theor. Wertes) nach 5-stündiger Reaktionszeit bei Zimmertemperatur und bei Rückflusskochung für die α-Ketone XII–XIX.

Substanz	Carbonyl (in % des theor. Wertes) gefunden nach 5-stündiger Reaktionszeit	
	bei Zimmertemperatur	unter Rückflusskochung
XII	52,1	93,5
XIII	58,0	94,7
XIV	10,1	97,5
XV	10,0	101,1
XVI	2,8	88,3
XVII	51,3	101,0
XVIII	74,4	99,8
XIX	1,2	87,1

BORHYDRID-METHODE

Während die untersuchten Carbonylverbindungen grosse Unterschiede in den Oximierungsgeschwindigkeiten zeigen (Abb. 2—4), sind sie durch Einwirkung von Kalium-borhydrid unter den im experimentellen Teil angegebenen Bedingungen vollständig reduzierbar. Eine Ausnahme bilden einige α-Carbonylverbindungen mit *p*-ständiger phenolischer Hydroxylgruppe, wie z. B. VI, VIII, IX und XIV. Sie benötigen zur vollständigen Reduktion längere Reaktionszeiten. Tabelle 3 zeigt eine Zusammenstellung der mit Hilfe der Borhydrid-Methode ermittelten Carbonylwerte für die wichtigsten Modellsubstanzen.

Zur Überprüfung der Spezifität der Borhydridreduktion wurden auch carbonylfreie Modellsubstanzen mit zum aromatischen Kern konjugierter Doppelbindung, mit Benzylalkylätherbindung, mit Esterbindung, mit Laktonbindung oder schliesslich mit freier Carboxylgruppe getestet (Tab. 4). Wie ersichtlich, werden unter den gewählten Bedingungen nur Ester- und Laktengruppen teilweise reduziert. Die Reduzierbarkeit dieser Gruppen wird durch Behandlung mit 0,1 N Natronlauge (Spaltung in Carboxyl- und Alkoholgruppe) aufgehoben. Da jedoch nach Alkalibehandlung und Wiederisolierung von Björkman-Lignin die Menge des bei der Borhydridreduktion verbrauchten Wasserstoffs ungefähr gleich gross wie beim unbehandelten Präparat ist, dürften derartige Struktur-elemente, wenn überhaupt, so nur in sehr geringer Menge im Lignin vorkommen.

Auch das Infrarot-Spektrum des Lignins, sowie die Ergebnisse des Studiums der Alkylierungsreaktion durch Einwirkung von alkoholischer Salzsäure [6] gaben keine Anhaltspunkte für das Vorkommen nennenswerter Mengen freier oder veresterter Carboxylgruppen im Fichtenholzlignin.

Tabelle 3. Mit Hilfe der Borhydrid-Methode ermittelte Carbonylgehalte verschiedener Modellsubstanzen.

Substanz	% Carbonyl	
	berechnet	gefunden
II	15,7	15,5
III	14,6	14,4
IV	12,6	15,3 *
V	14,4	14,6
VI	18,4	16,7 **
VII	16,8	16,8
VIII	16,8	6,3 **
IX	15,5	5,4 **
X	14,4	14,3
XI	14,3	14,7
XII	28,8	28,9
XIII	9,3	9,1
XIV	9,3	7,5 **
XV	8,9	8,8
XVI	8,4	8,5
XVII	15,6	15,8
XVIII	14,3	14,5
XIX	12,4	12,7
XX	9,9	10,2
XXI	9,7	9,4

* teilweise Reduktion der Estergruppe.
** Zur quantitativen Bestimmung ist eine bedeutende Verlängerung der Reaktionszeit erforderlich.

Bisher sind keine anderen, durch Borhydrid reduzierbaren Gruppen (als Carbonylgruppen) im Lignin aufgefunden worden. Es darf daher gefolgert werden, dass der hohe Wasserstoffverbrauch ausschliesslich durch Carbonylgruppen verursacht wird.

Tabelle 4. Wasserstoffverbrauch carbonylfreier Substanzen.

Substanz	Wasserstoffverbrauch (ml) von 1 Mmol	
	berechnet (für Reduktion aller) ev. reduzierbaren Gr.	gefunden
Ferulasäure	67,3	0,0
*Iso*eugenol-methyläther	22,4	0,0
Vanillylalkohol-methyläther	22,4	0,0
Pinoresinol	44,9	0,0
*Iso*eugenol-acetat	67,3	4,5
Conidendrin	44,9	24,3
Vanillinoxim	22,4	0,8
3,4-Dimethoxy-zimtaldehydoxim	44,9	1,4

Durch die Auffindung wesentlich grösserer Mengen Carbonylgruppen in "nativen" Ligninen, als bisher angenommen wurden, erhält das Problem der Aufteilung dieser Gruppen auf die drei Kohlenstoffatome der Propanseitenketten erhöhte strukturchemische Bedeutung. Wie bereits gezeigt wurde, erlaubt die Oximierungsreaktion eine grobe Differenzierung in zwei Kategorien verschiedener Reaktionsfähigkeit.

Die rasch verlaufende Phase der Oximierung könnte nach Aussage der Modellversuche (siehe oben) auf das Vorkommen von Aldehyd- und von "sterisch nicht gehinderten" β-Ketogruppen zurückzuführen sein. Die Hydroxylamin-Methode dürfte in ihrer bisherigen Ausführungsform (Zimmertemperatur, Extrapolation des Laugenverbrauchs auf die Zeit 0) — im Gegensatz zur Borhydrid-Methode — nur solche oder ähnliche rasch reagierende Carbonylgruppen im Lignin erfassen. Diese Deutung der Diskrepanz zwischen den Ergebnissen der beiden Bestimmungsmethoden wird auch durch den Befund gestützt, dass ein nach Ablauf der raschen Reaktionsphase isoliertes "oximiertes" Björkman-Lignin bei nachfolgender Borhydrid-Reduktion ungefähr soviel Wasserstoff verbraucht, wie der Differenz zwischen den Carbonylwerten der beiden Bestimmungsmethoden entspricht (Tab. 1). (Modelloxime erwiesen sich unter den Bedingungen der Bestimmungsmethode gegenüber Borhydrid stabil, siehe Tab. 4).

Die Zuordnung der langsam mit Hydroxylamin-Chlorhydrat reagierenden Carbonylgruppen zu bestimmten Strukturelementen des Lignins gestaltet sich schwieriger. Als einziger Strukturtyp, der in Bezug auf Oximierungsgeschwindigkeit mit der beim Lignin beobachteten langsamen Reaktionsphase vergleichbar ist, wurden bisher "sterisch gehinderte" α-Carbonylgruppen, wie z. B. in den Verbindungen XIV—XVI, sowie in XIX und XX (siehe Abb. 4), festgestellt.

Im "Dehydrierungspolymerisat" könnten derartige α-Ketogruppen eventuell durch sekundäre Oxydation benzylalkoholischer Hydroxylgruppen in entsprechenden Struktureinheiten entstanden sein (siehe z. B. den in einem Zwischenstadium der DHP-Herstellung isolierbaren Guajacyl-β-coniferyläther [11], sowie die im Zusammenhang mit oxydativen Abbauversuchen an einem DHP-Präparat diskutierten β-Arylstrukturen [12]). Die beiden anderen in einer Zwischenphase der DHP-Herstellung isolierbaren, dimeren Hauptprodukte, Pinoresinol und Dehydro-diconiferylalkohol [11-14], repräsentieren weitere mögliche Verknüpfungsstrukturen, die bei analoger Oxydation am α-ständigen Kohlenstoffatom Monoalkyl- bzw. Monoarylacetalgruppierungen (siehe unten) liefern würden.

Man wäre daher zunächst geneigt, die langsame Oximierungsphase des Lignins und damit den Unterschied in den Ergebnissen der beiden Bestimmungsmethoden auf derartige "sterisch gehinderte" α-Ketogruppen zurückzuführen. Gegen das Vorkommen bedeutender Mengen α-Carbonylgruppen sprechen jedoch spektrophotometrische Befunde. So konnte durch Messungen im Ultraviolett mit Hilfe der sogenannten $\Delta\varepsilon$-Methode [22] gezeigt werden, dass nur ein geringer Teil der phenolischen Einheiten des Lignins (in Brauns'"native lignin" aus *Picea mariana* 0,01—0,02/OCH_3 [23]) eine dem aromatischen Kern benachbarte Carbonylgruppen enthalten dürfte (siehe auch Ref.[24,21]). Auch in den nicht-phenolischen Einheiten des Lignins konnten auf spektrophotometrischem Weg keine grösseren Mengen α-Carbonylgruppen gefunden werden. Aus den Absorptionsänderungen im Ultraviolett, welche durch Reduktion

mit Borhydrid hervorgerufen werden, errechneten Adler und Mitarbeiter [25,26] die Frequenz derartiger Gruppen in Björkman-Lignin zu 0,05—0,06/OCH_3. Die Gesamtmenge α-ständiger Carbonylgruppen (in phenolischen und nicht-phenolischen Einheiten) beträgt nach dieser Bestimmungsmethode bei Björkman-Lignin nur 0,06—0,07/OCH_3 [26].

Eine Möglichkeit, diese spektrophotometrischen Resultate mit dem Vorkommen grösserer Mengen α-Carbonylgruppen im Lignin in Einklang zu bringen, besteht in der Annahme, dass diese Gruppen zu einem beträchtlichen Teil "maskiert", d. h. sowohl im Ultraviolett als auch im Infrarot nicht erkennbar vorliegen könnten (siehe oben, vgl.[27]). So wären z. B. Carbonylgruppen in Halbacetalform in Betracht zu ziehen, die wohl von der Borhydrid-Methode, nicht aber von der Hydroxylamin-Methode erfasst würden. Für das Vorkommen solcher Gruppen konnte jedoch in vorläufigen Versuchen bisher keine experimentelle Stütze erbracht werden. Durch Einwirkung von methanolischer Salzsäure auf Brauns"native lignin" und auf Björkman-Lignin bei Zimmertemperatur wurde nämlich die Menge der durch die Borhydrid-Methode bestimmbaren Carbonylgruppen nicht wesentlich vermindert (Tab. 1). Eine derartige Behandlung sollte eventuell vorhandene Halbacetalgruppen in Vollacetalgruppen überführen, welche auch gegenüber Borhydrid stabil sind. Die Richtigkeit dieser Schlussfolgerung müsste allerdings noch durch entsprechende Modellversuche gezeigt werden.

Die langsam oximierbaren Carbonylgruppen scheinen demnach in anderer Weise "maskiert" vorzuliegen. Zu ihrer Lokalisierung und Charakterisierung wird es zusätzlicher Modellstudien unter Heranziehung weiterer Carbonylreaktionen bedürfen.

EXPERIMENTELLER TEIL

Die verwendeten Modellsubstanzen und Ligninpräparate wurden nach in der Literatur beschriebenen Methoden dargestellt bzw. isoliert *.

Hydroxylamin-Methode. Rund 0,2 Millimole Modellsubstanz bzw. rund 200 mg Ligninpräparat wurden genau eingewogen und in 10 ml 95 %igem Äthanol (bei Ligninpräparaten, wenn erforderlich, unter Zusatz von etwas Wasser) gelöst. Die Lösung wurde auf pH 4,0 eingestellt (Glaselektrode) und zur Zeit Null einer in einem thermokonstant (25°C) gehaltenen Titrationsgefäss befindlichen Lösung von 138 mg (2 Millimole) Hydroxylamin-Chlorhydrat in 40 ml 95 %igem Äthanol vom pH 4,0 zugesetzt. Die bei konstanter Temperatur (25°C) unter ständigem Durchleiten eines mit Alkohol gesättigten Stickstoffstroms und unter magnetischer Umrührung vor sich gehende Oximierungsreaktion wurde durch automatische Titration (Autotitrator der Fa. Radiometer, Kopenhagen) des freigesetzten Chlorwasserstoffs mit 0,01 N Natronlauge laufend gemessen.

In der Abb. 1, welche den zeitlichen Verlauf der Oximierungen von Björkman-Lignin und von Brauns' "native lignin" wiedergibt, ist der Verbrauch an Natronlauge in Millilitern, in den Abb. 2—4, die sich auf die Oximierungen der Modellsubstanzen beziehen, in Prozenten des theoretischen Wertes gegen die Zeit aufgetragen.

Bei den Umsetzungen der Modellsubstanzen und Ligninpräparate mit Hydroxylamin-Chlorhydrat durch 5-stündiges Kochen unter Rückfluss wurden die gleichen Mengenverhältnisse wie oben angegeben angewandt. Das Reaktionsgemisch wurde nach Abkühlen auf 25°C in das Titrationsgefäss übergeführt und bis zum pH-Wert einer entsprechenden

* Die Substanzen XIV und XV wurden uns freundlicherweise von Herrn Prof. E. Adler, Göteborg, die Substanzen XVII—XIX von Herrn Doz. B. Lindgren, Stockholm, zur Verfügung gestellt, wofür auch an dieser Stelle bestens gedankt sei. Die Darstellungen dieser Verbindungen sind z. T. noch nicht veröffentlicht.

Blindprobe (erhalten durch 5-stündiges Kochen einer Lösung von 138 mg Hydroxylamin-Chlorhydrat im gleichen Alkohol-Wassergemisch unter Rückfluss) titriert.

Borhydrid-Methode. Rund 0,2 Millimole Modellsubstanz bzw. rund 100 mg Ligninpräparat wurden genau eingewogen, in den mittleren Teil des dreiteiligen Reaktionsgefässes [10] übergeführt und in 20 ml alkoholischer Borsäurelösung (9,27 g/l) gelöst bzw. suspendiert. Die Bestimmungen wurden im übrigen nach der von Lindberg und Theander [10] beschriebenen Arbeitsweise ausgeführt. Die Ligninpräparate gingen bei Zusatz von 20 ml Kaliumborhydridlösung (1,35 g/l 0,1 N Natronlauge) restlos in Lösung und wurden erst bei Abbruch der Bestimmung, d. h. bei Zusatz von 5 ml 2 N Schwefelsäure, wieder teilweise ausgefällt. Die Reaktionszeit betrug 5 Stunden, die Reaktionstemperatur 25°C (thermostatierter Raum).

Das als Differenz gegen eine entsprechende Blindprobe gemessene Volumen des verbrauchten Wasserstoffs wurde auf Normalbedingungen (760 Torr, 0°) reduziert und daraus der Carbonylgehalt der Probe errechnet (1 ml Wasserstoff entspricht 1,249 mg = 0,0446 Milliäquivalente Carbonyl). Das Verhältnis des bei der Reduktion verbrauchten Wasserstoffs zum Wert der Blindprobe schwankte zwischen ungefähr 1 : 10 und 1 : 6. Die mit Hilfe der Borhydrid-Methode ermittelten Carbonylwerte sind in den Tabellen 1 und 3 zusammengestellt.

LITTERATUR

1. Klason, P. *Ber.* **55** (1922) 448.
2. Brauns, F. E. *The Chemistry of Lignin*, New York 1952, Seite 255.
3. Gierer, J. und Söderberg, S. *Unveröffentlicht.*
4. Brauns, F. E. *J. Am. Chem. Soc.* **61** (1939) 2120; *Paper Trade J.* **111** (1940) 33; Brauns, F. E. und Brown, D. S. *Ind. Eng. Chem.* **30** (1938) 779.
5. Mitchell, Jr. J. In *Organic Analysis*, Interscience Publishers Inc., New York 1953, Vol. I, Seite 243.
6. Adler, E. und Gierer, J. *Acta Chem. Scand.* **9** (1955) 84.
7. Adler, E. und Walldén, I. *Privatmitteilung.*
8. Adler, E. und Ellmer, L. *Acta Chem. Scand.* **2** (1948) 839.
9. Lindberg, B. und Missiorny, A. *Svensk Papperstidn.* **55** (1952) 13.
10. Lindberg, B. und Theander, O. *Svensk Papperstidn.* **57** (1954) 83.
11. Freudenberg, K. *Angew. Chem.* **68** (1956) 508.
12. Freudenberg, K. *Angew. Chem.* **68** (1956) 84.
13. Freudenberg, K. *Fortschr. Chem. org. Naturstoffe* **11** (1954) 43.
14. Freudenberg, K. *Croatica Chem. Acta* **29** (1957) 189.
15. Adler, E. und Yllner, S. *Svensk Papperstidn.* **57** (1954).
16. Adler, E., Lindgren, B. und Saedén, U. *Svensk Papperstidn.* **55** (1952) 245.
17. Adler, E. und Lindgren, B. *Svensk Papperstidn.* **55** (1952) 563.
18. Kratzl, K. und Billek, G. *TAPPI* **40** (1957) 269.
19. Adler, E., Pepper, J. M. und Eriksoo, E. *Ind. Eng. Chem.* **49** (1957) 1391.
20. Ziegler, E. und Gartler, K. *Monatsh.* **79** (1948) 637; **80** (1949) 759.
21. Gierer, J. *Acta Chem. Scand.* **8** (1954) 1319.
22. Aulin-Erdtman, G. *Svensk Papperstidn.* **56** (1953) 287.
23. Aulin-Erdtman, G. und Hegbom, L. *Svensk Papperstidn.* **61** (1958) 187.
24. Goldschmid, O. *Anal. Chem.* **26** (1954) 1421.
25. Adler, E. *Privatmitteilung.*
26. Adler, E. und Marton, J. *Acta Chem. Scand.* **13** (1959) 75.
27. Erdtman, H. *Research* **3** (1950) 63.

Eingegangen am 11. August 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 138—143

Alkaline Hydrolysis of Glycosidic Linkages

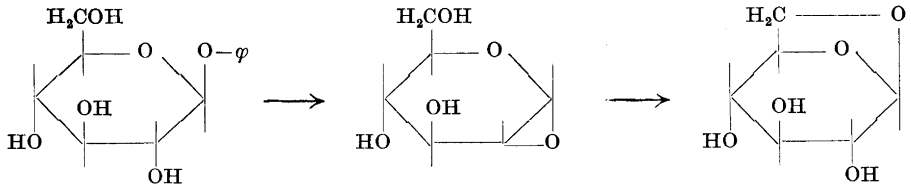
IV. The Action of Alkali on Some Glucopyranosides

JAN JANSON and BENGT LINDBERG

Institutionen för träkemi, Kungl. Tekniska Högskolan, Stockholm, Sweden

The alkaline hydrolysis of a number of glucopyranosides has been investigated. The results show that the hydrolysis may proceed by a number of different routes of comparable importance; one of those probably involves the intermediate formation of 1,2-anhydroglucose. Attempted transglycosidation of one β-glucoside and two β-xylosides by treatment with sodium methoxide in methanol at 170° yielded only traces of the corresponding methyl β-glycoside.

It is thought probable that the route by which 1,6-anhydro-D-glucose (III) is formed on the alkaline hydrolysis of phenyl β-D-glucoside (I) involves the intermediate formation of 1,2-anhydro-D-glucose (II). (For details of this and other reactions of glycosides with alkali, see Ref.[1])



I II III

Such a hypothesis offers an explanation of why the β-glucoside is much more sensitive to alkali than is the α-glucoside. The hydroxyl group at $C_{(2)}$ is in the *trans*-position to the phenoxyl group at $C_{(1)}$ and this makes it sterically possible for the first step in the reaction (I → II) to take place. McCloskey and Coleman [2] noted that phenyl 2,3-di-*O*-methyl-β-D-glucoside was quite stable to alkali. This may be explained in terms of the above mechanism. With different phenyl-β-glucosides, substituted in the *meta*- or *para*-position it was observed that the more acidic the phenol corresponding to the aglucone,

that is, the greater the electron attracting power of the phenoxyl group, the higher was the sensitivity to alkali [3].

It has been shown that alkyl glycosides are attacked by alkali only at higher temperatures [4]. Of the various methyl glycopyranosides studied, the "*trans*"-glycosides were always more reactive than were the "*cis*"-anomers, but this difference in reactivity was much less pronounced than in the case of the phenyl glycosides. The reactivity was also found to increase with the conformational instability of the most stable chair form of the glucoside (Part III) [5].

In the present paper the investigation of a number of glucopyranosides is reported. The method was the same as that previously used, that is treatment of the glucoside with 10 % aqueous sodium hydroxide at 170° and estimation of the percentage of unchanged starting material. The data for the various glucosides are summarised in Table 1.

Table 1. Rate and extent of hydrolysis of D-glucopyranosides with 10 % sodium hydroxide.

	Glucoside	Neutral residue, %			$10^3 \times k$ *
		6 h	24 h	48 h	
a	Methyl β-	98.2	88.3	73.9	2.6
b	Methyl 2-*O*-methyl β-	98.6	92.7	87.3	1.2
c	Methyl α-	96.4	93.8	89.6	1.0
d	Methyl 2-*O*-methyl α-	98.3	95.8	91.2	0.8
e	Ethyl β-	95.9	84.9	72.2	2.9
f	*Iso*propyl β-	93.8	80.1	61.6	3.9
g	*Tert.*-butyl β-	79.4	44.5	30.9	12.0
h	2-Methoxyethyl β-	95.6	90.6	81.3	1.7
i	2-Hydroxyethyl β-	89.7	73.6	58.0	4.5
j	2-Hydroxyethyl α-	97.0	90.1	81.0	1.8
k	2-Methoxyethyl α-	97.7	94.4	88.6	1.0

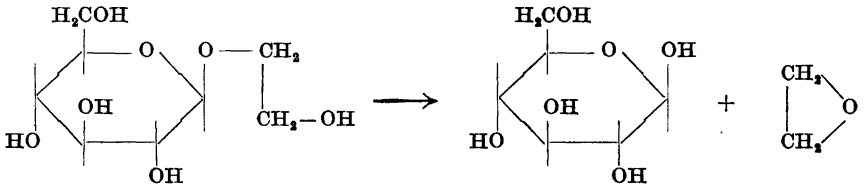
* Rate constants are expressed in Briggs logarithms and in hours.

If the hydroxyl at $C_{(2)}$ in methyl β-D-glucopyranoside (a in Table 1) is replaced by a methoxyl group (giving compound b), then the rate of alkaline hydrolysis is reduced by more than a half. In the α-series (compounds c and d) a similar substitution produced a much smaller effect. This observation, and the differences mentioned above between the reactivity of "*trans*"- and "*cis*"-glycosides, strongly support the view that, during the alkaline hydrolysis of *trans*-glycosides, an important route is that by way of the 1,2-anhydride. But as "*cis*"-glycosides and compound b show considerable reactivity, there must be other routes of importance.

The reaction velocities increase in the order methyl<ethyl<*iso*-propyl<*tert.*-butyl β-D-glucopyranoside (a,e,f,g resp.) and 2-methoxyethyl β-D-glucopyranoside (h) is even less reactive than is methyl-β-D-glucopyranoside. It is generally assumed that the electron attracting power decreases from methyl to *tert.*-butyl and therefore, by analogy with the phenyl glycosides, the reversed

order of reactivity would be expected. There are several competing mechanisms involved during alkaline hydrolysis and it is difficult to distinguish between the polar and the steric influence of alkyl groups. For these reasons, and without further studies, it is not at present possible to fully interpret the results.

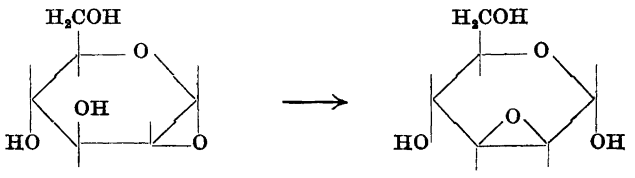
When cellobiitol was heated with alkali one of the main reaction products was 1,4-anhydro-D-glucitol [6]. This observation indicated the existence of another reaction route. The 1,4-anhydro-D-glucitol is probably formed by a nucleophilic attack on $C_{(4)}$ in the glucitol residue, either by the primary hydroxyl group at $C_{(1)}$ or by a hydroxyl group adjacent to that on $C_{(4)}$. In this last case there would be the intermediate formation of an ethylene oxide ring. Those possibilities have been studied by examining the alkaline hydrolysis of the α- and β-glucopyranosides both of ethylene glycol (monoglucosides, i and j) and of its monomethyl ether (compounds h and k). As expected, the glycol glucosides reacted much faster than did the glycol monomethyl ether glucosides. This indicated that in the hydrolysis of the former a major route involved the intermediate formation of an ethylene oxide ring (IV—V)



IV V

It is highly possible that this mechanism also operates under the conditions obtaining during sulphate and soda pulping and then leads to the hydrolysis of glycosidic linkages.

By treatment of suitable phenyl glycosides with alkali in the presence of an alcohol, transglycosidation reactions have been effected [1,7]. These reactions obviously proceed by way of the 1,2-anhydro sugars, and the possibility of transglycosidation reactions during the alkaline treatment of alkyl glycosides was inferred from previous investigations [6]. The formation of 1,6-anhydro sugars is of course an intramolecular transglycosidation. *Tert.*-butyl β-D-glucopyranoside was treated with sodium methoxide in methanol at 170° until about half of the starting material had been changed into acidic products. Only traces of methyl β-D-glucopyranoside and 1,6-anhydro-D-glucose could be chromatographically detected in the deionised reaction mixture. In similar



II VI

experiments with ethyl and *tert.*-butyl β-D-xyloside only traces of methyl β-D-xyloside were detected. These results are rather unexpected and the reason for the low yields of transglycosidation products is uncertain. One possible explanation is that the intermediate 1,2-anhydride (*e.g.* II) may preferably rearrange to a 2,3-anhydride (VII), which in turn is transformed into acidic products.

The rate constant for the hydrolysis of methyl β-D-glucopyranoside was in good agreement with that determined previously (Part III[5]) but the constant for methyl α-glucoside was found to be much higher than that recorded earlier. The amount of unchanged starting material in the hydrolysates of the various glucosides was previously determined gravimetrically. Those determinations suffered from the drawback that significant errors might be introduced due to imperfect drying or to the presence of small amounts of impurities praticularly when, as in case of methyl α-glucoside, only a small percentage of the material was hydrolysed. In the present work the quantitative determinations of the rate and degree of hydrolysis were made polarimetrically. This method would be expected to yield more reliable results especially for the α-glucosides with their high specific rotation. Some of the glycosides studied in this investigation had not been hitherto prepared. They, and the others, were prepared by conventional methods, outlined in the experimental part.

EXPERIMENTAL

All melting points are corrected. All distillations were carried out under reduced pressure. Unless otherwise stated, optical rotations were determined in aqueous solutions, $c \simeq 2$.

For chromatography, Whatman No. 1 paper and the following irrigants were used.

A. Ethyl acetate-acetic acid-water 3:1:1
B. Butanol-pyridine-water 6:2:3
C. Methyl ethyl ketone, saturated with water.

Those synthesis are described below which are of new glycosides or of glycosides prepared by methods other than those given in the literature.

2-Hydroxyethyl α-D-glucopyranoside. A mixture of glucose (57 g), glycol (500 g) and Amberlite IR 120 (H^+) (120 g) was heated on the steam bath with stirring for 72 h. The ion exchange resin was then filtered off and the solution was concentrated to a syrup, which was dissolved in 2 N aqueous sodium hydroxide (250 ml) and kept at 100° overnight. After deionisation and concentration a syrup was obtained which did not crystallise. Chromatographic investigation revealed that, amongst several compounds present, one was chromatographically indistinguishable from the expected β-glucoside. Part of the syrup was fractionated by carbon column chromatography, using aqueous ethanol as irrigant and the gradient elution technique. The main fraction did not crystallise but was chromatographically pure and both its R_F-value and its high specific rotation (98°) accorded with the high calculated approximate values for hydroxyethyl-α-glucoside. The β-anomer was obtained in lower yield from another fraction and had a rather higher mobility on electrophoresis in borate buffer.

A few months after the investigation of this substance was completed, it crystallised. Recrystallisation from ethyl acetate-ethanol yielded the pure substance, m. p. 99°—101°, $[\alpha]_D^{23}$ +139° (*c* 0.05, in water). (Found C 42.5; H 7.1. Calc. for $C_8H_{16}O_7$: C 42.9; H 7.2).

2-Methoxyethyl α-D-glucopyranoside was prepared in the same way as the above substance. It was purified by carbon column chromatography and gave a homogeneous syrup having $[\alpha]_D$ +94°.

Methyl 2-O-methyl-α-D-glucopyranoside. 2-*O*-methyl-D-glucose (6 g) prepared by the method of Lock and Richards [8], and *p*-toluene sulphonic acid (2.2 g) were dissolved in anhydrous methanol (20 ml) and the solution boiled overnight under reflux. The methanol was distilled off, the syrup dissolved in water, and acid removed by passing the solution through a column of IR 4B. The concentrated eluate was treated overnight with 2 N aqueous sodium hydroxide and after deionisation was again concentrated. The product was crystallised (1.9 g) from ethyl acetate. It had m. p. 146°–150° and $[\alpha]_D^{20}$ +155°.

Methyl 2-O-methyl-β-D-glucopyranoside. 2-*O*-Methyl-D-glucose (4 g), dissolved in a mixture of acetic anhydride (8 ml) and pyridine (25 ml), was heated on a steam bath for 30 min. The reaction mixture was taken to dryness and traces of pyridine were removed by co-distillation with acetic acid. The syrup was dissolved in a mixture of acetic acid (20 ml) and acetyl bromide (1.75 ml). This solution was cooled in ice-water and a solution of water (0.38 ml) in acetic acid (2 ml) was added over 30 min. The reaction mixture was kept at room temperature for 2 h. Then chloroform (25 ml) was added and the mixture, after being washed successively with ice-water, sodium hydrogen carbonate solution and water, was firstly dried over calcium chloride and calcium carbonate and then concentrated to a thick syrup (7.6 g). The syrup was dissolved in a mixture of anhydrous benzene (10 ml) and anhydrous methanol (20 ml). After the addition of anhydrous calcium sulphate (3 g) small portions of silver carbonate (9.5 g) were added and the flask was shaken for 90 min and then allowed to stand overnight. After filtration, the solution was concentrated, then treated with sodium hydroxide and the reaction mixture deionised as described above and concentrated to a syrup. The product, after crystallisation from ethyl acetate, had m. p. 95°–97°, $[\alpha]_D^{20}$ −38°. Yield 1.4 g.

Tert.-*butyl β-D-xyloside.* Tri-*O*-acetyl-α-D-xylopyranosylbromide (11.2 g) was dissolved in a mixture of benzene (15 ml) and *tert.*-butanol (15 ml). Silver carbonate (27 g) was added and the mixture was shaken overnight. After filtration and concentration the resultant syrup was dissolved in ethyl ether and the solution extracted with water. Crystalline 2,3,4-tri-*O*-acetyl-D-xylose, m. p. 136°–139°, $[\alpha]_D^{20}$ +45° (equilibr.) was isolated from the aqueous phase. The ether phase on concentration yielded a syrup which, on crystallisation from 50 % ethanol gave *tert.*-butyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (2.6 g). After recrystallisation the substance had m. p. 132°–134° and $[\alpha]_D^{25}$ −45° (*c*, 1.5 in chloroform). (Found C 54.4; H 7.2. Calc. for $C_{15}H_{24}O_8$: C 54.2; H 7.3.) The product on deacetylation yielded the free xyloside, which was crystallised from methyl ethyl ketone. M. p. 122°–123°, $[\alpha]_D^{25}$ −29°. (Found C 52.5; H 8.6. Calc. for $C_9H_{18}O_5$: C 52.4; H 8.8.)

Ethyl β-D-xylopyranoside was prepared by deacetylation of the corresponding acetate [9] and was crystallised from methyl ethyl ketone. M. p. 95°–96°, $[\alpha]_D^{25}$ −38°. (Found: C 47.0; H 7.6. Calc. for $C_7H_{14}O_5$: C 47.2; H 7.9.)

Alkaline hydrolysis. The samples (0.5 g) of glucoside were separately dissolved in 2.65 N sodium hydroxide (5 ml) and the solutions, in stainless steel bombs and under nitrogen, were heated at 170° in a thermostat oil bath for different times. When cold the solutions were deionised by filtration through a mixed bed of Amberlite IR 120 (H^+) and Dowex 2 (OH^-). The bed was thoroughly washed with water and the filtrate and washings were combined and concentrated. A chromatographic investigation, in the irrigants listed earlier, showed that the reaction mixtures contained unchanged starting material and, amongst the faint traces of other components, one which was chromatographically indistinguishable from 1,6-anhydro-D-glucose. The glycol glucosides yielded substantial amounts of ethylene glycol in addition. The recovery of unchanged starting material was determined polarimetrically.

A recovery of 98–100 % was achieved in control experiments in which the glucoside was treated as above but omitting the heating stage.

Transglycosidation reactions. Samples of the glycosides were separately dissolved in 2.65 N methanolic sodium methoxide and the solutions heated at 170° as previously. The reaction mixtures were then treated in the same way as those obtained by alkaline hydrolysis. The *tert.*-butyl β-glucoside, *tert.*-butyl β-xyloside and ethyl β-xyloside were heated for 48, 27 and 48 h, respectively, and the recoveries of unchanged starting materials were 43, 60 and 54 %, respectively. All products crystallised and, after recrystallisation, small

amounts of the corresponding methyl β-glycosides were chromatographically detected in the mother liquors. The glucoside gave in addition a compound chromatographically indistinguishable from 1,6-anhydro-D-glucose.

The authors are indebted to *Statens Tekniska Forskningsråd* for financial support.

REFERENCES

1. Ballou, C. E. *Advances in Carbohydrate Chem.* **9** (1954) 59.
2. Mc Closkey, C. M. and Coleman, G. H. *J. Org. Chem.* **10** (1945) 184.
3. Dyfverman, A. and Lindberg, B. *Acta Chem. Scand.* **4** (1950) 878.
4. Lindberg, B. *Svensk Papperstidn.* **59** (1956) 531.
5. Dryselius, E., Lindberg, B. and Theander, O. *Acta Chem. Scand.* **12** (1958) 340.
6. Dryselius, E., Lindberg, B. and Theander, O. *Acta Chem. Scand.* **11** (1957) 663.
7. Häggroth, S. and Lindberg, B. *Svensk Papperstidn.* **59** (1956) 870.
8. Lock, M. V. and Richards, G. N. *J. Chem. Soc.* **1955** 3024.
9. Asp, L. and Lindberg, B. *Acta Chem. Scand.* **4** (1950) 1447.

Received October 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 144—150

*iso*Thiocyanates XXXIV *. The Absolute Configuration of (–)-5-Vinyl-2-oxazolidinethione (Goitrin) and its Glucosidic Progenitor (Progoitrin)

ANDERS KJÆR, BO W. CHRISTENSEN and SVEND E. HANSEN

Organic Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen V, Denmark

The antithyroid factor, (–)-5-vinyl-2-oxazolidinethione (I), produced by enzymic hydrolysis of a glucoside (progoitrin) present in roots and seeds of, *e. g.*, rape and turnips, has been demonstrated to possess the absolute configuration depicted as (II). The salt of (–)-1-amino-3-buten-2-ol and (+)-α-bromocamphor-π-sulphonic acid, formerly employed for the synthesis of (I), has been transformed into the *dextro*rotatory acid oxalate of 1-amino-2-butanol.

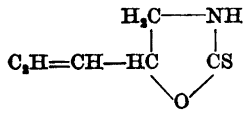
The latter salt has also been prepared from (–)-2-hydroxybutyric acid of known absolute configuration by a sequence of reactions not involving chemical bonds attached to the asymmetric carbon atom. (+)-1-Amino-2-butanol acid oxalate has been transformed into (–)-5-ethyl-2-oxazolidinethione of known absolute configuration.

On the likely assumption that enzymic hydrolysis of progoitrin proceeds with retention of configuration the above correlation further establishes the stereochemistry of the parent glucoside.

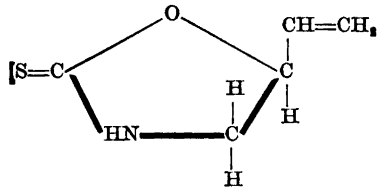
The presence of a goitrogenic factor in roots and seeds of various cruciferous species had long been suspected at the time Astwood *et al.*[1] succeeded in isolating from yellow turnip (rutabaga) a *levo*rotatory, crystalline compound to which the structure (I) was attributed and which upon ingestion elicited goitrous symptoms in animals and human subjects. The same authors [2] presented evidence for (I) being a secondary component produced by enzymic fission of a parent substance, possibly a glycoside. This view was supported by spectroscopic evidence in our laboratory and one of the stereoisomeric 2-hydroxy-3-butenyl *iso*thiocyanates, enzymically liberated from a glucoside, was suggested as a likely progenitor of (I)[3]. A similar conclusion was reached by Schultz and Wagner [4] who isolated a crystalline tetraacetate of the parent glucoside ('glukorapiferin') from seeds of a rape variety. Shortly thereafter, Greer [5] announced the isolation of progoitrin, the crystalline sodium salt of

* Part XXXIII of this series: *Acta Chem. Scand.* **12** (1958) 1693.

the glucosidic precursor of (I), which was enzymically hydrolyzed to glucose, sulphate and (-)-5-vinyl-2-oxazolidinethione (goitrin) *. The structure of the latter was substantiated by Ettlinger [6] who synthesized (I) by a series of reactions which left the configuration around the asymmetric carbon atom as the sole undecided structural feature. In connexion with current studies in this laboratory, knowledge of the absolute configuration of (I) became desirable. The present paper records experimental data supporting (II) as a correct expression for the *levo*rotatory 5-vinyl-2-oxazolidinethione (goitrin).

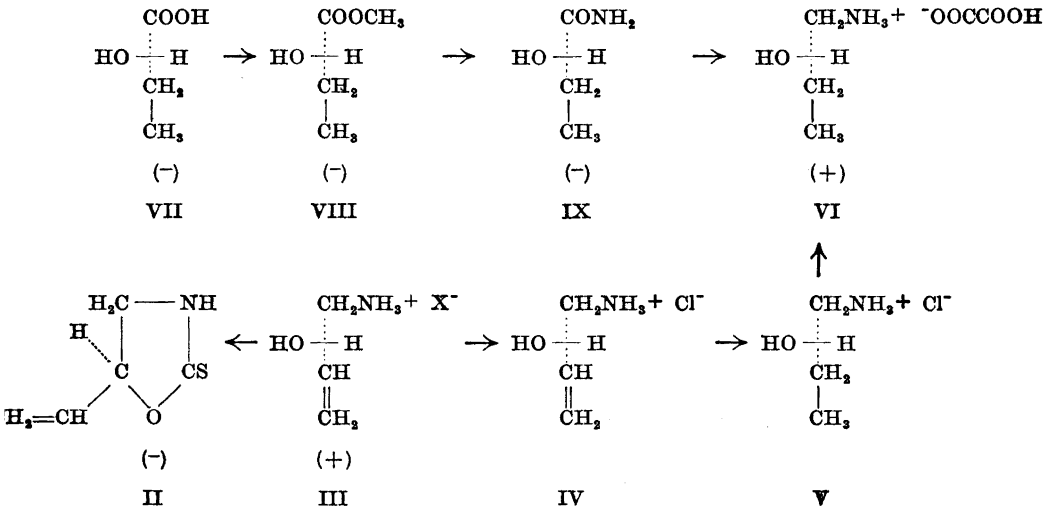


I



II

In his synthesis, Ettlinger [6] transformed the salt (III) of (-)-1-amino-3-buten-2-ol and (+)-α-bromocamphor-π-sulphonic acid into (-)-5-vinyl-2-oxazolidinethione (II). From the same salt, prepared in essentially the same way, we obtained by ion exchange the corresponding hydrochloride (IV). Catalytic hydrogenation of this afforded the hydrochloride of the corresponding 1-amino-2-butanol (V), which was, in turn, transformed into the *dextro*rotatory acid oxalate of the same amino-alcohol (VI).



* Greer, as well as Virtanen *et al.*[17], used the prefix L to denote this *levo*rotatory enantiomorph, a specification which should be dismissed in this context because of its generally accepted usage in expressing generic relationship within a particular configurational series. In the present case, however, such a correlation has not been previously established. Even if it had, the prefix would be an ambiguous or, at best, arbitrary specification.

The steric relationship of the salt (VI) was established by an alternative synthesis departing from a configurationally known compound. As such served *levo*rotatory 2-hydroxybutyric acid (VII), prepared by resolution of the racemic acid with brucine [7]. The acid was transformed into the previously unknown (-)-methyl 2-hydroxybutyrate (VIII) and thence into the more strongly *levo*rotatory 2-hydroxybutyramide (IX), which was reduced with lithium aluminium hydride to give an optically active 1-amino-2-butanol, characterized as the *dextro*rotatory acid oxalate (VI). The latter possessed the same rotation, both with regard to magnitude and sign, as the salt prepared as described above. Idential melting point, undepressed on admixture, and coinciding infra-red spectra further served to corroborate the identity of the two preparations.

The literature contains abundant evidence to support the assignment of the Fischer projection formula (VII) to the *levo*rotatory enantiomorph of 2-hydroxybutyric acid. Thus, Levene and Haller [8] correlated chemically, through the enantiomeric 2-butanols, (+)-lactic acid and (-)-2-hydroxybutyric acid.*

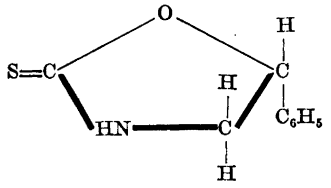
Szarvas [9] adduced good evidence for the configurational relationship of the two acids by rotatory dispersion studies, whereas Fredga *et al.*[10] demonstrated the same generic connexion by utilizing the quasi-racemate method. Supplementary evidence for (-)-2-hydroxybutyric acid being generically related to (+)-lactic acid is available by the fact that the rotations of both are similarly displaced on salt formation in accord with the general rule of Clough, which has been demonstrated to apply for monoasymmetric 2-hydroxy-substituted carboxylic acids [11]. Again, a direct correlation of (—)-hydroxybutyric acid with (—)-malic acid of ascertained configuration has been established by Horn and Pretorius [12] through an anodic coupling process.

The above correlations place the spatial representation of (—)-2-hydroxybutyric acids as (VII), and hence its absolute configuration, beyond any doubt. The same applies for the amine salt (VI) because the subsequent transformations into ester (VIII), amide (IX) and amine salt (VI) proceed without participation of bonds extending from the asymmetric carbon atom. In addition, Noyce and Denney [13] demonstrated an asymmetric center next to a carboxyl or carbonyl grouping to be unaffected by lithium aluminium hydride reduction. Again, inversion can safely be excluded in the catalytic hydrogenation and ion exchange reactions utilized in transforming the sulphonate (III) into the oxalate (VI). Although no rigorous proof is available of the mechanisms by which an optically active 2-amino-alcohol is transformed into an active 2-oxazolidinethione such as (II), there can hardly be any doubt that in the present case a base-induced cyclization of an intermediate *iso*thiocyanate represents the actual events. This further implies that no rupture of the C-O-linkage, or any other bond attached to the asymmetric center, occurs;

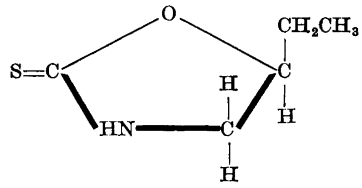
* In fact, the authors reported this acid as a *dextro*rotatory compound. Szarvas [9] subsequently demonstrated it to be slightly *levo*rotatory when measured in pure water, and further pointed out that Levene and Haller's measurement was performed in a solution of 0.58 M barium chloride and excess hydrochloric acid, resulting in an inversion of the sign of rotation. Similar conclusions were reached by other groups also [10, 12].

a conclusion which establishes the absolute configuration of goitrin (II) * relative to that of the salt (III) and, hence, of the acid (VII).

In previous communications from this laboratory the presence in seeds of *Barbarea vulgaris* R.Br.[15] and inflorescenses of *Reseda luteola* L.[16] of a glucoside (glucobarbarin) furnishing (–)-5-phenyl-2-oxazolidinethione (barbarin) upon enzymic hydrolysis, was demonstrated. Stereospecific synthesis of barbarin [16] further showed it to possess the absolute configuration depicted as (X)**



X



XI

It is interesting that goitrin and barbarin, both *levo*rotatory in methanolic solution, contain the substituents at the asymmetric carbon atom in opposite spatial positions. It hence became of interest to transform *levo*rotatory 1-amino-2-butanol acid oxalate (VI) into the corresponding 5-ethyl-2-oxazolidinethione possessing the absolute configuration (XI)***. The latter turned out to be *levo*rotatory in methanolic solution like the configurationally analogous goitrin. The ultra-violet absorption spectrum of (XI) was of the expected type and very similar to that of goitrin, a fact which supports the recent conclusion by Virtanen *et al.*[17] that (XI) can be excluded as the substance responsible for the absorption pattern observed in a milk fraction from a cow which was fed progoitrin-containing rape seed in amounts corresponding to 4.5 g of goitrin. Experimental details for the synthesis of (XI) will appear in a forthcoming communication.

The above determination of the absolute configuration of goitrin also establishes the configuration around the asymmetric carbon atom in the side-chain of progoitrin, because there can hardly be any doubt that cyclization of the intermediate 2-hydroxy-3-butenyl *iso*thiocyanate proceeds with full retention of configuration. The possible consequences of the above stereochemical studies for the biogenesis of the ring-producing *iso*thiocyanate glucosides are being considered at the present.

EXPERIMENTAL

All melting points are uncorrected and determined in capillary tubes in a slowly heated bath. Microanalyses were performed by Mr. P. Hansen at the Chemical Laboratory of the University of Copenhagen.

(±)-*1-Amino-3-buten-2-ol.* This unsaturated amino-alcohol was prepared by the route developed by Ettlinger [6] and the various synthetic reactions proceeded essentially as

* In the system for specification of absolute, asymmetric configuration proposed by Cahn *et al.*[14], goitrin (II) is (*S*)-5-vinyl-2-oxazolidinethione.

** (*R*)-5-Phenyl-2-oxazolidinethione in the specification system of Cahn *et al.*[14]

*** (*S*)-Ethyl-2-oxazolidinethione in the specification of Cahn *et al.*[14]

described. 3,4-Epoxy-1-butene, serving as the starting material, was prepared from butadiene as reported by Kadesch [18]. It was found advantageous, as proposed by Raciszewski *et al.*[19], to add a trace of pyrogallol to the 1-amino-3-buten-2-ol prior to distillation, resulting in a 74 % yield of distilled amino-alcohol based on the acid oxalate. Without polymerization inhibitor the yield was considerably lower (*cf.* also Ref.[19]) than that (79 %) reported by Ettlinger [6].

Salt of (—)-1-Amino-3-buten-2-ol and (+)-α-bromocamphor-π-sulphonic acid. Resolution of the above racemic amino-alcohol with (+)-α-bromocamphor-π-sulphonic acid (British Drug Houses) proceeded as reported by Ettlinger [6]. After recrystallization from wet ethyl acetate the monohydrate of the slightest soluble salt, m. p. 98—102°, possessed the rotation data: $[\alpha]_D^{21}$ +64° (H_2O, *c* 2.3), $[\alpha]_D^{21}$ +55° (0.5 % NaOH, *c* 2.1). A subsequent preparation, conducted at a time when the relative humidity in the laboratory was lower, resulted in isolation of the lower-melting [6], anhydrous salt.

Acid oxalate of (—)-1-amino-2-butanol (VI). The above bromocamphorsulphonate (3 g), dissolved in water (200 ml), was percolated slowly through a column containing an anion exchange resin (Amberlite IR-4B, about 120 mequiv.) in the chloride form. The combined filtrate and washing was concentrated to dryness when the hydrochloride (IV) remained as a viscous oil. Without further purification, the hydrochloride was dissolved in 96 % ethanol (20 ml), containing a few drops of water, and the solution was subjected to catalytic hydrogenation at atmospheric pressure in the presence of 10 % palladium charcoal (1 g). Hydrogen (140 ml) was rapidly taken up and after about 10 min. the absorption came to a stop. After filtration, the filtrate was concentrated *in vacuo* to an oil, consisting of (V), which was dissolved in water. The solution was made alkaline with NaOH, saturated with NaCl and subjected to continuous extraction with ether in a liquid-liquid extractor. To the ether-containing distillation flask was added oxalic acid dihydrate (950 mg) before extraction was initiated. After 3 h, the crystalline acid oxalate (500 mg) was removed by filtration. Two recrystallizations from absolute ethanol, with the use of a little charcoal, afforded a pure specimen of *dextro*rotatory acid oxalate of 1-amino-2-butanol (VI) (330 mg), m. p. 149—150°, $[\alpha]_D^{21}$ +11.3° (H_2O, *c* 0.69).

The substance showed no melting point depression on admixture with a specimen synthesized by another route described below. The infra-red spectra of the two preparations were coinciding throughout the wave-length range (650—4 000 cm^{-1}) examined.

(—)-2-Hydroxybutyric acid VII. 2-Bromobutyric acid (271 g), prepared in 76 % yield according to the directions for the synthesis of 2-bromo*iso*caproic acid [20], was transformed into (±)-2-hydroxybutyric acid in 67 % yield by the method of Bischoff and Walden [21].

The racemic acid (250 g), dissolved in water (1 300 ml), was resolved by means of brucine (966 g of anhydrous alkaloid base), as described by Guye and Jordan [7, 22]. The salt (410 g) was recrystallized from water (700 ml) to give a product (270 g), which possessed rotation values in accord with those reported [7]. To a solution of the salt in water (300 ml), conc. ammonia (50 ml) was added and the precipitated brucine was filtered off and washed. The filtrate was acidified with conc. HCl, saturated with NaCl, and subjected to continuous extraction with ether [9] for 6 h. After removal of the solvent from the dried ether solution, the crystalline (—)-2-hydroxybutyric acid remained (45 g). It was purified by distillation, b. p. 97° at 1.2 mm, and was obtained as hygroscopic, colourless needles (32 g), m. p. 53°, unchanged on recrystallization from a mixture of chloroform and pentane, $[\alpha]_D^{21}$ −15.3° (172 mg of acid in water, containing 1.3 equiv. of ammonia; total volume 5.00 ml). Literature values: m. p. 55—55.5° (recryst. from CCl_4)[10], 52.7—53.5° (from CCl_4: hexane) [12], $[\alpha]_D^{25}$ −15.9° (NH_4-salt in H_2O, *c* 2.7)[10].

(±)-Methyl 2-hydroxybutyrate. Because attempts to esterify 2-hydroxybutyric acid by the method of Clemmensen and Heitman [23] and by the customary hydrochloric acid-catalyzed procedure were unsatisfactory, due to the large amounts of by-products formed by intra- and inter-molecular condensations, special conditions were worked out with the racemic acid as a model in order to minimize the undesired side reactions. The following procedure was eventually adopted:

To a refluxing solution of anhydrous methanol (700 ml), containing dry HCl (6.7 g), was added in 7 portions, and with 0.5 h intervals, a total of 35 g of (±)-2-hydroxybutyric acid. The solution was refluxed for another 2 h when ether (100 ml) and enough water to

produce an aqueous phase of a few ml, were added, followed by solid $NaHCO_3$ until neutralization. The organic layer was removed by decantation, dried over Na_2SO_4 and freed of ether by distillation. The methyl ester distilled as a colourless liquid, b. p. 69° at 30 mm, n_D^{25} 1.4170. The yield was 19.8 g (50 %). Reppe [24] reports: b. p. 68° at 30 mm.

(−)-Methyl 2-hydroxybutyrate (VIII). The above procedure, applied to (−)-2-hydroxybutyric acid (32 g), afforded the corresponding optically active methyl ester (16.8 g) with the same b. p., n_D^{25} 1.4171, D_4^{24} 1.048, $[\alpha]_D^{24}$ −2.30° (neat). (Found: C 51.30; H 8.89. Calc. for $C_5H_{10}O_3$: C 50.85; H 8.53.)

(−)-2-Hydroxybutyramide (IX). A solution of (−)-methyl 2-hydroxybutyrate (10.0 g) in conc. aqueous ammonia (330 ml) was left standing in a closed vessel at room temperature for 3 days, whereupon it was concentrated *in vacuo* to a viscous oil. This was refluxed for one hour with sodium-dried ether (200 ml) which was decanted hot from undissolved material, and the process was repeated with a fresh portion of ether (200 ml). The combined ether solutions deposited on cooling overnight a crop of the hygroscopic amide as colourless small prisms (2.85 g). An additional crop of the same quality (2.26 g) separated from the concentrated mother liquor. An analytical specimen was secured by an additional recrystallization from anhydrous ether. Due to hygroscopicity it proved necessary to determine the m. p. in capillary tubes, sealed after drying over P_2O_5, m. p. 61−62°, $[\alpha]_D^{23}$ −45° (H_2O, *c* 1.15). (Found: C 46.65; H 8.72; N 13.40. Calc. for $C_4H_9NO_2$: C 46.60; H 8.80; N 13.59.) It is interesting to notice that the higher-melting racemic 2-hydroxybutyramide (m. p. 105° [25], 108° [26]) does not possess hygroscopic properties, a finding paralleled in the lactamide series.

Reduction of (−)-2-hydroxybutyramide to (VI). In a three-necked flask, provided with magnetic stirring, dropping funnel, nitrogen inlet and a Soxhlet-type extractor was placed a solution of lithium aluminium hydride (6.0 g) in dry ether (660 ml). (−)-2-Hydroxybutyramide (4.0 g) was placed in the extractor and washed down in the course of 2 h by the refluxing ether, during which time the reaction mixture was stirred in a slow stream of dry nitrogen. Excess reagent was then destroyed by addition of wet ether, whereafter a saturated solution (300 ml) of potassium sodium tartrate was introduced. The ether layer was combined with three 250 ml-extracts of the aqueous phase. The amine content in the dried ether solution was determined by titration to 1.21 g, corresponding to a 34 % yield.

Addition of the amine solution to an ethereal solution of oxalic acid (2.05 g) resulted in the separation of the acid oxalate in crystalline form (1.76 g), m. p. 150°. An analytical specimen was produced on recrystallization from absolute ethanol, m. p. 150°, alone or in admixture with the sample described above; $[\alpha]_D^{22}$ +11.8° (H_2O, *c* 0.71). (Found: C 40.30; H 7.27; N 7.91. Calc. for $C_6H_{13}NO_5$: C 40.21; H 7.31; N 7.82). As further criterion of identity of the two independently synthesized specimens served the coinciding infra-red spectra which, in addition to numerous expected bands, displayed an unidentified, characteristic peak at 718 cm^{-1} (KBr-disc).

Acid oxalate of (−)-1-amino-3-buten-2-ol. This salt was desired particularly for the purpose of studying its infrared spectrum. It was prepared from the above α-bromocamphor-π-sulphonate by liberation of the amino-alcohol with strong base, a process accompanied by unknown secondary reactions, followed by treatment with one equivalent of oxalic acid. The salt crystallized from absolute ethanol in colourless prisms, m. p. 134°, $[\alpha]_D^{23}$ −5.9° (H_2O, *c* 1.0). (Found: N 7.60. Calc. for $C_6H_{11}NO_5$: N 7.90). Reported m. p. for the racemic salt: 130.5−132° [6].

The infra-red spectrum, was very similar to that of the acid oxalate of the saturated amino-alcohol (VI), having in common with the latter the band at 718 cm^{-1}. Significant deviations were noted, however, particularly in the 800−1 100 cm^{-1} region.

The authors are indebted to Dr. J. Villadsen of the H. Topsøe Laboratory for the loan of a butadiene tank.

The work is part of investigations supported by *Statens Almindelige Videnskabsfond (The Danish State Research Foundation)* and *Carlsbergfondet (The Carlsberg Foundation)*. A special grant from *Kai Hansen's Fond* is gratefully aknowledged.

REFERENCES

1. Astwood, E. B., Greer, M. A. and Ettlinger, M. G. *J. Biol. Chem.* **181** (1949) 121.
2. Greer, M. A., Ettlinger, M. G. and Astwood, E. B. *J. Clin. Endocrinol.* **9** (1949) 1069.
3. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
4. Schultz, O.-E. and Wagner, W. *Arch. Pharm.* **289/61** (1956) 597.
5. Greer, M. A. *J. Am. Chem. Soc.* **78** (1956) 1260.
6. Ettlinger, M. G. *J. Am. Chem. Soc.* **72** (1950) 4792.
7. Jordan, C. 1re Thèse, *Dédoublement de l'acide butanoloique 2. et recherches sur les dérivés actifs de cet acide.* (Diss.) University, Genève 1895.
8. Levene, P. A. and Haller, H. L. *J. Biol. Chem.* **74** (1927) 343.
9. Szarvas, P. *Z. physik. Chem. A.* **188** (1941) 235.
10. Fredga, A., Tenow, M. and Billström, I. *Arkiv Kemi, Mineral. Geol.* **16A** (1943) No. 21.
11. Winitz, M., Bloch-Frankenthal, L., Izumiya, N., Birnbaum, S. M., Baker, C. G. and Greenstein, J. P. *J. Am. Chem. Soc.* **78** (1956) 2423.
12. Horn, D. H. S. and Pretorius, Y. Y. *J. Chem. Soc.* **1954** 1460.
13. Noyce, D. S. and Denney, D. B. *J. Am. Chem. Soc.* **72** (1950) 5743.
14. Cahn, R. S., Ingold, C. K. and Prelog, V. *Experientia* **12** (1956) 81.
15. Kjær, A. and Gmelin, R. *Acta Chem. Scand.* **11** (1957) 906.
16. Kjær, A. and Gmelin, R. *Acta Chem. Scand.* **12** (1958) 1693.
17. Virtanen, A. I., Kreula, M. and Kiisvaara, M. *Acta Chem. Scand.* **12** (1958) 580.
18. Kadesch, R. *J. Am. Chem. Soc.* **68** (1946) 44.
19. Raciszewski, Z. M., Spencer, E. Y. and Trevoy, L. W. *Can. J. Technol.* **33** (1955) 129.
20. Marvel, C. S. *Org. Syntheses* **21** (1941) 74.
21. Bischoff, C. A. and Walden, P. *Ann.* **279** (1894) 100.
22. Guye, P. A. and Jordan, C. *Bull. soc. chim. France* [3] **15** (1896) 474.
23. Clemmensen, E. and Heitman, A. H. C. *Am. Chem. J.* **42** (1909) 319.
24. Reppe, W. *Ann.* **596** (1955) 1, p. 69.
25. Bredereck, H., Gompper, R. and Theilig, G. *Chem. Ber.* **87** (1954) 537.
26. Mengelberg, M. *Chem. Ber.* **87** (1954) 1425.

Received October 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 151—158

The Addition of Dimethylamine to Propylene Sulphide

BERTIL HANSEN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

The product of the reaction between dimethylamine and propylene sulphide in ether has been studied in several ways, and found to be 2-dimethylamino-propanethiol. Based on a comparison with the opening of alkene oxide and alkene immonium rings, it is proposed that the opening of the propylene sulphide ring includes a cyclic sulphonium intermediate, $CH_3-CH-CH_2$ (S$^+$, R).

For the preparation of derivatives of α- and β-methyl-thiocholine esters 2-dimethylamino-propanethiol-(1) and 1-dimethylamino-propanethiol-(2) are useful starting materials [1]. As 2-dimethylamino-ethanethiol is easily obtained from ethylene sulphide and dimethylamine [2], it seemed obvious to investigate if one of the dimethylamino-propanethiols could be prepared from propylene sulphide and dimethylamine. This paper describes the work carried out to find out which of the two possible thiols I and II was formed in this reaction(1).

$$CH_3CH\text{-}CH_2 + (CH_3)_2NH \rightarrow \begin{cases} (CH_3)_2NCHCH_2SH & (I) \\ \quad\quad CH_3 & \\ (CH_3)_2NCH_2CHSH & (II) \\ \quad\quad CH_3 & \end{cases} \qquad (1)$$

The reaction between propylene sulphide and an amine is described in the literature, but nobody has shown how the ring ruptures. Reppe and Nicolai [3] say that they have obtained a primary mercaptan by treating propylene sulphide with *n*-butylamine. On the other hand Snyder *et al.*[4] guess that they got a secondary thiol when di-*n*-amylamine reacted with propylene sulphide, because they could show that the same amine with *iso*-butylene sulphide gave a tertiary mercaptan.

Regarding other reactions with propylene sulphide it has been found that hydrogen chloride, acetyl chloride and acetyl bromide [5] open the ring

at the centre carbon atom, while acetic anhydride [5] and lithium aluminium hydride [6] destroy the bond between the sulphur atom and the terminal carbon atom. There is, in other words, no general rule for the opening of the propylene sulphide ring which can be applied to reactions between amines and propylene sulphide.

EXPERIMENTAL

Propylene sulphide was prepared from propylene oxide and thiourea [1]. B. p. 74—76°.

Addition of dimethylamine to propylene sulphide. The reaction was carried out in anhydrous ether as described in Ref.[1] Physical data for the dimethylamino-propanethiol are b. p. 71°/88 mm; $n_D^{25} = 1.4538$; $d^{25} = 0.8693$ g/cm³.

The hydrochloride when crystallized from a mixture of *iso*propanol/ligroin gave needles which, after being dried in a vacuum desiccator, melted at 167°. (Found: C 38.6; H 9.1. Calc. for $C_5H_{14}ClNS$ (155.7): C 38.6; H 9.1.) The picrate, recrystallized from *iso*propanol/petroleum ether and dried in air, melted at 94.5°. (Found: C 38.1; H 4.5. Calc. for $C_{11}H_{16}N_4O_7S$ (348.3): C 37.9; H 4.6.)

1-Dimethylamino-2-chloropropane hydrochloride. See Ref.[1] M. p. 185°. Schultz and Sprague [7] observed 185—186°.

2-Dimethylamino-1-chloropropane hydrochloride was prepared from thionyl chloride and 2-dimethylamino-propanol-(1) which was obtained by reducing ethyl-α-dimethylamino-propionate with lithium-aluminium hydride. M. p. 102°. Schultz and Sprague [7] observed 103—104°.

Iso*thiuronium compounds.* Dimethylamino-chloropropane hydrochloride and thiourea were refluxed in abs. ethanol [1]. For the product from 1-dimethylamino-2-chloropropane hydrochloride see Ref.[1]

From 2-dimethylamino-1-chloropropane hydrochloride a crude product in 54 % yield was obtained, which, after recrystallization, melted at 152°. (Found: C 30.6; H 7.4. Calc. for $C_6H_{17}Cl_2N_3S$ (234.2): C 30.8; H 7.3.)

Hydrolysis of iso*thiuronium salts.* The reaction was carried out as described in Ref.[1] For the hydrolysis of the isothiuronium compound from 1-dimethylamino-2-chloropropane hydrochloride see Ref.[1] The hydrochloride of the hydrolysis product after reprecipitation by ether from a solution in absolute ethanol/ligroin (1:1) melted at 114—115°. (Found: C 38.5; H 9.4. Calc. for $C_5H_{14}ClNS$ (155.7): C 38.6; H 9.1.)

The picrate, after recrystallization from absolute ethanol, melted at 168°. Renshaw *et al.*[8] reported 159—166°. (Found: C 38.0; H 4.6. Calc. for $C_{11}H_{16}N_4O_7S$ (348.3): C 37.9; H 4.6.)

The *iso*thiuronium compound from 2-dimethylamino-1-chloropropane hydrochloride gave thiol in 48 % yield. B. p. 55.5°/27 mm. $n_D^{25} = 1.4678$. (Found: C 50.1; H 11.0. Calc. for $C_5H_{13}NS$ (119.2): C 50.4; H 10.8.)

S-Acetylation and N-methylation of the dimethylamino-propanethiols. The reactions with acetic anhydride and methyl bromide were carried out as reported in Ref.[1] The dimethylamino-propanethiol from propylene sulphide gave a product melting at 188°. The dimethylamino-propanethiol from the *iso*thiuronium salt gave a product melting at 168°. For analyses see Ref.[1]

Treatment of the dimethylamino-propanethiols with a Raney nickel catalyst. To 2 ml of dimethylamino-propanethiol in 100 ml of absolute ethanol Raney nickel catalyst, W 5[9], was added in portions, with shaking, until the gas evolution ceased. At the same time the red colour formed during the reaction disappeared. The catalyst was removed by filtration, and a saturated solution of picric acid in absolute ethanol was added. To avoid excess of picric acid the E.M.F. of a glass-calomel electrode system was plotted against the volume of the picric acid solution. When the curve showed that the equivalence point had almost been reached, the addition was stopped. The solution was evaporated to dryness under reduced pressure at a temperature below 40°. The residue was crystallized once from water. The yield of the crude product was about 50 %.

The dimethylamino-propanethiol from the *iso*thiuronium salt gave a product which melted at 110—115°. Recrystallization gave flakes which melted at about 110°. (Found: C 41.6; H 5.2. Calc. for $C_{11}H_{10}N_4O_7$ (316.3): C 41.8; H 5.1.)

The dimethylamino-propanethiol from propylene sulphide gave a product which melted at 130—150°. Recrystallization gave flakes which melted at about 110°. (Found: C 41.6; H 5.2. Calc. for $C_{11}H_{16}N_4O_7$ (316.3): C 41.8; H 5.1.)

Treatment of quaternary nitrogen compounds with a Raney nickel catalyst. One gram of the nitrogen compound and 20 g of catalyst were shaken for 1 h in 75 ml of absolute methanol. After filtration the solution was evaporated to dryness under reduced pressure at room temperature. The reaction was carried out with the two acetyl methyl-thiocholine iodides [1], acetyl choline iodide and choline iodide. In all cases the residues amounted to only a few mg.

Dimethylpropylamine picrates. One mole of propyl iodide was dissolved in 1.5 l of dry ether and cooled to −10°. Three moles of anhydrous dimethylamine were added, and the mixture was placed in an ice-water bath, which was allowed to come to room temperature. After 6 days a precipitate, consisting mainly of dimethylamine hydroiodide and possibly a little dimethyldipropylammonium iodide, was filtered off. The excess dimethylamine in the filtrate was bound as amide by adding acetic anhydride in portions until no increase in the temperature could be observed. The dimethylpropylamine was then precipitated with hydrogen chloride. The precipitate was filtered off by suction, washed with ether and dried in a vacuum desiccator. The picrate was obtained on cooling a solution of the crude hydrochloride and an equivalent amount of picric acid in the smallest possible amount of hot water. It was recrystallized once from water. Dimethyl-*n*-propylamine picrate: flakes, m. p. 109°. (Found: C 41.8; H 5.1. Calc. for $C_{11}H_{16}N_4O_7$ (316.3): C 41.8; H 5.1.) Dimethyl-*iso*propylamine picrate: needles, m. p. 239°. (Found: C 41.8 H 5.1. Calc. for $C_{11}H_{16}N_4O_7$ (316.3): C 41.8; H 5.1.)

Infra-red spectra. Samples were prepared from about 2 mg of the compound to be investigated and 300 mg of potassium bromide.

The spectra were recorded with a Perkin-Elmer spectrophotometer, Model 21, equipped with a rock-salt prism. The following settings of the instrument were used: resolution 927, response 2:1170, gain 6, scale 5 cm/μ, speed about 1 μ/min.

RESULTS

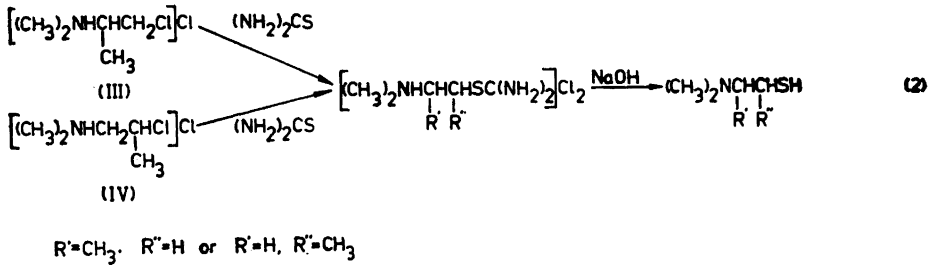
Preparative work. The addition of dimethylamine to propylene sulphide (reaction (1)) in ether gives a product which seems to consist of only one of the isomers I and II. It distils at constant temperature and the melting points of the hydrochloride and the picrate change only slightly on recrystallization (Table 1).

Table 1. Physical data for the products of the reactions (1) and (2) and derivatives.

Product of reaction	B.p./mm Hg	n_D^{25}	d^{25}g/cm^3	Melting-points			
				hydro-chloride	picrate	derivative *	
						pure	mixed with V
(1)	71°/88	1.4538	0.8693	167°	94.5°	187°	187°
(2)	78.5°/70	1.4684	0.9046	114—115°	168°	168°	135—140°

* Acetyl methyl-thiocholine bromide.

As the analytical literature gives no test that is characteristic for primary or secondary thiols, an attempt was made to synthesize I and II. A possible way for the dimethylamino-propanethiols was to synthesize them from the hydrochlorides of the corresponding chlorides, III and IV. However, when



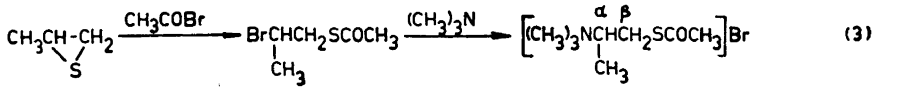
(2)

R'=CH_3. R''=H or R'=H, R''=CH_3

these compounds were refluxed with thiourea in ethanol the resulting *iso*-thiuronium compounds were found to be identical (from III m.p. 152°; from IV m.p. 154°). The refractive index of the thiols formed on hydrolysis of the *iso*thiuronium salts also indicated the formation of only one isomer (from III $n_D^{25} = 1.4678$; from IV $n_D^{25} = 1.4684$).

The fact that only one *iso*thiuronium salt was obtained from III and IV is not astonishing, as Schultz and Sprague [7] have found that III when melted rearranges into IV. As IV is the stable isomer, it seems most likely that an isomerisation occurs in the reaction between III and thiourea giving a compound with the *iso*thiuronium group bonded to the centre carbon atom. However, the possibility of an isomerisation in the reaction with IV could not be excluded at this point.

As the dimethylamino-propanethiols resulting from the reactions (1) and (2) have different physical properties themselves and as salts (Table 1), one of the thiols must have the structure I and the other II. By treatment first with acetic anhydride and then with methyl bromide they were transformed into N-(S-acetyl-mercaptopropyl)-trimethylammonium bromides (acetyl methyl-thiocholine bromides) and a comparison could thus be made with the product of reaction (3) (m.p. 187°). V was known to be acetyl α-methyl-thiocho-



(3)

(V)

line bromide, as Davies and Savige [5] have been able to elucidate the direction of the first step of reaction (3). From the values in Table 1 the conclusion can be drawn that the product of reaction (1) has the structure I and that (2) gives II. A study of the infra-red spectra of the acetyl methyl-thiocholine bromides confirms the correctness of the conclusion.

Degradations with a Raney nickel catalyst. In the hope of finding other ways of confirming the structures of the dimethylamino-propanethiols they were hydrogenated with a Raney nickel catalyst, a method that has been widely used for replacing the sulphur atom of organic sulphur compounds

with hydrogen atoms [10,11]. The amines formed were isolated as picrates and compared with the picrates of the tertiary amines of reaction (4).

$$2(CH_3)_2NH + RI \rightarrow RN(CH_3)_2 + [(CH_3)_2NH_2]\ I \qquad (4)$$

R = *n*-propyl or *iso*propyl

Contrary to the picrates of (4) (*n*-propyl m.p. 109°; *iso*propyl m.p. 239°) the two picrates from the hydrogenation reactions showed no distinct melting-points indicating that they were not pure. After one recrystallization from water both melted at about 110°. The melting-points and the analyses indicate a rearrangement of the *iso*propylamine derivative into the *n*-propyl one during the hydrogenation. Further confirmation for the rearrangement was obtained from the infrared spectra of the four picrates.

No rearrangement has been observed on treating benzoyl-L(—)-cystine with Raney nickel, and benzoyl-L(+)-alanine is formed [11]. A possible explanation of the difference between the reactions lies in the character of the unshared electron pair of the nitrogen atom, which in benzoyl-cystine is strongly attrac-

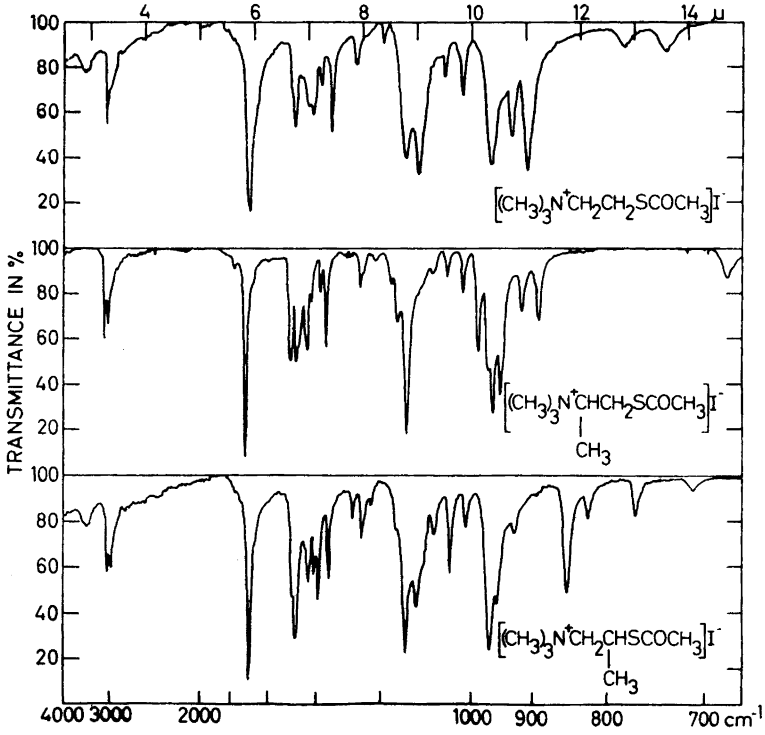


Fig. 1. Infra-red spectra of acetyl thiocholine iodide (first spectrum) and of the acetyl methyl-thiocholine iodides prepared from the dimethylamino-propanethiols derived from propylene sulphide and dimethylamine (second spectrum) and from S-(dimethylamino-propyl)-isothiuronium chloride hydrochloride.

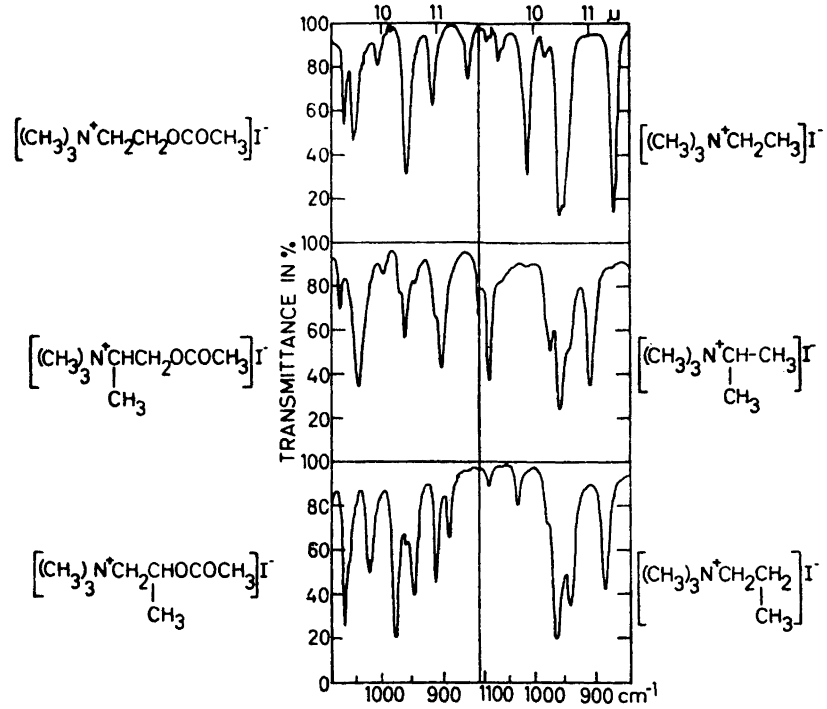


Fig. 2. Infra-red spectra of some derivatives of choline and of some tetraalkylammonium iodides.

ted by the benzoyl and the carboxylic groups and thus has a reduced ability to take part in reactions. If all the electron pairs of the nitrogen atom are occupied, as in a quaternary ammonium compound, no rearrangement would be expected. The acetyl methyl-thiocholine iodides prepared from the two dimethylamino-propanethiols [1] were therefore treated in ethanol solution with Raney nickel. The reactions were supposed to give *n*-propyl- and *iso*-propyltrimethylammonium iodide. However, when the solutions were evaporated to dryness at room temperature almost no residue was left, showing that the quaternary ammonium group must have been degraded by the Raney nickel. This behaviour was also found on treating choline iodide and acetyl choline iodide with Raney nickel. Consequently, the hydrogenation reactions could not give any information on the structures of the dimethylamino-propanethiols.

Infra-red spectra. The spectra of the acetyl methyl-thiocholine iodides derived from the two dimethylamino-propanethiols and of acetyl thiocholine iodide [2] are shown in Fig. 1. The compounds are differently substituted in the carbon chain between the nitrogen and the sulphur atoms and must therefore exhibit dissimilarities in the C—N^+ and C—S(—CO) bands. The C—S stretching bands which are expected to appear at 600—660 cm^{-1} (15—

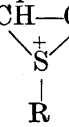
17 μ)[12] could not be recorded. As tetramethylammonium iodide has strong absorbtion in the range 900—1 000 cm^{-1} (11—10 μ) which can be ascribed to the C—N^+ bond, this range has also been recorded (Fig. 2) for some other compounds related in the same way as the thiocholine esters. In all cases the compounds with the simplest structure have the simplest spectra in this range, while the most complicated spectra are found for the compounds having an additional methyl group at the carbon atom adjacent to the nitrogen atom. This result shows that the infra-red spectra give some information about the actual structures and is a support for the formation of 2-dimethylamino-propanethiol-(1) in the addition of dimethylamine to propylene sulphide.

DISCUSSION

The only direct proof that the opening by dimethylamine of the propylene sulphide ring leads to a primary mercaptan (1) is the identification, by comparison of the melting-points, of the acetyl methyl-thiocholine bromide obtained from the reaction product with that obtained from the addition product of propylene sulphide and acetylbromide (3). This result is, however, more or less clearly supported by the other experiments, and nothing points to a rupture of the terminal C—S-bond. Only the rates of hydrolysis of the two acetyl methyl-thiocholine bromides, which have been studied by Heilbronn [13,14], seem strange. She found that the compound prepared from the product of (2) was hydrolyzed somewhat faster than V, while the reverse relationship was observed for the corresponding oxygen compounds. No explanation of the difference has been found.

It is interesting to compare the ring opening of propylene oxide and propylene sulphide. The propylene oxide ring is, in almost every reaction, destroyed at the terminal carbon atom, while the propylene sulphide ring generally ruptures at the centre carbon atom. The difference is probably due to different reaction mechanisms. The first step in the addition to propylene oxide is often a nucleophilic attack, for instance the additions of acetic acid [15], phenol [16] and methanol [17] which all are catalyzed by alkali.

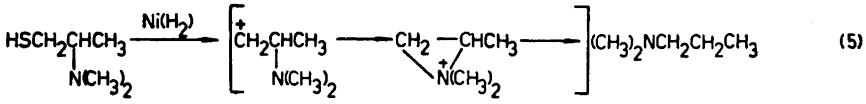
As sulphur atoms give onium compounds much more easily than oxygen atoms, it seems probable that additions to propylene sulphide involve the formation of an intermediate cyclic sulphonium ion, $CH_3CH—CH_2$,



which then opens at the centre carbon atom while capturing a negative ion. Support for this theory is obtained from ring ruptures of nitrogen compounds. Schultz and Sprague [7] found that (1-chloropropyl-(2))-dimethylamine cannot be distilled without rearrangement to (2-chloropropyl-(1))-dimethylamine. It is very likely that this rearrangement involves the formation of the cyclic quaternary ammonium ion, $CH_3CH—CH_2$, which opens at the centre carbon atom.

Another reaction showing that an *iso*propyl amino compound is less stable than a *n*-propyl amino compound is the reaction of 2-dimethylamino-propane-

thiol-(1) with Raney nickel catalyst, $Ni(H_2)$, which gives dimethyl-*n*-propylamine as described above. This reaction also throws some light on the mechanism of the removal of the sulphur atom by the Raney nickel catalyst. The



(5)

first step seems to be a rupture of the C—S-bond in such a way that the shared electron pair goes to the sulphur atom. Thus a carbonium ion is formed which can react with the unshared electron pair of the nitrogen atom, resulting in a cyclic quaternary immonium ion. This finally opens at the centre carbon atom.

The formation of a cyclic sulfonium ion intermediate in some addition reactions with propylene sulphides has also been proposed by Davies and Savige [5,18]. For the reaction between propylene sulphide and an amine they propose, however, a ring-opening at the terminal carbon atom. It is certainly difficult to find a source for the first hydrogen ions required for the intermediate sulphonium ion $CH_3CH(\overset{+}{S}H)CH_2$, but once some mercaptan has formed it can give rise to the hydrogen ions necessary.

To Professor G. Ljunggren, the Head of this Institute, I wish to express my gratitude for his kind interest in this work.

REFERENCES

1. Hansen, B. *Acta Chem. Scand.* **13** (1959) 159.
2. Hansen, B. *Acta Chem. Scand.* **11** (1957) 537.
3. Reppe, W. and Nicolai, F. *German Patent 631,016*. May 20, 1936.
4. Snyder, H. R., Stewart, J. M. and Ziegler, J. B. *J. Am. Chem. Soc.* **69** (1947) 2672.
5. Davies, W. and Savige, W. E. *J. Chem. Soc.* **1950** 317.
6. Bordwell, F. G., Andersen, H. M. and Pitt, B. M. *J. Am. Chem. Soc.* **76** (1954) 1082.
7. Schultz, E. M. and Sprague, J. M. *J. Am. Chem. Soc.* **70** (1948) 48.
8. Renshaw, R. R., Dreisbach, P. F., Ziff, M. and Green, D. *J. Am. Chem. Soc.* **60** (1938) 1765.
9. *Org. Syntheses Coll. Vol. III* (1955) 176.
10. Bougault, J., Cattelain, E. and Chabrier, P. *Bull. soc. chim. France* [5] **7** (1940) 781.
11. Mozingo, R., Wolf, D. E., Harris, S. A. and Folkers, K. *J Am. Chem. Soc.* **65** (1943) 1013.
12. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules* Methuen & Co. **Ltd.**, London 1954, p. 291.
13. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1481.
14. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1492.
15. Fraenkel-Conrat, H. and Olcott, H. S. *J. Am. Chem. Soc.* **66** (1944) 1420.
16. Sexton, A. R. and Britton, E. C. *J. Am. Chem. Soc.* **70** (1948) 3606.
17. Adams, R. M. and Vanderwerf, C. A. *J. Am. Chem. Soc.* **72** (1950) 4368.
18. Davies, W. and Savige, W. E. *J. Chem. Soc.* **1951** 774.

Received October 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 159—162

The Preparation of Thiocholine Esters

II. Esters of α- and β-Methyl-thiocholine

BERTIL HANSEN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

The paper describes the preparation of α-methyl- and β-methyl-thiocholine halide esters of the lower fatty acids and of benzoic acid. The α-methyl compounds were prepared from propylene sulphide either by addition of acid bromide and then trimethylamine or preferably by addition of dimethylamine, acid anhydride and finally methyl halide. The last two steps were also used for the synthesis of the β-methyl compounds from 1-dimethylamino-propanethiol-(2).

The preparation of thiocholine esters in two ways from ethylene sulphide has been described earlier [1]. This paper gives methods for the syntheses of α- and β-methyl-thiocholine esters, which were of interest for biochemical, physicochemical [2,3] and pharmacological comparisons with the thiocholine esters.

The 1-dimethylamino-propanethiol-(2) required for the preparation of the β-methyl-thiocholine derivatives was synthesized as described by Renshaw *et al.*[4] from 1-dimethylamino-2-chloropropane hydrochloride and thiourea. The melting-point of the intermediate *iso*thiuronium compound could not, even after several recrystallizations, be raised above 154°, and the value 201—2° found by Renshaw *et al.*[4] seems doubtful.

An attempt to prepare 2-dimethylamino-propanethiol-(1) in the same way from 2-dimethylamino-1-chloropropane hydrochloride and thiourea failed and resulted in 1-dimethylamino-propanethiol-(2)[5]. However, the 2-dimethylamino-propanethiol-(1) was easily obtained as the only product from the addition of dimethylamine to propylene sulphide in ether solution [5].

The esters of the α- and β-methyl-thiocholine halides were prepared in good yields from the thiols by esterification with acid anhydride and quaternization with methyl halide. Another method for the α-methyl-thiocholine esters was based on the addition of an acid bromide to propylene sulphide, which gave a 2-bromo-*n*-propyl-thiolo ester [6]. This was quaternized with trimethylamine.

All the α-methyl-thiocholine compounds, independent of the method of preparation, at once formed crystals, whereas some of the β-methyl-thiocholine

iodides could not be obtained in crystalline form. Thus the esters of the *iso*-butyric, *n*-butyric and *n*-valeric acids were obtained as oils of which the last-mentioned partially crystallized after one or two days. The melting-points of the aliphatic esters of the α-methyl-thiocholine were higher than those of the corresponding β-methyl-thiocholine derivatives. The compounds were found to be hygroscopic, and even a few minutes exposure to moist air produced, in some cases, enough hydrolysis to give, some time later, a smell of the acid component. Owing to the formation of iodine the iodides assumed a yellow discolouration when stored in light. The solubility of the salts decreased in water and increased in ethanol with increasing molecular weight.

The acetyl and benzoyl β-methyl-thiocholine iodides have been described by Renshaw *et al.*[4] The melting-points given by them were found to be a few degrees below those now reported.

Ivin [7] has prepared a compound which, according to him, is acetyl α-methyl-thiocholine iodide. The compound was obtained by treating propylene sulphide with acetyl iodide and then adding trimethylamine. It gave acceptable nitrogen and sulphide analyses, but its iodine content was in accordance with a wrongly calculated value (found 35.5, calc. 35.3; correctly calc. 41.9). Furthermore the melting-point 238° is much higher than that for the compound prepared in this institute, 164°. It thus seems clear that Ivin's compound cannot have been acetyl α-methyl-thiocholine iodide or that he by mistake has reported some erroneous values.

EXPERIMENTAL

Propylene sulphide. Propylene oxide and thiourea gave propylene sulphide (b.p. 74—76°; yield 58 %) when treated as described for the preparation of ethylene sulphide[1].

Esters of 2-bromo-propanethiol-(1). The esters were obtained from propylene sulphide and acid bromides in the same way as the esters of 2-bromo-ethanethiol-(1)[1]. The boiling points and yields are given in Table 1. The products were not analysed.

Table 1. Esters of 2-bromo-propanethiol-(1).

Acid	Formula	Mol.wt	B.p./mm Hg	Yield in %
Acetic	C_5H_9BrOS	197.1	72—73°/6 *	73
Propionic	$C_6H_{11}BrOS$	211.1	93—96°/9	66
n-Butyric	$C_7H_{13}BrOS$	225.1	99.5—100.5°/8	53
n-Valeric	$C_8H_{15}BrOS$	239.2	125—126.5°/10	53

* Davies and Savige [6] found 45°/0.2.

Esters of α-methyl-thiocholine bromide. The esters of 2-bromo-propanethiol-(1) were allowed to react in dry acetone (the valeric derivative in dry acetone/ligroin 1:1) with excess of trimethylamine. The precipitates were recrystallized, the acetic ester from dry ethanol/ligroin and the other ones from dry acetone/ligroin. Analyses and melting-points are given in Table 2.

Table 2. Esters of α-methyl-thiocholine bromide.

Acid	Formula	Mol. wt.	M. p.	C		H		Br		Yield in %
				calc.	found	calc.	found	calc.	found	
Acetic	$C_8H_{18}BrNOS$	256.2	187°	37.5	37.2	7.1	7.0	31.2	31.3	63
Propionic	$C_9H_{20}BrNOS$	270.2	154°	40.0	39.9	7.5	7.5	29.6	29.3	63
n-Butyric	$C_{10}H_{22}BrNOS$	284.3	170°	42.3	42.2	7.8	7.6	28.1	28.3	54
n-Valeric	$C_{11}H_{24}BrNOS$	298.3	179°	44.3	44.0	8.1	8.1	26.8	26.9	67

2-Dimethylamino-propanethiol-(1). A solution of 46 g (0.63 mole) of propylene sulphide dissolved in 65 ml of dry ether was cooled to 0° and 75 ml (1.05 mole) of anhydrous dimethylamine were added. The mixture was stored at room temperature for two days and then distilled through a 25 cm Widmer column, the excess of dimethylamine and the ether being removed at ordinary pressure and the 2-dimethylamino-propanethiol-(1) being distilled under reduced pressure. Yield 51.5 g (69 %) at 71°/88 mm; $n_D^{25} = 1.4538$; $d^{25} = 0.8693$ g/cm³. (Found: C 50.4; H 10.8. Calc. for $C_5H_{13}NS$ (119.2): C 50.4; H 11.0.)

1-Dimethylamino-2-chloropropane hydrochloride was prepared from thionyl chloride and 1-dimethylamino-propanol-(2), obtained by adding dimethylamine to propylene oxide. M. p. 185°. Schultz and Sprague [8] observed 185—186°.

S-(1-Dimethylaminopropyl-(2))-isothiuronium chloride hydrochloride. One mole of 1-dimethylamino-2-chloropropane hydrochloride and 1.02 mole of thiourea in 600 ml of absolute ethanol were refluxed for 65 h. The solution was cooled to room temperature and ethyl acetate then added in small portions until the first permanent opalescence was obtained. The vessel was scratched with a glass rod and two days later crystals had formed. They were removed by suction, washed with absolute ethanol/ethyl acetate (1:1) and dried in air. Yield 122 g (52 %). After being recrystallized from *n*-propanol and dried at 75° the compound melted at 154°. (Found: C 30.4; H 7.3. Calc. for $C_6H_{17}Cl_2N_3S$ (234.2): C 30.8; H 7.3.)

By performing the reaction in *n*-propanol Renshaw *et al.*[4] obtained the *iso*thiuronium compound in 92 % yield. Their method was tried without success, nor could their melting-point, 201—202°, be reproduced.

1-Dimethylamino-propanethiol-(2). One mole of the *iso*thiuronium compound was dissolved in 280 ml of water. The solution was covered with ether in an extraction apparatus. With cooling, a solution of 2.0 moles of sodium hydroxide in 80 ml of water was added in portions. The reaction mixture was continously extracted with ether until there was no further change in the volume of the water phase. The ether solution was dried with calcium chloride and distilled through a 25 cm Vidmer column, the ether at ordinary and the thiol under reduced pressure. Yield 64 %; b. p. 78.5°/70 mm; $n_D^{25} = 1.4684$; $d^{20} = 0.9046$ g/cm³.

Esterification of the dimethylamino-propanethiols. The reactions between the thiols and the acid anhydrides were carried out as described for 2-dimethylamino-ethanethiol [1]. The boiling ranges are given in Table 3.

Table 3. B.p./mm Hg of esters of x-dimethylamino-propanethiol-(y).

Thiol \ Acid	Acetic	Propionic	*n*-Butyric	*Iso*butyric	*n*-Valeric	Benzoic
x = 1, y = 2	76—82°/11	78—88°/10	74—85°/1.8	86—92°/12	67—82°/0.3	120—125°/0.63
x = 2, y = 1	64.5—66°/11	67—81°/12	80—88°/9	85—94°/10	53—60°/0.07	88—96°/0.01

Table 4. Esters of x-methyl-thiocholine halides.

x	Acid	Formula	Mol. wt.	M. p.	C		H		Halogen		Yield * in %
					calc.	found	calc.	found	calc.	found	
α	Acetic	$C_8H_{18}BrNOS$	256.2	188°	37.5	37.6	7.1	7.1	31.2	31.1	—
β	»	$C_8H_{18}BrNOS$	256.2	168°	37.5	37.5	7.1	7.2	31.2	—	—
α	»	$C_8H_{18}INOS$	303.2	164°	31.7	31.8	6.0	5.9	41.9	41.8	50
β	»	$C_8H_{18}INOS$	303.2	150°**	31.7	31.7	6.0	6.0	41.9	41.8	70
α	Propionic	$C_9H_{20}INOS$	317.2	155°	34.1	34.3	6.4	6.2	40.0	39.6	61
β	»	$C_9H_{20}INOS$	317.2	132°	34.1	34.0	6.4	6.3	40.0	39.9	65
α	*n*-Butyric	$C_{10}H_{22}INOS$	331 3	130°	36.3	36.3	6.7	6.7	38.3	38.8	62
β	»	$C_{10}H_{22}BrNOS$	284.3	164°	42.3	41.8	7.8	7.8	28.1	28.3	67
α	*Iso*butyric	$C_{10}H_{22}INOS$	331.3	158°	36.3	35.9	6.7	6.5	38.3	38.3	82
β	»	$C_{10}H_{22}BrNOS$	284.3	140°	42.3	42.4	7.8	7.8	28.1	—	32
α	*n*-Valeric	$C_{11}H_{24}INOS$	345.3	152°	38.3	38.4	7.0	6.8	36.8	—	62
β	»	$C_{11}H_{24}INOS$	345.3	74°	38.3	38.1	7.0	7.0	36.8	36.5	31
α	Benzoic	$C_{13}H_{20}INOS$	365.3	136°	42.7	42.4	5.5	5.4	34.8	—	54
β	»	$C_{13}H_{20}INOS$	365.3	194°***	42.7	42.5	5.5	5.6	34.8	—	76

* Calculated on the thiol. ** Renshaw *et al.*[4] reported 144—145°. *** Renshaw *et al.*[4] found 185—186°.

Esters of methyl-thiocholine iodides and bromides. The esters of the dimethylaminopropanethiols were quaternized with methyl bromide or iodide according to the method used earlier[1]. Of the α-methyl esters the acetic, butyric and benzoic compounds were recrystallized from dry acetone/ligroin (2:1). The acetyl β-methyl-thiocholine bromide was recrystallized from dry acetone/absolute ethanol (3:1) and the benzoic ester from water. Table 4 contains the melting-points, analyses and yields of the compounds.

All melting-points have been determined with a »Kofler Hot Bench».

My thanks are due to the Head of this Institute, Professor G. Ljunggren, for his interest in this work.

REFERENCES

1. Hansen, B. *Acta Chem. Scand.* **11** (1957) 537.
2. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1481.
3. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1492.
4. Renshaw, R. R., Dreisbach, P. F., Ziff. M. and Green, D. *J. Am. Chem. Soc.* **60** (1938) 1765.
5. Hansen, B. *Acta Chem. Scand.* **13** (1959) 151.
6. Davies, W. and Savige, W. E. *J. Chem. Soc.* **1950** 317.
7. Ivin, S. Z. *Zhur. Obshcheĭ Khim. (J. Gen. Chem.)* **28** (1958) 177.
8. Schultz, E. M. and Sprague, J. M. *J. Am. Chem. Soc.* **70** (1948) 48.

Received October 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 163—178

Composition of Products Formed by Thermal Neutron Fission of ^{235}U

III. Isotopic Composition and Atomic Weight of the Fission Product Elements

JAN PRAWITZ

Research Institute of National Defence, Dept. 1, Sundbyberg 4, and Institute of Inorganic and Physical Chemistry, University of Stockholm, Sweden

The isotopic composition and atomic weight of the fission product elements have been calculated for seven different irradiation times between one day and two years, two cooling times, 100 and 3 000 days, and three thermal neutron fluxes 10^{12}, 10^{13} and 10^{14} n cm^{-2} sec^{-1}. Some applications of the difference in composition between the natural and fission product elements are discussed.

Since more accurate nuclear data of the fission products are available, many calculations of their properties have appeared in the literature [1–12]. Most of these works only consider the radioactive isotopes. Calculations on the stable isotopes and their poisoning effect in thermal reactors have been performed by Blomeke and Todd [9], Robb *et al.*[7], Lock [3], and Walker [8]. Part II[12] of this series considers the fission products, both radioactive and stable, from a chemical point of view. Atomic weights of artificially produced elements in the transmercury region are discussed by Glass *et al.*[16]

At the moment of "shut down" of a reactor the fission fragments are highly radioactive, but after moderate cooling times the major part of them has become stable. After 100 days 80 % and after 10 years 90 % of the nuclei are stable or have half-lives longer than 10^{10} years. The fission product elements have other isotopic compositions and therefore also differing atomic weights than the natural elements. These two properties are functions of the irradiation and cooling time. Except for the effect of neutron absorption by the fission products themselves the isotopic composition is independent of the thermal neutron flux.

Presentation. In this paper the isotopic abundances in atomic per cent and the atomic weights have been calculated for all fission product elements for seven irradiation times, (1 and 7 days, 1, 2 and 6 months, 1 and 2 years) and two cooling times, 100 days, a practical moment for fuel reprocessing, and 3 000

Table 1. Isotopic composition of the fission product elements in atomic per cent for seven different irradiation times and two different cooling times. Thermal neutron flux $\leq 10^{14}$ n cm^{-2} sec^{-1}. In those cases where the neutron flux influences the results, values are given for three fluxes, 10^{12}, 10^{13}, and 10^{14} n cm^{-2} sec^{-1}, equal flux for values on the same line. If the compositions after 3 000 days cooling time are not independent of the irradiation time, values are given for two irradiation times: 7 days and depending on the neutron fluxes (10^{12}, 10^{13}, or 10^{14} n cm^{-2} sec^{-1}) 2 years, 6 months, or 1 month. Isotopic abundances of the natural elements are also given. In the half life column y stands for years, d for days, and h for hours.

Fission product										
Isotope	Half-life for radioactive isotope	Cooling time 100 days. Irradiation time 1 day	7 days	1 month	2 months	6 months	1 year	2 years	Cooling time 3 000 days Irradiation time $\leq$2 y	Natural element
1	2	3	4	5	6	7	8	9	10	11
^{3}He										10^{-4}
^{4}He					100				100	100
—										
^{70}Ge										20.55
^{72}Ge					0.6				0.6	27.37
^{73}Ge					4.0				4.0	7.67
^{74}Ge					16				16	36.74
^{76}Ge					80				80	7.67
—										
^{75}As					100				100	100
—										
^{74}Se										0.87
^{76}Se										9.02
^{77}Se					2.4				2.4	7.58
^{78}Se					5.1				5.1	23.52
^{79}Se	65 000 y				9.1				9.1	
^{80}Se					17.3				17.3	49.82
^{82}Se					66				66	9.19
—										
^{79}Br		7.9×	8.1×	9.0×	10×	15×	22×	36×10^{-5}	2.3 × 10^{-3}	50.52
^{81}Br					100				100	49.48
—										
^{78}Kr										0.354
^{80}Kr										2.27
^{82}Kr					0.058				0.060	11.56
		*			14.1				14.5 14.6	
^{83}Kr		** 14.1	14.1	14.1	14.1	13.8			14.5 14.2	11.55
		*** 14.1	14.1	13.7					14.5 14.1	
		*			25.8				26.6 26.7	
^{84}Kr		** 25.8	25.8	25.8	25.8	26.0			26.6 27.0	56.90
		*** 25.8	25.8	26.1					26.6 26.9	
^{85}Kr	10.27 y	7.43	7.43	7.42	7.34	7.31	7.23	7.01	4.5	
^{86}Kr		52.5	52.5	52.5	52.5	52.5	52.8	52.9	54.4	17.37

1	2	3	4	5	6	7	8	9	10	11
—										
^{85}Rb		28.8	28.8	28.8	28.9	28.9	29.0	29.2	31.3	72.15
^{87}Rb	6.2×10^{10} y	71.2	71.2	71.2	71.1	71.1	71.0	70.8	68.7	27.85
—										
^{84}Sr										0.56
^{86}Sr		2.9 ×	2.9 ×	2.9 ×	3.0 ×	3.2 ×	3.3 ×	3.4×10^{-4}	4.1×10^{-4}	9.86
^{87}Sr										7.02
^{88}Sr		33.5	33.7	34.2	34.8	36.4	37.5	38.3	46.8	82.56
^{89}Sr	50.4 d	12.5	12.1	10.6	9.04	5.25	2.72	1.40		
^{90}Sr	27.7 y	54.0	54.3	55.1	56.1	58.2	59.7	60.2	53.2	
—										
^{89}Y		65.5	66.8	70.6	74.6	84.7	91.2	95.3	99.97	100
^{90}Y	64.2 h	0.029	0.029	0.029	0.029	0.030	0.030	0.030	0.026	
^{91}Y	58.3 d	34.4	33.1	29.4	25.4	15.2	8.67	4.49		
—										
^{90}Zr		0.13	0.14	0.17	0.21	0.26	0.37	0.60	5.4	51.46
^{91}Zr		12.5	12.8	13.5	14.2	16.1	17.2	17.9	17.8	11.23
^{92}Zr		18.9	18.9	18.9	18.9	18.9	19.0	19.0	18.0	17.11
^{93}Zr	9.5×10^{5} y	20.3	20.3	20.3	20.3	20.3	20.4	20.4	19.4	
^{94}Zr		20.8	20.8	20.8	20.9	20.9	20.9	20.9	20.0	17.40
^{95}Zr	65 d	7.20	6.91	6.19	5.26	3.20	1.83	0.93		
^{96}Zr		20.0	20.0	20.0	20.1	20.1	20.1	20.1	19.2	2.80
—										
^{93m}Nb	3.65 y	2.6 ×	2.7 ×	3.1 ×	4.0 ×	8.7 ×	21 ×	63×10^{-5}	16	
^{93}Nb		5.4 ×	5.9 ×	6.9 ×	8.7 ×	19×	48×	159×10^{-5}	84	100
^{95m}Nb	90 h	0.12	0.12	0.11	0.11	0.10	0.09	0.09	$\sim10^{-10}$	
^{95}Nb	35 d	99.9							$\sim10^{-7}$	
—										
^{92}Mo										15.86
^{94}Mo										9.12
^{95}Mo		12.5	13.0	14.2	15.9	19.6	22.2	23.8	25.4	15.70
^{96}Mo		2.7 ×	2.7 ×	2.6 ×	2.6 ×	2.5 ×	2.4 ×	2.3×10^{-3}	2.3×10^{-3}	16.50
^{97}Mo		28.4	28.2	27.8	27.3	26.0	25.2	24.7	24.1	9.45
^{98}Mo		28.2	28.1	27.7	27.1	25.9	25.1	24.6	24.0	23.75
^{100}Mo		30.9	30.7	30.3	29.7	28.3	27.4	26.9	26.2	9.62
—										
^{99}Tc	2.12×10^{5} y	100							100	
—										
^{96}Ru										5.50
^{98}Ru										1.91
^{99}Ru										12.70
^{100}Ru										12.69
^{101}Ru		43.4	43.4	43.8	44.1	44.9	45.5	45.9	47.0	17.01
^{102}Ru		33.1	33.2	33.4	33.7	34.3	34.7	35.1	35.9	31.52
^{103}Ru	40 d	4.24	4.03	3.35	2.68	1.33	0.70	0.36		
^{104}Ru		15.8	15.8	15.9	16.1	16.3	16.5	16.7	17.1	18.67
^{106}Ru	366.6 d	3.52	3.51	3.46	3.39	3.09	2.66	2.02	0.01	
—										
^{103}Rh		100							100	100
—										
^{102}Pd										0.96
^{104}Pd										10.97
^{105}Pd		79	79	79	78	77	74	71	64	22.2
^{106}Pd		5	5	5	6	8	11	14	23	27.3
^{107}Pd	5×10^{6} y				10				9	
^{108}Pd					4				3	26.7
^{110}Pd					1				0.9	11.8

1	2	3	4	5	6	7	8	9	10	11
—										
^{107}Ag										51.35
^{109}Ag					100				100	48.65
—										
^{106}Cd										1.215
^{108}Cd										0.875
^{110}Cd					4×10^{-4}				4×10^{-4}	12.39
^{111}Cd					33.3				33.3	12.75
^{112}Cd					20.4				20.4	24.07
		* 24.1	23.9	23.4	22.8	20.5	17.7	13.4	23.9 13.4	
^{113}Cd		** 24.0	22.6	18.6	14.7	7.1			22.6 7.1	12.26
		*** 22.9	14.4	4.6					14.4 4.6	
		* 22.2	22.2	23.0	23.3	25.6	28.5	33.0	22.2 33.0	
^{114}Cd		** 22.3	23.7	27.8	31.5	39.3			23.7 39.3	28.86
		*** 23.3	31.9	41.5					31.9 41.5	
^{115m}Cd	43 d	0.26	0.25	0.21	0.17	0.08	0.04	0.02		
^{116}Cd										7.58
—										
^{113}In										4.23
^{115}In	6×10^{14} y				100				100	95.77
—										
^{112}Sn										0.95
^{114}Sn										0.65
^{115}Sn					0.61				0.61	0.34
^{116}Sn					11				11	14.24
^{117}Sn					11				11	7.57
^{118}Sn					11				11	24.01
^{119m}Sn	250 d	10.3	10.2	9.9	9.5	8.1	6.5	4.4		
^{119}Sn		3.3	3.4	3.7	4.1	5.5	7.1	9.2	13.6	8.58
^{120}Sn					13				13	32.97
^{122}Sn					16				16	4.71
^{123m}Sn	136 d	0.8	0.8	0.7	0.7	0.5	0.4	0.2		
^{124}Sn					23				23	5.98
—										
^{121}Sb		24	24	24	24	25	26	27	28 31	57.25
^{123}Sb		30	30	30	30	31	33	35	37 41	42.75
^{125}Sb	2.0 y	46	46	46	46	44	42	38	35 28	
—										
^{120}Te										0.089
^{122}Te										2.46
^{123}Te										0.87
^{124}Te										4.61
^{125m}Te	58 d	0.026	0.027	0.028	0.030	0.033	0.031	0.031		
^{125}Te		0.03	0.03	0.04	0.04	0.07	0.11	0.19	0.82	6.99
^{126}Te		3.6	3.6	3.6	3.6	3.6	3.7	3.7	3.7	18.71
^{127m}Te	105 d	0.63	0.62	0.57	0.52	0.37	0.24	0.13		
^{128}Te		3.6	3.6	3.6	3.6	3.6	3.7	3.7	3.7	31.79
^{129m}Te	41 d	2.4	2.2	1.8	1.4	0.72	0.38	0.19		
^{130}Te		90	90	91	91	91	92	92	92	34.49
—										
^{127}I		17.3	17.3	17.3	17.2	17.2	17.4	17.5	17.8	100
^{129}I	1.72×10^{7} y	82.6	82.6	82.7	82.8	82.8	82.6	82.5	82.2	
^{131}I	8.06 d	0.067	0.052	0.025	0.013	0.004	0.002	0.001		
—										

1	2		3	4	5	6	7	8	9	10		11
^{134}Xe												0.096
^{136}Xe												0.090
^{128}Xe			———			2×10^{-3}			———	2×10^{-3}		1.919
^{129}Xe												26.44
^{130}Xe												4.08
		*	13.6	13.4	13.3	13.3	13.3	13.3	13.3	13.4	13.3	
^{131}Xe		**	12.8	12.0	11.9	11.8	11.7			12.0	11.7	21.18
		***	11.8	11.0	10.5					11.0	10.5	
		*	20.1	19.7	19.6	19.6	19.6	19.6	19.6	19.7	19.6	
^{132}Xe		**	18.9	17.7	17.5	17.5	17.4			17.7	17.4	26.89
		***	17.5	16.2	16.5					16.2	16.5	
		*	36.3	35.6	35.5	35.5	35.5	35.5	35.5	35.6	35.5	
^{134}Xe		**	34.2	31.9	31.6	31.6	31.5			31.9	31.5	10.44
		***	31.6	29.3	29.0					29.3	29.0	
		*	30.1	31.3	31.5	31.6	31.6	31.6	31.6	31.3	31.6	
^{136}Xe		**	34.0	38.4	39.0	39.1	39.2			38.4	39.2	8.87
		***	39.1	43.6	44.2					43.6	44.2	
—												
		*	45.2	37.2	36.1	35.9	35.8	35.9	36.1	39.5	38.1	
^{133}Cs		**	47.1	43.2	42.7	42.6	42.6			46.3	45.7	100
		***	50.6	50.1	50.0					54.4	54.1	
		*			0.01	0.03	0.09	0.16	0.33	—	0.002	
^{134}Cs	2.19 y	**			0.1	0.3	1.0			—	0.008	
		***		~0.1	1.4					—	0.010	
		*	12.5	28.1	30.3	30.6	30.8	30.9	31.1	29.8	32.8	
^{135}Cs	2.1×10^6 y	**	9.0	16.6	17.5	17.7	17.8			17.8	19.0	
		***	2.05	3.24	3.41					3.5	3.7	
		*	42.1	34.7	33.6	33.5	33.3	33.1	32.9	30.7	29.0	
^{137}Cs	30.0 y	**	43.9	40.2	39.8	39.7	39.6			35.9	35.3	
		***	47.2	46.7	46.6					42.2	42.1	
—												
^{130}Ba												0.101
^{132}Ba												0.097
										* —	0.03	
^{134}Ba										** —	0.2	2.42
										*** —	0.2	
^{135}Ba												6.59
^{136}Ba			0.088	0.088	0.088	0.088	0.087	0.087	0.086	0.077		7.81
^{137}Ba			0.54	0.56	0.62	0.70	1.0	1.5	2.5	13.0	14.0	11.32
^{138}Ba			99.2	99.2	99.2	99.2	98.9	98.4	97.3	87.0	85.7	71.66
^{140}Ba	12.8 d		0.37	0.32	0.19	0.11	0.04	0.02	0.01			
—												
^{138}La	2×10^{11} y											0.089
^{139}La			———			100			———	100		99.911
—												
^{136}Ce												0.193
^{138}Ce												0.250
^{140}Ce			36.3	36.5	37.1	37.8	40.0	41.4	43.9	51.1		88.48
^{141}Ce	32.8 d		4.34	4.26	3.41	2.63	1.23	0.72	0.35			
^{142}Ce	5.1×10^{15} y		34.8	35.0	35.4	36.0	37.8	39.5	41.9	48.8		11.07
^{144}Ce	284.5 d		24.4	24.4	24.0	23.6	21.5	18.5	13.9	0.03		

1	2	3	4	5	6	7	8	9	10	11
—										
^{141}Pr		99.30	99.40	99.64	99.79	99.93	99.96	99.98	100	100
^{143}Pr	13.95 d	0.70	0.60	0.36	0.21	0.07	0.04	0.02		
—										
							*0.006	0.01	— 0.01	
^{143}Nd					** 0.005	0.02			— 0.02	27.13
			*** 0.01	0.04					0.01 0.03	
		* 36.0	36.0	35.8	35.5	34.5	33.2	31.5	28.6 28.3	
^{145}Nd		** 36.0	36.0	35.7	35.4	34.0			28.6 28.3	12.20
		*** 36.0	36.0	35.4					28.6 28.3	
		* 7.3	7.4	8.0	8.8	11.7	14.8	18.8	26.6 26.8	
^{144}Nd	2.2×10^{15} y	** 7.3	7.4	8.0	9.0	12.3			26.6 27.1	23.87
		*** 7.3	7.4	8.4					26.6 26.9	
^{145}Nd		24.1	24.1	23.9	23.7	23.0	22.2	21.1	19.0	8.30
^{146}Nd		18.4	18.4	18.2	18.0	17.5	16.9	16.1	14.5	17.18
^{146}Nd	11.6 d	0.037	0.030	0.017	0.010	0.003	0.002	0.001		
^{148}Nd		10.2	10.2	10.1	10.0	9.7	9.4	8.9	8.0	5.72
^{150}Nd		4.0	4.0	4.0	3.9	3.8	3.7	3.5	3.2	5.60
—										
^{147}Pm	2.66 y	———	———	———	100	———	———	———	100	
—										
^{144}Sm										3.16
^{147}Sm	1.25×10^{11}y	6.9	7.0	7.9	8.8	12.4	17.3	24.6	52	15.07
							* 0.1	0.2	— 0.1	
^{148}Sm					** 0.2	0.5			— 0.1	11.27
				*** 0.3					— 0.2	
		* 54.3	53.8	50.1	45.8	32.2	20.7	10.5	28 7	
^{149}Sm		** 54.2	50.1	30.1	17.6	5.8			26 3	13.82
		*** 53.6	33.5	9.0					17 5	
		* 0.01	0.5	3.7	7.5	18.7	27.6	33.3	2 21	
^{150}Sm		** 0.1	4.2	23.7	45.1	45.1			2 25	7.47
		*** 0.8	20.8	44.8					10 23	
		* 21.4	21.3	20.9	20.3	18.2	15.8	12.0	10 7	
^{151}Sm	93 y	** 21.3	20.9	18.4	15.8	9.1			10 5	
		*** 21.3	17.3	7.5					8 4	
		* 13.7	13.8	13.9	14.1	14.4	15.2	16.0	7 10	
^{152}Sm		** 13.7	14.2	16.6	18.8	23.6			7 13	26.63
		*** 13.8	17.7	27.9					9 15	
^{154}Sm		3.70	3.70	3.67	3.63	3.47	3.29	2.98	1.9	22.53
—										
									8.0 6.3	
^{151}Eu		0.2	0.2	0.2	0.1	0.2	0.7	0.9	8.0 3.7	47.86
									6.7 3.1	
^{153}Eu		91	91	91	92	93	94	95	92 94	52.14
		* 0.001	0.01	0.05	0.1	0.3	0.6	1.3	0.007 0.8	
^{154}Eu	16 y	** 0.01	0.1	0.5	1.0	2.9			0.07 2	
		*** 0.1	1	4.5					0.7 3	

1	2	3	4	5	6	7	8	9	10		11
		* 8.4	8.4	8.1	7.6	7.0	5.8	4.2	0.3	0.1	
^{155}Eu	1.7 y	** 8.1	8.0	7.0	5.9	3.4			0.3	0.1	
		*** 7.9	5.7	2.5					0.2	0.1	
		* 0.041	0.036	0.024	0.016	0.007	0.005	0.003			
^{156}Eu	15.4 d	** 0.041	0.040	0.034	0.028	0.016					
		*** 0.044	0.066	0.079							
—											0.20
^{152}Gd							* 0.2	0.4	—	2	
^{154}Gd									—	**3.8	2.15
									—	***5.8	
		* 8.2	8.6	8.9	9.1	10.3	9.4	7.1	44	24	
^{155}Gd		** 8.1	7.5	6.1	4.4	2.3			42	16	14.73
		*** 7.5	4.5	1.6					30	12	
		* 29	29	30	31	34	39	46	18	37	
^{156}Gd		** 30	31	38	43	54			19	44	20.47
		*** 32	42	59					31	48	
		* 36	34	29	26	12	5.8	2.7	21	2	
^{157}Gd		** 34	24	8.1	3.8	1.1			15	1	15.68
		*** 25	5.2	0.9					4	0.8	
		* 20	22	26	28	38	40	39	13	31	
^{158}Gd		** 21	31	42	43	38			19	31	24.87
		*** 30	42	37					31	30	
		* 6.5	6.5	6.3	6.2	5.8	5.3	4.8	} 4		
^{160}Gd		** 6.4	6.4	5.8	5.4	4.5					21.90
		*** 6.3	5.5	4.4							
—											
		* 100	100	100	100	99.99	99.99	99.99	} 100		
^{159}Tb		** 100	100	99.98	99.96	99.92					100
		*** 100	99.96	99.83							
		* 10^{-5}	4.10^{-4}	0.002	0.004	0.008	0.011	0.013			
^{160}Tb	72.3 d	** 10^{-4}	0.004	0.018	0.035	0.078					
		*** 10^{-3}	0.036	0.17							
^{161}Tb	7.15 d	20 ×	20 ×	8 ×	4 ×	1.4 ×	0.7 ×	0.3×10^{-5}			
—											
^{156}Dy											0.0524
^{158}Dy											0.0902
		* 10^{-4}	0.013	0.054	0.11	0.37	0.75	1.7	0.018	1.9	
^{160}Dy		** 0.001	0.13	0.54	1.08	3.5			0.18	4.5	2.294
		*** 0.01	1.2	4.8					1.6	6.9	
		* 100	99.99	99.95	99.9	99.6	99.2	98.3	99.98	98.1	
^{161}Dy		** 100	99.9	99.5	98.9	96.5			99.8	95.5	18.88
		*** 99.99	98.8	95.2					98.4	93.1	
^{162}Dy											25.53
^{163}Dy											24.97
^{164}Dy											28.18

* Values on this line are calculated for a thermal neutron flux of 10^{12} n cm^{-2} sec^{-1}.
** Values on this line are calculated for a thermal neutron flux of 10^{13} n cm^{-2} sec^{-1}.
*** Values on this line are calculated for a thermal neutron flux of 10^{14} n cm^{-2} sec^{-1}.

days, a moment when the isotopic composition in most cases has become stabilized and independent of the irradiation times considered (See Part II[12], Fig. 8). In those cases where the thermal neutron flux influences the result three fluxes, 10^{12}, 10^{13} and 10^{14} n cm^{-2} sec^{-1}, have been considered. For the two higher fluxes the calculation has been limited to irradiation times up to 6 and 1 month, respectively, because the burn up makes longer times impractical.

The isotopic compositions are summarized in Table 1 together with the natural isotopic abundances taken from Strominger *et al.*[13]. If no value is given for an isotope after a time, the value is either very small or the isotope does not belong to the fission products. The calculated atomic weights are given in Table 2 together with the atomic weights (1955) of the natural elements. The atomic weights are independent of the irradiation time except for seven elements, which are to be found in Table 3.

For a few elements with highly neutron absorbing isotopes, the compositions are also after 3 000 days cooling time strongly dependent on the irradiation time. In these cases two values are given for 7 days, and depending on neutron flux, 2 years, 6 months, or 1 month.

Method of calculation. The number of atoms of the fission product isotopes were calculated for the different irradiation and cooling times and neutron fluxes mentioned above with the modified Rubinson [14] formulae given in Part II[12]. The calculations assume constant thermal neutron flux, constant fission rate, and no fission product removal during irradiation. The required data of fission yields, half-lives, and neutron absorption cross sections are taken from Part I[15]* of this series except for a few more recent data. The isotopic abundances within the elements are then easily computed. By multiplying the abundances of the isotopes of an element with their atomic masses, adding the results, and dividing by 1.000275 the atomic weight on the chemical scale of the element is obtained. Nuclide masses are taken from Wapstra and Duckworth [17]. For five isotopes, where no data are available, the integral mass numbers are used. This uncertainty is negligible because the difference between the integral mass numbers and the corresponding measured nuclide masses in the fission product region is <0.1 %, and the abundances of these elements are low. For some elements, monoisotopic both in Nature and as fission products, the atomic weights adopted for the natural elements have been used.

Errors. The accuracy of the results depends directly on the accuracy of the parameters used. In this work fission yields and cross sections give the largest contributions to the errors, while half-lives and nuclide masses have little influence. The best values of the abundances have a standard deviation of 1 %, while others may have 20 %. However, most values have a standard deviation better than 10 %. Such a deviation will in general only cause a minor deviation in the atomic weights, but is larger than the difference between the physical and the chemical scale, except in the case of elements consisting of mainly one isotope, where the small errors of the nuclide masses dominate.

* This compilation is in good agreement with that of Katcoff [18], but uses a fission yield of 6.32 % for ^{140}Ba as a standard value instead of Katcoff's 6.44 %. However, the choice of standard value has no or little effect on calculated isotopic compositions.

DISCUSSION

The main difference in isotopic composition between the natural and the fission product elements is the lack of light stable isotopes for most of the fission product elements. The reason for this is that such a light isotope is shielded from build-up from beta-decaying radioactive fission products by a stable isobar with lower atomic number. These fission product elements, therefore, have higher atomic weights than the natural ones. Furthermore, long-lived radioactive isotopes not occurring in Nature due to too short half-lives cause considerable differences in the atomic weights. The elements Tc and Pm with no stable isotopes are included among the fission products. Zn and Ga are not considered here, because only short-lived isotopes of these elements belong to the fission products. The reported results for the element Dy may be of limited value, because the isotope ^{162}Dy not yet reported as a fission product, may be formed in quantities as large as 10 % of ^{161}Dy.

Some fission product elements differ from those in Nature by consisting of one isotope only. These isotopes are ^{81}Br, ^{99}Tc, ^{109}Ag, ^{115}In, and ^{139}La. Other pure isotopes may be produced by chemical separation at a moment when they, or their radioactive precursors, make up 100 % of a chemical element, or else by many consecutive separations at special moments. Such procedures will be investigated in a later work.

About 25 % of the fissions result in stable Xe-isotopes. If the known world reserve of uranium in ores (25 Mton [20]), *i. e.* both ^{235}U and ^{238}U, was burnt up

Table 2. Atomic weights of the fission product elements after an irradiation time $\leq$ 2 years. Cooling time 100 and 3 000 days. Thermal neutron flux $\leq 10^{14}$ *n* cm^{-2} sec^{-1}. Atomic weights of natural elements (1955) are included.

Element	Fission product Cooling time in days 100	3 000	Natural element	Element	Fission product Cooling time in days 100	3 000	Natural element
He	4.003		4.003	In	114.909		114.82
Ge	75.5		72.60	Sn	120		118.70
As	74.91		74.91	Sb	124.6	123.4	121.76
Se	81.0		78.96	Te	129.7	129.6	127.61
Br	80.9198		79.916	I	128.56	128.55	126.91
Kr	84.9		83.80	Xe	See Table 3		131.30
Rb	86.34	86.29	85.48	Cs	134.9	134.8	132.91
Sr	89.11	88.97	87.63	Ba	138.0	137.9	137.36
Y	See Table 3	88.92	88.92	La	138.92		138.92
Zr	See Table 3	93.0	91.22	Ce	See Table 3	140.89	140.13
Nb	94.911	92.910	92.91	Pr	140.92		140.92
Mo	See Table 3	97.3	95.95	Nd	See Table 3	144.7	144.27
Tc	98.911		—	Pm	146.920		—
Ru	102		101.1	Sm	150	148	150.35
Rh	102.91		102.91	Eu	153.0	152.8	152.0
Pd	105.4	105.6	106.4	Gd	157	156	157.26
Ag	108.909		107.880	Tb	158.93		158.93
Cd	See Table 3		112.41	Dy	160.98		162.51

Table 3. Atomic weights of seven fission product elements after various irradiation times. Cooling time 100 days. Thermal neutron flux $\leq 10^{14}$ n cm^{-2} sec^{-1}.

Element	Irradiation time						
	1 day	7 days	1 month	2 months	6 months	1 year	2 years
Y	89.60	89.57	89.50	89.42	89.22	89.09	89.00
Zr	93.4	93.4	93.4	93.3	93.2	93.2	93.2
Mo	97.9	97.9	97.9	97.8	97.7	97.6	97.5
Cd§ *	112.3	112.3	112.3	112.3	112.3	112.4	112.4
**	112.3	112.3	112.4	112.4	112.5		
***	112.4	112.4	112.5				
Xe§ *	133.7	133.7	133.7	133.7	133.7	133.7	133.7
**	133.8	134.0	134.0	134.0	134.0		
***	134.0	134.1	134.2				
Ce	141.63	141.63	141.62	141.60	141.53	141.45	141.31
Nd	144.9	144.9	144.8	144.8	144.8	144.8	144.7

§ The Cd and Xe values also hold for cooling times >100 days, but they depend upon the the thermal neutron flux.
* Values on this line are calculated for a neutron flux of 10^{12} n cm^{-2} sec^{-1}.
** Values on this line are calculated for a neutron flux of 10^{13} n cm^{-2} sec^{-1}.
*** Values on this line are calculated for a neutron flux of 10^{14} n cm^{-2} sec^{-1}.

and the Xe produced was distributed in the atmosphere, the natural atomic weight of Xe would not be notably changed. However, if the uranium in the oceans (2 100 Mton) was burnt up, the atomic weight of Xe would change from 131.3 to 131.5. As Xe belongs to the rare elements in Nature the above result indicates that fission products evenly distributed among the natural elements will not cause a change of the atomic weights. However, concentrated deposits may cause local variations.

If ^{12}C instead of natural O is used as standard value for the atomic weights, this will change the values for six fission product elements only (Br, Nb, Tc, Ag, In, and Pm).

Applications. Since the first self-sustaining nuclear reactor was realized in the Chicago Pile-1 on December 2, 1942 [19], large amounts of fission products have been accumulated. Their isotopic composition differs from the natural elements which means different and sometimes useful nuclear properties. Isotope dilution, activation, and nuclear magnetic resonance analysis methods are favoured, when the desired isotope is present in high concentration and especially when undesired isotopes are not present at all. Mo with high neutron absorption cross section and Pd with low may be useful in nuclear energy facilities. Another useful construction metal is Zr, and as a fission product (yield 35 %) it is completely free from Hf but slightly radioactive due to the isotope ^{93}Zr with 950 000 years half-life. The cross section of fission product Cd generated during 7 days irradiation at a neutron flux of 10^{13} n cm^{-2} sec^{-1} is

4 700 barns, while natural Cd has 2 550 barns. The fission product Ag, consists of 100 % ^{109}Ag, whereas in Nature only 49 % ^{109}Ag, may be useful for the production of 253 days ^{110}Ag. A detailed penetration of Table 1 would certainly show further applications similar to the examples above. Some other utilizations have been pointed out by Glueckauf [21].

Acknowledgements. The author wishes to thank Prof. G. Ljunggren for his large interest in and support of these calculations, Prof. A. Ölander, Doc. J. Rydberg, Doc. D. Dyrssen, Tekn.lic. L. W. Holm, Fil.mag. Brita Bernström, Ass. S. Andersson, and Fil.kand. B. Collén for valuable discussions in the planning of this work and for criticism of the manuscript. The author also wishes to thank Mr. C. Roering for correcting the English text.

REFERENCES

1. Hunter, H. F. and Ballou, N. E. *Nucleonics* **9** No. 5 (1951) C-1.
2. Faller, I. L., Chapman, T. S. and West, I. M. *ANL-4807*, USA 1952.
3. Lock, C. J. L. *A. E. R. E. Report C/R-1715*, Harwell 1955.
4. Moteff, J. *General Electric Co Report APEX-134*, USA 1953.
5. Moteff, J. *Nucleonics* **13** No. 5 (1951) 28.
6. Clark, F. H. *NDA-27—39*, USA 1954.
7. Robb, W. L., Sampson, J. B., Stehn, J. R. and Davidson, J. K. *Nucleonics* **13** No. 12 (1955) 30; Sampson, J. B. *et al. KAPL-1226*, USA 1954.
8. Walker, W. H. *CRRP-626*, USA 1956.
9. Blomeke, J. O. and Todd, M. F. *ORNL-2127*, USA 1957.
10. Bolles, R. C. and Ballou, N. E. *Report USNRDL-456*, San Fransisco 1956.
11. Löw, K. M. and Björnerstedt, R. *Arkiv Fysik* **13** (1957) 85.
12. Prawitz, J. and Rydberg, J. *Acta Chem. Scand.* **12** (1958) 377 (Part II).
13. Strominger, D., Hollander, J. M., and Seaborg, G. T. *Revs. Modern Phys.* **30** (1958) 585.
14. Rubinson, W. *J. Chem. Phys.* **17** (1949) 542.
15. Prawitz, J. and Rydberg, J. *Acta Chem. Scand.* **12** (1958) 369 (Part I).
16. Glass, R. A., Thompson, S. G. and Seaborg, G. T. *J. Inorg. & Nuclear Chem.* **1** (1955) 3.
17. Wapstra, A.H. *Handbuch der Physik*, Springer-Verlag, Berlin, 1958, Vol. 38, p. 1; Duckworth, H.E. *Progress in Nuc ear Physics* **6** (1957) 139; *Revs. Mod. Phys.* **29** (1957) 767.
18. Katcoff, S. *Nucleonics* **16** No. 4 (1958) 78.
19. Fermi, E. *Am. J. Phys.* **20** (1952) 537.
20. Putnam, P. C. *Energy in the Future.* D. Van Nostrand Co. Inc., 1953, p. 214.
21. Glueckauf, E. *Endeavour* **14** (1955) 83.

Received September 9, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 174—177

Synthesis of 4-Antipyryl Alkamine Ethers *

JAAKKO HUKKI and JORMA MYRY

Lääketehdas Orion Oy, Helsinki, Finland

A number of basic ethers have been prepared by the reaction of 4-hydroxyantipyrine with alkamine halides commonly used for the production of antihistamines.

Only a few ethers of 4-hydroxyantipyrine are reported in the literature. Of these, two alkyl ethers (a methyl and an ethyl ether) are described by Knorr and Pschorr [1]. The rest are nitrophenyl and substituted nitrophenyl ethers prepared by Kaufmann *et al.*[2] In all these cases, the method of etherification was the same. 4-Hydroxyantipyrine was allowed to react with the requisite alkyl or nitrophenyl halide in the presence of sodium methoxide or ethoxide.

In the hope of obtaining compounds with a simultaneous antipyretic and antihistaminic effect, some alkamine ethers of 4-hydroxyantipyrine were prepared for pharmacologic evaluation. The present paper is concerned with the synthesis of these compounds.

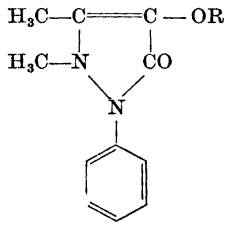
An attempt to use 4-bromoantipyrine for the etherification failed, obviously owing to the poor reactivity of the "aromatically" substituted bromine atom. After reflux with dimethylaminoethanol or its sodium compound in dry toluene for 24 h, the 4-bromoantipyrine was recovered unchanged.

The method used by Knorr and Pschorr [1], with 4-hydroxyantipyrine as starting material, although successfully employed by us for the preparation of 4-antipyryl benzyl ether, did not work with alkamine halides. In spite of the markedly phenolic character of the 4-hydroxyantipyrine, the formation of N,N,N′,N′-tetra-alkylpiperazinium dihalides quite outbalanced the etherification reaction. An attempt to carry out the condensation of 4-hydroxyantipyrine with an alkamine halide in the presence of pyridine was also unsuccessful. In this case, an alkamine pyridinium halide hydrohalide was formed, as observed already by Kratzl and Berger [3] when studying the corresponding condensation with 4-aminoantipyrine.

The etherification could be effected satisfactorily, if the sodium compound of 4-hydroxyantipyrine was prepared by means of sodium amide in dry toluene and the condensation carried out under anhydrous conditions (method A).

* New features disclosed in this paper are covered by a pending Finnish patent application.

Table 1. Alkamine ethers of 4-hydroxyantipyrine



R	Free amine	Mono-oxalate								
		Formula	Yield[e] %	M.P. °C	Analyses					
					Calc.			Found		
					C	H	N	C	H	N
$(CH_3)_2NCH_2CH_2$-	M.p.65°C[a,b]	$C_{17}H_{23}O_6N_3$	84	153[c]	55.88	6.35	11.50	55.47	6.44	11.57
$(C_2H_5)_2NCH_2CH_2$-	Oil	$C_{19}H_{27}O_6N_3$	66	123[d]	58.00	6.92	10.68	57.81	7.08	10.68
$(CH_3)_2NCH_2CH(CH_3)$-	Low melting solid[a]	$C_{18}H_{25}O_6N_3$	74	170[d]	56.98	6.64	11.08	56.57	6.76	10.86
$(CH_3)_2NCH_2CH_2CH_2$-	Oil	$C_{18}H_{25}O_6N_3$	71	158[d]	56.98	6.64	11.08	56.70	6.94	10.99
$H_2C(CH_2—CH_2)N—CH_2CH_2$-	Oil	$C_{20}H_{27}O_6N_3$	69	156[c]	59.24	6.71	10.37	59.01	6.98	10.25

[a] From petroleum ether. [b] From this compound a dihydrochloride, m.p. 180° (from ethanol), was also obtained. [c] From ethanol. [d] From acetone-methanol. [e] Calculated on the basis of 4-hydroxyantipyrine.

It turned out, however, that the best results in regard to both the yield and purity of the products could be obtained simply by refluxing 4-hydroxyantipyrine with the appropriate alkamine halide hydrohalide in dry acetone in the presence of anhydrous potassium carbonate (method B).

The ethers obtained were viscous liquids or low-melting solids, which were difficult to purify. For analysis they were converted into their mono-oxalates. Some data relating to these new compounds, all prepared according to method B, are presented in Table 1.

For further characterization, ultraviolet absorption spectra of the ethers were determined. These are represented in Fig. 1. For comparison, the corresponding spectra of 4-hydroxy-, -ethoxy- and -benzyloxyantipyrine are included.

One of the ethers, 4-antipyryl 2-dimethylaminoethyl ether, was allowed to react with methyl iodide at room temperature to give the corresponding quaternary ammonium salt. It has been shown by Knorr [4] that at 60° antipyrine also reacts with methyl iodide to form 1-phenyl-2,3-dimethyl-5-methoxypyrazolium-2 iodide. In order to decide, which of these two courses the reaction had taken, a test was made with antipyrine for comparison. It turned out that under the same reaction conditions, no iodide formation was observed

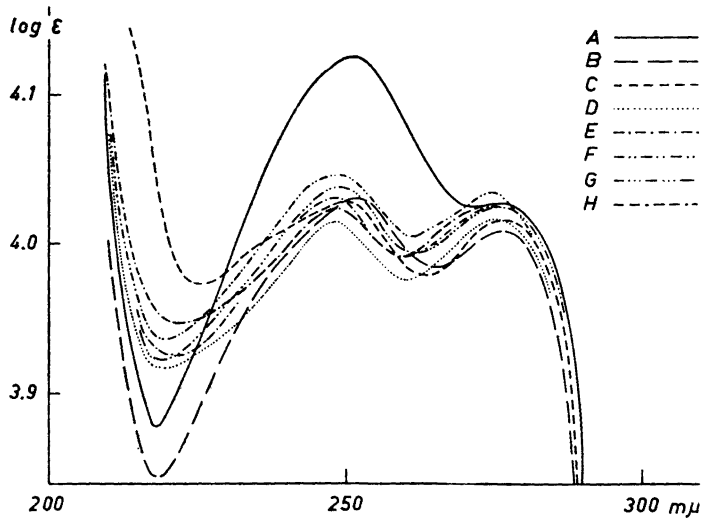


Fig. 1. Ultraviolet absorption spectra of 4-hydroxyantipyrine (A) and the following of its ethers: ethyl (B), benzyl (C), 2-dimethylaminoethyl (D), 2-diethylaminoethyl (E), 3-dimethylaminopropyl (F), 2-dimethylamino*iso*propyl (G) and 2-(N-piperidino)ethyl (H).

between antipyrine and methyl iodide after 3 days at room temperature, while from 4-antipyryl 2- dimethylaminoethyl ether an almost quantitative yield of the salt was obtained within one day. Accordingly, the salt in question must be β-(4-antipyryloxy)ethyltrimethylammonium iodide.

EXPERIMENTAL

All melting points were determined on a Kofler hot stage and are corrected. The microanalyses were performed by the Microanalytisches Laboratorium im Max-Planck-Institut, Mülheim (Ruhr), Germany. The ultraviolet data, from the Analytical Laboratory of Lääketehdas Orion Oy, are for solutions in ethanol (concentration 0.010—0.025 g per liter).

4-Hydroxyantipyrine. This was prepared from 4-dimethylaminoantipyrine (pyramidon) by the general route devised by Bockmühl [5]. Because no detailed information is to be found in the literature, a description of the procedure used by us is given:

Pyramidon (0.5 mole) was dissolved in a mixture of 200 ml of conc. hydrochloric acid (37.8 %) and 85 ml of water. This solution was added dropwise and with continuous agitation to a solution of sodium nitrite (2.0 mole) in 250 ml of water, while the temperature was kept below 0°. After the addition, the mixture was allowed to stand in the refrigerator overnight to complete the crystallization of the resultant intermediate, 1-diketobutyryl-1-phenyl-2-methyl-2-nitrosohydrazide monohydrate. This was filtered off, washed with 200 ml of cold water and then added to a solution of 130 g of sodium bisulfite in 700 ml of water. The mixture was allowed to stand at room temperature overnight. The crystallized 4-hydroxyantipyrine was filtered, washed with water and dried. The yield was 92 %, m. p. 182°.

4-Antipyryl alkamine ethers. Method A: 4-Hydroxyantipyrine (0.1 mole) and sodium amide (0.1 mole) in 300 ml of anhydrous toluene were heated and stirred for 4 h at 80°, all moisture being excluded. To this mixture a carefully dried solution of the appropriate

alkamine chloride (0.12 mole) in toluene was added. The mixture was heated at 80° for 25 h. The residue, after evaporation of the solvent *in vacuo*, was dissolved in chloroform, washed with dilute sodium hydroxide and water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the ether remained as a viscous liquid. This was dissolved in ethanol, oxalic acid was added and the resulting oxalate was precipitated from the solution with ethyl ether.

Method B: A mixture of 4-hydroxyantipyrine (0.1 mole), an alkamine chloride hydrochloride (0.1 mole) and freshly ignited, fine-ground potassium carbonate (0.3 mole) was added to 350 ml of dry acetone. The mixture was refluxed for 2 days with continuous agitation. After cooling, the anorganic salts were filtered off. The filtrate was evaporated *in vacuo*, after which the remainder was worked up as in A.

4-Benzyloxyantipyrine. To a solution of 0.01 mole of sodium ethoxide in ethanol 0.01 mole of benzyl chloride and 0.01 mole of 4-hydroxyantipyrine were added. The mixture was refluxed for 4 h, after which it was evaporated to dryness and the remainder worked up as in the case of alkamine ethers. The yield was 79 %, m. p. 71—72° (from a mixture of petroleum ether and acetone). With method B, a yield of 72 % was obtained. (Found: C 73.63; H 6.29; N 9.71. Calc. for $C_{18}H_{18}N_2O_2$: C 73.45; H 6.16; N 9.71.)

4-Ethoxyantipyrine. To a solution of 0.25 mole of 4-hydroxyantipyrine in an equivalent amount of 0.1 N aqueous sodium hydroxide, 0.25 mole of diethyl sulfate was added gradually with shaking. After half an hour's heating at 60—70°, the mixture was extracted with chloroform and the chloroform solution worked up as in method A. The yield was 65 %, m. p. 53—54° (from a mixture of petroleum ether and acetone)(lit[1]. m. p. 60).

β-(4-Antipyryloxy)ethyltrimethylammonium iodide. To a solution of 2.75 g (0.01 mole) of 4-antipyryl 2-dimethylaminoethyl ether in 30 ml of absolute ether was added 1.42 g (0.01 mole) of methyl iodide. After one day standing at room temperature, the crystals formed were filtered off, washed with 10 ml of absolute ether and dried. The theoretical yield was obtained, m. p. 187°. After recrystallization from a mixture of acetone and methanol the salt was analyzed. (Found: C 45.90; H 5.73; N 10.12; I 30.38. Calc. for $C_{16}H_{24}O_2N_3I$: C 46.05; H 5.80; N 10.07; I 30.42.)

REFERENCES

1. Knorr, L. and Pschorr, R. *Ann.* **293** (1896) 49.
2. Kaufmann, H., Seher, A. and Pankoke, H. *Arch. Pharm.* **284** (1951) 330.
3. Kratzl, K. and Berger, K. *Monatsh.* **89** (1958) 160.
4. Knorr, L. *Ann.* **293** (1896) 1.
5. Bockmühl, M. *Med. u. Chem. Abhandl. med. chem. Forschungstätten IG Farbenind.* **3** (1936) 294.

Received October 18, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 178—185

Ozonolysis of *p*-Benzoquinone II

ERLING BERNATEK and KNUT ASBJØRN STRAUMSGÅRD

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The ozonolysis of *p*-benzoquinone in chloroform at 3—4° has been studied. By decomposition of the ozonized solution with water the following compounds were formed: Formic acid, glyoxylic acid, oxalic acid, maleic acid, mesoxalic dialdehyde, formaldehyde and carbon dioxide. A quantitative study of these compounds has been undertaken, and possible mechanisms for their formation are presented.

In a preliminary communication [1] (Part I of this series) one of the present authors discussed some aspects of the ozonolysis of *p*-benzoquinone in chloroform. The main result was the tentative establishment of the unsaturated aldehyde CHOCOCH=CHCOCHO as a decomposition product. An earlier investigation by Fichter, Jetzer and Leepin [2] had led to the identification of formic acid among the products of this ozonolysis.

It soon became clear that the ozonolysis of *p*-benzoquinone was a rather complex affair and that the reaction products might be numerous. We have therefore performed a more detailed investigation of the products which occurred when the ozonolysis was carried out under certain specified conditions. It is appropriate to remark at this point that the qualitative and quantitative composition of the reaction mixture seems to be highly susceptible to even minor alterations in reaction conditions. We have thus in the present investigation not isolated any unsaturated aldehyde although the conditions of ozonolysis were not very different from those of the earlier investigation.

During the ozonization an ozonide separated as described before [1,2], but no attempts were made to characterize this unstable and highly hydrolysable substance. If kept in air at room temperature for instance, it soon became a dark syrup in which after some time long needles of oxalic acid appeared.

The ozonized mixture was decomposed by shaking with cold water when the ozonide quickly went into solution and the active oxygen disappeared. The aqueous extract had a strong acidic reaction but contained only traces of hydrochloric acid (which have resulted from decomposition of the chloroform). In order to identify and determine the amount of volatile acids the aqueous solution was distilled at room temperature by passing nitrogen through it at low pressure and collecting the distillate in aqueous sodium hydroxide.

Only formic acid could be found. A neutral substance in the decomposition solution was isolated by removing the acidic products in a column of a weakly basic ion-exchange resin. In the neutral eluate there was a substance which instantly reduced Tollens' reagent and which through its *p*-nitrophenylhydrazone was identified as mesoxalic dialdehyde. As it was found that the reaction mixture also contained non-volatile acid substances, the ion-exchange column after removing quantitatively the mesoxalic dialdehyde with water, was treated with 5 % aqueous formic acid. The eluate contained a carbonyl compound which reacted with 2,4-dinitrophenylhydrazine to give a derivative in the form of yellow needles. The melting point of this product (190° decomp.) was not changed after recrystallization so it may be concluded that only one carbonyl compound had been eluted with the dilute formic acid, *viz.*, glyoxylic acid. Elution of the ion-exchange column with 2 N hydrochloric acid afforded finally oxalic and maleic acids. These two acids were, however, more easily isolated directly from the decomposition solution.

Beside the two aldehydes, mesoxalic dialdehyde and glyoxylic acid, a third aldehyde was found but only in very small amounts. The derivative with dimedone and the colour with chromotropic acid proved that this third aldehyde was formaldehyde. It could be shown that this compound did not result from decomposition of the solvent or of impurities.

During the decomposition of the ozonized solution with water an evolution of carbon dioxide could be noticed. The aqueous extract gave only a very faint reaction on hydrogen peroxide.

In order to get a better picture of the fate of benzoquinone during ozonolysis and the mode of formation of the fragments, it was necessary to undertake a quantitative determination of the products. Some of the analytical methods available were not very accurate but it is felt that a fairly good estimate of the relative amounts in which the above-mentioned degradation products occurred has been secured.

Formic acid was determined as mentioned before by distillation into aqueous sodium hydroxide at low temperature and pressure.

Mesoxalic dialdehyde was determined by evaporation of the eluate from the ion-exchange column and weighing the residue. This is clearly a minimum value as there always is a certain loss of substance in the column. Furthermore the aldehyde is somewhat volatile with water vapour and in addition to this only that part of the eluate which contained appreciable amounts of the aldehyde was evaporated. To concentrate the total volume of the neutral eluate would have been almost a practical impossibility.

Glyoxylic acid could be precipitated as the dinitrophenylhydrazone but the precipitation was far from quantitative. To avoid this source of error and to avoid the loss in the column glyoxalic acid was determined by difference. The decomposition solution was in this case evaporated directly in a vacuum desiccator over potassium hydroxide to constant weight. From this value were subtracted the amounts of the non-volatile products mesoxalic dialdehyde, oxalic acid and maleic acid. The amount found in this way must be a maximum value since contaminations and eventual minor amounts of unidentified substances are included and — which is important — because the mesoxalic dialdehyde is represented by a minimum value.

Oxalic acid was precipitated in accordance with the usual analytical method as the calcium salt.

Maleic acid was determined in the filtrate from the oxalate determination by precipitation as the barium salt. This salt has the drawback to be somewhat soluble in water so the filtrate had to be measured and the amount of dissolved barium maleate calculated. An alternative method of analysis was to oxidize the concentrated decomposition solution

with hydrogen peroxide. Maleic acid is much slower oxidized by this reagent than the other compounds present. The residue after evaporating the oxidation mixture consists of pure maleic acid. Some loss is inevitable in this last procedure but the two methods give results of the same order of magnitude.

Several methods were tried for the determination of carbon dioxide. Absorption in barium hydroxide followed by a rapid filtration of the precipitated barium carbonate was chosen.

In Table 1 the analytical results are given in relation to one mole of benzoquinone and in addition the percentages of recovered carbon are calculated.

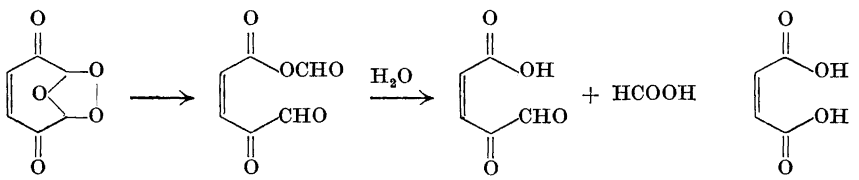
Table 1.

Product	Mole/mole benzoquinone	Recovered carbon, %
Formic acid	1.65	27.3
Mesoxalic dialdehyde	0.13	7.7
Glyoxylic acid	1.16	39.0
Oxalic acid	0.03	1.4
Maleic acid	0.06	4.5
Carbon dioxide	0.16	2.7

It will be seen that account can be made for about 83 % of the carbon in the starting material. The deficiency of about 17 % may in part be explained by imperfection of our analytical methods, but it is also highly probable that some of the benzoquinone is oxidized directly to water and carbon dioxide during the ozonization. As ozone is not absorbed quantitatively by benzoquinone some of it escapes with the effluent oxygen and interferes with attempts to determine directly formed carbon dioxide. In addition to this comes a small amount of material still contained in the chloroform layer after extraction with water. Per gram of benzoquinone this amounts to about 20 mg.

The maleic acid can only be derived from a monozonide of benzoquinone. In the diozonide of course both carbon-carbon double bonds are broken and no maleic acid can result. During ozonolysis there was always passed through the solution ozone in excess of that calculated for reaction with both double bonds but nevertheless some monozonide must have existed. This is evidently due to the already mentioned incomplete absorbtion of ozone by benzoquinone and to the fact that some ozone is decomposed by the solvent before it can react with the quinone.

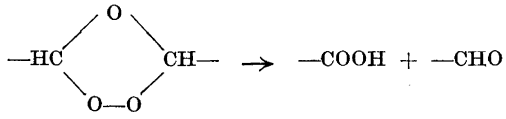
The monozonide of benzoquinone may be a normal ozonide like I.



I II III IV

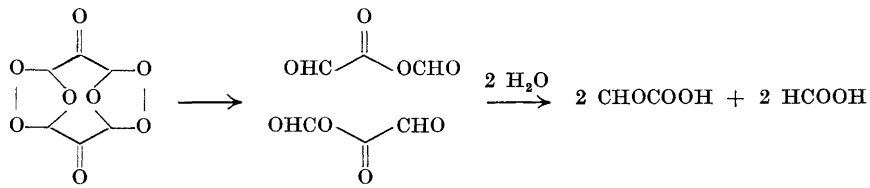
It is well known that normal ozonides like other peroxy compounds may undergo rearrangements, which in our case would lead from I to II. Another possibility is that benzoquinone *via* anomalous ozonolysis more directly is converted to II. The latter structure will be recognized as a mixed anhydride between formic acid and the unsaturated ketoaldehydic acid III. By treatment with water these two acids will be set free. Transformation of III into maleic acid (IV) obviously involves an oxidation. This may be effected by ozonide still present or, since maleic acid is formed only in minor amounts, by those small quantities of hydrogen peroxide present in the decomposition solution or maybe already during the ozonization. The aldehyde group is most probably oxidized to formic acid, less likely it seems to carbon dioxide and water. This last statement is in part based upon the fact that Dallwigk and Briner [3] who investigated the ozonolysis of cinnamaldehyde found that the glyoxylic acid which was formed by treatment of the ozonide with water was further oxidized to formic acid and carbon dioxide by undecomposed ozonide.

When discussing the formation of the other products several reaction paths must be taken into consideration, the most conspicious of which are the two already mentioned, *viz.*, formation of the normal ozonide with subsequent rearrangement and anomalous ozonolysis. Further may be mentioned direct hydrolytic cleavage of the ozonide and the so-called "acid-rearrangement" which is well known to occur among aldozonides and which may be represented as follows:



Still more numerous are of course the combinations of these reactions which may be possible with a diozonide. To postulate such combinations will, however, be rather speculative and therefore left out of serious consideration.

The main products, formic acid and glyoxylic acid, which are formed in rather overwhelming molar quantities as compared with the other products are most probably formed by symmetrical * double rearrangement of the normal diozonide or by symmetrical anomalous ozonolysis:

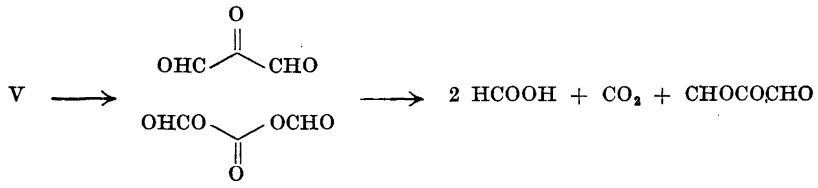


V VI

* Symmetrical in the sense that the two migrating oxygen atoms become adjacent to different carbonyl groups. In the same sense unsymmetrical rearrangement means that the two oxygens become adjacent to the same carbonyl group.

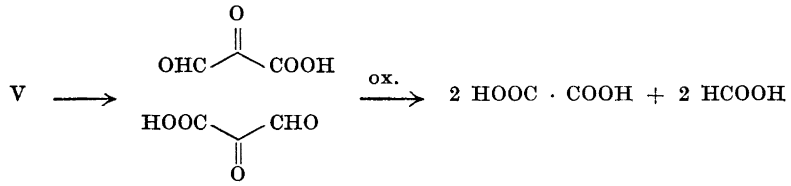
It is difficult to discern between the two mentioned possibilities, but it may be concluded that at least a part of the reaction goes *via* the normal diozonide as the peroxidic substance which separated during ozonization could be kept for some time at 0° without decomposition. At room temperature, however, it dissolved in the chloroform after a short time. Simultaneously carbon monoxide began to evolve which can be taken as an indication of the actual existence of structures like VI. Mixed anhydrides of formic acid are known to decompose with the evolution of carbon monoxide under anhydrous conditions. If 0.58 mole of benzoquinone undergoes the above degradation there will be produced the 1.16 moles of glyoxylic acid actually found and 1.16 moles of formic acid.

Carbon dioxide and mesoxalic dialdehyde are formed in roughly equimolecular amounts (especially if we take into account that the found amount of mesoxalic dialdehyde is a minimum value) and very likely through an unsymmetrical double rearrangement of the normal ozonide or by unsymmetrical anomalous ozonolysis:



Assuming that 0.16 moles of benzoquinone reacts in this way the products will be 0.16 moles of carbon dioxide, 0.16 moles of mesoxalic dialdehyde and 0.32 moles of formic acid.

Oxalic acid contains carbon in a higher oxidation state than the other products (except carbon dioxide) and its formation is difficult to explain without further oxidation of the decomposition products. A possible mechanism is the symmetrical double acid-rearrangement of the normal diozonide:



Oxidation of the primarily formed mesoxalic monoaldehyde probably takes place during the ozonization. If the rearrangement starts at an early stage of the reaction, ozone and small amounts of water formed by complete breakdown of benzoquinone may easily convert the ketoaldehydic acid to oxalic acid and formic acid. 0.015 moles of benzoquinone reacting as described give 0.03 moles of oxalic acid and 0.03 moles of formic acid.

When adding together the mentioned molar amounts of formic acid and remembering that 0.06 moles of maleic acid are accompanied by 0.12 moles

of formic acid we get the total of 1.63 moles of formic acid. This figure corresponds very well with the found amount of 1.65 moles and therefore lends support to the soundness of the presented paths of reaction. Addition of the consumed molar amounts of benzoquinone gives 0.82 moles. (Compare *ca.* 83 % recovered carbon).

The problem of formaldehyde formation has not been discussed yet, mainly because the amount of this aldehyde is so small that it does not affect the overall picture of degradation. If we regard the molecular formulae for benzoquinone and formaldehyde $C_6H_4O_2$ and CH_2O, it will be noticed that carbon in both compounds has the formal oxidation stage zero. That means that the particular carbon atom in the benzoquinone skeleton going to form formaldehyde must be severed retaining the mean formal oxidation stage. Obviously this can only be attained by hydrolytic cleavage of at least one carbon-carbon bond, for instance in the following way:

$$HOH + —CO—CHO \longrightarrow —COOH + HCHO$$

The fragment to be hydrolysed must be a rearrangement product from one of the ozonides.

EXPERIMENTAL

Ozonization procedure. The source of ozone was an apparatus built in this institute and consisted of ten Berthelot tubes; voltage 11 000 V. Through the apparatus was led a stream of dry tank oxygen at the rate of 30 l/h. At this rate of flow a yield close to 2 g O_3/h was obtained which corresponds to an ozone concentration of *ca.* 4.8 % (weight).

A good commercial grade of chloroform was used as a solvent. This was dried with calcium chloride and distilled with rejection of rather large head and tail fractions. The *p*-benzoquinone was a recrystallized Eastman-Kodak product.

As reaction vessel a gas washing bottle with fritted bottom for the distribution of gas was employed. During ozonization the bottle was placed in an ice-bath. The ozonization mixture (usually 5 g of quinone in 150 ml of chloroform or 1 g of quinone in 50 ml of chloroform) would then during the reaction attain a temperature of 3 to 4°. The end point of the reaction could not be determined by the sudden appearance of unreacted ozone (as ozone was passing through the solution already from the outset) but could be judged with fair accuracy from the disappearance of the yellow quinone colour. At this point an ozonide had separated at the walls of the vessel as a white sticky mass, the solution being light greenish. If this mixture was kept at room temperature the separated ozonide went into solution. Strips of filter paper moistened with a palladium chloride solution, which were suspended in the reaction vessel above the liquid, blackened in a short time.

The reaction mixture was decomposed by shaking with ice-cold water and the chloroform layer was extracted a few more times with additional amounts of water. The ozonide went quickly into solution and the chloroform thereafter gave no reaction on active oxygen. On the other hand the aqueous solution (hereafter referred to as "decomposition solution") gave a faint reaction on hydrogen peroxide with titanium reagent. If the extracted chloroform was dried with sodium sulphate and evaporated to dryness there remained a solid brownish residue amounting to about 20 mg/g benzoquinone. The nature of this residue has so far not been investigated.

Formic acid. Some of the decomposition solution was distilled and the distillate exactly neutralized with aqueous sodium hydroxide. To this was added a hot concentrated solution of S-benzylthiouronium chloride. The mixture was immediately cooled and inoculated with a small crystal of S-benzylthiouronium formate. In a few hours white crystals had separated which had m. p. 146°. Mixed m. p. with an authentic sample showed no depression.

For the quantitative determination aliquots of the decomposition solutions from ozonization of 1.000 g portions of benzoquinone were distilled. The distillations were in this

case performed at low pressure and room temperature while a slow stream of nitrogen was passed through the solution. Formic acid was absorbed directly in a 0.1000 N solution of sodium hydroxide and the resulting mixture back-titrated with hydrochloric acid. As a mean of the determinations it was found that 1.000 g of benzoquinone produced 15.2 mmoles of formic acid.

Mesoxalic dialdehyde. The decomposition solution from 5.00 g of benzoquinone was added to the same volume of ethanol and the mixture passed through a column of De-Acidite G (3 × 30 cm). Before use the ion exchange resin had been treated with 1 N sodium hydroxide and washed neutral, first with tap water and finally with distilled water. The ethanolic decomposition solution was left in the column for about one hour and thereafter eluted with 50 % ethanol. About 500 ml of a neutral eluate which showed a strong reaction with Tollens' reagent was collected. A faint but very persistent tailing was characteristic of the elution and the volume of eluate actually containing traces of a neutral carbonyl compound was very large. The 500 ml of eluate was evaporated to dryness *in vacuo* and formed a dry brittle mass of only faint colour. It could not be brought to crystallization and was difficult to purify in this state. The mean yield of the product was 500 mg. In order to prepare the *p*-nitrophenylhydrazone, an aqueous solution of the aldehyde was added to a hot solution of *p*-nitrophenylhydrazine in glacial acetic acid and the mixture heated on a water-bath for about one hour when a dark red substance separated. This substance was digested three times with glacial acetic acid and the undissolved part recrystallized from nitrobenzene. The dark brick-red derivative had no melting point but decomposed at about 297°; the decomposition point was, however, rather dependent upon the rate of temperature increase. (Found: C 52.0; H 3.8; N 24.4. Calc. for $C_{21}H_{17}N_9O_6$: C 51.3; H 3.5; N 25.7.) The analytical values were rather high in carbon and low in nitrogen content, a fact which is attributed to the tenacious retaining of nitrobenzene by the tris-*p*-nitrophenylhydrazone. An authentic sample of mesoxaldialdehyde was prepared by ozonization of phorone and a derivative with *p*-nitrophenylhydrazine was made as described above. Also here a decomposition point of about 297° was found and the analytical figures were close to those already mentioned. (Found: C 52.0; H 3.7; N 24.1.) The infrared spectra of the two derivatives proved that they were identical.

From the first acetic acid extract of our original *p*-nitrophenylhydrazone mixture a dark red substance separated. Decomposition occurred in the range 260 to 270°. (Found: C 50.0; H 3.8; N 21.8. Calc. for $C_9H_7N_3O_4$ (mono-*p*-nitrophenylhydrazone): C 48.9; H 3.2; N 19.0. Calc. for $C_{15}H_{12}N_6O_5$ (bis-*p*-nitrophenylhydrazone): C 50.6; H 3.4; N 23.6).

Glyoxylic acid. After removing the mesoxalic dialdehyde completely the ion-exchange column was treated with 5 % aqueous formic acid. In the eluate a carbonyl compound appeared which reacted with 2,4-dinitrophenylhydrazine to give yellow needles; m. p. 190° decomp. Recrystallized twice from water the melting point was unchanged. Mixed melting point with an authentic sample of glyoxylic acid 2,4-dinitrophenylhydrazone was undepressed. (Found: C 37.3; H 2.8; N 22.1. Calc. for $C_8H_6O_6N_4$: C 37.8; H 2.4; N 22.1.) The eluate and an aqueous solution of phenylhydrazine hydrochloride gave brownish orange needles. Recrystallized from water (charcoal) they became light yellow; m. p. 137—138° decomp. (Found: C 58.5; H 4.9; N 16.6. Calc. for $C_8H_8N_2O_2$: C 58.5; H 4.9; N 17.1.)

The decomposition solution from 1.000 g of benzoquinone was evaporated in a vacuum desiccator over potassium hydroxide to constant weight. Weight of the residue 1.197 g. From this value was deducted the sum of the found amounts of mesoxalic dialdehyde, oxalic acid and maleic acid, in all 202 mg, which leaves us 995 mg of glyoxylic acid monohydrate, *i. e.* 800 mg of the anhydrous acid.

Oxalic acid. The decomposition solution from 1.000 g of benzoquinone was added to a solution of 2 g calcium chloride in 50 ml of water and dilute aqueous ammonia added until pH reached *ca.* 6. The crystalline precipitate was collected after one hour and dried at 110°: 52 mg of calcium oxalate monohydrate which corresponds to 32 mg of oxalic acid. A larger amount of calcium oxalate prepared in this way was dissolved in 2 N hydrochloric acid and extracted with ether. The dried ethereal extract was evaporated when white crystals remained. These had m. p. 101°, undepressed upon admixture of an authetic sample of oxalic acid.

Maleic acid. The filtrate from the oxalate precipitation was concentrated to a small volume and a hot solution of 0.5 g of barium chloride in a few ml of water added when a

white precipitate was formed. The mixture was cooled to about 15° and filtered rapidly by suction. In a typical run the dried barium maleate weighed 44 mg and the filtrate from this salt measured 20 ml. Now it is found in the literature [4] that 100 parts of water at 14.5° dissolves 0.584 parts of barium maleate monohydrate. The filtrate contains therefore 108 mg of the anhydrous salt and the total of 153 mg of barium maleate corresponds to 72 mg of maleic acid. From the barium salt maleic acid could be set free and isolated. It was identified by the melting point which was undepressed upon admixture of an authentic sample.

Maleic acid could also be determined more directly. The decomposition solution from 1.000 g of benzoquinone was mixed with 2 ml of 40 % hydrogen peroxide and evaporated slowly on a water-bath. The residue weighed 70 mg, but was somewhat contaminated with a yellow peroxidic syrup. A further 0.5 ml of hydrogen peroxide was therefore added together with some water and the mixture again evaporated to dryness. The residue consisted this time of pure, white maleic acid, but weighed only 50 mg.

Carbon dioxide. 1.000 g of benzoquinone was ozonized as usual and the dissolved ozone swept out quantitatively with oxygen. Water was added and the liberated carbon dioxide was carried into a solution of barium hydroxide by the oxygen stream. The precipitated barium carbonate was rapidly filtered off by suction, dried and weighed. Found: 294 mg of barium carbonate which corresponds to 66 mg of carbon dioxide.

Formaldehyde. 260 ml of the chloroform ordinarily used in these experiments were treated with ozone for 6 h and thereafter extracted five times with 25 ml portions of water. Especially the first extract gave a positive reaction on formaldehyde with chromotropic acid reagent. Further it gave a small crystalline precipitate with an ethanolic solution of dimedone. This product had m. p. 189.5° which was undepressed upon admixture of an authentic sample.

500 ml of chloroform were extracted five times with the same volume of water, dried over calcium chloride and distilled. A part of the distillate was treated for 6 h with ozone and extracted with water. The aqueous extract gave a negative test for formaldehyde. If a small amount of ethanol was added to the purified chloroform and treated with ozone as before, formaldehyde reappeared in the aqueous extracts.

Benzoquinone was dissolved in the purified chloroform and ozonized as usual. The decomposition solution was passed through an ion-exchange column and the neutral eluate collected. With an ethanolic solution of dimedone it gave sparingly a precipitate of white needles. Recrystallized from a small amount of ethanol they had m. p. 190.5° which was undepressed upon admixture of an authentic sample of formaldehyde dimedone derivative. Also the test with chromotropic acid reagent was positive.

REFERENCES

1. Bernatek, E. *Acta Chem. Scand.* **8** (1954) 1943.
2. Fichter, Fr., Jetzer, M. and Leepin, R. *Ann.* **395** (1913) 1.
3. Dallwigk, E. and Briner, E. *Helv. Chim. Acta* **33** (1950) 2186.
4. Meyer, H. *Nachweis und Bestimmung organischer Verbindungen. Lehrbuch der organisch-chemischen Methodik*, Julius Springer Verlag, Berlin 1933, Vol. 2, p. 142.

Received June 18, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 186—202

Short Communications

Least Square Method for Computor Calculation of Stability Constants *

JAN RYDBERG** and JAMES C. SULLIVAN

Chemistry Division, Argonne National Laboratory Lemont, Illinois, USA

Although a number of investigators have been concerned with the problem of calculation of stability constants, the methods presented have in general been approximation techniques (*cf. e. g.* Ref.[1]). With the growing use of high-speed computors, it becomes feasible to re-examine some of the data in the literature using a least square technique to calculate such stability constants. In addition, the use of such a computor enables the experimentalist to assign standard deviations to constants so calculated. This feature of computor calculation is a valuable contribution, since it provides an objective measure of the precision associated with such constants.

The necessity for such an approach is rather pointedly illustrated when one considers the compilation prepared by Bjerrum, Schwarzenbach and Sillén [2], where, for example, in the system Fe(III)-SCN^- there are 35 different investigations tabularized. In the absence of any measure of precision (not considering systematic errors) of the calculated stability constants, the user of this compilation is forced to *ad hoc* considerations.

The authors have therefore started a series of studies in order to reveal the advantage and precision of using least square methods in combination with high-speed digital computors for the calculation of stability constants. The results of our studies will be given in a series of papers dealing with the application of the method to systems containing simple complexes (one central atom and one type of ligand, *e. g.* MA_n), composite complexes (one central atom and several different types of ligands, *e. g.* MA_nB_p) and polynuclear complexes (several central atoms of same kind for each kind of ligand, *e. g.* M_mA_n) as studied by means of solvent extraction, ion exchange, spectrophotometric and e.m.f. methods.

In the present paper, as an example, the treatment of a simple system will be briefly described.

The U(IV)-acetylacetone system

This system, which has been studied by means of solvent extraction [3], was chosen partly because of its familiarity to one of the authors, and partly because of its regular behavior in the present treatment.

Outline of the mathematical treatment. U(IV), here abbreviated to M^{+4}, is assumed to form a series of complexes, MA_n^{4-n}, with acetylacetone, HA, in aqueous solutions. The uncharged complex MA_4 can be extracted into inert organic solvents like C_6H_6, and it is assumed that this complex is the only extractable one of M. The distribution ratio, q, of all species of M between the organic and aqueous solvents will then be

$$q = \frac{[M]_{org}}{[M]_{aq}} = \frac{\lambda_N \cdot \varkappa_N [A^-]^N}{\sum_0^n \varkappa_n [A^-]^n} \qquad (1)$$

where λ_N is the distribution constant of the uncharged complex MA_N between the organic and aqueous phases, and $\varkappa_n$ is the over-all stability constant for the n:th complex:

* Based on work performed under the auspices of the U.S. Atomic Energy Commission.

** On leave of absence from the University of Stockholm, and Research Institute of National Defence, Stockholm, Sweden.

$$\varkappa_n = \frac{[MA_n^{N-n}]}{[M^N]\,[A^-]^n} \qquad (2)$$

If we introduce the following notations

$$y = \frac{[M]_{aq}}{[M]_{org}}\ ,\ x = [A^-],\ a_n = \frac{\varkappa_n}{\lambda_N \varkappa_N}\ ,\ \text{and}\ z = \frac{[M]_{aq}}{[M]_{org}}\,[A]^N = y\,x^N$$

into eqn. (1), it will yield

$$z = \sum_0^n a_n x^n \qquad (3)$$

This can be solved for a_n, provided n combinations of $z(x)$ are available. Usually the number L of experimental points is $\gg n$, and the L equations of type 3 can therefore be solved with a least square technique. However, x and z are experimentally determined quantities and therefore have associated with them errors, which makes it necessary to properly weight the equations. The details of the procedures will be described in a later paper.

Computer operations. In the U(IV) case it was assumed that the errors in $[A^-]$ could be neglected compared to the errors in $[M]_{org}$ and $[M]_{aq}$, which will be called σ_{org} and σ_{aq}. The computer was therefore programmed for these five kinds of data: $[A^-]$, $[M]_{org} \pm \sigma_{org}$ and $[M]_{aq} \pm \sigma_{aq}$, which were punched on cards together with information of N, L and maximum number of n. In the programming, the computor was ordered, 1) to print the best combination of positive a_n for a minimum number of n, 2) to print the standard deviations of a_n, 3) to print the z_i values, calculated according to eqn. (1) with the a_n given by the computor, for the various input x_i values, 4) to print the standard deviations of these z_i, 5) to print the over-all accuracy of the computation. This latter information is given in the form of S_{min}/k*, which should have a value between 1.5 and 0.5 for reliable data.

For $n = N = 4$, and $L = 35$, the time of the whole operation (*i. e.* putting in program and data cards, running the computor and printing out) is about 5 min for the computer used (an I.B.M. 704).

Results of the computations. In the U(IV)-acetylacetone system [3], it was felt that there was no obvious way to unambigously establish the analytical errors in the determinations of $[M]_{org}$ and $[M]_{aq}$. Therefore, the error in log q was estimated from the spread of the individual points; see Fig. 1. For this purpose, points with very close lying log $[A^-]$ values were chosen. From 6 such groups, comprising altogether 14 points, at various log $[A^-]$, an average error of ± 0.04 in log q was estimated, which corresponds to a ± 10 % error in q on a linear scale. For the sake of simplicity, and also in order to make the data more suitable to the input form of the computor, the whole error was attributed to $[M]_{aq}$. Thus the data put into the machine were $[M]_{org} = q \pm 0.00$, $[M]_{aq} = 1.00 \pm 0.10$, and $[A^-]$.

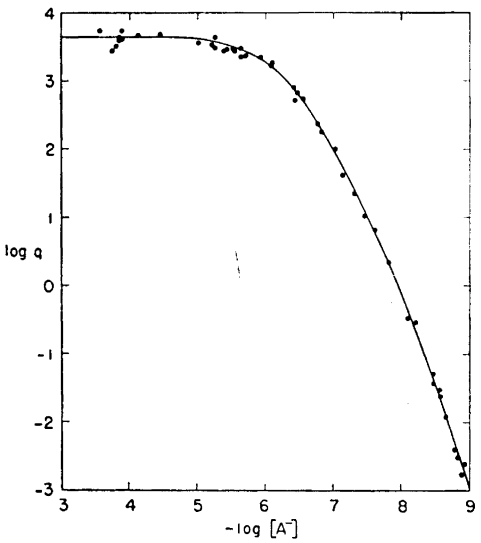


Fig. 1.

* The significance of the function

$$S_{min}/k = \frac{\sum \omega_i \sum (a_n x_i^n - z_i)^2}{L - N - 2},$$

where ω_i is the weight of the point i, will be fully discussed in a subsequent paper.

Table 1. Equilibrium constants calculated by the least square method.

Reaction	Constants
$U^{4+} + A^- \rightleftharpoons UA^{3+}$	$(1.04 \pm 0.69)\ 10^9$
$UA^{3+} + A^- \rightleftharpoons UA_2^{2+}$	$(1.80 \pm 0.64)\ 10^8$
$UA_2^{2+} + A^- \rightleftharpoons UA_3^{+}$	$(3.31 \pm 0.72)\ 10^6$
$UA_3^{+} + A^- \rightleftharpoons UA_4$	$(0.95 \pm 0.19)\ 10^6$
UA_4 (aq.) $\rightleftharpoons UA_4$ (C_6H_6)	$(4.38 \pm 0.25)\ 10^3$

In addition to the 10 % error in $[M]_{aq}$, a number of other errors were also tested. The S_{min}/k values of these runs were 15.5 (for a 5 % error), 3.9 (10 % error), 1.0 (20 % error) and 0.4 (30 % error), indicating that the 10 % error first assigned to the experimental points was too small.

In this particular system, it turned out that the best fit of the experimental data to the curve given by eqn. (1) is obtained when all constants $\varkappa_1$,.....$\varkappa_4$ and λ_4 are present. The stepwise formation constants k_n as well as the distribution constant λ_4 are given in Table 1. It is seen that the standard deviation is very large for k_1 (about 70 %), but diminishes successively up to k_4 (about 20 %). The precision in λ_4 is unusually good. With these values (or rather $\varkappa_n = \prod_1^n k_n$), the curve in Fig. 1 is calculated.

In Table 2, the log $\varkappa_n$ and log λ_4 values are given in order to compare them with the constants manually calculated in the original paper [3]. There, two different sets of constants were given for the two-parameter method; here, the average values are given, and the errors are the spread between these two values. When comparing the constants, the agreement between the constants calculated with the least square method and those of the two-parameter method is rather good, while it is less good for the ligand number method. Since both these latter methods are graphical in nature, the better agreement of the two-parameter method probably stems from the fact that the experimental data in that method are used directly for the estimation of the stability constants, and that the sequence-values of the stability constants are of the suitable two-parameter type. In the ligand number method, the graphical derivation of the smoothed curve through the experimental points makes possible the introduction of large errors.

From Table 2 it is further seen that the precision assigned to the constants obtained by the two graphical methods is far too small. While all errors in the primary data are considered in the least square computations, only errors in a very narrow range of data can be regarded for estimating the errors of the constants obtained by the graphical methods. This illuminates one of the statements made in the introduction, namely the inherent difficulty of the approximation methods in making valid estimates of the precision of the stability constants.

Table 2. Comparison of equilibrium constants (log $\varkappa_n$) obtained with three different calculation procedures.

Reaction	Least square method	Ligand number method	Two-parameter method
$U^{4+} + A^- \rightleftharpoons UA^{3+}$	9.02 ± 0.29	8.6 ± 0.2	9.05 ± 0.1
$U^{4+} + 2A^- \rightleftharpoons UA_2^{2+}$	17.27 ± 0.26	17.0 ± 0.2	17.02 ± 0.15
$U^{4+} + 3A^- \rightleftharpoons UA_3^{+}$	23.79 ± 0.26	23.4 ± 0.1	23.92 ± 0.1
$U^{4+} + 4A^- \rightleftharpoons UA_4$	29.77 ± 0.29	29.5 ± 0.1	29.76 ± 0.1
UA_4 (aq.) $\rightleftharpoons UA_4$ (C_6H_6)	3.64 ± 0.025	3.62 ± 0.05	3.62 ± 0.05

1. Sullivan, J. C. and Hindman, J. C. *J. Am. Chem. Soc.* **74** (1952) 6091.
2. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants. Part I: Organic Ligands. Part II: Inorganic Ligands.* The Chemical Society, Burlington House, London W. 1, 1957 and 1958.
3. Rydberg, J. and Rydberg, B. *Arkiv Kemi* **9** (1956) 81.

Received November 12, 1958.

The Presence of Pyruvate as a Source of Error in Ammonia Determination by Means of Nessler's Reagent

G. STEENSHOLT and L. ROTNES

Department of Biochemistry, University of Oslo, Norway

A simple and rapid method for estimating the pyruvate-activated glutaminase II activity of rat liver was described by Beaton [1] in 1954. During experiments with glutaminase II, using exactly the same procedure [1], we found that sodium pyruvate reduced the colour of Nessler's reagent even in the presence of Archibald's persulphate-gluconate stabilizer [2]. Comparing standard curves made with and without pyruvate, we found about 50 % reduction when using Vanselow's [3] Nessler reagent. Utilizing Kolthoff's [4] Nessler reagent, the reduction amounted to about 24 %.

Standard curves were made according to Beaton [1], using the same quantities of pyruvate as in the enzyme activity determinations. A set of tubes was prepared as shown in Table 1.

Table 1. Preparation of standard curves. Each tube contained 1 ml veronal-acetate buffer, pH 7.7 (31.8 μmole sodium pyruvate) and 1 ml glutamine solution (20.4 μmole). Tube 1 is the blank.

Tube No.	Water ml	$(NH_4)_2SO_4$ standard solution containing 3.598 μmole per ml ml
1	2.0	0
2	1.8	0.2
3	1.6	0.4
4	1.5	0.5
5	1.4	0.6
6	1.2	0.8

A similar set of tubes was prepared in identically the same manner, except that the pyruvate was omitted. After incubation for 1 h at 37°C, 2 ml 0.7 N sulphuric acid and 2 ml 7 % sodium tungstate were added. A 4 ml sample was removed from each tube. To each sample were then added 4 ml of a freshly prepared Archibald's stabilizer diluted 2 : 1 with water. 1 ml of Nessler's reagent was rapidly added and after 5 min the colour was read at 450 mμ in a Coleman spectrophotometer. Three different sodium pyruvate preparations were tried. One preparation had been obtained from Boehringer & Soehne, Mannheim, and two were prepared according to Greenstein [5]. They gave the same results in all our experiments. The results obtained are shown in Fig. 1.

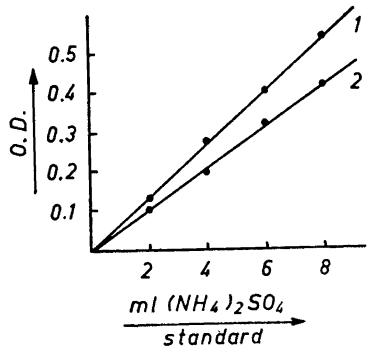


Fig. 1. The reduction of the colour of Kolthoff's form of Nessler's reagent. Curve 1, samples without pyruvate read against blank without pyruvate. Curve 2, samples containing pyruvate read against blank containing pyruvate.

When estimating glutaminase II activity we might use curve 2 adding adequate amounts of pyruvate to the blank after incubation. According to Refs. [6,7] 80—90 % of the pyruvate is recovered at the end of the incubation period. In experiments with varying pyruvate concentrations we found that the reduction in colour intensity reached its maximum at a pyruvate concentration of approximately 50 % of that used in the experiments reported in Table 1.

A reasonable procedure consists in adding 80 % of the initial amount of pyruvate to the blank after adding the sulphuric acid on termination of the incubation. This preserves the rapidity and convenience of the method, especially when series of analyses have to be carried out.

1. Beaton, J. R. *Proc. Soc. Exptl. Biol. Med.* **87** (1954) 238.
2. Archibald, R. M. *J. Biol. Chem.* **154** (1944) 643.
3. Vanselow, A. P. *Ind. Eng. Chem. Anal. Ed.* **12** (1940) 516.
4. Kolthoff, I. M. and Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*, 3rd Ed. p. 634.
5. Greenstein, J. P. and Leuthardt, F. M. *Arch. Biochem.* **17** (1948) 105.
6. Errera, M. *J. Biol. Chem.* **178** (1949) 483.
7. Meister, A. and Tice, S. V. *J. Biol. Chem.* **187** (1950) 173.

Received December 4, 1958.

On the Relative Donor Strengths of $POCl_3$ and $PO(CH_3)_3$. A Ramanspectroscopic Study

PER-OLOF KINELL, INGVAR LINDQVIST and MARGARETA ZACKRISSON

Institute of Physical Chemistry and Institute of Chemistry, University of Uppsala, Uppsala, Sweden

A very important problem in the study of addition compounds is the determination of the relative acceptor and donor strengths of the reacting molecules.

The relative donor strenghts of two donor molecules D_1 and D_2 are only defined for a given acceptor molecule A. The free energy changes of the two reactions $A(g) + D_1(g) = AD_1(g)$ and $A(g) + D_2(g) = AD_2(g)$ should be compared in an exact study. If liquid or solid phases appear in either of these reactions the discussion is complicated by the large variations in cohesive energies. These depend very much upon the dipole moments of the different molecules, and bear no obvious relation to the strengths of the acceptor-donor bonds. The same difficulty prevails if a solvent with an appreciable dipole moment is used for the study. In an ideal solvent the differences between the solvation energies for D_1 and AD_1, and for D_2 and AD_2 should be equal. It is rather difficult to find such a solvent but the use of different types of solvents which may interact more or less favourably with the different molecules might lead to a rather safe assessment of the relative donor strengths.

If such solvents can be selected a suitable method of studying the reactions taking place remains to be found so that at least the order of donor strength can be determined. The molecular spectra are then of an especial interest although they do not seem to have been used much for this purpose. Infrared spectra and Raman spectra each have their advantages and disadvantages in this connection. IR is sensitive to low concentrations of the dissolved molecules if the solvent has a low absorption in the spectral regions where the molecules to be studied absorb. Raman spectra are more difficult to measure in dilute solutions but have fewer absorption bands with less risk for overlap between the spectra of the different molecules or with the solvent spectrum. In this first study we have investigated the usefulness of the Raman method.

For this purpose we have tried to find a test pair of donor molecules with well known donor functions for which a rather safe prediction about the relative donor strengths could be made, so that the difficult search for a number of different, suitable solvents could be omitted as an unnecessary precaution. This requirement is fulfilled by the pair $POCl_3$ and $PO(CH_3)_3$. The donor function of the oxygen atom in such compounds has recently been definitely established by X-ray and spectroscopic work[1,2]. It can also be safely predicted that $PO(CH_3)_3$ should be a better donor molecule than $POCl_3$. The larger electronegativity of chlorine causes a drift of electrons from oxygen in $POCl_3$ compared with $PO(CH_3)_3$ and thus a lower dipole moment and a lower tendency to donate an electron pair.

1,2-Dichlorethane was used as a solvent and the following solutions were studied (mole fractions within brackets): $POCl_3$ (0.10), $PO(CH_3)_3$ (0.10), $SbCl_5$ + $POCl_3$ (0.10 + 0.10), $SbCl_5$ + $PO(CH_3)_3$ (0.02 + 0.02) and $SbCl_5$ + $POCl_3$ + $PO(CH_3)_3$ (0.02 + 0.02 + 0.02). The solution of $SbCl_5 \cdot POCl_3$ was supersaturated. The preparation of the mixture of all three molecular species must be made in such an order that $POCl_3$ and $PO(CH_3)_3$ are not permitted to react with each other. The products of that complicating reaction are not known but the reaction is prevented by the presence of $SbCl_5$.

1. Beaton, J. R. *Proc. Soc. Exptl. Biol. Med.* **87** (1954) 238.
2. Archibald, R. M. *J. Biol. Chem.* **154** (1944) 643.
3. Vanselow, A. P. *Ind. Eng. Chem. Anal. Ed.* **12** (1940) 516.
4. Kolthoff, I. M. and Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*, 3rd Ed. p. 634.
5. Greenstein, J. P. and Leuthardt, F. M. *Arch. Biochem.* **17** (1948) 105.
6. Errera, M. *J. Biol. Chem.* **178** (1949) 483.
7. Meister, A. and Tice, S. V. *J. Biol. Chem.* **187** (1950) 173.

Received December 4, 1958.

On the Relative Donor Strengths of $POCl_3$ and $PO(CH_3)_3$. A Ramanspectroscopic Study

PER-OLOF KINELL, INGVAR LINDQVIST and MARGARETA ZACKRISSON

Institute of Physical Chemistry and Institute of Chemistry, University of Uppsala, Uppsala, Sweden

A very important problem in the study of addition compounds is the determination of the relative acceptor and donor strengths of the reacting molecules.

The relative donor strenghts of two donor molecules D_1 and D_2 are only defined for a given acceptor molecule A. The free energy changes of the two reactions $A(g) + D_1(g) = AD_1(g)$ and $A(g) + D_2(g) = AD_2(g)$ should be compared in an exact study. If liquid or solid phases appear in either of these reactions the discussion is complicated by the large variations in cohesive energies. These depend very much upon the dipole moments of the different molecules, and bear no obvious relation to the strengths of the acceptor-donor bonds. The same difficulty prevails if a solvent with an appreciable dipole moment is used for the study. In an ideal solvent the differences between the solvation energies for D_1 and AD_1, and for D_2 and AD_2 should be equal. It is rather difficult to find such a solvent but the use of different types of solvents which may interact more or less favourably with the different molecules might lead to a rather safe assessment of the relative donor strengths.

If such solvents can be selected a suitable method of studying the reactions taking place remains to be found so that at least the order of donor strength can be determined. The molecular spectra are then of an especial interest although they do not seem to have been used much for this purpose. Infrared spectra and Raman spectra each have their advantages and disadvantages in this connection. IR is sensitive to low concentrations of the dissolved molecules if the solvent has a low absorption in the spectral regions where the molecules to be studied absorb. Raman spectra are more difficult to measure in dilute solutions but have fewer absorption bands with less risk for overlap between the spectra of the different molecules or with the solvent spectrum. In this first study we have investigated the usefulness of the Raman method.

For this purpose we have tried to find a test pair of donor molecules with well known donor functions for which a rather safe prediction about the relative donor strengths could be made, so that the difficult search for a number of different, suitable solvents could be omitted as an unnecessary precaution. This requirement is fulfilled by the pair $POCl_3$ and $PO(CH_3)_3$. The donor function of the oxygen atom in such compounds has recently been definitely established by X-ray and spectroscopic work[1,2]. It can also be safely predicted that $PO(CH_3)_3$ should be a better donor molecule than $POCl_3$. The larger electronegativity of chlorine causes a drift of electrons from oxygen in $POCl_3$ compared with $PO(CH_3)_3$ and thus a lower dipole moment and a lower tendency to donate an electron pair.

1,2-Dichlorethane was used as a solvent and the following solutions were studied (mole fractions within brackets): $POCl_3$ (0.10), $PO(CH_3)_3$ (0.10), $SbCl_5$ + $POCl_3$ (0.10 + 0.10), $SbCl_5$ + $PO(CH_3)_3$ (0.02 + 0.02) and $SbCl_5$ + $POCl_3$ + $PO(CH_3)_3$ (0.02 + 0.02 + 0.02). The solution of $SbCl_5 \cdot POCl_3$ was supersaturated. The preparation of the mixture of all three molecular species must be made in such an order that $POCl_3$ and $PO(CH_3)_3$ are not permitted to react with each other. The products of that complicating reaction are not known but the reaction is prevented by the presence of $SbCl_5$.

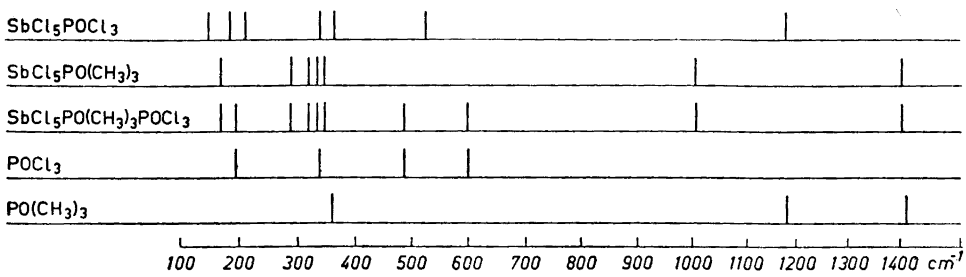


Fig. 1. The Raman spectra of the different solutions.

The Raman spectra were taken with a redesigned spectrograph of the type described by Kinell [3]. The photographic technique using Ilford Zenith plates was applied. The spectra were excited by the 4 358 Å Hg-line, and saturated $NaNO_2$ solution was used to filter away the UV part of the Hg-spectrum.

The results are shown in the simplified Fig. 1 where the spectrum of 1,2-dichlorethane is omitted together with those bands which overlap with it (the intensities are not indicated).

The interpretation is obvious: all the $SbCl_5$ in the ternary mixture has reacted with $PO(CH_3)_3$ to form $SbCl_5 \cdot PO(CH_3)_3$, leaving $POCl_3$ unreacted in the solution. This confirms that $PO(CH_3)_3$ is a better donor molecule than $POCl_3$ in the reaction with $SbCl_5$ (at these concentrations in 1,2-dichlorethane, strictly speaking).

The very distinct determination of the relative donor strengths achieved by this method in this case gives good hope for its application to problems with a less obvious solution. Such work is in progress in these Institutes.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Finally we wish to thank Professor S. Claesson and Professor G. Hägg for all facilities put at our disposal.

1. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134.
2. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **80** (1958) 4775.
3. Kinell, P. O. *Thesis*, Uppsala 1953.

Received December 11, 1958.

Corrections to "A Group Separation Method for Gamma Spectrometry of Complex Radionuclide Mixtures" *

K SAMSAHL **

Nuclear Chemistry Laboratory, AB Atomenergi, Stockholm, Sweden

P. 1293, line 11 from below,

for

Besides its powerful solving and oxidizing action on many elements it will for example reduce possible higher valences of manganese to the divalent state and does not effect oxidation of trivalent chromium in hydrochloric acid. As a result chromium, if not initially present as chromate, and manganese can easily be quantitatively separated from other elements.

read

Besides its powerful, solving and oxidizing action on many elements it will for example reduce possible higher valences of manganese to the divalent state and chromates to trivalent chromium in hydrochloric acid. As a result chromium and manganese can easily be quantitatively separated from other elements.

* *Acta Chem. Scand.* **12** (1958) 1292.

** Present address: Eurochemic, C. E. N., Mol, Belgium.

Received December 20, 1958.

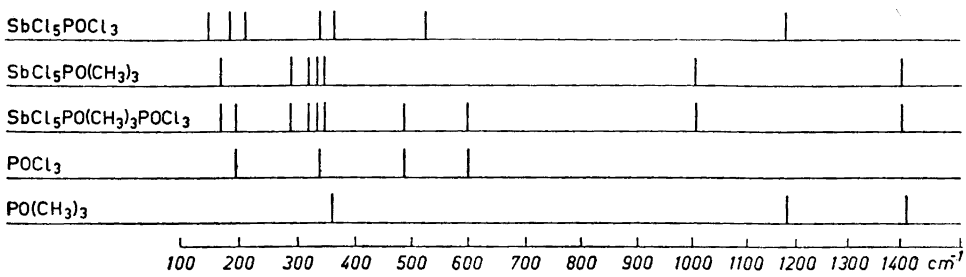


Fig. 1. The Raman spectra of the different solutions.

The Raman spectra were taken with a redesigned spectrograph of the type described by Kinell [3]. The photographic technique using Ilford Zenith plates was applied. The spectra were excited by the 4 358 Å Hg-line, and saturated $NaNO_2$ solution was used to filter away the UV part of the Hg-spectrum.

The results are shown in the simplified Fig. 1 where the spectrum of 1,2-dichlorethane is omitted together with those bands which overlap with it (the intensities are not indicated).

The interpretation is obvious: all the $SbCl_5$ in the ternary mixture has reacted with $PO(CH_3)_3$ to form $SbCl_5 \cdot PO(CH_3)_3$, leaving $POCl_3$ unreacted in the solution. This confirms that $PO(CH_3)_3$ is a better donor molecule than $POCl_3$ in the reaction with $SbCl_5$ (at these concentrations in 1,2-dichlorethane, strictly speaking).

The very distinct determination of the relative donor strengths achieved by this method in this case gives good hope for its application to problems with a less obvious solution. Such work is in progress in these Institutes.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Finally we wish to thank Professor S. Claesson and Professor G. Hägg for all facilities put at our disposal.

1. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134.
2. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **80** (1958) 4775.
3. Kinell, P. O. *Thesis*, Uppsala 1953.

Received December 11, 1958.

Corrections to "A Group Separation Method for Gamma Spectrometry of Complex Radionuclide Mixtures" *

K SAMSAHL **

Nuclear Chemistry Laboratory, AB Atomenergi, Stockholm, Sweden

P. 1293, line 11 from below,

for

Besides its powerful solving and oxidizing action on many elements it will for example reduce possible higher valences of manganese to the divalent state and does not effect oxidation of trivalent chromium in hydrochloric acid. As a result chromium, if not initially present as chromate, and manganese can easily be quantitatively separated from other elements.

read

Besides its powerful, solving and oxidizing action on many elements it will for example reduce possible higher valences of manganese to the divalent state and chromates to trivalent chromium in hydrochloric acid. As a result chromium and manganese can easily be quantitatively separated from other elements.

* *Acta Chem. Scand.* **12** (1958) 1292.

** Present address: Eurochemic, C. E. N., Mol, Belgium.

Received December 20, 1958.

Electroorganic Preparations

VII. Reduction of a Compound Containing a Trifluoromethyl Group

HENNING LUND

Leo Pharmaceutical Products, Copenhagen, Denmark

Several compounds are reducible at the dropping mercury electrode due to an electrochemical fission of a carbon-halogen bond. The reductive splitting of such bonds have been studied by means of polarography and controlled potential reductions and in most cases the halogens have been iodine, bromine or chlorine. Apparently only in the reduction of one compound,[1] phenacyl fluoride, the electrochemical fission of an aliphatic carbon-fluorine bond has been reported. In this communication the polarography and reduction of a compound containing a trifluoromethyl group is investigated.

The trifluoromethyl group which has received considerable attention as a substituent in various drugs is not generally reducible at the dropping mercury electrode, as no reduction between 0 and −2.0 V *vs.* S.C.E. was observed for trifluoroacetic acid, trifluoromethylbenzene, 3-trifluoromethylaniline, or 3-trifluoromethyl-4-sulphamyl-aniline. However, the introduction of two sulphamyl groups in 3-trifluoromethylaniline renders the compound reducible. In Table 1 is reported the polarographic behaviour of 6-trifluoromethyl-7-sulphamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide (6-TFM).

Table 1. Diffusion currents in μA and half-wave potentials *vs.* S.C.E. for 6-trifluoromethyl-7 - sulphamyl - 3,4 - dihydro - 1,2,4 - benzothiadiazine-1,1-dioxide. Concentration 0.60 × 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	i_d μA	$E_{\frac{1}{2}}$ V
6.28	8.4 *	1.67
7.35	7.7	1.67
8.30	7.1	1.67
8.80	7.0	1.67$_5$
9.75	6.7	1.70
10.50	6.6	1.75
10.75	6.5	1.78

* merges with the hydrogen wave.

The compound yields in the pH-interval 6 to 11 a well-defined, diffusion controlled, irreversible wave. By comparison of the wave height with that of 2,4-disulphamyl-5-bromo-aniline, which was assumed to be reduced in a two-electron reduction to 2,4-disulphamyl-aniline, it was found that the wave height of 6-TFM corresponded to a comsumption of 5−6 electrons per molecule. As seen from Table 1 the half-wave potentials of 6-TFM are constant at pH-values lower than about 8.5, whereas they at higher pH-values become more negative. This pH-dependence of the half-wave potentials is connected with the presence of the two sulphamyl groups. Each sulphamyl group contains a weakly acidic hydrogen atom. The *pK*-values of the groups are 8.9 and 10.7. At pH-values lower than 8.5 the undissociated sulphamyl groups strongly attract electrons from the benzene ring and the trifluoromethyl group, thus making an attack of electrons more easy. At pH-values higher than 8.5 an increasing part of the sulphamyl groups are dissociated into anions which attract electrons much less than does the undissociated sulphamyl group. The potential required to promote an attack of electrons thus becomes more negative.

A similar dependence of the half-wave potentials upon pH has been found for many halogen compounds containing a dissociable group, such as α-halogenated acids [2,3], iodophenols [4], and iodoanilines [4]. A plot of the half-wave potentials of these compounds against pH normally yields an S-shaped curve with one pH-independent branch in acid solution and one in alkaline solution. The pK_a value of the acid is found in the rising part of the curve; the midpoint of the S-shaped curve is generally found at a pH somewhat higher than the pK_a of the acid. This is probably due to recombination of the anion with hydrogen ions at the electrode surface.

As the half-wave potential of both the acid and the anion are pH-independent one might have expected a pH-interval in which two waves were observed due to the reduction of the acid and of the anion, respectively. This is the case in the reduction of, *e. g.*, pyruvic acid [5]. The pH at which the wave due to the reduction of the acid is of equal height as that due to the reduction of the anion is somewhat higher than the pK_a of pyruvic acid and is determined by the rate of the recombination at

the electrode surface [5]. A difference between pyruvic acid and the halogenated acids lies in the fact that the reduction of the reducible group in pyruvic acid (the carbonyl group) generally is pH-dependent in acid solution, whereas the reduction of a carbon-halogen bond as such is pH-independent. It seems that the half-wave potentials of the halogen compounds by some as yet undetermined feature of the electrode process kinetics reflects the average electronic interaction between the dissociable group and the carbon atom, at which the fission of the carbon-halogen bonds takes place.

Controlled potential reduction of 6-TFM and its parent disulphonamide, 5-trifluoromethyl-2,4-disulphamyl-aniline, showed that the electrode reaction was a six-electron reduction and the methyl compounds, 6-methyl-7-sulphamyl - 3,4 - dihydro - 1,2,4 - benzothiadiazine-1,1-dioxide and 5-methyl-2,4-disulphamyl-aniline, respectively, were isolated from the reaction mixture. A photometric determination with zirconiumeriochrome cyanine of the fluoride ions produced showed the presence of about 95 % of the total original fluorine content as fluoride ion. The overall reaction thus is

$$R—CF_3 + 6e^- + 3\ H_2O \rightarrow R—CH_3 + 3\ F^- + 3\ OH^-$$

Although a stepwise reduction of a polyhalogenated carbon atom is the most common reaction path a few examples of polarographic waves due to a complete reduction of a polyhalogenated compound are reported. Chloral hydrate [6] thus produces a single wave which corresponds to a reduction to acetaldehyde.

Elving and Leone [1] point to the fact that the carbon-fluorine bonds in F_3CH are considerably shorter than in FCH_3, which is reflected in the greater stability of the polyfluoromethanes. They conclude that if this behaviour is generally true for the trifluoromethyl group, the stepwise fission of the carbon-halogen bonds would not be observed as in the case of trichloro- and tribromoacids, but all of the bonds would probably be ruptured in a single step at a potential more negative than that at which the one carbon-fluorine bond in the monofluoro compound would be reduced. The results of the present investigation fully substantiate the conclusion of Elving and Leone.

As 6-TFM can be determined spectrophotometrically after alkaline hydrolysis, diazotization and coupling we have a means of determining in a solution both the concentration of the primary, aromatic amine and of trifluoromethyl groups bonded to a benzene ring containing two sulphamyl groups. This has been applied to the determination of 6-TFM in the urine from persons given 6-TFM perorally, and has shown that the concentrations of the amine and the trifluoromethyl compound found in the urine were the same, which strongly suggests that the trifluoromethyl group remains intact during the passage through the body.

Experimental. The polarograph was a recording polarograph Radiometer Type PO 3a. The capillary delivered 2.16 mg of mercury per second at a corrected mercury height of 48.5 cm. The drop time 3.90 sec (water, open circuit). The capillary constant $m^{2/3}t^{1/6} = 2.13$ $mg^{2/3}\ sec^{-\frac{1}{2}}$. The apparatus used for the controlled potential reductions was an electromechanical potentiostat of the Lingane-Jones type.

The buffer solutions used were below pH 8 sodium phosphate-citric acid buffers and from pH 8 to 11 sodium borate buffers. The medium employed was 40 % alcohol containing 1 M potassium chloride and 0.01 % gelatine. The pH-values were measured with a glass electrode; in the medium used they have only relative value.

Reduction of 6-trifluoromethyl-7-sulphamyl-3,4 - dihydro - 1,2,4 - benzothiadiazine - 1,1 - dioxide. A suspension of 1.0 g of 6-TFM in 180 ml 30 % aqueous methanol containing 20 g of potassium hydrogen carbonate was reduced at a cathode potential of —1.70 V *vs.* S.C.E.; the reduction was completed after the consumption of 6.3 electrons per molecule. Small amounts of hydrochloric acid were added during the electrolysis to compensate for the hydrogen ions used in the reduction; the consumption of hydrogen ions was calculated from the reading of the coulometer. After completion of the reduction the reaction mixture was extracted several times with ethyl acetate, which was evaporated. The residue was recrystallized from dilute methanol yielding 600 mg of a compound with an IR-spectrum identical with that of actual 6-methyl-7-sulphamyl-3,4 - dihydro - 1,2,4 - benzothiadiazine - 1,1 - dioxide. (Found: C 34.53; H 4.04; N 15.12; S 22.99. Calc. for $C_8H_{11}N_3O_4S_2$: C 34.64; H 3.99; N 15.16; S 23.13.)

From a similar reduction of 5-trifluoromethyl-2,4-disulphamyl-aniline were isolated 650 mg of a compound with an IR-spectrum identical with that of 5-methyl-2,4-disulphamyl-aniline. (Found: C 31.79; H 4.22; N 15.92; S 24.02. Calc. for $C_7H_{11}N_3O_4S_2$: C 31.69; H 4.18; N 15.84; S 24.17.)

1. Elving, P. J. and Leone, J. T. *J. Am.Chem. Soc.* **79** (1957) 1546.
2. Elving, P. J., Rosenthal, I. and Kramer, M. K. *J. Am. Chem. Soc.* **73** (1951) 1717.
3. Rosenthal, I., Albright, C. H. and Elving, P. J. *J. Electrochem. Soc.* **99** (1952) 227.
4. Gergely, E. and Iredale, T. *J. Chem. Soc.* **1951** 3502.
5. Brdicka, R. and Wiesner, K. *Collection Czechoslov. Chem. Communs.* **12** (1947) 138.
6. Elving, P. J. and Bennett, C. E. *J. Electrochem. Soc.* **101** (1954) 520.

Received December 17, 1958.

Separation of Hg^{++} from Active Carbon by Extraction with EDTA

HARALD HOVDEN

Norsk Hydros Forskningslaboratorium, Porsgrunn, Norway

In the synthesis of vinyl chloride from acetylene and hydrogen chloride active carbon impregnated with mercuric chloride is used as a catalyst. The catalyst usually contains 8—10 % by wt. of mercuric chloride. The mercuric chloride content of catalyst samples drawn from a reactor which has been in use for a certain length of time, varies between zero and a value higher than the original one.

A producer of vinyl chloride is of course highly interested in a rapid and fairly accurate method for the determination of the mercury content of the catalyst. The present work was initiated some 20 months ago, at which time no publications dealing with this special problem could be found in available literature.

In our first experiments we investigated several procedures based on initial oxidation of the carbon, *e.g.* combustion in a Parr bomb, oxidation with fuming sulphuric acid and potassium persulphate and the method described by Schöniger[1,2] where the sample is burned in a conical flask filled with oxygen. By all these methods, however, the recovery of mercury was too low. This was also the case when the catalyst was extracted with acetone, while extraction with nitric acid or a mixture of nitric and hydrochloric acid seemed more promising. Another possible way of separating the mercury from the carbon may be distillation in a quartz apparatus, a method worked out for the determination of mercury in organic matter.[3]

During our further work, however, we were led to the conclusion that the most convenient method would be direct extraction of the mercury by means of EDTA. Check analysis by the aqua regia/H_2O_2 extraction method described in Ref.[4] have shown that this extraction is complete also when applied on spent catalyst samples. In the extract the mercury content is easily determined photometrically as mercury dithizonate. For a more detailed description of the dithizone procedure, Ref.[5] may be consulted.

Experimental. All of the solutions are made up with glass distilled water.

Buffer solution, pH = 10. 70 g NH_4Cl and 610 ml 12 N NH_4OH, diluted to 1 000 ml.

EDTA-solution. Disodiumsalt of ethylenediaminetetraacetic acid, 0.1 M.

Procedure. 20—30 mg of ground catalyst is transferred to a centrifuge tube together with 5 ml of EDTA-solution and 15 ml of buffer solution and extracted for 30 min with moderate shaking. The complexing of Hg^{++} with EDTA is considered a very rapid reaction and it therefore seems probable that complete extraction is achieved in considerably less than 30 min. The extraction is followed by settling of the carbon, which is most conveniently done by means of centrifuging. 5 ml of the supernatant liquid is pipetted into a volumetric flask and the volume is adjusted to 100 ml. An aliquot of this solution is used for the subsequent determination with dithizone.

1. Schöniger, W. *Mikrochim. Acta* **1955** 123.
2. West, T. S. *Chem. Age, London* **80** (1958) 193.
3. *Chem. Age, London* **78** (1957) 595.
4. Singliar, M. and Kapisinska, V. *Chem. zvesti* **11** (1957) 411.
5. Iwantscheff, G. *Das Dithizon und seine Anwendung in der Mikro- und Spuren-Analyse*, Verlag Chemie, Weinheim 1958.

Received December 15, 1958.

From a similar reduction of 5-trifluoromethyl-2,4-disulphamyl-aniline were isolated 650 mg of a compound with an IR-spectrum identical with that of 5-methyl-2,4-disulphamyl-aniline. (Found: C 31.79; H 4.22; N 15.92; S 24.02. Calc. for $C_7H_{11}N_3O_4S_2$: C 31.69; H 4.18; N 15.84; S 24.17.)

1. Elving, P. J. and Leone, J. T. *J. Am.Chem. Soc.* **79** (1957) 1546.
2. Elving, P. J., Rosenthal, I. and Kramer, M. K. *J. Am. Chem. Soc.* **73** (1951) 1717.
3. Rosenthal, I., Albright, C. H. and Elving, P. J. *J. Electrochem. Soc.* **99** (1952) 227.
4. Gergely, E. and Iredale, T. *J. Chem. Soc.* **1951** 3502.
5. Brdicka, R. and Wiesner, K. *Collection Czechoslov. Chem. Communs.* **12** (1947) 138.
6. Elving, P. J. and Bennett, C. E. *J. Electrochem. Soc.* **101** (1954) 520.

Received December 17, 1958.

Separation of Hg^{++} from Active Carbon by Extraction with EDTA

HARALD HOVDEN

Norsk Hydros Forskningslaboratorium, Porsgrunn, Norway

In the synthesis of vinyl chloride from acetylene and hydrogen chloride active carbon impregnated with mercuric chloride is used as a catalyst. The catalyst usually contains 8—10 % by wt. of mercuric chloride. The mercuric chloride content of catalyst samples drawn from a reactor which has been in use for a certain length of time, varies between zero and a value higher than the original one.

A producer of vinyl chloride is of course highly interested in a rapid and fairly accurate method for the determination of the mercury content of the catalyst. The present work was initiated some 20 months ago, at which time no publications dealing with this special problem could be found in available literature.

In our first experiments we investigated several procedures based on initial oxidation of the carbon, *e.g.* combustion in a Parr bomb, oxidation with fuming sulphuric acid and potassium persulphate and the method described by Schöniger[1,2] where the sample is burned in a conical flask filled with oxygen. By all these methods, however, the recovery of mercury was too low. This was also the case when the catalyst was extracted with acetone, while extraction with nitric acid or a mixture of nitric and hydrochloric acid seemed more promising. Another possible way of separating the mercury from the carbon may be distillation in a quartz apparatus, a method worked out for the determination of mercury in organic matter.[3]

During our further work, however, we were led to the conclusion that the most convenient method would be direct extraction of the mercury by means of EDTA. Check analysis by the aqua regia/H_2O_2 extraction method described in Ref.[4] have shown that this extraction is complete also when applied on spent catalyst samples. In the extract the mercury content is easily determined photometrically as mercury dithizonate. For a more detailed description of the dithizone procedure, Ref.[5] may be consulted.

Experimental. All of the solutions are made up with glass distilled water.

Buffer solution, pH = 10. 70 g NH_4Cl and 610 ml 12 N NH_4OH, diluted to 1 000 ml.

EDTA-solution. Disodiumsalt of ethylenediaminetetraacetic acid, 0.1 M.

Procedure. 20—30 mg of ground catalyst is transferred to a centrifuge tube together with 5 ml of EDTA-solution and 15 ml of buffer solution and extracted for 30 min with moderate shaking. The complexing of Hg^{++} with EDTA is considered a very rapid reaction and it therefore seems probable that complete extraction is achieved in considerably less than 30 min. The extraction is followed by settling of the carbon, which is most conveniently done by means of centrifuging. 5 ml of the supernatant liquid is pipetted into a volumetric flask and the volume is adjusted to 100 ml. An aliquot of this solution is used for the subsequent determination with dithizone.

1. Schöniger, W. *Mikrochim. Acta* **1955** 123.
2. West, T. S. *Chem. Age, London* **80** (1958) 193.
3. *Chem. Age, London* **78** (1957) 595.
4. Singliar, M. and Kapisinska, V. *Chem. zvesti* **11** (1957) 411.
5. Iwantscheff, G. *Das Dithizon und seine Anwendung in der Mikro- und Spuren-Analyse*, Verlag Chemie, Weinheim 1958.

Received December 15, 1958.

Synthesis of *o*-Phthalic Acid Di-Esters of Choline and Related Compounds

SVERRE ØKSNE

Norwegian Defence Research Establishment, Toxicological Group, Lilleström, Norway

The preparation and physiological effects of a large number of choline esters and other types of quaternary alkylammonium compounds are dealt with in the literature.[1,2] Fusco *et al.*[3] prepared the bis-choline esters of the three phthalic acids and Bovet-Nitti[4] reported that β-diethylaminoethyl phthalate bis-ethiodide (306 I.S.) was a powerful inhibitor of pseudocholinesterase but not of true cholinesterase.

$$o\text{-}C_6H_4(COOCH_2CH_2\overset{+}{N}(C_2H_5)_3)_2\ 2I^-$$

306 I.S.

As part of an investigation on the inhibition of cholinesterases carried out by our institute,[5] the bis-methiodides and bis-ethiodides of the tertiary amines I, II and III were prepared.

$o\text{-}C_6H_4R_2$ I R = $COOCH_2CH_2N(CH_3)_2$
II R = $COOCH(CH_3)CH_2N(CH_3)_2$
III R = $COOCH(CH_3)CH_2N(C_2H_5)_2$

Experimental *. *Phthalyl chloride.* This was prepared as described by Kyrides[6], from phthalic anhydride and thionyl chloride. B. p. 119—122°/4 mm.

β-Dimethylaminoethyl phthalate (I)[3]. A solution of phthalyl chloride (0.04 mole) in dry ether (75 ml) was added dropwise to a solution of 2-dimethylaminoethanol (0.1 mole) in dry ether (50 ml) under vigorous stirring and cooling in cold water. The mixture was heated under reflux for 2 h and left overnight at room temperature. After evaporating the ether under reduced pressure, the residue was dissolved in water (20 ml). A chilled solution of potassium carbonate (15 g) in water (20 ml) was then added, and the mixture was extracted with five portions of 25 ml of ether. The combined ether extracts were dried for two days with anhydrous potassium carbonate, filtered and the ether evaporated under reduced pressure. Distillation of the residue *in vacuo* gave a light yellow oil. Yield 7.3 g (59 %); b. p. 168—169°/0.55 mm. (Reported by Fusco: 187°/2.5 mm.) No analyses were taken of this compound.

α-Methyl - β - dimethylaminoethyl phthalate (II). The ester II was prepared from 10.3 g (0.1 mole) of 1-dimethylamino-2-propanol and 8.1 g (0.04 mole) of phthalyl chloride in the same way as described for the preparation of I. Distillation *in vacuo* gave a colourless oil. Yield 7.0 g (52.5 %); b. p. 132—134°/0.1 mm. The dipicrate of II was prepared with a saturated solution of picric acid in absolute ethanol,

* Melting points and boiling points are uncorrected.

Table 1. Chemical data of the bis-quaternary methiodides and ethiodides with the general formula $o\text{-}C_6H_4(COOCHR'CH_2\overset{+}{N}(R'')_2R''')_2 2I^-$

No.	R′	R″	R‴	M. p. °C	Found % C[a]	Found % H[a]	Found % N[a]	Found % I	Calculated % C	Calculated % H	Calculated % N	Calculated % I
IV[b]	H	CH_3	CH_3	230[c]				41.5				42.8
V	H	CH_3	C_2H_5	217	38.9	5.65	4.55	40.6	38.7	5.53	4.52	40.9
VI	CH_3	CH_3	CH_3	249	39.2	5.58	4.53	39.7	38.7	5.53	4.52	40.9
VII	CH_3	CH_3	C_2H_5	222	40.5	5.89	4.47	38.8	40.7	5.90	4.32	39.1
VIII	CH_3	C_2H_5	C_2H_5	207	44.7	6.68	4.23	35.5	44.3	6.58	3.98	36.0
IX	CH_3	C_2H_5	CH_3	190	42.4	6.29	4.16	36.9	42.6	6.26	4.14	37.5

a The average of two analyses.
b 330 I.S. first prepared by Fusco.
c The same melting point is reported by Fusco.

and recrystallized from dry ethanol. M. p. 136—146°. (Found: N 13.92. Calc. for $C_{30}H_{30}O_{18}N_8$: N 14.10.)

α - Methyl - β - diethylaminoethyl phthalate (III). The ester III was prepared from 26.2 g (0.2 mole) of 1-diethylamino-2-propanol and 16.2 g (0.08 mole) of phthalyl chloride following the same procedure as for I. The ester seemed to be partly decomposed by distillation. It was a yellow oil. B.p. 145—147°/0.05 mm. The dipicrate was recrystallized from absolute ethanol. M. p. 148—149.5°. (Found: N 13.50 and 12.73. Calc. for $C_{34}H_{42}O_{18}N_8$: N 13.18.)

Bis-methiodides and bis-ethiodides of I, II and III. The bis-quaternary alkyliodides of I, II and III were prepared in an acetone solution. The alkyliodides formed were dissolved in hot methanol and ethyl acetate was added slowly until a precipitate appeared. The solution was then chilled overnight in a refrigerator. For the preparation of the compounds IV, V and VI a solution of the tertiary amine and excess of methyl iodide or ethyl iodide in acetone was left overnight at room temperature. Compound VII was formed when a solution of II in acetone was heated with ethyl iodide under reflux for 3 h. In preparing the ethiodide of III (VIII), the reaction mixture was left at room temperature for eight days, and the methiodide of III (IX) was prepared by keeping the reaction mixture at 37° for three days. All the quaternary compounds (IV—IX) prepared, decomposed on melting. They were analysed for iodine by potentiometric titration with silver nitrate.

Melting points and analytical data of the bis-quaternary compounds are listed in Table 1.

1. Craig, L. E. *Chem. Rev.* **42** (1948) 285.
2. Brücke, F. *Pharmacol. Rev.* **8** (1956) 265.
3. Fusco, R., Palazzo, G., Chiavarelli, S. and Bovet, D. *Gazz. chim. ital.* **78** (1948) 511.
4. Bovet-Nitti, F. *Rend. ist. super. sanita* **12** (1949) 138.
5. Barstad, J. A. B., Skramstad, K. H. and Öksne, S. To be published in *Arch. int. Pharmacodyn.*
6. Kyrides, L. P. *J. Am. Chem. Soc.* **50** (1937) 206.

Received December 13, 1958.

M. Delépine's Nitrido-Iridium(III,IV) Sulphate Complexes with Nitrogen Bound to Three Iridium Atoms

CHR. KLIXBÜLL JØRGENSEN

Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark

Delépine [1] prepared green salts from $(NH_4)_3IrCl_6$ boiled with concentrated sulphuric acid. These salts are aquo acids, forming red-brown hydroxo complexes in alkaline solution by a reversible reaction, consuming one OH^- per Ir. They are distinctly different from Lecoq de Boisbaudran's blue-green sulphates,[2] formed by boiling, *e.g.*, K_3IrCl_6 with H_2SO_4 without the presence of NH_4^+. The latter salts can have the composition $K_{10}Ir_3(SO_4)_9O,H_2O$ (private communication from Professor Delépine) and may have further H_2O of crystallization. Delépine [3] found later that the green salts, prepared in the presence of NH_4^+, contain one nitrogen atom for each three iridium atoms. Then, he proposed one of two formulae:

I. $K_4[N\{Ir(H_2O)(SO_4)_2\}_3]$
II. $K_4H_2[N\{Ir(H_2O)(SO_4)_2\}_3]$

I contains two tetravalent and one trivalent iridium atom, while II assumes that all three iridium atoms are trivalent. Since Pascal and Pacault found that the nitrogen salts are diamagnetic, while Lecoq de Boisbaudran's salts are paramagnetic and according to the analysis contains one Ir(IV) and two Ir(III), it was concluded that II was probably true. During the study of the absorption spectra of Delépine's many interesting complexes,[4] the present author was surprised by the strong colour of a pure Ir(III) salt as II, and further by the presence of two hydrogen ions even in the hydroxo salts $K_7H_2[N\{Ir(OH)(SO_4)_2\}_3]$. This note shall present evidence that I is correct. The magnetochemical criterion is not very certain; even compounds with an odd number of electrons may be diamagnetic in certain cases (*e.g.* NO at low temperatures) where the gyromagnetic factor g accidentally is zero. But the case of I with an even number of electrons has a great chance for

and recrystallized from dry ethanol. M. p. 136—146°. (Found: N 13.92. Calc. for $C_{30}H_{30}O_{18}N_8$: N 14.10.)

α - Methyl - β - diethylaminoethyl phthalate (III). The ester III was prepared from 26.2 g (0.2 mole) of 1-diethylamino-2-propanol and 16.2 g (0.08 mole) of phthalyl chloride following the same procedure as for I. The ester seemed to be partly decomposed by distillation. It was a yellow oil. B.p. 145—147°/0.05 mm. The dipicrate was recrystallized from absolute ethanol. M. p. 148—149.5°. (Found: N 13.50 and 12.73. Calc. for $C_{34}H_{42}O_{18}N_8$: N 13.18.)

Bis-methiodides and bis-ethiodides of I, II and III. The bis-quaternary alkyliodides of I, II and III were prepared in an acetone solution. The alkyliodides formed were dissolved in hot methanol and ethyl acetate was added slowly until a precipitate appeared. The solution was then chilled overnight in a refrigerator. For the preparation of the compounds IV, V and VI a solution of the tertiary amine and excess of methyl iodide or ethyl iodide in acetone was left overnight at room temperature. Compound VII was formed when a solution of II in acetone was heated with ethyl iodide under reflux for 3 h. In preparing the ethiodide of III (VIII), the reaction mixture was left at room temperature for eight days, and the methiodide of III (IX) was prepared by keeping the reaction mixture at 37° for three days. All the quaternary compounds (IV—IX) prepared, decomposed on melting. They were analysed for iodine by potentiometric titration with silver nitrate.

Melting points and analytical data of the bis-quaternary compounds are listed in Table 1.

1. Craig, L. E. *Chem. Rev.* **42** (1948) 285.
2. Brücke, F. *Pharmacol. Rev.* **8** (1956) 265.
3. Fusco, R., Palazzo, G., Chiavarelli, S. and Bovet, D. *Gazz. chim. ital.* **78** (1948) 511.
4. Bovet-Nitti, F. *Rend. ist. super. sanita* **12** (1949) 138.
5. Barstad, J. A. B., Skramstad, K. H. and Öksne, S. To be published in *Arch. int. Pharmacodyn.*
6. Kyrides, L. P. *J. Am. Chem. Soc.* **50** (1937) 206.

Received December 13, 1958.

M. Delépine's Nitrido-Iridium(III,IV) Sulphate Complexes with Nitrogen Bound to Three Iridium Atoms

CHR. KLIXBÜLL JØRGENSEN

Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark

Delépine [1] prepared green salts from $(NH_4)_3IrCl_6$ boiled with concentrated sulphuric acid. These salts are aquo acids, forming red-brown hydroxo complexes in alkaline solution by a reversible reaction, consuming one OH^- per Ir. They are distinctly different from Lecoq de Boisbaudran's blue-green sulphates,[2] formed by boiling, *e.g.*, K_3IrCl_6 with H_2SO_4 without the presence of NH_4^+. The latter salts can have the composition $K_{10}Ir_3(SO_4)_9O,H_2O$ (private communication from Professor Delépine) and may have further H_2O of crystallization. Delépine [3] found later that the green salts, prepared in the presence of NH_4^+, contain one nitrogen atom for each three iridium atoms. Then, he proposed one of two formulae:

I. $K_4[N\{Ir(H_2O)(SO_4)_2\}_3]$
II. $K_4H_2[N\{Ir(H_2O)(SO_4)_2\}_3]$

I contains two tetravalent and one trivalent iridium atom, while II assumes that all three iridium atoms are trivalent. Since Pascal and Pacault found that the nitrogen salts are diamagnetic, while Lecoq de Boisbaudran's salts are paramagnetic and according to the analysis contains one Ir(IV) and two Ir(III), it was concluded that II was probably true. During the study of the absorption spectra of Delépine's many interesting complexes,[4] the present author was surprised by the strong colour of a pure Ir(III) salt as II, and further by the presence of two hydrogen ions even in the hydroxo salts $K_7H_2[N\{Ir(OH)(SO_4)_2\}_3]$. This note shall present evidence that I is correct. The magnetochemical criterion is not very certain; even compounds with an odd number of electrons may be diamagnetic in certain cases (*e.g.* NO at low temperatures) where the gyromagnetic factor g accidentally is zero. But the case of I with an even number of electrons has a great chance for

being diamagnetic; the platinum group complexes with bridge atoms between the heavy atoms seem to utilize all possibilities of spin-pairing.[5]

The green salts do not react with most reducing agents. However, the present author found that vanadous ions (prepared from NH_4VO_3 and Zn in HCl) reduces the green solution to a straw-yellow colour, having only a weakly increasing absorption in the ultraviolet. Metallic zinc may act directly on the green solution in H_2SO_4. Uranium(III) in 2 M HCl reduces also quantitatively, and H_2O_2 restores at least 90 % of the original green colour, as measured on a recording Cary MS-11 spectrophotometer. The band maxima (shoulders in parenthesis) are at wavelengths in mμ with the extinction coefficients ε given per iridium atom present:

Green aquo form		Red-Brown hydroxo form		Blue-green Lecoq de Boisbaudran's salt	
(661)	ε = 450	653	ε = 470	(800)	ε = 350
582	530	562	910	701	770
439	1 260	452	1 500	306	1 250
(356)	465	335	710		
(311)	850	(298)	1 300		
(276)	1 220	269	1 850		

The special type of absorption bands in polynuclear complexes with the same metal in different oxidation states have often smaller wavenumbers than the electron transfer bands of mononuclear complexes.[4] The bands of the iridium complexes given above, are more narrow than the bands of iron(II,III) cyanides or molybdenum-(V,VI) blue. This may simply be connected with the fact that we have well-defined iridium compounds. The wavenumbers of the first bands are smaller [6] than of $IrCl_6^{--}$ and resemble those of $IrBr_6^{--}$. The absorption spectra are probably related to the formation of molecular orbitals of the nitrogen lone-pair (mainly a p_z-orbital) interacting with some of the iridium d-electrons, as will be discussed later.

The stability of the bonding of the nitrogen atom to the three iridium atoms, which even may survive the reduction to three Ir(III), is rather surprising. Werner prepared many cobalt (III) with NH- and NH_2-bridges, but the bonding of three metal atoms is rather exceptional. By X-ray crystallography, Scavnicar and Grdenic [7] found that $HgO,2HgCl_2$ contains oxygen atoms, surrounded in a plane by three rectilinear HgCl groups. The nitrogen-atom in Delépine's green salts may be nearly co-planar with the iridium atoms, forming a structure of the type of triphenylamine without tendency to co-ordinate a fourth group. That such »nitrido» complexes are formed in concentrated H_2SO_4, is rather remarkable. The oxygen atom (which also may be two OH^-) in Lecoq de Boisbaudran's salts may also co-ordinate three Ir. These salts are less stable; with alkalis or by standing with water, they precipitate violet, basic salts, and they are easily reduced by vanadium(II). On the other hand, the green nitride salt does not decompose to any great extent, if stored in aqueous solution at 25°C for 15 months.

Analysis. Since it is only possible to reduce the green nitrogen compound with ions, evolving hydrogen with water, it is not easy to make a direct titration, involving the reduction of iridium. However, the vanadium content [8] of the mixed $(NH_4)_2$ (V; Zn) $(H_2O)_6$ $(SO_4)_2$ was found by potentiometric titration with $KMnO_4$ in 1 M H_2SO_4 (showing distinct potential shifts at each of the oxidized forms (III), (IV), (V)) to have the effective molecular weight M = 2 090 (while a pure V (II) salt would have M = 387). To each 1 ml of a solution, 0.5 M in HCl and 0.0150 M in total Ir from the green salt, was added 10, 15, 20, 25, and 30 mg of the V (II), Zn (II) salt, which is remarkably stable in air [8]. The solutions were diluted to 10 ml with 0.5 M HCl, and the decrease of ε at several wavelengths was found to be a linear function of the reducing agent added, corresponding to complete reduction for 20.5 mg. This is 98 % of the value expected for a Ir (III, IV, IV) compound, proving the formula I discussed above.

Acknowledgements. I am very much indebted to Professor Marcel Delépine of College de France for many valuable informations and for the precious samples of iridium complexes.

1. Delépine, M. *Bull. soc. chim. France* [4] **5** (1909) 359, 1084, 1126.
2. Lecoq de Boisbaudran. *Compt. rend.* **96** (1883) 1406.
3. Delépine, M. in Pascal, P. *Traité de la Chimie Minérale*, Vol. 11, Paris 1932, p. 485.
4. Jørgensen, C. Klixbüll *Acta Chem. Scand.* **11** (1957) 151, 166.

5. Nyholm, R. S. *Quelques Problemes de Chimie Minérale. X Conseil de Chimie Solvay*, Bruxelles 1956, p. 225.
6. Jørgensen, C. Klixbüll *Acta Chem. Scand.* **10** (1956) 518.
7. Scavnicar, S. and Grdenic, D. *Acta Cryst.* **8** (1955) 275.
8. Jørgensen, C. Klixbüll *Acta Chem. Scand.* **12** (1958) 1537.

Received December 22, 1958.

Cuprous Chloride Catalysed Dimerisation of Methyl Propiolate

LARS SKATTEBÖL

SINTEF, Norges Tekniske Högskole, Trondheim, Norway

In an attempt to prepare methyl deca-2:4-diynoate by the so-called Glaser coupling [1] of methyl propiolate with *n*-hept-1-yne, Christensen and Sörensen [2] were able to obtain only tetradeca-6:8-diyne and two crystalline substances which analysed for $C_8H_8O_5$ and $C_8H_8O_6$, respectively. In order to determine the structures of these two anomalous products the Glaser coupling of methyl propiolate was investigated.

The reaction of methyl propiolate in slightly acidic aqueous cuprous chloride-ammonium chloride solution, with air as the oxygen source, gave a crystalline compound in 36 % yield. The substance

$$H_3COOC \cdot C \equiv C - H + HC \equiv C \cdot COOCH_3 \xrightarrow[NH_4Cl]{Cu_2Cl_2} H_3COOC \cdot C \equiv C \cdot CH = CH \cdot COOCH_3$$

(I)

was obtained as colourless thick needles from ether-petroleum ether, m.p. 57°. (Found: C 57.40; H 5.04. Calc. for $C_8H_8O_4$; C 57.14; H 4.80). U.V.-absorption, in ethanol: λ_{max} 2 660, 2 540, 2 440 (inflexion) Å. ε 12 900, 17 500, 16 300. The infrared spectra showed the presence of acetylenic- and ethylenic bonds in addition to bands characteristic of an ester. A strong band at 958 cm^{-1} indicated that the double bond had the *trans* configuration. [3] On the basis of this evidence we concluded that the substance was dimethyl hex-2-en-4-yne-1:6-dioate (I). This compound has been reported in the literature. [4] There are, however, small discrepancies in the melting points and ultraviolet spectra of the two compounds, but these may be due to a difference in the configuration about the double bond.

The formation of the compound can be explained by addition of one molecule of methyl propiolate to another, thus being an example of a substituted acetylene undergoing the Nieuwland dimerisation. [5] Accordingly oxygen should not play any part in the reaction. This was confirmed by running the reaction under pure nitrogen, when practically the same yield of (I) was obtained.

Just after the completion of this work, Akhtar and Weedon [6] published a report on the reaction of substituted copper acetylides with oxygen in hot acetic acid. They obtained the corresponding vinyl acetylenic compounds in good yields.

Further work on this reaction is in progress.

Acknowledgement. I am indebted to Professor N. A. Sörensen for his interest in this work.

1. Glaser, C. *Ann.* **154** (1870) 159; *Ber.* **2** (1869) 422. See also Raphael, R. A. *Acetylenic Compounds in Organic Synthesis*, London 1955, p. 127.
2. Christensen, P. Koch and Sörensen, N. A. *Acta Chem. Scand.* **6** (1952) 893.
3. Allan, J. L. H., Meakins. G. D. and Whiting, M. C. *J. Chem. Soc.* **1955** 1874.
4. Heilbron, I. M., Jones, E. R. H. and Sondheimer, F. *J. Chem. Soc.* **1949** 604.
5. Nieuwland, J. A. and Vogt, R. R. *The Chemistry of Acetylene*. New York 1945, p. 160.
6. Akhtar, M. and Weedon, B. C. L. *Proc. Chem. Soc.* **1958** 303.

Received December 18, 1958.

5. Nyholm, R. S. *Quelques Problemes de Chimie Minérale. X Conseil de Chimie Solvay*, Bruxelles 1956, p. 225.
6. Jørgensen, C. Klixbüll *Acta Chem. Scand.* **10** (1956) 518.
7. Scavnicar, S. and Grdenic, D. *Acta Cryst.* **8** (1955) 275.
8. Jørgensen, C. Klixbüll *Acta Chem. Scand.* **12** (1958) 1537.

Received December 22, 1958.

Cuprous Chloride Catalysed Dimerisation of Methyl Propiolate

LARS SKATTEBÖL

SINTEF, Norges Tekniske Högskole, Trondheim, Norway

In an attempt to prepare methyl deca-2:4-diynoate by the so-called Glaser coupling [1] of methyl propiolate with *n*-hept-1-yne, Christensen and Sörensen [2] were able to obtain only tetradeca-6:8-diyne and two crystalline substances which analysed for $C_8H_8O_5$ and $C_8H_8O_6$, respectively. In order to determine the structures of these two anomalous products the Glaser coupling of methyl propiolate was investigated.

The reaction of methyl propiolate in slightly acidic aqueous cuprous chloride-ammonium chloride solution, with air as the oxygen source, gave a crystalline compound in 36 % yield. The substance

$$H_3COOC \cdot C \equiv C - H + HC \equiv C \cdot COOCH_3 \xrightarrow[NH_4Cl]{Cu_2Cl_2} H_3COOC \cdot C \equiv C \cdot CH = CH \cdot COOCH_3$$

(I)

was obtained as colourless thick needles from ether-petroleum ether, m.p. 57°. (Found: C 57.40; H 5.04. Calc. for $C_8H_8O_4$; C 57.14; H 4.80). U.V.-absorption, in ethanol: λ_{max} 2 660, 2 540, 2 440 (inflexion) Å. ε 12 900, 17 500, 16 300. The infrared spectra showed the presence of acetylenic- and ethylenic bonds in addition to bands characteristic of an ester. A strong band at 958 cm^{-1} indicated that the double bond had the *trans* configuration. [3] On the basis of this evidence we concluded that the substance was dimethyl hex-2-en-4-yne-1:6-dioate (I). This compound has been reported in the literature. [4] There are, however, small discrepancies in the melting points and ultraviolet spectra of the two compounds, but these may be due to a difference in the configuration about the double bond.

The formation of the compound can be explained by addition of one molecule of methyl propiolate to another, thus being an example of a substituted acetylene undergoing the Nieuwland dimerisation. [5] Accordingly oxygen should not play any part in the reaction. This was confirmed by running the reaction under pure nitrogen, when practically the same yield of (I) was obtained.

Just after the completion of this work, Akhtar and Weedon [6] published a report on the reaction of substituted copper acetylides with oxygen in hot acetic acid. They obtained the corresponding vinyl acetylenic compounds in good yields.

Further work on this reaction is in progress.

Acknowledgement. I am indebted to Professor N. A. Sörensen for his interest in this work.

1. Glaser, C. *Ann.* **154** (1870) 159; *Ber.* **2** (1869) 422. See also Raphael, R. A. *Acetylenic Compounds in Organic Synthesis*, London 1955, p. 127.
2. Christensen, P. Koch and Sörensen, N. A. *Acta Chem. Scand.* **6** (1952) 893.
3. Allan, J. L. H., Meakins. G. D. and Whiting, M. C. *J. Chem. Soc.* **1955** 1874.
4. Heilbron, I. M., Jones, E. R. H. and Sondheimer, F. *J. Chem. Soc.* **1949** 604.
5. Nieuwland, J. A. and Vogt, R. R. *The Chemistry of Acetylene*. New York 1945, p. 160.
6. Akhtar, M. and Weedon, B. C. L. *Proc. Chem. Soc.* **1958** 303.

Received December 18, 1958.

Scavenging of Free Radicals and Radiation Protection by Nitric Oxide in Plant Seeds

B. SPARRMAN, L. EHRENBERG and A. EHRENBERG

Institute for Organic Chemistry and Biochemistry, University; and Biochemical Department, Nobel Medical Institute, Stockholm, Sweden

Under anoxic conditions nitric oxide (NO) is noteworthily untoxic to living tissue. In an exploratory study (unpublished) a treatment of resting barley seeds with NO at atmospheric pressure for several days was found neither to induce growth inhibition nor to raise the mutation rate significantly. Being a simple, fairly stable and easily diffusing free radical, *i. e.*, with possible ability to combine quantatively with free radicals in the tissue, NO is of potential value in radiobiological experiments. NO reacts in a 1:1 ratio with reactive free radicals [1]. This fact makes its modifying influence on radiation sensitivity more suited to interpretation on a quantitative basis than the corresponding [2-5] effects of the diradical oxygen, which might start chain reactions [1] and, in addition, affects the radiation sensitivity *via* its metabolic action [6].

In the present investigation the reactions of NO with free radicals was used to shed light on the mechanism by which water exerts an action on the radiation sensitivity of plant seeds. This effect consists of a decrease of the radiation sensitivity with increasing water content with a minimum at some value between 12 and 20 % water, dependent on the system studied (*cf.* Ehrenberg and Ehrenberg [7] and quoted literature).

In the latter publication measurements of electron spin resonance (esr) demonstrated that the same amount of free radicals is produced initially, irrespective of the water content, when seeds of *Agrostis stolonifera* are exposed to X-rays. In very dry seeds the radicals remain unchanged but, as the water content increases, an increasing proportion of the radicals decays in a second order reaction. The fact that the growth inhibition induced at the different water contents is found to be proportional to the (semiconstant) level of radical concentration after the decay, indicates that the protective action of water is due to an increasing recombination of free radicals.

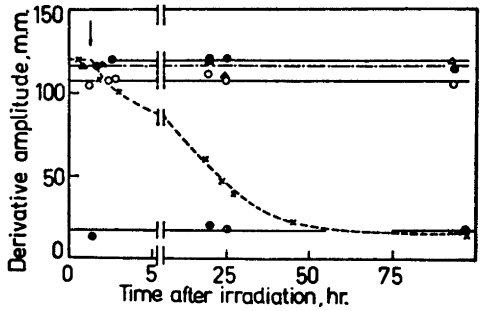


Fig. 1. Time dependence of derivative amplitude of esr absorption in dry seeds (4.5 % H_2O) of *Agrostis stolonifera*, X-irradiated and stored in different atmospheres. ◑ unirradiated seeds stored in N_2; ● irradiated in air, stored in air; ○ irradiated in N_2, stored in air; △ irradiated in N_2, stored in N_2; × irradiated in N_2, stored in NO. The arrow indicates the moment when the gas was changed over the three latter samples.

In the present studies we determined the rate of entrance into the seeds of NO and/or its rate of reaction with radiation produced paramagnetic centers. Dry seeds (4.5 % H_2O) were irradiated with 150 000 r of X-rays (185 kV, 2 mm Al filtration, 800 r min^{-1}) in N_2. After determination of the esr absorption the N_2 filling of the glass tube was replaced by NO, and the decay of the signal was followed for a few days. Total decay down to the control value [8] required about 48 h at 20°C (Fig. 1), at higher water contents the reaction was more rapid. The fact that the signal height was equal to the (stable) N_2 value when N_2 was replaced by air after irradiation or when irradiation was performed in the presence of air, indicates that free oxygen is not present in the tissue [7] or that the radicals are changed into an equal amount of peroxy radicals.

In a second experiment seeds were irradiated at different water contents, and the influence of the gas (N_2, NO, or air) present during irradiation was studied by determination of the dose causing 50 % reduction of the seedling growth (measurement at 15 mm control height). In order to secure penetration of NO into the tissue

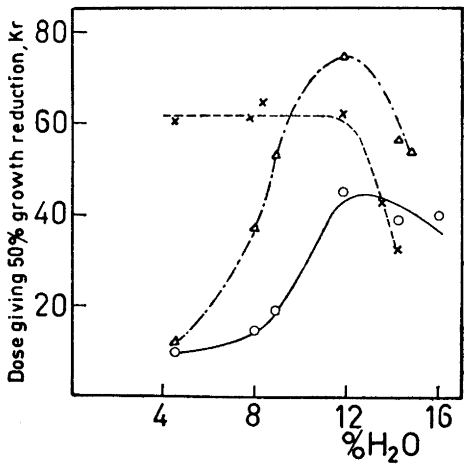


Fig. 2. The dose of X-rays required for 50 % growth inhibition of seeds of *Agrostis stolonifera* as a function of their water content. The seeds were irradiated and stored (see text) in air O, in N_2 △, and in NO ×.

and a quantitative reaction with the radicals, the seeds were kept in the different gases for 48 h, and irradiation was performed in the middle of that period.

In Fig. 2 the radiation sensitivities are shown. In N_2 and air a clear minimum sensitivity (*i. e.*, maximum of 50 % dose) is registered at 12 % H_2O. In the presence of NO, however, about maximal protection was found at all water contents below 12 %, and under these conditions esr signals close to the control values were invariously found.

This influence of the radical scavenger, NO, connects directly the radiation produced free radicals with a large part of the biological effect. Within this range of water content the destruction of radicals leads to a protection, either this occurs by a recombination (which is facilitated by the presence of hydration water) or by reaction with a scavenger. The sensitivity increasing effect of oxygen does not necessarily depend on immediate reactions with radicals, since the air was not removed by evacuation before sowing. Thus the seeds irradiated in air had a higher oxygen content than the N_2 seeds, at the start of germination. Under similar conditions after-effects of O_2 have been observed [9].

At water contents above the minimum, where the radiosensitivity is again higher, NO increases the radiosensitivity in a manner similar to that of O_2, and in agreement with results of other investigators [2-5]. When the H_2O content exceeds 12 %, more loosely bound H_2O becomes present, permitting new types of reactions. Below 12 % H_2O, the reaction constant for radical recombination is independent of the water content [7], indicating that H_2O is exclusively present as a single, more or less complete layer of hydration water. Above 12 % H_2O, the reaction constant increases with increasing water content.

These preliminary investigations are being continued on a quantitative basis.

The investigation was aided by grants from the *Swedish Agricultural Research Council*, the *Swedish Natural Science Research Council*, and the *Magnus Bergvall Foundation*.

1. Ziegler, K., Orth, Ph. and Weber, K. *Ann.* **504** (1933) 131; Ziegler, K. and Ewald, L. *Ann.* **504** (1933) 162; Ziegler, K., Ewald, L. and Seib, A. *Ann.* **504** (1933) 182.
2. Howard-Flanders, P. *Nature* **180** (1957) 1191.
3. Howard-Flanders, P. *Radiation Research* **9** (1958) 131.
4. Gray, L. H., Green, F. O. and Hawes, C. A. *Nature* **182** (1958) 952.
5. Kihlman, B. A. *Exptl. Cell Research* **14** (1958) 639.
6. Ehrenberg, L. and Nybom, N. *Acta Agr. Scand.* **4** (1954) 396.
7. Ehrenberg, A. and Ehrenberg, L. *Arkiv Fysik* **14** (1958) 133.
8. Zimmer, K. G., Ehrenberg, L. and Ehrenberg, A. *Strahlenther.* **103** (1957) 3.
9. Caldecott, R. S., Johnson, E. B., North, D. T. and Konzak, C. F. *Proc. Natl. Acad. Sci.* **43** (1957) 925.

Received January 17, 1958.

Structure of the Hexathionate Ion in the *trans*-Dichloro-dien-cobalt(III) Salt

OLAV FOSS and KJARTAN MARØY

Chemical Institute, University of Bergen, Bergen, Norway

In the potassium barium salt, $K_2Ba(S_6O_6)_2$, the sulphur chain of the hexathionate ion has a *cis-cis* configuration as in the S_8 ring of orthorhombic sulphur.[1] It has now been found that the extended *trans-trans* rotational isomer occurs in the crystals of *trans*-dichloro-dien-cobalt(III) hexathionate monohydrate.

This salt, $(Coen_2Cl_2)_2S_6O_6.H_2O$, is orthorhombic, with the unit cell dimensions:[2] a = 12.12 Å, b = 19.13 Å, c = 6.43 Å, and two formula units per unit cell. The space group is C_{2v}^8 — $Pba2$, which requires that the hexathionate ions and the water molecules lie on twofold axes of symmetry.

The intensities of 149 observed $hk0$ reflections out of 187 attainable with Fe$K\alpha$ radiation were estimated visually from zero-layer Weissenberg photographs, taken of a prism {110} with edges 0.08 × 0.09 mm (μr approx. 0.7). The cobalt x and y coordinates were found from the $hk0$ Patterson map, the Co-Co $2x,2y$ vector being also the centre vector of the centro-symmetric cation and the peak therefore large. Using cobalt as a searcher atom, the two chlorine atoms were located by means of the vector convergence method.[3] The cobalt and chlorine contributions gave the probable signs of one fourth (37) of the strongest $hk0$ reflections, and a Fourier synthesis based on these indicated the positions of the three sulphur atoms of the asymmetric unit and thereby the signs of 40 additional reflections; none of the signs changed later. The second Fourier map allowed the location of the lighter atoms, and Fourier refinement and next Fourier difference refinement were then carried out.

The $hk0$ Fourier map is shown in Fig. 1, and the x and y coordinates of the heavier atoms are listed in Table 1, together with the temperature constants B used in the calculation of structure factors. The B values, assigned on the basis of the differ-

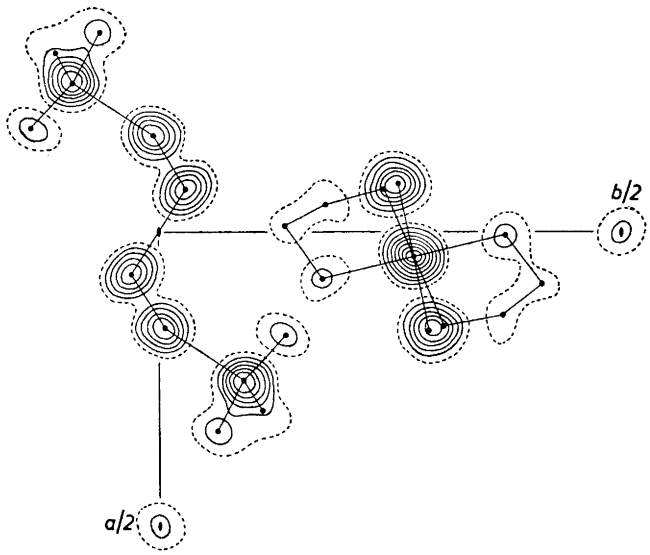


Fig. 1. Electron density projection of *trans*-dichloro-dien-cobalt(III) hexathionate monohydrate along the c axis. Contours are at intervals of 4 e · Å^{-2} for the cobalt and chlorine atoms and 3 e · Å^{-2} for the other atoms, beginning with 4 e · Å^{-2} (dashed).

Table 1. Atomic coordinates, in fractions of cell edges, and temperature constants B, in Å^2. Origin on a twofold axis.

	x	y	B
Co	0.0410	0.2757	
Cl_1	0.1660	0.2930	3.5
Cl_2	—0.0840	0.2584	
S_1	0.2530	0.0917	3.0
S_2	0.1638	0.0050	3.5
S_3	0.0722	—0.0289	4.0

ence maps, indicate an increased thermal vibration of atoms at the middle of the sulphur chain relative to the ends. Ignoring contributions from the 17 hydrogen atoms of the asymmetric unit, the reliability index R is 0.10 for the $hk0$ data.

The z coordinates of the atoms will be determined later from a projection along the a axis. The middle sulphur-sulphur bond of the hexathionate ion lies across the twofold axis and the length is independent of the sulphur z coordinate; the x and y coordinates of S_3 give a length of 2.07 ± 0.02 Å for this bond.

1. Foss, O., Hordvik, A. and Palmork, K. H. *Acta Chem. Scand.* **12** (1958) 1339.
2. Foss, O. and Palmork, K. H. *Acta Chem. Scand.* **12** (1958) 1337.
3. Beevers, C. A. and Robertson, J. H. *Acta Cryst.* **3** (1950) 164.

Received December 29, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 203—210

Studies on Succinic Acids

II.* Equilibrium Constants for the Interconversion of *Meso*- and *Racem*-α,α'-dialkylsuccinic Acids

LENNART EBERSON

Department of Organic Chemistry, Chemical Institute, University of Lund, Sweden

This paper deals with the determination of the equilibrium constants of the reaction *meso* acid $\rightleftharpoons$ *racemic* acid for α,α'-dimethyl-, α,α'-diethyl-, and α,α'-di*iso*propylsuccinic acid at 170° in the solvents dioxane, water, and 5 N aqueous hydrochloric acid. In certain cases the interpretation of the results is complicated by the occurrence of simultaneous anhydrization, but it is clearly established that the *racemic* forms are relatively more stable, contrary to the findings of several other investigators. The stability order found can be explained by assuming the existence of an intramolecular hydrogen bond which selectively stabilizes one of the *racemic* acid conformations. A similar stabilizing effect is not probable for any of the *meso* acid conformations.

It has been known for a long time that diastereoisomers of α,α'-disubstituted succinic acids can be converted into each other under certain conditions, *e. g.* by heating with hydrochloric acid at elevated temperatures. Wagner-Jauregg [1] has cited a number of cases of such interconversions, which all point to the *meso* forms being the most stable ones. Linstead and Whalley [2] have stated without experimental proof that the *meso* form of α,α'-dimethylsuccinic acid should be the more stable isomer. Later on, Golden and Linstead [3] have semiquantitatively investigated the equilibration of *mesoid* and *racemoid* α-methyl-α'-ethylsuccinic acid under varying conditions. In two cases the equilibrium mixture contained more than 50 % of the *racemoid* form, *i. e.*, the stability order was in line with that of the corresponding anhydrides and contrary to the cases cited above. Barton and Cookson [4] used the data of Wagner-Jauregg as evidence for the stability order of *erythro* and *threo* isomers predicted from the conformational point of view. According to this reasoning the *erythro (meso)* form should be the more stable one.

* Part I. *Acta Chem. Scand.* 13 (1959) 40.

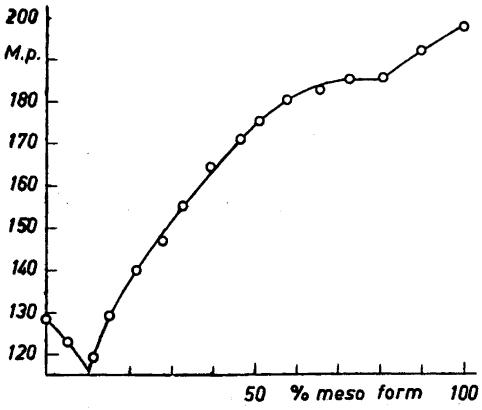


Fig. 1. Melting point diagram for *meso*- and *racem*-α,α′-dimethylsuccinic acid.

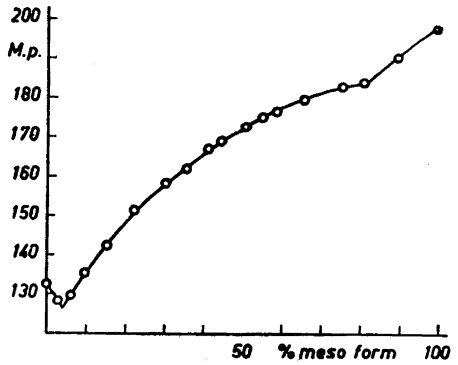


Fig. 2. Melting point diagram for *meso*- and *racem*-α,α′-diethylsuccinic acid.

However, a critical examination of the original literature [5–13] on equilibrations of α,α′-dialkylsuccinic acids revealed that the table set up by Wagner-Jauregg was based on an unreliable experimental material of great inhomogeneity. Thus, in most cases it has not been established whether the inversions are reversible or not, the analysis of the equilibrated samples has been made by

Table 1. Equilibrium constants for the reaction *meso*

Succinic acid	Melting point of equilibrium mixture, mean value and maximum deviation					
	In dioxane		In water		In 5 N aq. HCl	
Racem-α,α′-dimethyl-	156.0 157.0	157.4 ± 1.4	171.0 172.8	172.9 ± 1.9	169.6 170.6	169.9 ± 1.3
Meso-α,α′-dimethyl-	157.8 158.8		173.2 174.6		168.6 170.6	
Racem-α,α′-diethyl-	143.4 144.0	143.0 ± 1.0	166.6 167.0	166.7 ± 1.3	159.0 161.4	161.2 ± 2.2
Meso-α,α′-diethyl-	142.4 142.0		165.4 167.6		161.2 163.0	
Racem-α,α′-di*iso*propyl-	Difficult to measure		181.0 183.0	182.0 ± 1.0	Difficult to measure	
Meso-α,α′-di*iso*propyl-			181.0 183.0			
Racem-α,α′-di*iso*propyl- [a]						
Meso-α,α′-di*iso*propyl- [a]						

[a] Measured by potentiometric titration.

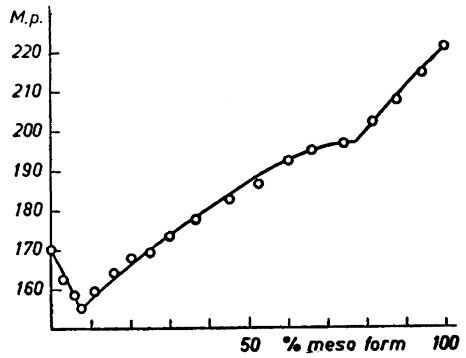


Fig. 3. Melting point diagram for *meso*- and *racem*-α,α'-di*iso*propylsuccinic acid.

preparative methods, which only qualitatively give the direction of the changes, the temperatures and times used for equilibration have been extraordinarily varying, and the inversions have been carried out with strong hydrochloric acid as solvent, which might influence the equilibrium state. Besides, in several cases the equilibria actually were at about 50 % inversion [7,8] and in one case (α,α'-di*iso*propylsuccinic acid) in favour of the *racemic* acid [11].

This paper, which is part of an investigation on the stereochemistry of alkylsubstituted succinic acids, describes an attempt to measure the equi-

acid $\rightleftharpoons$ *racemic* acid at 170° in various solvents.

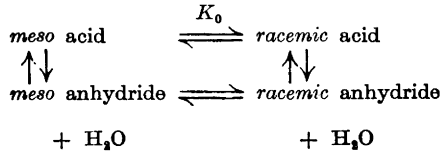
% *meso* acid in the equilibrium mixture			Equilibrium constant K_0		
In dioxane	In water	In 5 N aq. HCl	In dioxane	In water	In 5 N aq. HCl
34.3 ± 1.2	48.2 ± 2.0	45.2 ± 1.2	1.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
15.0 ± 1.0	41.5 ± 2.0	34.5 ± 3.0	(5.7 ± 0.5)	1.4 ± 0.1	1.9 ± 0.3
	42.2 ± 2.0			1.4 ± 0.1	
25 ± 5	43 ± 2	22 ± 2	(3.0 ± 1.0)	1.3 ± 0.1	(3.5 ± 0.5)

librium constants for the interconversion of the *meso* and *racemic* forms of α,α′-dimethyl-, α,α′-diethyl-, and α,α′-di*iso*propylsuccinic acids at 170° in the solvents dioxane, water, and 5 N hydrochloric acid. The composition of the equilibrium mixtures was determined by thermal analysis and/or by potentiometric titration in the case of α,α′-di*iso*propylsuccinic acid. The shape of the melting point curves and the states of the equilibria are such as to permit the evaluation of the constants in an unambiguous manner. The melting point diagrams are shown in Figs 1, 2, and 3, and the results of the inversion experiments in Table 1.

These data clearly indicate that the inversions really are reversible and that the equilibrium mixtures in all cases contain 50 % or more of the *racemic* acid. Furthermore, there is a trend to an increase of the constant when the steric requirements of the substituents are enhanced. Unfortunately, the two forms of α,α′-di-(*tert.*-butyl)-succinic acid do not equilibrate at 170°, but experiments carried out at 220—230° showed that both isomers are converted into the *racemic* anhydride with no observable contamination of *meso* acid. The values for α,α′-di*iso*propylsuccinic acid are probably somewhat too small as the analytical method necessitated treatment of the equilibrated samples with aqueous base and subsequent acidification with dilute hydrochloric acid, filtration, and washing with water. This procedure should be expected to influence the final result in direction towards lower constants, as the *meso* acid is less soluble in water than the *racemic* acid.

DISCUSSION

The reason for putting some values in Table 1 in parentheses is the complication introduced in these cases by the formation of anhydride during equilibration, which makes the significance of the corresponding equilibrium constants somewhat questionable. The procedure has been to convert the mixture of acid and anhydride into acids by treatment with water or aqueous base and then analyzing the product of hydrolysis as outlined above. The measured constant will then refer to the system *meso* material ⇌ *racemic* material. This behaviour immediately actualizes the question: Could not the stability order found be explained in all cases, even when anhydride formation is not observed, by assuming the existence of the equilibrium in aqueous or dioxane



solution at 170°? In that case the driving force would be the formation of the *racemic* anhydride, which is the stable form of the anhydrides. When the equilibrium has been frozen in and the system returned to room temperature, the anhydrides would have the possibility of passing into the acids again during the analytical work, except when their rate of hydrolysis is low. There is

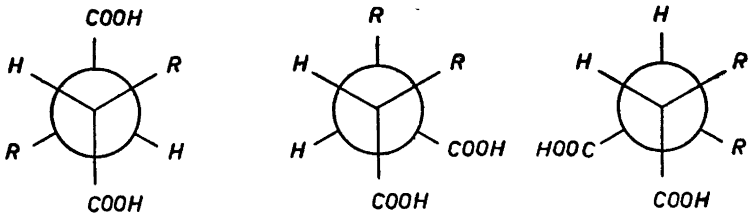
evidence for the existence of an equilibrium between acid and anhydride in aqueous solution at elevated temperatures in a number of cases [14]. For example, steam-distillation of an aqueous solution of tetramethylsuccinic acid gives tetramethylsuccinic anhydride, which is then slowly hydrolyzed by cold water again.

It would hardly be possible to develop a method for the unambiguous determination of an eventual equilibrium between acid and anhydride at 170° which does not interfere with the reaction. However, the fact that equilibration of the α,α'-dimethylsuccinic acids in absolute dioxane yields acid mixtures with no contamination of anhydride makes it probable that the equilibrium *meso* acid $\underset{\rightleftharpoons}{\overset{K_0}{}}$ *racemic* acid is not influenced to any appreciable extent by anhydrization in those cases where it has not been observed.

Koskikallio [15] has measured the rate of hydrolysis of acetic anhydride in dioxane containing 0.07 % of water and found a second order rate constant of about 10^{-5} l · mole^{-1} · sec^{-1} at 100° and 10^{-7} l · mole^{-1} · sec^{-1} at 40°. Comparison of the rate constants for the hydrolysis of acetic anhydride in various solvent mixtures with those of succinic anhydride under similar conditions indicates that they always are of the same magnitude [15]. The rate constants of the hydrolysis of the two α,α'-dimethylsuccinic anhydrides in water at 25° in turn are of roughly the same order as those of acetic and succinic anhydride [14,16]. If now the equilibration of α,α'-dimethylsuccinic acids in dioxane would lead to partial anhydride formation, the anhydride portion would not have time to be hydrolyzed during the working-up period (about 4 h), provided the above reasoning holds. In fact, even if an average rate constant of 10^{-3} l · mole^{-1}·sec^{-1} for hydrolysis during this period is allowed, the half-life time would still be about 4 h. Thus it can be concluded with tolerable certainty that those equilibrium constants which have not been put in parentheses actually refer to the reaction *meso* acid $\underset{\rightleftharpoons}{\overset{K_0}{}}$ *racemic* acid, and that the others must be interpreted with regard to the complication discussed above.

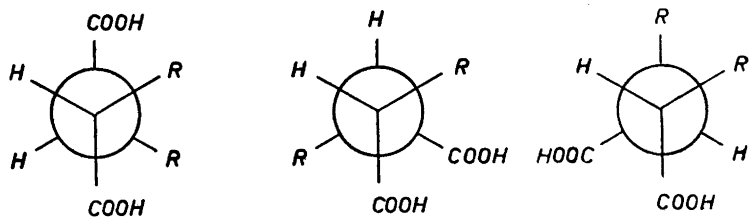
As already mentioned the results of the earlier investigations in this field have been explained on the basis of conformational analytical considerations [2,4]. Accordingly, the *meso* acid would be the more stable isomer, as in its most favoured conformation (I a) all substituents are situated in *trans* staggered positions, thereby allowing a minimum of non-bonded interactions.

The conformations of the *racemic* acids (II, a—c) are all energetically less favoured than I a, provided these interactions are *repulsive*, *i. e.*, it must be assumed that the carboxyl groups behave like alkyl groups in this connection. This reasoning cannot be true, as the relative stabilities of the acids reported in this paper are the same as those of their anhydrides. The departure from the expected behaviour must be due to *attractive* forces between the carboxyl groups, and it is plausible to assume that intramolecular hydrogen bonding is responsible for this effect. The *racemic* acid conformation II b would then be the most favourable arrangement, as it contains a ring system with two *trans*-situated alkyl substituents. The concept of a seven-membered hydrogen-bonded ring system existing in aqueous solution is perhaps not quite acceptable on these grounds alone, and the topic will be discussed more in detail in



I a *I b* *I c*

The conformations of the *meso* acid.



II a *II b* *II c*

The conformations of the *racemic* acid.

papers III[17] and IV[18] of this series, where dissociation constants and infra-red data will be presented. In fact, the infra-red spectra give further evidence for the existence of an intramolecular hydrogen bond in solutions of peralkyl-substituted succinic acids.

EXPERIMENTAL

Materials. Commercial dioxane of *purissimum* quality was purified according to Vogel [19] but the treatment with sodium was repeated once more. The α,α'-dialkylsuccinic acids were prepared and purified as described in paper I [20].

Determination of the melting point diagrams. It has been pointed out earlier that the melting points of alkyl-substituted succinic acids are rather sensitive to the method of determination and the heating conditions owing to the decomposition into anhydride and water near the melting point [20]. However, if the melting point was determined in a sealed capillary, it was found to be satisfactorily reproducible. Naturally the true melting point is not measured by this procedure but instead a mixed melting point for a complex system with the components acid, anhydride, and water. The good reproducibility points to an equilibrium being established, but this problem requires further investigation before it can be fully discussed. For analytical purposes the method is satisfactory provided all determinations are made as identical as possible. Each point of the diagrams was obtained in the following way:

The mixture of the *meso* and *racemic* acid (total 20 mg) was accurately weighed into a small weighing-glass, and one ml of analytical grade acetone was added. If the acids did not dissolve immediately, the mixture was warmed for a few seconds, until complete solution was achieved. The acetone was evaporated in a slow stream of air and the residue finally dried over concentrated sulphuric acid *in vacuo* for 10—12 h. The crystal mass was finely ground and introduced into melting point capillaries (two determinations

were made for each mixture) which were sealed in a micro-burner. The whole capillary must be immersed into the heating-block in order to avoid distillation or sublimation during the heating period. The capillary was introduced into the melting point apparatus at a temperature about 10° below the melting point, which was first roughly estimated in the Kofler apparatus. The heating rate was 1°/min. The melting point was taken as the temperature at which the last crystals disappeared, and this value was generally reproducible within ± 1°.

Equilibration of meso- *and* racem-*α,α'-dialkylsuccinic acids.* Samples of *meso* and *racemic* acid, 20 mg of each, were introduced into glass ampoules made from 10 mm Pyrex tubing and with a volume of about 3 ml. The solvent (dioxane, water, or 5 N hydrochloric acid), 2.0 ml, was added and the ampoules were sealed in a burner. The thermostat consisted of a large brass block which was heated electrically by means of two heating spirals of Kanthal wire, and was fitted with a contact thermometer (graded in 0.1° in the range 150−200°) and a calibrated thermometer (gradation as above). The block was very carefully isolated with diabas wool and placed in a sheet container. Preliminary experiments had indicated that equilibration occurred with reasonable speed at 170°. With the described apparatus this temperature was maintained within 0.1°, which was quite sufficient for the purpose of this study. The sealed ampoules were placed in a small steel-bomb, which fitted snugly into a hole in the brass block. After some experiments the times for reaching equilibrium were established at 120 h with dioxane as solvent, and 48 h with water or 5 N hydrochloric acid. After the heating period the bomb was removed and rapidly cooled in ice-water (this procedure lasts about 1 min), the ampoules were opened and the contents poured into small beakers. The ampoules were rinsed with a few ml of dioxane. The solvent was then evaporated *in vacuo* over concentrated sulphuric acid and sodium hydroxide pellets for 4 h in the case of dioxane and 12 h in the case of water and 5 N hydrochloric acid. The procedure then differed somewhat for different acids.

For α,α'-dimethylsuccinic acid the residue in all cases consisted of a mixture of the acids with no contamination of anhydride, which is shown by the fact that another treatment with water and subsequent evaporation did not change the melting point.

For α,α'-diethylsuccinic acid the residue from equilibration in dioxane was a mixture of water and anhydride, which first had to be converted into acids by treatment with water and subsequent evaporation. From the equilibrations in water and 5 N hydrochloric acid mixtures of the acids were directly obtained. Consequently, the melting points of these mixtures, determined as described above in connection with the melting point diagrams directly give the composition of equilibrium for α,α'-dimethylsuccinic and α,α'-diethylsuccinic acid.

For α,α'-di*iso*propylsuccinic acid the residue was in two cases a mixture of acid and anhydride. As the anhydride portion could not be hydrolyzed by water alone a small amount of aqueous potassium hydroxide was added. When the anhydride had dissolved, the acid mixture was precipitated by the addition of a few drops of dilute hydrochloric acid, the crystals were filtered, washed with some water and finally dried *in vacuo*. However, the melting points of these mixtures were too low to be compatible with the melting point diagrams, which was probably due to the presence of minute amounts of anhydride. Fortunately, the dissociation constants of *meso*- and *racem*-α,α'-di*iso*propylsuccinic acid in 50 % ethanol (see paper III[17]) are of an order to permit analysis by means of potentiometric titration. Experiments with mixtures of known composition gave results with an accuracy of about ± 2 %. As is seen from Table 1, the two methods of analysis give agreeing results in the case where both have been applied.

The author wishes to express his sincere gratitude to Professor Erik Larsson for his kind interest in this work. A grant from *Kungliga Fysiografiska Sällskapet* is gratefully acknowledged.

[REFERENCES

1. Freudenberg, K. *Stereochemie* p. 873, F. Deuticke, Leipzig and Wien 1933.
2. Linstead, R. P. and Whalley, M. *J. Chem. Soc.* **1954** 3722.
3. Golden, J. H. and Linstead, R. P. *J. Chem. Soc.* **1958** 1732.
4. Barton, D. H. R. and Cookson, R. C. *Quart. Revs. (London)* **10** (1956) **44.**

5. Bone, W. A. and Perkin, W. H. *J. Chem. Soc.* **69** (1896) 253.
6. Auwers, K. and Fritzweiler, R. *Ann.* **298** (1897) 154.
7. Bischoff, C. A. *Ber.* **21** (1888) 2102.
8. Bentley, W. H., Perkin, W. H. and Thorpe, J. F. *J. Chem. Soc.* **69** (1896) 270.
9. Küster, W. and Haas, K. *Ann.* **346** (1906) 23.
10. Bone, W. A. and Sprankling, C. H. G. *J. Chem. Soc.* **77** (1900) 1299.
11. Bone, W. A. and Sprankling, C. H. G. *J. Chem. Soc.* **77** (1900) 654.
12. Auwers, K. and Schlosser, F. *Ann.* **292** (1896) 162.
13. Stobbe, H. and v. Vigier, V. *Ber.* **37** (1904) 2662.
14. Verkade, P. E. *Rec. trav. chim.* **40** (1921) 199.
15. Koskikallio, J. *Ann. Acad. Sci. Fennicae Ser.* **A II** No 57 (1954).
16. Böeseken, M. M. J., Schweizer, A., and van der Want, G. F. *Rec. trav. chim.* **31** (1912) 86.
17. Eberson, L. *Acta Chem. Scand.* **13** (1959) 211.
18. Eberson, L. *Acta Chem. Scand.* **13** (1959) 224.
19. Vogel, A. I. *A Textbook of Practical Organic Chemistry*, Longmans, Green and Co London, New York, and Toronto 1951, p. 175.
20. Eberson, L. *Acta Chem. Scand.* **13** (1959) 40.

Received December 24, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 211—223

Studies on Succinic Acids

III.* Application of the Kirkwood-Westheimer Theory to the Anomalously High K_1/K_2-Ratios Exhibited by Certain Alkylated Succinic Acids

LENNART EBERSON

Department of Organic Chemistry, Chemical Institute, University of Lund, Sweden

The first and second dissociation constants of a number of 1,2-dicarboxylic acids have been measured in 50 % ethanol. It was found that *racem*-α,α'-dialkylsuccinic acids, containing alkyl groups with large steric requirements, and tetraalkylsuccinic acids have K_1/K_2-ratios of very great magnitudes, the ratio for *racem*-α,α'-di-(*tert.*-butyl)-succinic acid being the highest ever observed. By application of the Kirkwood-Westheimer theory to these molecules it is found that electrostatic effects alone cannot be responsible for this anomaly, but together with the assumption of an intramolecularly hydrogen-bonded monoanion the theory is in satisfactory agreement with experimental data.

The ratio between the first and second dissociation constants of a dibasic acid has been the basis of several attempts to calculate the distance between the dissociating protons [1–6]. This topic has been reviewed by Brown, McDaniel, and Häfliger (up to 1953) [7] and earlier by Adell [8] and Kortüm [9]. The high K_1/K_2-ratios exhibited by certain highly alkylated short-chain dicarboxylic acids which could not be accounted for by the original simple theory have given rise to a more complicated treatment by Kirkwood and Westheimer [10–12]. This theory treats the molecules as spherical or ellipsoidal cavities of low dielectric constant (assigned a value of 2.0, as for *n*-paraffines), imbedded in a medium of high dielectric constant, *i. e.*, the concept of an "effective" dielectric constant, which is a function of the size and shape of the molecule, was introduced. According to this treatment, the accommodation of alkyl groups in the carbon chain increases the size of the molecule, thereby allowing a greater part of the electrostatic effect to be transmitted through the cavity of low dielectric constant. With this theory reasonable values of the interprotonic

* Part II. *Acta Chem. Scand.* 13 (1959) 203.

distances could be calculated even for acids with high K_1/K_2-ratios, though it involved a number of important assumptions regarding the size and shape of the molecules. For instance, the calculations were based on molecules with the carboxyl groups in *trans* position, though chemical evidence pointed to other models with the carboxyl groups in *gauche* position. Furthermore, the theory has been critisized for the fact that it does not explain the influence of solvent and temperature on the K_1/K_2-ratio in a satisfactory manner [13, 14]. Peek and Hill [15] have considered the effect of rotation about the central carbon-carbon bond in succinic acid, and stated that the Kirkwood-Westheimer theory, together with an acceptable value of the energy barrier against internal rotation (about 2 kcal/mole) was in good agreement with experiment.

Davison [16], Hunter [17], McDaniel and Brown [18], and Brown, McDaniel, and Häfliger [7] have discussed hydrogen bonding as a factor in the ionization of dicarboxylic acids and proposed that an anomalously high K_1/K_2-ratio was best explained by assuming the existence of an intramolecular hydrogen bond in the monoanion. As a summary of the discussions of these authors the formation of such a structure should be favoured by the following factors:

1. The fixation of the carboxyl groups in *cis*-position.
2. The prevention of rotation of the carboxyl groups.
3. The existence of steric strain in the molecule, which should in part be relieved by the formation of a cyclic structure.
4. Lowering of the dielectric constant of the solvent.

The investigations by, among many others, Wotiz and Merrill [19] on highly branched malonic acids, Cookson and Trevett [20] on a dicarboxylic acid derived from a *Delphinium* alkaloid and having the carboxyl groups rigidly held in positions near each other, and Mizutani [21] on the strength of maleic acid in methanol-water mixtures of varying composition, confirm these statements.

However, Westheimer and Benfey [22] recently have shown that the ratio K_1/K_E, where K_E is the ionization constant of the monomethyl ester of the acid in question, forces an upper limit upon the effect of hydrogen bonding on the K_1/K_2-ratio, so that it should contribute only to a fraction of the total observed effect.

These discussions have been based on essentially the same material, published before 1940, and surprisingly few new data have appeared since that time. In paper I of this series [23] the author has described the preparation of a number of α,α'-dialkyl- and tetraalkyl-substituted succinic acids with structures suitable for obtaining more information regarding the factors which influence the K_1/K_2-ratio. The thermodynamic dissociation constants of these acids have now been measured in 50 % ethanol by weight by the electrometric titration method in cells with liquid junctions. The experimental conditions were mainly those used by Speakman [24, 25] in the determination of K_1 and K_2 for a few dibasic acids in aqueous ethanol, but a simpler calculation method was used. The use of 50 % ethanol was necessary, as a number of the investigated acids were almost insoluble in water. Succinic, *cis*- and *trans*-caronic acid, maleic and fumaric acid have been included for comparison. The author has previously reported the preparation of a number of α,α'-dialkylglutaric acids [26, 27], from which the two forms of α,α'-di-(*tert.*-butyl)-glutaric acid were

Table 1. Thermodynamic dissociation constants of diacids in 50 % aqueous ethanol at 20°.

Acid	pK_1	pK_2	ΔpK	K_1/K_2 in 50 % aq. ethanol	K_1/K_2 in water [7]
Succinic	5.44	7.35	1.91	81	19.2
Racem-α,α′-dimethylsuccinic	5.04	8.17	3.13	1 350	181
Meso-α,α′-dimethylsuccinic	4.97	7.58	2.61	410	147
Racem-α,α′-diethylsuccinic	4.76	9.22	4.46	29 000	1 220
Meso-α,α′-diethylsuccinic	5.37	7.43	2.06	115	672
Racem-α,α′-di*iso*propylsuccinic	3.66	11.44	7.78	60 000 000	
Meso-α,α′-di*iso*propylsuccinic	5.98	8.10	2.12	130	
Racem-α,α′-di-(*tert.*-butyl)-succinic	3.58	13.12	9.54	3 500 000 000	
Meso-α,α′-di-(*tert.*-butyl)-succinic	6.43	8.29	1.86	73	
Racem-α,α′-di*neo*pentylsuccinic [a]	5.22	9.56	4.34	22 000	
Racem-α,α′-bis-(trimethylsilylmethyl)-succinic [a]	4.96	9.96	5.00	100 000	
Racem-α,α′-di*cyclo*hexylsuccinic	3.83	11.59	7.76	58 000 000	
Meso-α,α′-di*cyclo*hexylsuccinic	6.06	8.35	2.29	195	
Cis-caronic	3.33	10.68	7.35	22 000 000	929 000
Trans-caronic	5.45	7.24	1.79	62	31.8
Maleic	2.22	8.82	6.60	4 000 000	20 200
Fumaric	4.37	6.19	1.82	66	23.2
Tetramethylsuccinic	4.84	10.13	5.29	200 000	6 130
α,α′-dimethyl-α,α′-diethylsuccinic	4.86	10.16	5.30	200 000	
Tetraethylsuccinic	4.78	11.42	6.64	4 400 000	
α,α′-diethyl-α,α′-di*iso*propylsuccinic	4.55	12.61	8.06	110 000 000	
α,α,α′,α′-bis-(ethylene)-succinic	5.80	8.78	2.98	960	
α,α,α′,α′-bis-(trimethylene)-succinic	5.68	9.55	3.87	7 400	
α,α,α′,α′-bis-(tetramethylene)-succinic	5.87	9.83	3.96	9 100	
α,α′-di-(*tert.*-butyl)-glutaric, high-melting	6.64	8.34	1.70	50	
α,α′-di-(*tert.*-butyl)-glutaric, low-melting	6.68	8.35	1.67	47	

a. The *meso* form of this acid was almost insoluble in 50 % ethanol. The dissociation constants in 75 % ethanol were estimated from the titration curves as the pH-values at 25 and 75 % neutralization. The K_1/K_2-ratio was of the order 1 000.

Table 2. Thermodynamic dissociation constants for monomethyl esters in 50 % aqueous ethanol at 20°.

Monomethyl succinate	pK_E	pK_1	K_1/K_E in 50 % aq. ethanol	K_1/K_E in water [7,28]
Racem-α,α′-dimethyl-	5.98	5.04	8.7	2.7
Meso-α,α′-dimethyl-	5.92	4.97	8.9	3.1
Racem-α,α′-diethyl-	6.01	4.76	17.8	
Meso-α,α′-diethyl-	6.04	5.37	4.7	
Racem-α,α′-di*iso*propyl-	6.63	3.66	930	20
Meso-α,α′-di*iso*propyl-	6.40	5.98	2.6	1.7
Racem-α,α′-di-(*tert.*-butyl)-	7.44	3.58	7 300	
Meso-α,α′-di-(*tert.*-butyl)-	6.83	6.43	2.5	
Tetramethyl-	6.80	4.84	91	27.0
Tetraethyl-	7.35	4.78	370	

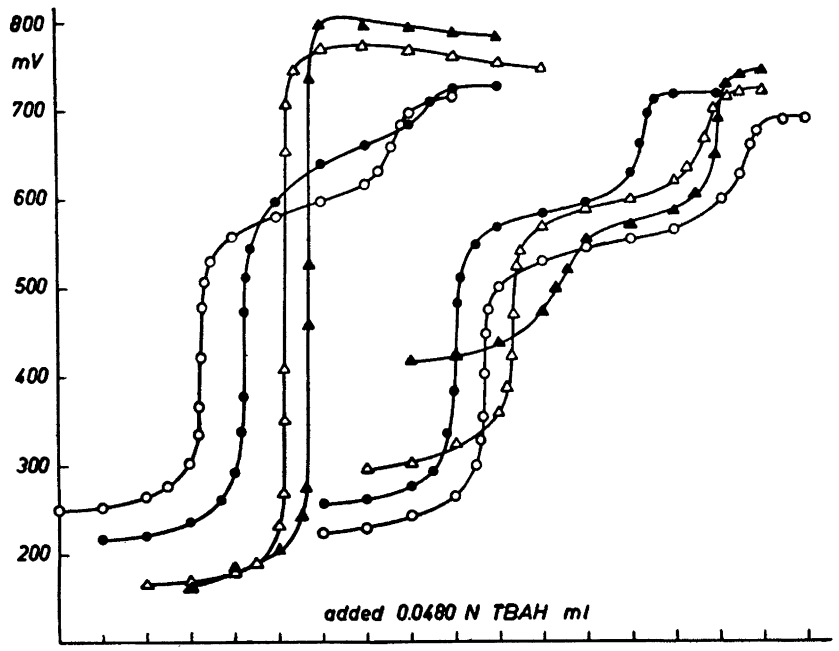


Fig. 1. Titration of α,α'-dialkylsuccinic acids with TBAH.
○—○—○—○—○ α,α'-dimethylsuccinic acid
●—●—●—●—● α,α'-diethylsuccinic acid
△—△—△—△—△ α,α'-di*iso*propylsuccinic acid
▲—▲—▲—▲—▲ α,α'-di-(*tert.*-butyl)-succinic acid
The *racemic* acid titration curves are the left ones.

chosen for pK-measurement, representing glutaric acids with a large bulk of alkyl substituents. The values of pK_1, pK_2, ΔpK and K_1/K_2 are shown in Table 1 together with the results of earlier determinations in water.

The monomethyl esters of *meso*- and *racem*-α,α'-dimethyl-, *meso*- and *racem*-α,α'-diethyl-, *meso*- and *racem*-α,α'-di*iso*propyl-, *meso*- and *racem*-α,α'-di-(*tert.*-butyl)-, tetramethyl-, and tetraethylsuccinic acid have been prepared and their pK_E-values determined in 50 % aqueous ethanol, as shown in Table 2. Furthermore, some of the acids listed in Table 1 were titrated electrometrically in pyridine with tetrabutylammonium hydroxide (TBAH)* in methanol-benzene solution. This titration method has recently been applied to dibasic acids [29–31] and has been found to give two sharp inflection points in the titration curves of a number of acids, namely *m*- and *p*-hydroxybenzoic, malic,

* These titrations were carried out at the Department of Analytical Chemistry, University of Lund.

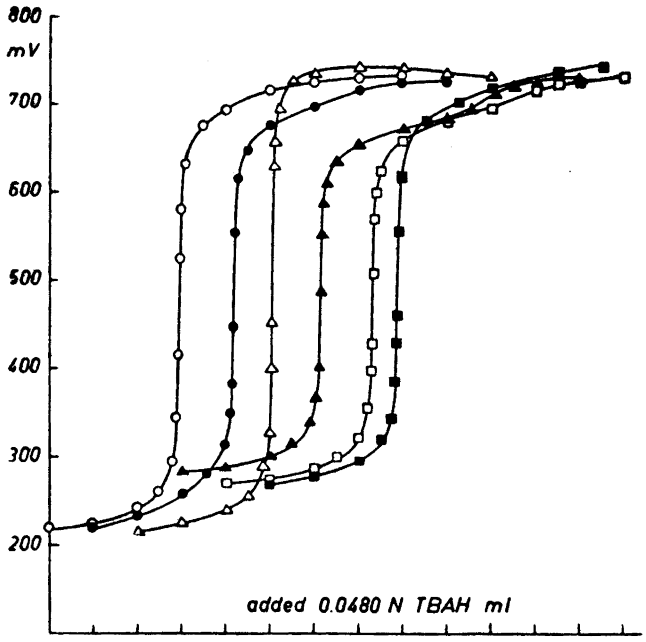


Fig. 2. Titration of tetraalkylsuccinic acids with TBAH.

○—○—○—○—○ tetramethylsuccinic acid
●—●—●—●—● α,α′-dimethyl-α,α′-diethylsuccinic acid
△—△—△—△—△ tetraethylsuccinic acid
▲—▲—▲—▲—▲ α,α,α′,α′-bis-(ethylene)-succinic acid
□—□—□—□—□ α,α,α′,α′-bis-(trimethylene)-succinic acid,
■—■—■—■—■ α,α,α′,α′-bis-(tetramethylene)-succinic acid.

oxalic, malonic, maleic, fumaric, succinic, *o*-phthalic, and sulphuric acid. Salicylic acid titrated as a monobasic acid and had a very large break in the titration curve. The results of the TBAH-titrations are given in the form of titration curves in Figs 1 and 2.

The most striking fact in Table 1 is the enormous increase of the K_1/K_2-ratio of the *racemic* acids when the steric requirements of the alkyl substituents are increased, the value for *racem*-α,α′-di-(*tert.*-butyl)-succinic being the highest ever observed, while on the contrary the corresponding *meso* forms have ratios of about the same relatively small magnitude. The K_1/K_E-values listed in Table 2 show the same general trend. Among the tetra-alkylated acids the trend is a substantial increase of the K_1/K_2-ratio with higher "degree of alkylation" and a much greater ratio for the open-chain acids than the cyclic ones. As is seen from Fig. 1, this corresponds to a change in titration curve type for the *racemic* acids from a curve with two inflection points to a curve with one

very strong break (compare salicylic acid). The curves of the *meso* acids all have two inflection points. Fig. 2 shows the titration curves of the tetra-alkylated acids, which all have very strong breaks for the first equivalence point but the second ones either very small or totally absent. Also in 50 % ethanol *racem*-α,α'-di-(*tert.*-butyl)-succinic acid titrates as a monobasic acid, thus resembling the highly branched malonic acids investigated by Wotiz and Merrill [19].

DISCUSSION

Kirkwood and Westheimer based their calculations of interprotonic distances in symmetrical dibasic acids on the formula

$$\log \frac{K_1}{\sigma K_2} = \frac{e^2}{2.303\, k\, T\, R\, D_E} \qquad (1)$$

where K_1 and K_2 are the first and second dissociation constants, e the electronic charge, k the Boltzmann constant, R the interprotonic distance, and σ a statistical factor, in this instance equal to four. D_E is the effective dielectric constant and is a function of the shape of the molecule, the positions of the protons within the molecule, the dielectric constant of the solvent, and the dielectric constant of the acid molecule. For the calculations of D_E it was necessary to assume the shape of the molecule to be either a sphere or a prolate ellipsoid of revolution, owing to the mathematical difficulties associated with other models. In the former case D_E is calculated as a function of the positions of the protons within the sphere, in the latter as a function of the excentricity of the ellipsoid. An intersection of the spherical model is shown schematically in Fig. 3.

In this Figure b is the radius of the sphere (estimated from the partial molar volume obtained according to Traube [32]), r the distance from the center of the sphere to the ionizable protons, ϑ the angle between the distances r, and R the interprotonic distance. In the case of the ellipsoidal model the mathematical treatment requires the protons to be placed at the foci of an ellipsoid of revolution with the excentricity λ_0. The interfocal distance is R, and λ_0 is obtained from the formula

$$\lambda_0^3 - \lambda_0 = \frac{6\, V}{\pi\, R^3} \qquad (2)$$

where V is the partial molar volume.

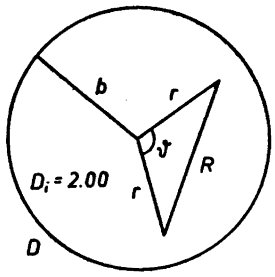


Fig. 3.

For a number of succinic acids (*meso-* and *racem-α,α'*-diethylsuccinic acid, tetramethylsuccinic acid) the spherical model was used and in other cases (*meso-* and *racem-α,α'*-dimethylsuccinic acid, succinic acid) the ellipsoidal one, but in all cases the carboxyl groups were supposed to be *trans* to each other [12].

Now there is evidence that a number of the acids investigated in this paper and also some of those treated by Kirkwood and Westheimer should have *gauche* carboxyl groups *in their most favoured conformations.* Thus equilibration of *meso-* and *racem-α,α'*-dialkylsuccinic acids indicates that the *racemic* form is the more stable isomer [33] contrary to the stability order predicted by conformation analytical reasoning [34,35]. The presence of an intramolecular hydrogen bond in the preferred conformation of the *racemic* acid could explain this anomaly. The extreme ease of anhydrization of certain succinic acids [23] also makes it reasonable to assume that the carboxyl groups have been forced into *gauche* position, partly by the possibility of an intramolecularly hydrogen-bonded ring structure being formed, which should relieve part of the steric strain in the open-chain acid, partly by the orienting effects of large alkyl groups. For example in *α,α'*-di-(*tert.*-butyl)-succinic acid the bulky alkyl groups should entirely determine the conformation of the molecule, and consequently in the *meso* acid the carboxyl groups become orientated *trans* to each other, and in the *racemic* from *gauche* to each other.

Table 3 gives the calculated ΔpK-values in 50 % ethanol for a number of the acids investigated in this paper. D_E-functions were worked out for the solvent 50 % ethanol *. These curves are shown in Figs 4 and 5. The geometrical

Table 3. Comparison between calculated and found ΔpK-values in 50 % ethanol.

Succinic acid	Calculated ΔpK (spherical model)			Calculated ΔpK (ellipsoidal model)	Found ΔpK
	COOH-groups in *gauche* position. Cos ϑ = + 0.07	COOH-groups in *trans* position. Cos ϑ = — 1.00	Corrected for intramolecular H-bonding. COOH-groups *gauche*	COOH-groups in *trans* position	
Racem-α,α'-dimethyl-	1.70	1.17	2.98	1.50	3.13
Meso-α,α'-dimethyl-	1.70	1.17	3.00	1.50	2.61
Racem-α,α'-diethyl-	2.19	1.34	4.09	1.73	4.46
Meso-α,α'-diethyl-	2.19	1.34		1.73	2.06
Racem-α,α'-di*iso*propyl-	2.93	1.60	8.26	1.98	7.78
Meso-α,α'-di*iso*propyl-	2.93	1.60		1.98	2.12
Racem-α,α'-di-(*tert.*-butyl)-	3.64	1.84	10.76	2.23	9.54
Meso-α,α'-di-(*tert.*-butyl)-	3.64	1.84		2.23	1.86
Tetramethyl-	2.19	1.34	5.51	1.73	5.29
Tetraethyl-	3.64	1.84	8.17	2.23	6.64

* The author wishes to express his thanks to Laborator C.-E. Fröberg and Fil.mag. E. Larsson at the Institute of Numerical Analysis, University of Lund, for their kind help with the calculation of the D_E-function in the ellipsoidal case.

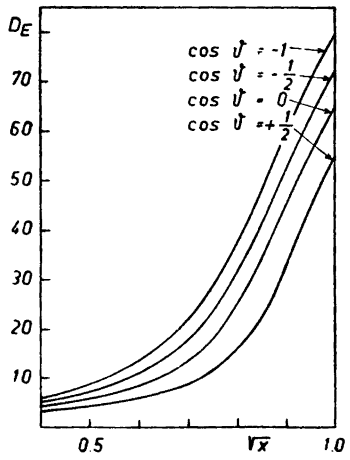


Fig. 4. Plot of D_E *versus* $\sqrt{x} = r/b$ for $\cos\vartheta = +\frac{1}{2}, 0, -\frac{1}{2}, -1$. $D_i = 2.00$; $D = 50.4$.

relations between the carboxyl groups were obtained from the bond lengths and angles in succinic acid. The proton was placed on the extension of the bond between the carboxyl carbon atom and its nearest carbon neighbor at a distance of 1.45 Å from the former one. The factors for the correction of the effect of hydrogen bonding in the monoanion were calculated from the K_1/K_E-values in Table 2 according to Westheimer and Benfey. This correction is calculated for the case that hydrogen bonding exists in the monoanion but not in the free acid and its monomethyl ester. As mentioned above, available data [33, 36] point to the existence of an intramolecular hydrogen bond also in the diacid, which should reduce the magnitude of the correction. However, with regard to the approximate nature of this theory, no attempt was made to estimate this effect. It is also quite obvious that a measured Δ pK in reality does not refer to a single conformation but to an equilibrium mixture between several rotation isomers of acids and ions with or without interactions between

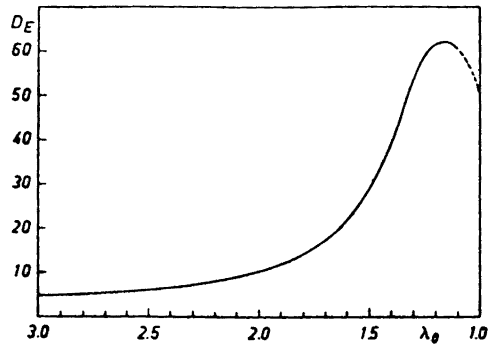
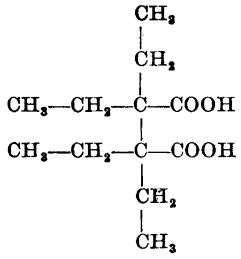


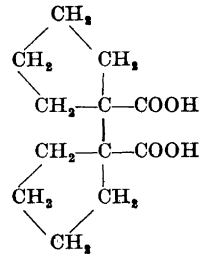
Fig. 5. Plot of D_E *versus* λ_ϑ. $D_i = 2.00$; $D = 50.4$.

the carboxyl groups. By comparing calculated and measured ΔpK-values it should, however, be possible to get an idea about which conformations are the most preferred ones.

Turning to Table 3, it must first be established by examination of molecular models whether the various conformations approximate the shapes of spherical or ellipsoidal molecules. It is found that the spherical model is best suited for conformations with *gauche* carboxyl groups and that the ellipsoidal model with the protons at the foci is difficult to associate with the shape of these molecules. For the conformations with *trans* carboxyl groups it was similarly found that both models could be used. By inspection of Table 3 it is obvious that a preferred conformation with *gauche* carboxyl groups together with the assumption of an intramolecular hydrogen bond in the monoanion is in satisfactory agreement with the observed ΔpK-values for the *racem*-α,α'-dialkyl- and tetraalkylsuccinic acids. Electrostatic effects alone cannot account for the large K_1/K_2-ratios in these cases. Likewise, it is probable that the *meso* acids have *trans* carboxyl groups in their most favoured conformations, though *meso*-α,α'-dimethylsuccinic acid appears to be capable of existing also in a conformation with *gauche* carboxyl groups.

Thus, the main cause of an anomalously high K_1/K_2-ratio appears to be steric in origin. If the steric properties of a dibasic acid are such as to permit a close approach of the carboxyl groups, the pure electrostatic effect transmitted through the solvent and the molecule itself will increase. Besides, formation of an intramolecular hydrogen bond will be possible, which further magnifies the K_1/K_2-ratio. In the case of highly alkylated dibasic acids it is tempting to compare this effect with the empirically found rule that accommodation of alkyl substituents in a carbon chain capable of ring closure facilitates ring formation [37]. The theoretical background of this rule is not entirely understood, but probably steric strain in the open-chain compound, which is relieved in part in the cyclic structure, together with the existence of preferred conformations with the reacting groups in *gauche* position, is responsible for this effect. The behaviour of the tetraalkylated succinic acids investigated in this paper is consistent with this hypothesis. For example, if in tetraethylsuccinic acid the ethyl groups on each carbon atom are bent back and united with a C—C bond we obtain the structure of $\alpha,\alpha,\alpha',\alpha'$-bis-(tetramethylene)-succinic acid





By analogy with the investigations on the base strengths of triethylamine and quinuclidine carried out by Brown and Sujishi [38] it may be concluded that the

steric strain should be diminished by this structural change. Actually a much smaller K_1/K_2-ratio is found for $\alpha,\alpha,\alpha',\alpha'$-bis-(tetramethylene)-succinic acid *.

Lastly, it must be pointed out that the above discussion has neglected the effect of interactions between solvent and solute and of medium changes in the vicinity of the carboxyl groups. As very little is known about the conditions at the boundary surfaces it is, however, difficult to include such a discussion. At present the Kirkwood-Westheimer theory, together with the assumption of an intramolecularly hydrogen-bonded monoanion for molecules with carboxyl groups near each other, accounts for the observed effects, though in a crude manner. Specifically, the theory constitutes a valuable tool for the assignment of conformations in dibasic acids.

EXPERIMENTAL

Materials. Commercial absolute ethanol was freed from water by the magnesium ethylate method and distilled. Equal weights of the ethanol and carbon dioxide-free water were mixed and stored in a container protected from the carbon dioxide of the air. To prepare the standard sodium hydroxide solution the calculated volume of 50 % aqueous carbonate free sodium hydroxide for obtaining a 0.05 N solution was added, followed by the amount of absolute ethanol needed for restoring the original solvent composition.

The preparation and purification of the acids have been described (except those mentioned below) by the author in earlier papers. Before pK-measurement they were recrystallized once more from the appropriate solvents and dried *in vacuo* over concentrated sulphuric acid. Succinic acid was analytical grade reagent, *cis*- and *trans*-caronic acid were prepared and purified according to Fredga and Sikström [39] and commercial samples of maleic and fumaric acid were recrystallized twice from benzene and water, respectively.

The monomethyl esters listed in Table 3 were prepared according to either of the following methods. *Meso*-α,α'-di-(*tert.*-butyl)-succinic acid which does not form an anhydride, was converted into the monosilver salt (contaminated by the disilver salt) and

Table 4. Melting points and analyses of substituted monomethyl succinates.

Monomethyl succinate	M.p. °C	M. p. lit. °C	Analysis					
			Found			Calc.		
			C	H	neutr. equiv.	C	H	neutr. equiv.
Racem-α,α'-dimethyl-	35—37	38 [40]						
Meso-α,α'-dimethyl-	45—47	49 [40]						
Racem-α,α'-diethyl-	liquid [a]		56.9	8.7	188	57.4	8.6	188
Meso-α,α'-diethyl-	60—63		57.3	8.8	189	57.4	8.6	188
Racem-α,α'-di*iso*propyl-	63—66		61.0	9.5	217	61.1	9.3	216
Meso-α,α'-di*iso*propyl-	120—121		60.8	9.4	217	61.1	9.3	216
Racem-α,α'-di-(*tert.*-butyl)-	106—108		63.9	10.0	246	63.9	9.9	244
Meso-α,α'-di-(*tert.*-butyl)-	135—137		63.3	10.1	243	63.9	9.9	244
Tetramethyl-	66—67	63 [40]						
Tetraethyl-	52—55	46—48 [41]						

a. B.p. 115—119°/1 mm.

* *Cf.* Kuhn's [43] results in his study on intramolecular hydrogen bonding in alkylsubstituted ethanediols, which are quite analogous to those presented here.

treated with methyl iodide. A fair yield of the monomethyl ester was obtained, which was recrystallized twice from methanol.

The other acids were converted into anhydrides by boiling with acetyl chloride, the excess of acetyl chloride was removed *in vacuo*, and the crude anhydride allowed to react with the calculated amount of sodium methylate in absolute methanol. The solution was evaporated to dryness in a vacuum desiccator at room temperature and the residue acidified with dilute hydrochloric acid. The monomethyl ester was then isolated and purified by distillation or recrystallization from aqueous methanol. Melting points and analyses are shown in Table 4.

Method. Speakman [24] has developed a graphical method for the evaluation of the dissociation constants of dibasic acids from pH-measurements which is especially well adapted for acids with small K_1/K_2-ratios, say < 50. As the purpose of the present investigation was more to get an idea of the relative magnitudes of K_1 and K_2 for a series of dibasic acids rather than to make very precise measurements, and as the K_1/K_2-ratios in question are generally $\gg 50$, the author has used the simple formulae (3) and (4) for the calculations of K_1 and K_2,

$$K_1 = \frac{a_{H^+} (b + [H^+]) f_1}{(a - b - [H^+]) f_0} \quad (3)$$

$$K_2 = \frac{a_{H^+} (b - a - [OH^-]) f_2}{(2a - b + [OH^-]) f_1} \quad (4)$$

where a_{H^+} is the hydrogen ion activity, as measured by the glass electrode, a the total molar concentration of acid, b the molar concentration of added base, and f_0, f_1, and f_2 the activity coefficients of diacid, monoanion, and dianion, respectively.

For acids with $K_1/K_2 > 10^3$ the calculations of K_1 and K_2 are totally independent of each other, and they were based on 5—6 pH-measurements in each of the ranges of 15—35 and 65—85 % neutralization. For acids where $50 < K_1/K_2 < 10^3$ it can be shown that the influence of the separate dissociation stages on each other is negligible in this connection if K_1 and K_2 were computed from pH-measurements in the ranges of 10—20 and 80—90 % neutralization. As has been shown by Bacarella *et al.*[42] the glass electrode correctly measures a_{H^+} in aqueous organic solvents, provided that certain conditions for the handling of the glass electrode are fulfilled. Thus, in this work the glass electrode was standardized before use with an acetate buffer of pH 4.11 and a borate buffer of pH 9.08. It was then equilibrated with 50 % ethanol for several hours before the beginning of the electrometric titrations. It was stored in 50 % ethanol when not in use. No correction was made for the liquid junction potentials. As $[H^+]$ normally makes only a small contribution to the expressions $(b + [H^+])$ and $(a - b - [H^+])$ a_{H^+} was used in its place. $[OH^-]$ could not be neglected in a few cases where K_2 was very small, and it was calculated from the expression $[OH^-] = K_w/[H^+]$. A rough estimate of K_w (7.2×10^{-16}) was obtained from titration of the pure solvent with standard sodium hydroxide. No correction was made for the presence of ethoxide ions, a and b were corrected for dilution during the titration, f_0 was assumed to be unity, and f_1 and f_2 were computed by the Debye-Hückel limiting law, $\log f_i = -A z_i^2 \sqrt{I}$, where $A = 1.01$ in 50 % ethanol at 20°, and I was approximated as $(b + [H^+])$ when $a < b$ and as $(2b - a)$ when $b > a$ [24, 25].

The dissociation constants of the monomethyl esters were calculated as for monobasic acids.

The accuracy of the pK-values of Tables 1 and 2 is estimated at ± 0.15 unit in pK except for those pK_2-values which have been corrected for $[OH^-]$, where the error may be as high as ± 0.30 unit. For comparison within the material of this paper the accuracy is probably rather better. Speakman [25] has determined the pK-values of succinic acid in 50 % ethanol by weight at 20° and found $pK_1 = 5.48 \pm 0.05$ and $pK_2 = 7.24 \pm 0.05$ (calculated on the basis of molalities). For comparison, the pH-data for succinic acid were also put into the equations given by Speakman and then the pK-values graphically determined. This method gave $pK_1 = 5.43$ and $pK_2 = 7.36$, which is in satisfactory agreement with those calculated according to formulae (3) and (4).

pH-Measurements. Solutions of the acids in 50 % ethanol (0.004—0.005 M), were electrometrically titrated with standard sodium hydroxide in the same solvent (about 0.05 N), and pH-readings were made for various volumes of added base in the ranges mentioned above. The following cell was used

Glass electrode	solution under investigation	satd. KCl	satd. KCl	Hg	Hg_2Cl_2

The glass electrode was Radiometer Type G 202 B. The titrations were run in a carbon dioxide-free atmosphere and the temperature was 20 ± 0.5°. The titrations were run up to the equivalence points to check the purity of the acids; neutralization equivalents always agreed with the calculated ones within ± 1 %.

The author wishes to express his sincere gratitude to Professor Erik Larsson for his kind interest in this work.

REFERENCES

1. Bjerrum, N. *Z. physik. Chem.* **106** (1923) 219.
2. Gane, R. and Ingold, C. K. *J. Chem. Soc.* **1931** 2153.
3. Jones, I. and Soper, F. G. *J. Chem. Soc.* **1936** 133.
4. Adell, B. *Über die elektrolytische Dissoziation von Dicarbonsäuren in Alkalichloridlösungen* (Diss.) Lund 1938.
5. Adell, B. *Z. physik. Chem. (A)* **185** (1939) 161.
6. Adell, B. *Svensk Kem. Tidskr.* **53** (1941) 89.
7. Brown, H. C., McDaniel, D. H. and Häfliger, O. in Braude, E. A. and Nachod, F. C. *Determination of Organic Structures by Physical Methods*, Academic Press Inc. New York 1955, p. 622.
8. Adell, B. *Elementa* **26** (1943) 20.
9. Kortüm, G. *Elektrolytlösungen*, Akademische Verlagsgesellschaft, Leipzig **1941**, p. 377.
10. Kirkwood, J. G. and Westheimer, F. H. *J. Chem. Phys.* **6** (1938) 506.
11. Kirkwood, J. G. and Westheimer, F. H. *J. Chem. Phys.* **6** (1938) 513.
12. Westheimer, F. H. and Shookhoff, M. W. *J. Am. Chem. Soc.* **61** (1939) 555.
13. Wynne-Jones, W. F. K. and Rushbrooke, G. S. *Trans. Faraday Soc.* **40** (1944) 99.
14. Westheimer, F. C. and Kirkwood, J. G. *Trans. Faraday Soc.* **43** (1947) 77.
15. Peek, H. M. and Hill, T. L. *J. Am. Chem. Soc.* **73** (1951) 5304.
16. Hunter, L. *Chem. & Ind. London* **1953** 155.
17. Davison, W. H. T. *Chem. & Ind. London* **1953** 408
18. McDaniel, D. H. and Brown, H. C. *Science* **118** (1953) 370.
19. Wotiz, J. H. and Merrill, H. E. *J. Am. Chem. Soc.* **80** (1958) 866.
20. Cookson, R. C. and Trevett, M. E. *J. Chem. Soc.* **1956** 3864.
21. Mizutani, M. *Z. physik. Chem.* **118** (1925) 318.
22. Westheimer, F. H. and Benfey, O. T. *J. Am. Chem. Soc.* **78** (1956) 5309.
23. Eberson, L. *Acta Chem. Scand.* **13** (1959) 40.
24. Speakman, J. C. *J. Chem. Soc.* **1940** 855.
25. Speakman, J. C. *J. Chem. Soc.* **1943** 270.
26. Eberson, L. *Acta Chem. Scand.* **12** (1958) 314.
27. Eberson, L. *Acta Chem. Scand.* **12** (1958) 1906.
28. Bone, W. A. and Sprankling, C. H. G. *J. Chem. Soc.* **77** (1900) 1299.
29. Harlow, G. A., Noble, C. M. and Wyld, G. E. A. *Anal. Chem.* **28** (1956) 787.
30. Cundiff, R. H. and Markunas, P. C. *Anal. Chem.* **28** (1956) 792.
31. Harlow, G. A. and Wyld, G. E. A. *Anal. Chem.* **30** (1958) 69.
32. Traube, J. *Samml. chem. u. chem.-tech. Vorträge* **4** (1899) 255.
33. Eberson, L. *Acta Chem. Scand.* **13** (1959) 203.
34. Linstead, R. P. and Whalley, M. *J. Chem. Soc.* **1954** 3722.
35. Barton, D. H. R. and Cookson, R. C. *Quart. Revs. (London)* **10** (1956) 44.
36. Eberson, L. *Acta Chem. Scand.* **13** (1959) 224.

37. Newman, M. S. *Steric Effects in Organic Chemistry*, J. Wiley & Sons, New York **1956**, p. 460.
38. Brown, H. C. and Sujishi, S. *J. Am. Chem. Soc.* **70** (1948) 2878.
39. Fredga, A. and Sikström, Å. *Arkiv Kemi* **8** (1956) 433.
40. Walker, J. *J. Chem. Soc.* **61** (1892) 696.
41. Walker, J. and Walker, A. P. *J. Chem. Soc.* **87** (1905) 961.
42. Bacarella, A. L., Grunwald, E., Marshall, H. P. and Purlee, E. L. *J. Phys. Chem.* **69** (1958) 856.
43. Kuhn, L. P. *J. Am. Chem. Soc.* **80** (1958) 5950.

Received December 24, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 224—235

Studies on Succinic Acids

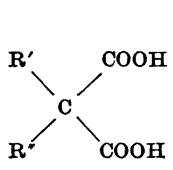
IV.* Evidence for the Existence of Intramolecular Hydrogen Bonding in Certain Highly Alkylated Succinic Acids by Infra-Red Spectra

LENNART EBERSON

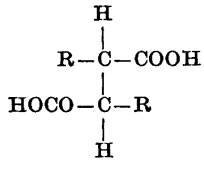
Department of Organic Chemistry, Chemical Institute, University of Lund, Sweden

The infra-red spectra of *racem*-α,α'-di*iso*propyl-, *racem*-α,α'-di-(*tert.*-butyl)-, *racem*-α,α'-di*cyclo*hexyl-, and tetraethylsuccinic acid and their monopotassium salts have been found to be consistent with an intramolecularly hydrogen-bonded structure. All these acids have very large K_1/K_2-ratios. The infra-red spectra of *meso*-α,α'-di*iso*-propyl-, *meso*-α,α'-di-(*tert.*-butyl)-, and *meso*-α,α'-di*cyclo*hexylsuccinic acid and their monopotassium salts are compatible with a structure containing one carboxyl group and one carboxylate ion which are essentially independent of each other.

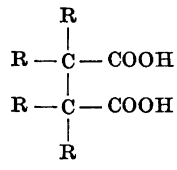
There is chemical and physico-chemical evidence for the existence of an intramolecular hydrogen bond in certain dibasic acids and their monoanions [1–4]. These include highly branched malonic acids (I), *racem*-α,α'-dialkylsuccinic acids (II) with bulky alkyl groups, tetraalkylsuccinic acids (III), β,β-dialkylglutaric acids (IV), *cis*-1,2-*cyclo*propanedicarboxylic acids (V), *cis*-1,2-dicarboxyethylenes (VI), and others.



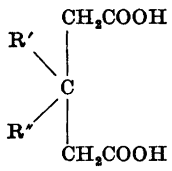
I



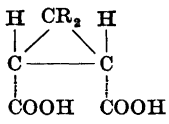
II



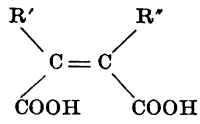
III



IV



V



VI

* Part III, *Acta Chem. Scand.* **13** (1959) 211.

In all these cases the steric relationships probably play an important role. For example, it is well known that the accomodation of alkyl groups in a carbon chain capable of ring closure will increase the ease of formation and stability of the corresponding cyclic compound [5]. In an alkylated dibasic acid the formation of a cyclic structure can be accomplished by the formation of an intramolecular hydrogen bond, provided the arrangement of alkyl groups will be energetically favoured. In the other types of dibasic acids the carboxyl groups are rigidly held at distances which make hydrogen bonding possible. The most

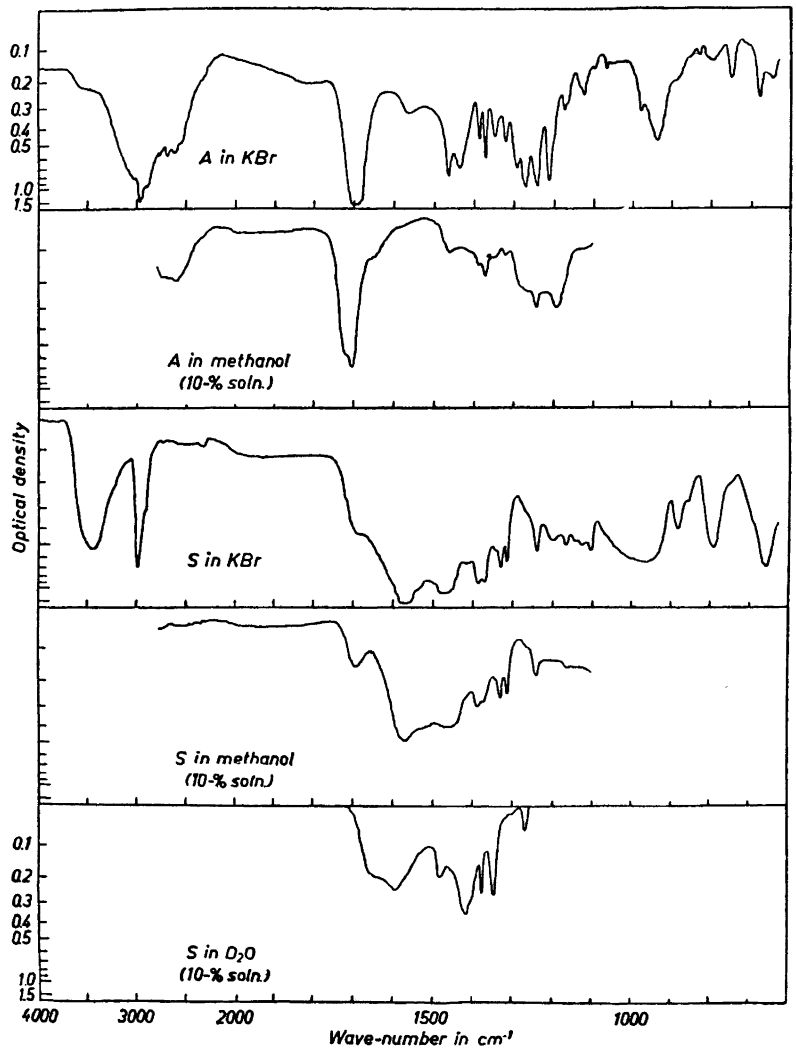


Fig. 1. Infra-red spectra of *racem*-α,α'-di*iso*propylsuccinic acid (A) and its monopotassium salt (S).

important arguments for the existence of an intramolecular hydrogen bond in dibasic acids of these kinds are:

1. They have anomalously high ratios between their first and second dissociation constants, which cannot be explained on the basis of electrostatic effects alone [1,3].

2. Measurements of the equilibrium constants of the reaction *meso*-acid ⇌ *racem*-acid for α,α'-dialkylsuccinic acids indicate that the *racemic* form is the

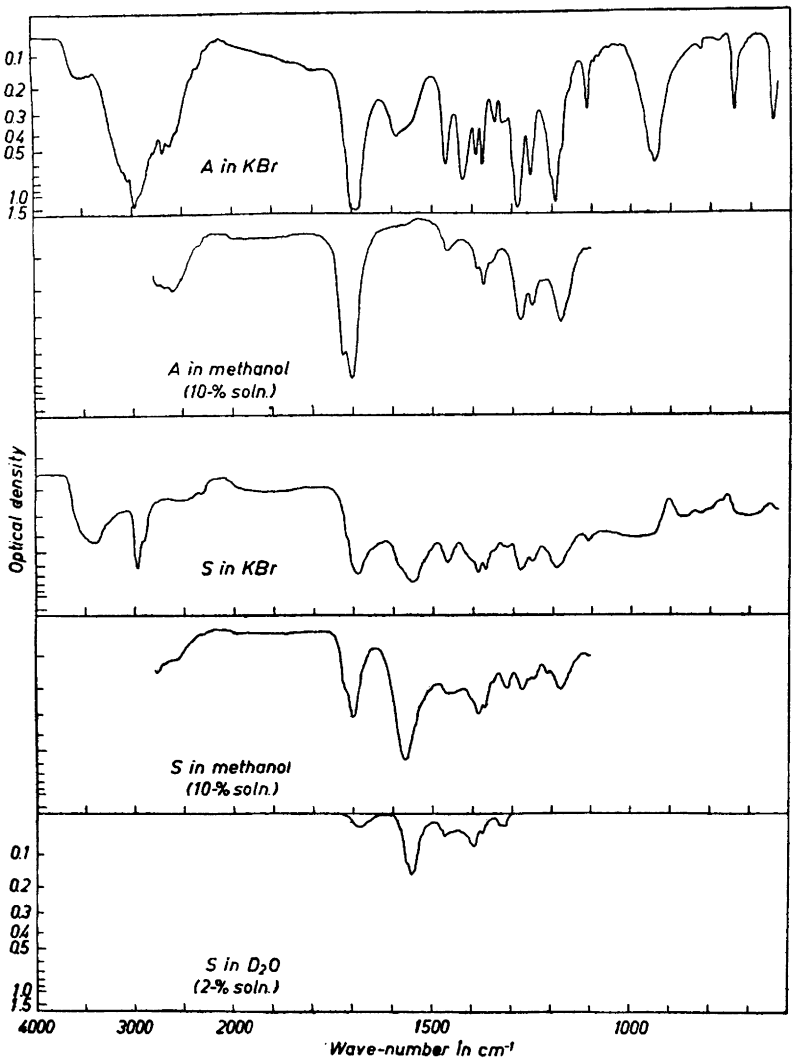


Fig. 2. Infra-red spectra of *meso*-a,a'-di*iso*propylsuccinic acid (A) and its monopotassium salt (S).

more stable isomer [2], *i. e.*, the stability order is the reverse of that predicted from conformational analytical considerations.

3. They are very easily anhydrized, some of them even in aqueous solution or in the solid state [4].

Shahat [6] has shown by X-ray analysis that in crystalline maleic acid there exists an unsymmetrical hydrogen bond. Cardwell *et al.*[7] have investigated maleic acid and its monopotassium salt in the solid state by means of infra-red

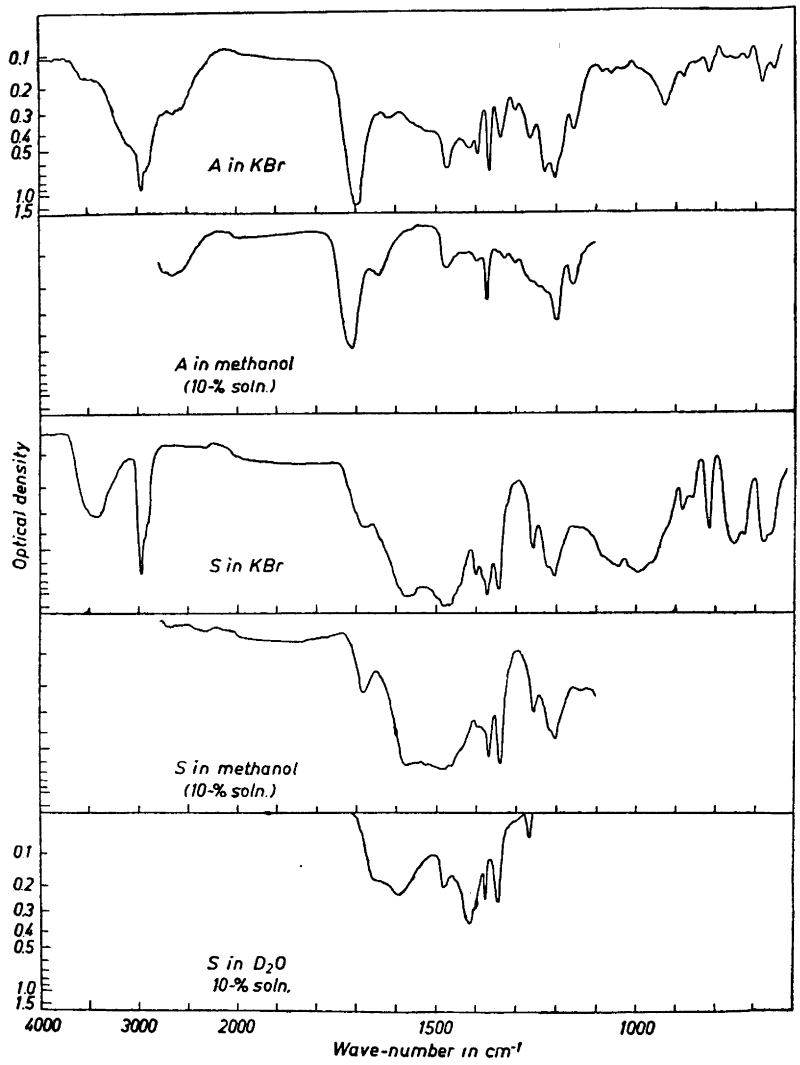


Fig. 3. Infra-red spectra of *racem*-α,α'-di-(*tert.*-butyl)-succinic acid (A) and its monopotassium salt (S).

spectra and found evidence for an intramolecular hydrogen bond in both acid and salt. In the latter case the absence of the carbonyl band indicated that the hydrogen bond might be symmetric.

The purpose of the present investigation was to study the infra-red spectra under various conditions for a series of alkylsubstituted succinic acids and their monopotassium salts. Four acids, *racem*-α,α'-di*iso*propyl-, *racem*-α,α'-di-(*tert.*-butyl)-, *racem*-α,α'-di*cyclo*hexyl-, and tetraethylsuccinic acid, which exhibit very high K_1/K_2-ratios (4×10^6—3.5×10^9 in 50 % aqueous ethanol) and three acids, the *meso* forms of the above acids, with low K_1/K_2-ratios (about 100) have been selected, and the infra-red spectra of the acids and their monopotassium salts have been examined in the solid state and in methanolic solution. Some of the salts were also studied in deuterium oxide solution. Methanol is the only solvent which meets the requirements of being transparent in the double bond stretching frequency region and of dissolving both

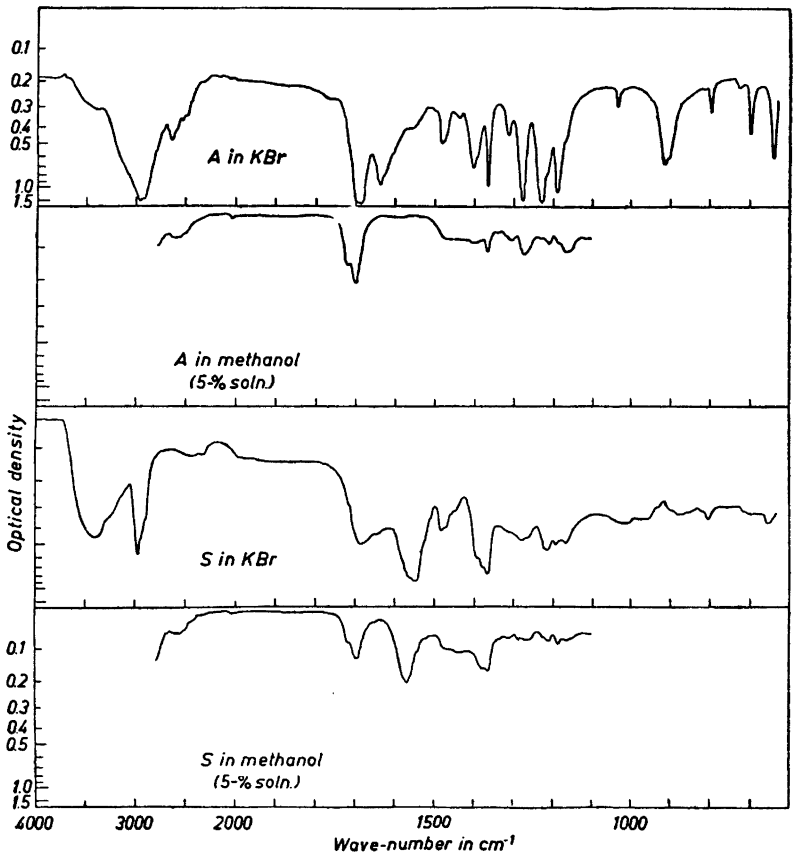


Fig. 1. Infra-red spectra of *meso*-α,α'-di-(*tert.*-butyl)-succinic acid (A) and its monopotassium salt (S).

acids and salts in sufficient concentrations. The discussion will be limited to a consideration of the carbonyl stretching frequency and the carboxylate asymmetric stretching frequency, as the other bands associated with these groups either are difficult to recognize or fall in a region where the solvent has a large absorption. The results are shown in Figs 1—7 and in Table 1, which gives the wave-numbers of the bands in the double bond region together with the approximate molar extinction coefficients in methanolic solution.

At a first glance it is seen that the spectra of the low-ratio acids and their salts are consistent with the structure VII for the acids and VIII for the monoanions.

Thus, in the solid state the carbonyl stretching frequency of the acids has a single peak at about 1 695 cm^{-1}, and no significant shift occurs in the spectra of the corresponding salts. In methanolic solution the situation is similar;

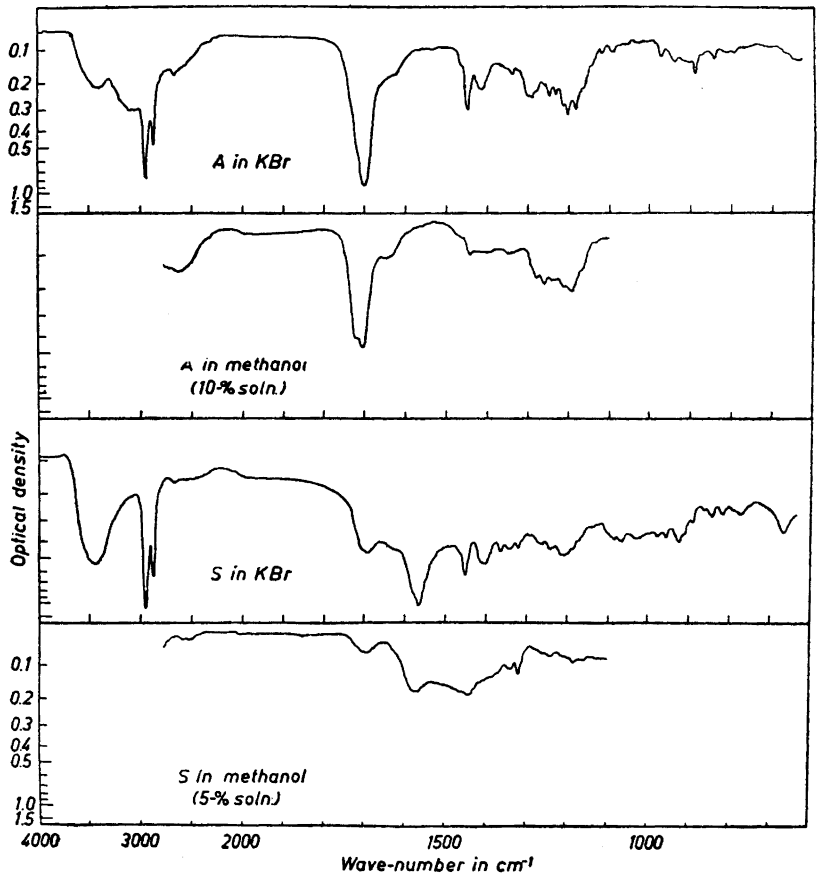
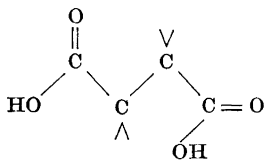
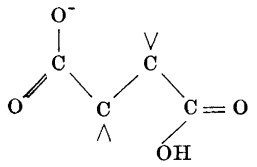


Fig. 5. Infra-red spectra of *racem-a,a'*-di*cyclo*hexylsuccinic acid (A) and its monopotassium salt (S).



VII



VIII

however, the carbonyl band has split into two peaks at about 1 720 and 1 700 cm^{-1}. These peaks are not shifted in the spectra of the salts in methanol, but the molar extinction coefficients are reduced to about half their values in the acids. The carboxylate asymmetric stretching frequency occurs in the solid state at 1 550—1 560 cm^{-1} and in methanol it is shifted to about 1 570 cm^{-1}. The only *meso* acid salt which has been studied in deuterium oxide solution

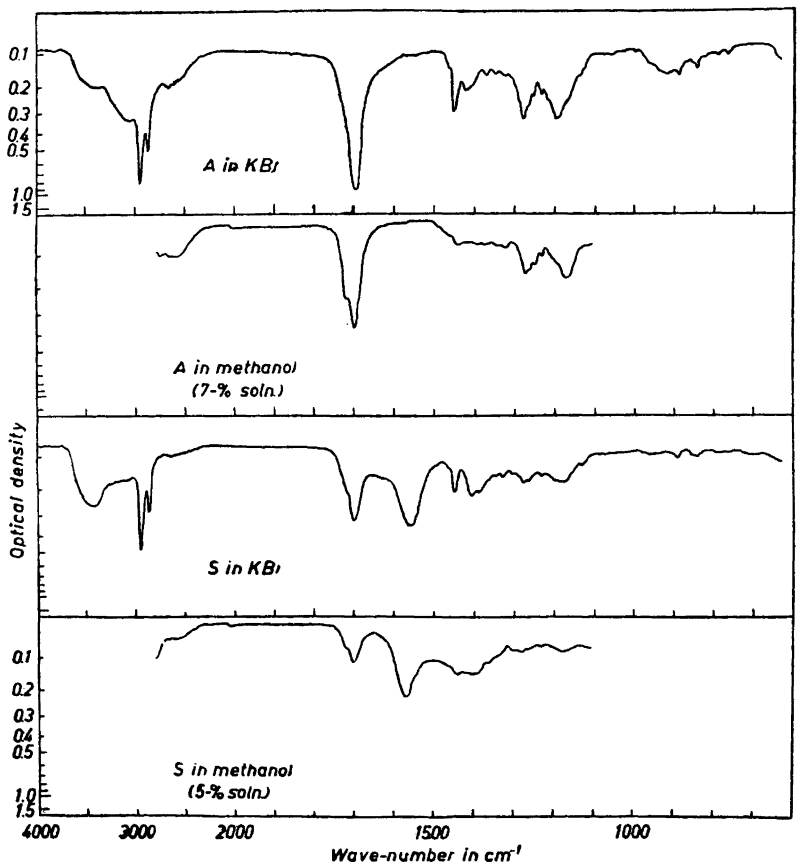


Fig. 6. Infra-red spectra of *meso*-α,α'-di*cyclo*hexylsuccinic acid (A) and its mono-potassium salt (S).

(the others are too slightly soluble) shows a single carbonyl peak at 1 680 cm^{-1} and the carboxylate band at 1 550 cm^{-1}, *i. e.*, both bands have shifted about 20 cm^{-1} towards lower wave-numbers as compared with the spectra in methanol. Summarizing, the double bond region of a low-ratio acid salt can be interpreted as a superposition of the carboxyl group and carboxylate ion spectra, and consequently the structure of the monoanions is best represented by VIII without interactions between the carboxyl groups.

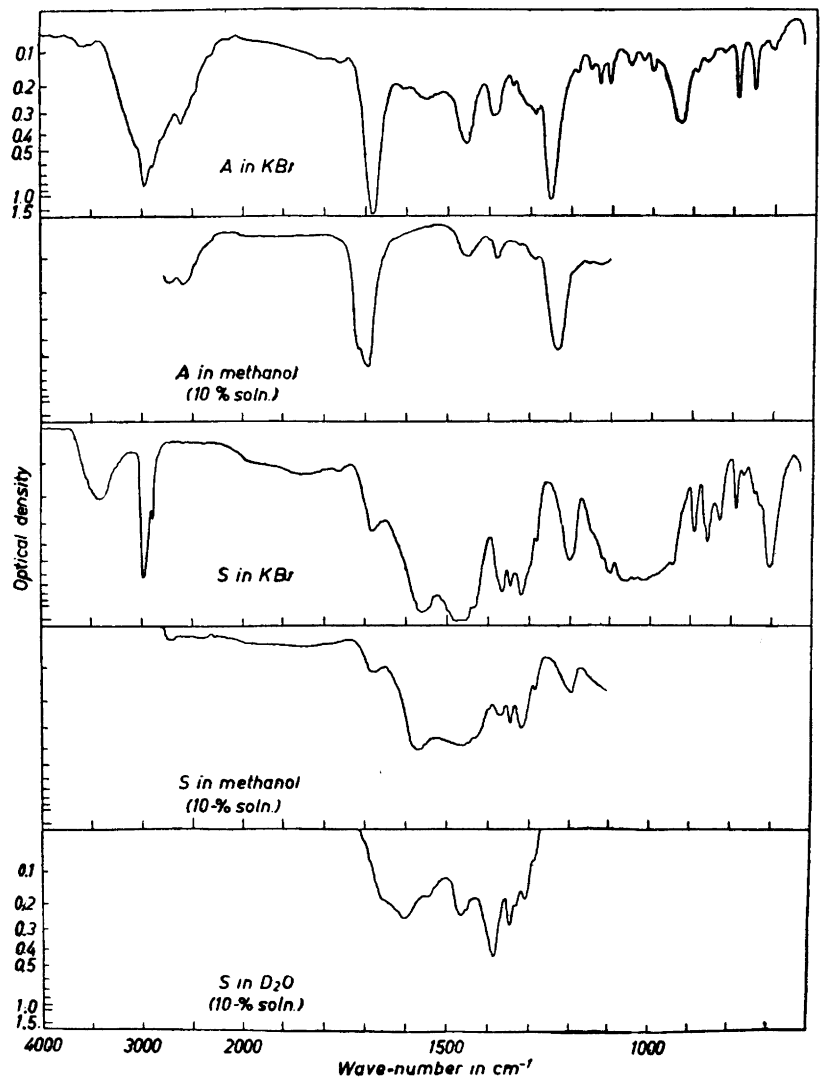


Fig. 7. Infra-red spectra of tetraethylsuccinic acid (A) and its monopotassium salt (S).

Table 1. Carbonyl frequencies of alkylsubstituted

Succinic acid	Carbonyl stretching frequency		
	In KBr, wave number in cm^{-1}	In methanol	
		wave number in cm^{-1}	Molar extinction coefficient ε
Racema-α,α'-di*iso*propyl-	1 695 vs	1 718 sh 1 704 1 650 sh	440 480 50
monopotassium salt	1 680 sh	1 690	110
Meso-α,α'-di*iso*propyl-	1 692 vs	1 723 1 701	350 510
monopotassium salt	1 687 sb	1 720 sh 1 700	150 250
Racem-α,α'-di-(*tert.*-butyl)-	1 695 vs 1 615 w	1 716 sh 1 708 1 640	390 110
monopotassium salt	1 675 mb	1 682	80
Meso-α,α'-di-(*tert.*-butyl)-	1 690 vs 1 635 s	1 720 1 700	330 500
monopotassium salt	1 687 sb	1 718 sh 1 695	170 270
Racem-α,α'-di*cyclo*hexyl-	1 700 s 1 625 sh	1 720 1 705 1 650	410 450 70
monopotassium salt	1 690 sb	1 695	110
Meso-α,α'-di*cyclo*hexyl-	1 695 s	1 717 1 697	330 540
monopotassium salt	1 697 m	1 717 1 698	130 240
Tetraethyl-	1 683 vs	1 716 1 691	360 550
monopotassium salt	1 682 m	1 678	70

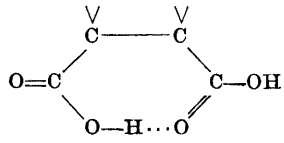
vs = very strong; s = strong; m = medium; w = weak; sh = shoulder; b = broad.

A similar interpretation is not possible for the spectra of the high-ratio acids and their salts. In methanolic solution all the *racem*-acids but not tetraethylsuccinic acid show a carbonyl band of low intensity at about 1 650 cm^{-1} (*racem*-α,α'-di-(*tert.*-butyl)-succinic acid has a weak band at 1 615 cm^{-1} also

succinic acids and their monopotassium salts.

In D_2O wave number in cm^{-1}	Carboxylate ion, asymm. stretching frequency				$\varepsilon_{C=O}$ (acid) / $\varepsilon_{C=O}$ (salt)
	In KBr, wave number in cm^{-1}	In methanol		In D_2O wave number in cm^{-1}	
		wave number in cm^{-1}	Molar extinction coefficient ε		
1 645 sh	1 570 vsb	1 568	400	1 590	4.4
1 680	1 550 sb	1 568	470	1 550	2.0
1 650 sh	1 570 vsb	1 575	290	1 590	5.0
	1 550 sb	1 568	450		1.9
	1 566 vs	1 573	360		4.1
	1 558 m	1 568	440		2.2
1 650 sh	1 560 vsb	1 568	340	1 605	7.9

in the solid state). This band must be due to a carbonyl group taking part in an intramolecular hydrogen bond (IX). A band at 1 630 cm^{-1} in solid maleic acid has been explained in the same way [7]. Accordingly, the peaks at 1 720 and 1 700 cm^{-1} are assigned to the other carbonyl group which may take



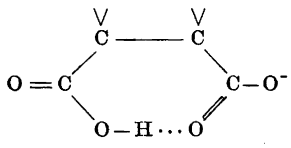
IX

part in an external hydrogen bond. The cause of the splitting around 1 700 cm^{-1} is probably coupling of the carbonyl frequencies[8-10], and not the existence of monomers and polymers in solution as the relative intensities of the two bands are independent of concentration.

The spectra of the *high*-ratio acid salts show the following main features. In both the solid state and in solution the salts have a large, almost continuous absorption in the region 1 600—1 400 cm^{-1}. In methanolic solution the carbonyl frequency has shifted by 10—25 cm^{-1} towards lower wave-numbers as compared with those of the acids, which is indicative of an intramolecular hydrogen bond [11]. The carbonyl band has been considerably reduced in intensity and its molar extinction coefficient is only a small fraction of that in the acid. In the hydrogen maleate ion the carbonyl band has completely disappeared [7], which points to the existence of a symmetric hydrogen bond in this ion with a structure intermediate between the extreme structures X a and X b.

X a X b

The fact that there actually is found a carbonyl peak in the spectra of the salts in question, though of appreciably reduced intensity, suggests that a structure for the monoanion with an unsymmetric intramolecular hydrogen bond (XI) is best compatible with the spectral data. In the deuterium oxide solution of a high-ratio acid salt the carbonyl peak has shifted towards lower wave-numbers and the carboxylate peak towards higher wave-numbers. This behaviour lends further support to an intramolecularly hydrogen-bonded structure.



XI

EXPERIMENTAL

The acids investigated in this paper were prepared and purified as described previously [4]. The monopotassium salts were prepared by dissolving accurately weighed amounts of acids and potassium carbonate (analytical grade reagent) in water or aqueous

ethanol by slight warming, filtering, and then evaporating *in vacuo* at room temperature. The last traces of solvent were removed by warming at 110° for 3 h. The purity was checked by analysis for carbon and hydrogen. It is by no means certain that a solid salt spectrum actually refers to the hydrogen potassium salt, but these spectra are given for comparison. The discussion above is based on the salt spectra in methanolic solution, where only the ion species HA^- is present.

The infra-red spectra were recorded by a Perkin Elmer model 21 spectrophotometer equipped with a sodium chloride prism. The solid samples of the acids and salts were examined in pressed potassium bromide plates. The wave-numbers given in Table 1 are accurate within ± 5 cm^{-1}.

The author wishes to express his sincere gratitude to Professor E. Larsson for his kind interest in this work.

The infra-red part of this work was carried out at the Chemical Institute, University of Copenhagen. I am greatly indebted to Professor B. Bak for placing these facilities at my disposal and to Civilingenjör D. Christensen for valuable discussions.

Grants from *Kungliga Fysiografiska Sällskapet* and *Matematisk-naturvetenskapliga Fakulteten*, University of Lund, are gratefully acknowledged.

REFERENCES

1. Brown, H. C., McDaniel, D. H. and Häfliger, O. in Braude, E. A. and Nachod, F. C. *Determinations of Organic Structures by Physical Methods*, Academic Press Inc. New York 1955, p. 622.
2. Eberson, L. *Acta Chem. Scand.* **13** (1959) 203.
3. Eberson, L. *Acta Chem. Scand.* **13** (1959) 211.
4. Eberson, L. *Acta Chem. Scand.* **13** (1959) 40.
5. Newman, M. S. *Steric Effects in Organic Chemistry*, J. Wiley & Sons, New York 1956, p. 460.
6. Shahat, M. *Acta Cryst.* **5** (1952) 763.
7. Cardwell, H. M. E., Dunitz, J. D., and Orgel, L. E. *J. Chem. Soc.* **1953** 3740.
8. Schotte, L. and Rosenberg, A. *Arkiv Kemi* **7** (1954) 479.
9. Schotte, L. and Rosenberg, A. *Arkiv Kemi* **8** (1955) 551.
10. Schotte, L. *Arkiv Kemi* **9** (1956) 397.
11. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules*, Methuen & Co Ltd, London 1958.

Received December 24, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 236—240

The Metabolism of 7-Ketolithocholic Acid-24-^{14}C in the Rat *

Bile Acids and Steroids 70

BENGT SAMUELSSON**

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The metabolism of intraperitoneally administered 7-ketolithocholic acid-24-^{14}C has been studied in bile fistula rats. This acid is partly transformed into 3α,6β,7β-trihydroxycholanic acid (Acid I) and 3α,7β-dihydroxycholanic acid (ursodeoxycholic acid). The results are discussed in relation to the metabolism of chenodeoxycholic acid.

Studies of the metabolism of chenodeoxycholic acid in the rat liver have shown that this acid is transformed into two more polar acids [1], which have recently been identified by Doisy *et al.* as 3α, 6β, 7β-trihydroxycholanic acid (Acid I) and 3α,6β,7α-trihydroxycholanic acid (Acid II) [2,3]. In the former metabolite an inversion of the original 7α-hydroxylgroup has occurred. Experiments with 7β-tritiochenodeoxycholic acid have shown that the 7β-hydroxy-acid is formed with loss of the tritium labell [4], and it was thus of interest to study the metabolism of carboxylabelled 7-ketolithocholic acid in the rat liver. Similar studies have been reported by Doisy *et al.*[5], who found that 7-ketolithocholic acid, intragastrically administered to rats, was converted into chenodeoxycholic acid, ursodeoxycholic acid, Acid I and Acid II.

EXPERIMENTAL

7-Ketolithocholic acid-24-^{14}C. 66.9 mg of methyl chenodeoxycholate-24-^{14}C[6] were treated with ethylchlorocarbonate in dioxane and pyridine [7], and the 3α-cathylate oxidized with 26 mg of sodium dichromate in aqueous acetic acid for 16 h at 25°C. After saponification the product was chromatographed with phase system F[9], yielding 50.5 mg of 7-ketolithocholic acid — 24—^{14}C. Specific activity 1.85 μC/mg. M.p. 200—202°.

* A preliminary report of this work was read at the meeting of the Danish Biochemica Society in Copenhagen, June 1957 [11].

** Present address: Department of Chemistry, Karolinska Institutet, Stockholm 60, Sweden.

Table 1. Recrystallizations of the radioactive material contained in peak A in the chromatogram shown in Fig. 2.

Inactive bile acid added	Crystallizing solvent	Weight mg	c.p.m. per mg
3α,6β,7β-Trihydroxy-cholanic acid (Acid I)		50	410
	Acetone/light petroleum	40	415
	Methanol/water	31	390
	Acetone	23	395
	Methanol/ethyl acetate	19	410
	Acetone/light petroleum	12	400

0.8 mg of this acid was neutralized with sodium hydroxide and injected intraperitoneally in 0.9 % sodium chloride solution into each of two 200—250 g white male rats of the institute stock on which the common bile duct had been cannulated 24 h before the administration. The rats had free access to white bread and oats and 0.9 % sodium chloride solution. The bile was collected, hydrolyzed, extracted with ether and chromatographed on hydrophobic supercel as described earlier [8,9]. The following systems were used.

System	Moving phase	Stationary phase
C[8].	150 ml of methanol	15 ml of *iso*octanol
	150 ml of distilled water	15 ml of chloroform
F[9].	165 ml of methanol	45 ml of chloroform
	135 ml of distilled water	5 ml of heptane

4 ml of the stationary phase were used per 4.5 g hydrophobic supercel. All chromatograms were run at a constant temperature of +23°C.

Reference substances used for identification of the metabolites

3α,6β,7β-Trihydroxycholanic acid (Acid I) was isolated from rat bile after hydrolysis and chromatography with phase system C. 0.084 g was obtained from the bile of 10 rats, collected during the first 12 h after the bile duct cannulation, at which time the concentration of this acid is maximal. M. p. 226—227°, $[\alpha]_D^{23} = +62° \pm 2°$ (*c* 0.51, methanol). The m. p. was not depressed by synthetic Acid I, prepared through hydroxylation of methyl-3α-acetoxy-Δ^6-cholenate with osmium tetroxide [3].

Table 2. Recrystallizations of the radioactive material contained in peak B in the chromatogram shown in Fig. 1.

Inactive bile acid added	Crystallizing solvent	Weight mg	c.p.m. per mg
Ursodeoxycholic acid		60	690
	Acetic acid/water	50	710
	Ethyl acetate/light petroleum	44	730
	Acetone/water	35	700
	Methanol/water	22	725
	Ethyl acetate/light petroleum	14	705

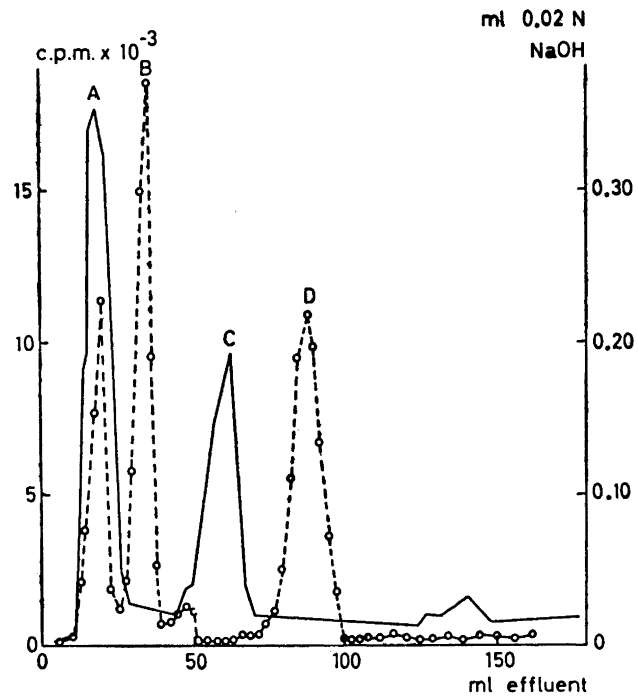


Fig. 1. Chromatography of acids from hydrolyzed bile, excreted during 24 h following intraperitoneal administration of 0.8 mg of 7-ketolithocholic acid —24—^{14}C. Column: 4.5 g hydrophobic supercel. Phases: Type F, see page 237. Solid line: Titration values. Broken line: Radioactivity.

Ursodeoxycholic acid was prepared through reduction of 7-ketolithocholic acid with sodium in *n*-propanol and chromatography with phase system F[10]. M. p. 201—202°, $[\alpha]_D^{23} = +56° \pm 2°$ (*c* 0.50, methanol).

RESULTS AND DISCUSSION

The carboxylabelled 7-ketolithocholic acid was injected intraperitoneally into two rats with bile fistulas and the bile was collected daily. The administered ^{14}C was almost completely recovered in the bile within the first 24 h. A chromatographic separation of the hydrolyzed bile with phase system F is shown in Fig. 1. The front peak (A) was rechromatographed with phase system C, Fig. 2. The activity is then eluted immediately after the band of inactive cholic acid. The radioactive material (105—140 ml) was diluted with inactive Acid I and the identity established by recrystallizations from different solvents and determination of the specific activity of the material from each recrystallization. (Table 1). About 2—3 % of the total radioactivity was eluted at about 50 ml, but the amount of this material was insufficient for identifica-

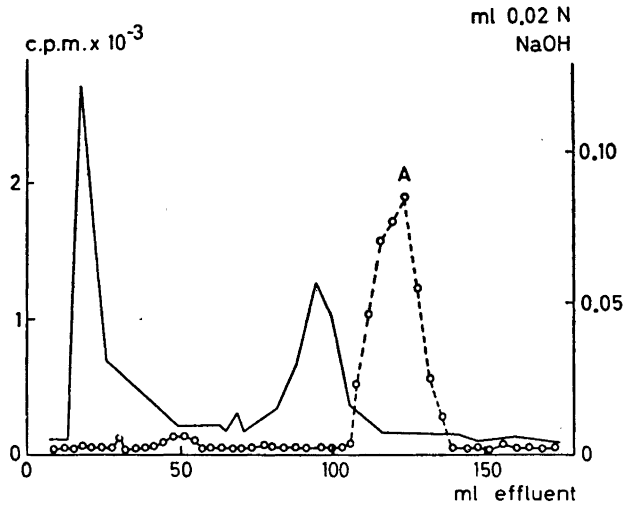


Fig. 2. Chromatography of peak A in the chromatogram shown in Fig. 1. Column: 4.5 g hydrophobic supercel. Phases: Type C, see page 237. Solid line: Titration values. Broken line: Radioactivity.

tion. With phase system C, Acid II (65—85 ml) is eluted before and Acid I (105—140 ml) after cholic acid (85—105 ml). The second peak (B) in the chromatogram shown in Fig. 1 was diluted with inactive ursodeoxycholic acid and recrystallized from different solvents. There was no depression of the specific activity (Table 2). Peak C in the chromatogram in Fig. 1 represents inactive chenodeoxycholic acid, normally present in the bile, and peak D consists of unchanged 7-ketolithocholic acid-24-^{14}C. This compound was also identified through isotope dilution. The composition of the labelled products was calculated from determinations of the total activity of the material and the ^{14}C contained in peak A, B and D. The results from two rats are shown in Table 3.

The ketogroup of 7-ketolithocholic acid is thus reduced in the liver to a 7β-hydroxylgroup and any formation of the 7α-epimer could not be demon-

Table 3. Percentage composition of the labelled products excreted in the bile during 24 hours after intraperitoneal administration of 7-ketolithocholic acid-24-^{14}C to two rats with bile fistulas.

Compound	Per cent		
	Rat I	Rat II	Average
3α,6β,7β-Trihydroxycholanic acid	24.2	29.3	26.8
Ursodeoxycholic acid	38.1	33.4	35.8
7-Ketolithocholic acid	32.8	33.7	33.3
Total	95.1	96.4	95.9

strated. The pathway for the formation of 3α,6β,7β-trihydroxycholanic acid (Acid I) may either be an initial hydroxylation of 7-ketolithocholic acid in the 6β-position and a later reduction of the 7-ketone, or a 6-hydroxylation of ursodeoxycholic acid. The facts that 7-ketolithocholic acid and ursodeoxycholic acid are only formed in small amounts in the metabolism of chenodeoxycholic acid, and that they are only partly metabolized, when injected intraperitoneally into a bile fistula rat [4], suggest that these acids constitute only a minor pathway in the formation of Acid I from chenodeoxycholic acid. Furthermore, evidence for the direct conversion of Acid II into Acid I will be presented in a subsequent publication [4].

This work is part of an investigation supported by the *Medical Faculty of the University of Lund* and by a research grant (H 2842) to Prof. S. Bergström from the *National Institutes of Health, United States Public Health Service.*

REFERENCES

1. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **8** (1954) 611.
2. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **226** (1957) 667.
3. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 573.
4. Samuelsson, B. *To be published in Acta Chem. Scand.*
5. Mahowald, T. A., Mao Wu Yin, Matschiner, J. T., Hsia, S. L., Doisy, E. A., Jr., Elliott, W. H. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 581.
6. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
7. Fieser, L. F., Herz, J. E., Klohs, M. W., Romero, M. A. and Utne, T. *J. Am. Chem. Soc.* **74** (1952) 3309.
8. Bergström, S. and Norman, A. *Proc. Soc. Exptl. Biol. Med.* **83** (1953) 71.
9. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
10. Samuelsson, B. *To be published in Acta Chem. Scand.*
11. Bergström, S., Lindstedt, S., Samuelsson, B. and Corey, E. J. *Acta Chem. Scand.* **11** (1957) 1096.

Received October 27, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 241—248

Low Temperature Heat Capacities and Thermodynamic Properties of the Iron Selenides $Fe_{1.04}Se$, Fe_7Se_8 and Fe_3Se_4 from 5 to 350°K

FREDRIK GRØNVOLD

Kjemisk Institutt A, Universitetet i Oslo, Blindern, Norway

and

EDGAR F. WESTRUM, JR.

Department of Chemistry, University of Michigan, Ann Arbor, Michigan, USA

Heat capacities of three iron-selenide phases representing the tetragonal PbO-like structure with composition $Fe_{1.04}Se$ and the hexagonal and monoclinic NiAs-like structures with compositions Fe_7Se_8 and Fe_3Se_4, respectively, were measured in the range 5 to 350°K. Heat capacity values, enthalpy, and entropy increments are tabulated at different temperatures. At 298.15°K the entropies $(S°-S_0^\circ)$ are 8.437 eu for $Fe_{0.5103}Se_{0.4897}$ (*i. e.*, $Fe_{1.04}Se$), 9.780 eu for $Fe_{0.4667}Se_{0.5333}$ (*i. e.*, Fe_7Se_8), and 9.554 eu for $Fe_{0.4286}Se_{0.5714}$ (*i. e.*, Fe_3Se_4). A λ-type transition with a maximum at 34°C is found in Fe_3Se_4, associated with an order-disorder process of supposedly ferrimagnetic to paramagnetic nature. The entropy increment of transition is about 2 eu per mole of Fe_3Se_4.

It has been shown by Hägg and Kindström [1] that an intermediate phase with composition FeSe and a tetragonal structure of the PbO-type exists in the iron-selenium system below a temperature between 600 and 300°C. In addition, another intermediate phase with homogeneity range from about 50 to 57.5 atomic percent selenium exists in samples quenched from 600°C. This phase has a NiAs-like structure which is hexagonal only in the iron-rich range and becomes monoclinic above 54 atomic percent selenium. By combining X-ray and density data, Hägg and Kindström concluded that the solid solution takes place by subtraction of iron atoms with increasing selenium content. Thus, the structure deforms when about 20 % of the iron lattice sites are unoccupied. This extended solid solution exists only at high temperatures and the iron-rich selenide with PbO-type structure and composition $Fe_{1.04}Se$ is formed below about 490°C according to Grønvold and Haraldsen [2].

The iron-rich composition limit of the $Fe_{1-x}Se$-phase goes towards a higher selenium content as the temperature is lowered, and at about 350°C the limit is at $Fe_{0.88}Se$ or Fe_7Se_8. Furthermore, a two-phase region is observed in the range $Fe_{0.87}Se$ to $Fe_{0.80}Se$.

As the temperature is lowered another phenomenon is also observed, *i.e.*, the ordering of the vacancies after the iron atoms. Bertaut [3] has studied the ordering process in Fe_7S_8 with NiAs-like structure and found that the decrease in internal energy for an ordered arrangement of holes compared to a random distribution is so large that all ionic defect structures are expected to have ordered distribution of the holes. This picture has to be modified for phases like the FeSe-phase which are not typical ionic compounds. It is apparent that the concentration of holes must exceed a certain value for the ordering — and thus the superstructure formation — to take place at a given temperature. With an increasing number of holes another superstructure might be formed, and so on. In case of the FeSe-phase two such superstructure phases have been established by Okazaki and Hirakawa [4], one in the composition range Fe_7Se_8 to Fe_6Se_7, the other in the range Fe_4Se_5 to Fe_3Se_4.*

In addition to the phases mentioned, an iron diselenide also exists at lower temperatures. It was found [5] in samples heat-treated for 4 months at 250°C

In respect to their magnetic properties, the iron selenides are very interesting [6]. Ferrimagnetism is associated with the FeSe-phase, with a Néel temperature at about 150°C for samples in the range $Fe_{1.00}Se$ to $Fe_{0.79}Se$. By further increase in selenium content the Néel point decreases rapidly and reaches 30°C by $Fe_{0.74}Se$. The magnetization intensity at —195°C has its maximum at $Fe_{0.89}Se$, confirming the presence of only one phase in this region. The sharp and almost linear drop on the iron-rich side can be interpreted as due to a two phase region with a paramagnetic phase with composition $Fe_{1.04}Se$, and the curved region from $Fe_{0.79}Se$ to $Fe_{0.74}Se$ as the homogeneity range of the Fe_3Se_4-phase.

More recent work by Hirone and Chiba [7] by X-ray, thermal and magnetic methods confirms the non-existence of stoichiometric FeSe and places the selenium-rich composition limit of the tetragonal phase at $Fe_{1.05}Se$ and the iron-rich limit of the hexagonal phase at $Fe_{0.88}Se$ at temperatures below 350°C. They also found ferrimagnetism associated with the hexagonal phase and a spontaneous magnetization of 0.20 Bohr magnetons. The Néel point is at 174°C and increases gradually to 192°C with increasing selenium content, and then rapidly falls to 40°C at $Fe_{0.77}Se$.

The magnetic properties of single crystals of Fe_7Se_8 and Fe_3Se_4 were studied by Hirakawa [8] using a torque magnetometer. For Fe_7Se_8 the direction of easy magnetization is in the (00.) plane at room temperature, but changes into the [001] direction below about 150°K. No such change

* Note added in proof: Okazaki (*J. Phys. Soc. Japan* **14** (1959) 112) has just reported a triclinic superstructure for Fe_7Se_8 in samples annealed at 290°C and slowly cooled. The present authors have observed a similarly deformed structure on Fe_7Se_8 samples subjected to annealing after having been in contact with the atmosphere. The sample studied here did not show any line splitting characteristic of a monoclinic or triclinic cell, and its superstructure is probably based upon an orthorhombic cell with doubled *c*-axis.

Table 1. Heat capacities of iron selenides, cal "mole"$^{-1}$ °K^{-1}.

T,°K	C_p	T,°K	C_p	T,°K	C_p
		$Fe_{1.042}Se$			
		(formula weight $Fe_{0.5103}Se_{0.4897}$ = 67.17 g)			
Series I		289.71	6.628	15.77	0.1864
51.58	1.639	299.16	6.692	16.89	0.2188
56.71	1.872	308.73	6.754	18.07	0.2552
63.13	2.167	318.32	6.817	19.29	0.2957
69.08	2.656	327.94	6.873	20.65	0.3412
74.43	2.805	337.51	6.928	22.30	0.3998
80.50	2.919	347.23	6.982	24.17	0.4683
87.17	3.185			26.26	0.5472
94.34	3.746	Series II		29.47	0.6758
101.47	3.686	7.02	0.023	32.30	0.7919
109.61	3.951	7.77	0.030	35.32	0.9188
118.34	4.210	8.48	0.039	38.30	1.047
127.34	4.452	9.31	0.049	41.49	1.185
136.29	4.668	10.15	0.061	45.04	1.342
144.93	4.870	11.03	0.074	48.40	1.495
154.15	5.055	11.97	0.092	54.52	1.781
163.86	5.238			60.40	2.017
173.52	5.402	Series III		64.63	2.233
182.90	5.541	5.60	0.012	67.59	2.366
192.20	5.687	6.38	0.016	70.56	2.489
201.68	5.804	7.25	0.024	73.53	2.625
211.17	5.910	8.06	0.034	76.75	2.759
223.65	6.055	8.91	0.044	80.36	2.914
232.96	6.130	9.76	0.055	83.94	3.057
242.36	6.247	10.67	0.069	87.50	3.198
251.68	6.332	11.62	0.086	91.11	3.328
261.19	6.408	12.63	0.1040	94.92	3.462
270.80	6.487	13.65	0.1274	99.11	3.604
280.33	6.559	14.67	0.1543	103.51	3.751
		Fe_7Se_8			
		(formula weight $Fe_{0.4667}Se_{0.5333}$ = 68.18 g)			
5.91	0.016	39.01	1.536	166.24	5.687
6.86	0.023	43.23	1.803	176.54	5.818
7.92	0.034	48.46	2.128	187.24	5.948
8.90	0.050	53.75	2.438	197.70	6.062
9.95	0.068	59.40	2.748	207.95	6.168
11.11	0.091	65.61	3.063	218.00	6.272
12.35	0.1220	68.25	3.186	228.16	6.369
13.58	0.1585	74.25	3.448	238.43	6.474
14.84	0.1994	81.67	3.766	248.51	6.570
16.13	0.2459	89.51	4.058	258.43	6.661
17.45	0.2990	97.40	4.307	268.37	6.757
18.84	0.3582	105.92	4.551	278.34	6.852
20.34	0.4296	114.67	4.773	288.43	6.951
22.02	0.5117	123.20	5.036	298.72	7.050
23.91	0.6108	131.46	5.133	308.98	7.148
26.14	0.7353	139.76	5.287	319.12	7.253
28.86	0.8944	148.64	5.435	331.24	7.389
32.66	1.129	152.28	5.495	344.70	7.544
35.41	1.305	158.23	5.580		

Table 1 (continued)

T,°K	C_p	T,°K	C_p	T,°K	C_p
		Fe_3Se_4			
		(formula weight $Fe_{0.4286}Se_{0.5714}$ = 69.06 g)			
Series I		327.01	6.913	53.74	2.165
		337.29	6.890	59.00	2.472
81.54	3.621	347.55	6.898	64.70	2.795
88.16	3.899			70.75	3.103
95.16	4.152	Series II		77.06	3.405
102.57	4.397			83.85	3.713
110.73	4.651	5.57	0.009	274.20	7.149
119.66	4.892	6.70	0.014	283.64	7.269
129.18	5.128	7.58	0.019	291.43	7.392
138.85	5.331	8.53	0.025	297.63	7.513
148.51	5.545	9.62	0.034	301.85	7.587
159.09	5.731	10.68	0.045	304.07	7.629
169.51	5.907	11.72	0.058	305.72	7.666
180.21	6.064	12.77	0.0737	306.83	7.678
190.88	6.212	13.85	0.0922	307.93	7.647
194.78	6.266	14.99	0.1171	309.03	7.608
204.34	6.373	16.23	0.1467	310.14	7.552
213.76	6.501	17.62	0.1856	311.24	7.446
223.01	6.600	19.11	0.2333	312.33	7.342
232.23	6.699	20.69	0.2906	313.42	7.228
241.53	6.804	22.44	0.3607	314.52	7.145
250.85	6.903	24.45	0.4500	316.19	7.057
260.04	6.996	26.82	0.5638	318.42	6.992
269.14	7.099	29.55	0.7079	320.66	6.956
278.38	7.212	32.71	0.8847	322.91	6.936
287.76	7.349	36.30	1.097	325.16	6.920
297.22	7.524	40.51	1.353	329.10	6.903
306.81	7.612	44.84	1.620	336.77	6.893
316.74	7.088	49.04	1.881	346.44	6.896

was observed for Fe_3Se_4. The saturation magnetizations at liquid air temperature for Fe_7Se_8 and Fe_3Se_4 were 68 and 80 gauss cm^{-3}, respectively.

The structural and magnetic properties of the iron selenides are thus rather sensitive to changes in temperature, and in order to get a further understanding of the causes of these changes, a thermodynamic study of the iron-selenium system is being carried out. In this paper will be reported the results of heat-capacity measurements on three single-phase samples, one representing the tetragonal FeSe-phase with composition $Fe_{1.042}Se$, and two others representing the $Fe_{1-x}Se$ phase with NiAs-like structure and compositions Fe_7Se_8 and Fe_3Se_4, or $Fe_{0.875}Se$ and $Fe_{0.750}Se$, respectively.

EXPERIMENTAL

A. Preparation of the samples. The iron selenides were synthesized from high purity iron and selenium. "Ferrum reductum pro analysi" from Merck was reduced with dry purified hydrogen gas at 1 000°C until constant weight was attained. A spectrographic analysis showed the presence of about 0.01 % Ni and Si and about 0.001 % Mn. The high-purity selenium was a gift from Bolidens Gruvaktiebolag, and contained these

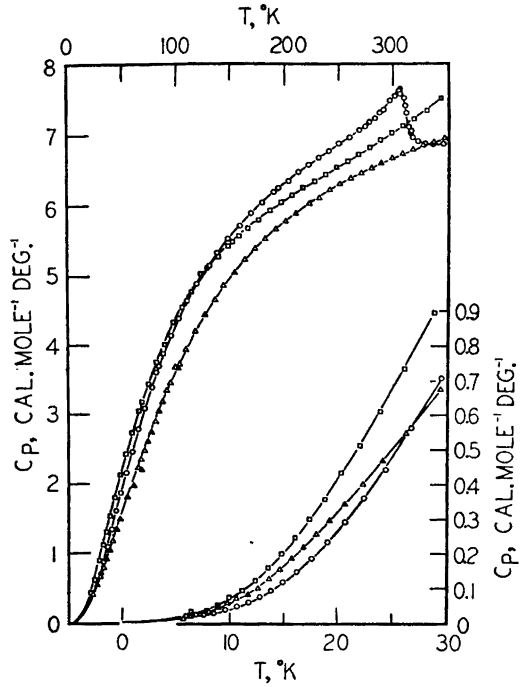


Fig. 1. Heat capacities of iron selenides on a gram formula weight basis: △ represent $Fe_{0.5103}Se_{0.4897}$, □ represent $Fe_{0.4667}Se_{0.5333}$ and O represent $Fe_{0.4286}Se_{0.5714}$.

impurities (in ppm) according to their analysis: Cl(2), Fe(0.8), K(0.3), Na(0.4), non-volatile matter (12). The following elements were not detected (the numbers indicate the sensitivity limit in ppm): Ag(0.03), Al(0.3), As(1), Bi(0.1), Ca(1), Cr(0.3), Cu(0.1), Hg(0.5), Mg(0.3), Mn(0.1), Ni(0.3), Pb(0.3), S(5), Sb(1), Si(1), Sn(0.3), Te(1), Zn(1).

Accurately-weighed quantities of the elements corresponding to the compositions $FeSe_{0.96}$, Fe_7Se_8 and Fe_3Se_4 were heated in evacuated and sealed silica tubes. Because of the transitions in the solid selenides which sometimes causes cracking of the silica tubes on cooling, the silica tubes were put into larger silica tubes which were also evacuated and sealed. The samples were fused for 4 h at 1 050°C in an electric muffle furnace, cooled to room temperature and fragmented under dry nitrogen in an agate mortar. They were then homogenized at 350°C for 30 days and cooled down to room temperature over another 30 days. By means of X-ray powder photographs the samples were proved to be of identical nature to those obtained earlier [2].

B. *Cryostat and calorimeter.* The Mark I cryostat and technique employed for low-temperature adiabatic calorimetry are being described elsewhere [9]. The copper calorimeter (laboratory designation W-7) has a capacity of 40.33 cm^3; it is gold-plated inside and out and has only four vanes. A separate series of measurements were carried out to determine the heat capacity of the empty calorimeter, using the same thermometer and heater and exactly the same amount of indium-tin solder for sealing and Apiezon-T grease for thermal contact with the thermometer and heater. It represented from 11 to 35 % of the total heat capacity observed.

Temperatures were measured with a capsule-type platinum resistance thermometer (laboratory designation A-3) contained in an axial well in the calorimeter. A 150-ohm constantan heater was wound on a cylindrical copper tube surrounding the resistance thermometer. The thermometer has been calibrated by the National Bureau of Standards,

and the temperatures are judged to correspond with the thermodynamic temperature scale within 0.03°K from 10 to 90°K and within 0.04°K from 90 to 350°K. Precision is considerably better, and the temperature increments are probably correct to a millidegree after corrections for quasi-adiabatic drift.

The thermometer resistance and the power input were measured with a calibrated White double-potentiometer, calibrated resistances and a calibrated standard cell. An electric timer, operated by a calibrated tuning fork and amplifier, was automatically started at the beginning of the heating period and stopped at the end. The calorimeter was loaded with sample and evacuated, and helium was added at 4 cm Hg pressure at about 25°C to provide thermal contact between sample and calorimeter. It was then sealed, placed in the cryostat and cooled. The mass of sample used was 107.795 g $Fe_{1.04}Se$, 118.961 g Fe_7Se_8 and 132.015 g Fe_3Se_4.

RESULTS

The heat-capacity determinations are listed in Table 1 in chronological order, and expressed in terms of the thermochemical calorie, defined as 4.1840 abs joules. The ice point is taken to be 273.15°K, and the atomic weights of iron and selenium as 55.85 and 78.96, respectively. The data are expressed in terms of one mole of mixture ("mole"), equivalent to the gram formula weight of Fe_ySe_{1-y}; *i.e.*, 67.17 g $Fe_{1.04}Se$, 68.18 g Fe_7Se_8 and 69.06 g Fe_3Se_4. An analytically-determined curvature correction for the finite temperature increments was applied to the observed values of $\Delta H/\Delta T$. The approximate temperature increments can usually be inferred from the adjacent mean temperatures in Table 1.

The heat-capacity *versus* temperature curves are shown in Fig. 1. For $Fe_{1.04}Se$ it has the usual sigmoid shape up to 350°K, while for Fe_7Se_8 the curve shows pretransition effects and for Fe_3Se_4 a transition actually takes place at about 307°K or 34°C.

Values of C_p, $S°—S_0°$ and $(H°—H_0°)/T$ for the three samples are listed in Table 2 at selected temperatures. The reason for not giving the free-energy function is the uncertainty about complete ordering. The enthalpy and entropy increments were computed by numerical integration, using graphically interpolated values of heat capacity. The heat-capacity values are considered to have a probable error of 0.1 % above 25°K and 1 % at 10°K. The effects of nuclear spin and of isotope mixing are not included in the entropy function. The estimated probable error in the entropy and enthalpy functions is 0.1 % above 100°K, but some of the values are given to an additional digit because of their significance when the entropies or enthalpies at different temperatures or compositions are compared.

The entropy increment associated with the λ-transition in Fe_3Se_4 at 34°C is about 2 cal $mole^{-1}$ deg^{-1}. The transition temperature corresponds to that found [6,7] for the change from paramagnetism to ferrimagnetism in $Fe_{0.74}Se$ and the nature of the transition seems thus established.

It is known from X-ray work [4] that the structure of Fe_3Se_4 has an ordered distribution of vacancies, just as Fe_7S_8[3], Cr_3S_4[10] and $Ni_{0.80}Se$[11], but less is known about the valence states of the atoms. According to the electrostatic treatment of Yosida [12], Bertaut [3] and Lotgering [13], the distribution of Fe^{2+}

Table 2. Thermodynamic properties of iron selenides, cal "mole"$^{-1}$ °K^{-1}.

	$Fe_{1.042}Se$			Fe_7Se_8			Fe_3Se_4		
	(formula weight $Fe_{0.5103}Se_{0.4897}$ = 67.17 g)			(formula weight $Fe_{0.4667}Se_{0.5333}$ = 68.18 g)			(formula weight $Fe_{0.4286}Se_{0.5714}$ = 69.06 g)		
T, °K	C_p	$S°-S_0°$	$\frac{H°-H_0°}{T}$	C_p	$S°-S_0°$	$\frac{H°-H_0°}{T}$	C_p	$S°-S_0°$	$\frac{H°-H_0°}{T}$
10	0.058	0.0195	0.0146	0.069	0.0230	0.0172	0.037	0.0125	0.0093
15	0.164	0.0606	0.0448	0.205	0.0739	0.0549	0.117	0.0405	0.0302
20	0.319	0.1284	0.0934	0.412	0.1597	0.1170	0.265	0.0928	0.0690
25	0.500	0.2186	0.1563	0.671	0.2787	0.2012	0.475	0.1736	0.1284
30	0.698	0.3269	0.2297	0.963	0.4264	0.3035	0.733	0.2824	0.2072
35	0.905	0.4499	0.3113	1.278	0.5984	0.4200	1.021	0.4167	0.3025
40	1.120	0.5849	0.3990	1.600	0.7901	0.5474	1.322	0.5725	0.4110
45	1.341	0.7295	0.4913	1.914	0.9967	0.6818	1.631	0.7460	0.5294
50	1.567	0.8825	0.5876	2.221	1.214	0.8205	1.940	0.9339	0.6550
60	2.023	1.208	0.7888	2.781	1.670	1.1015	2.530	1.340	0.9190
70	2.473	1.554	0.9975	3.266	2.136	1.3769	3.065	1.771	1.1878
80	2.897	1.912	1.2085	3.699	2.601	1.6404	3.550	2.213	1.4532
90	3.289	2.277	1.4185	4.071	3.059	1.8906	3.968	2.656	1.7102
100	3.636	2.642	1.6231	4.385	3.504	2.1246	4.319	3.092	1.9540
110	3.962	3.004	1.8211	4.658	3.935	2.3427	4.626	3.519	2.1831
120	4.253	3.361	2.0118	4.896	4.351	2.5457	4.901	3.933	2.3981
130	4.516	3.712	2.1945	5.105	4.751	2.7347	5.149	4.336	2.6004
140	4.755	4.056	2.3689	5.291	5.136	2.9107	5.372	4.726	2.7905
150	4.973	4.391	2.5354	5.457	5.507	3.0750	5.560	5.103	2.9694
160	5.167	4.718	2.6938	5.602	5.864	3.2286	5.750	5.468	3.1377
170	5.341	5.037	2.8445	5.736	6.208	3.3723	5.913	5.822	3.2963
180	5.499	5.347	2.9876	5.860	6.539	3.5071	6.060	6.164	3.4458
190	5.642	5.648	3.1236	5.975	6.859	3.6340	6.201	6.496	3.5871
200	5.775	5.941	3.2528	6.084	7.169	3.7538	6.333	6.817	3.7211
210	5.898	6.226	3.3759	6.190	7.468	3.8673	6.456	7.129	3.8485
220	6.013	6.503	3.4932	6.292	7.758	3.9752	6.571	7.432	3.9696
230	6.123	6.772	3.6051	6.392	8.040	4.0781	6.682	7.727	4.0851
240	6.226	7.035	3.7122	6.488	8.314	4.1765	6.788	8.013	4.1956
250	6.316	7.291	3.8146	6.583	8.581	4.2708	6.894	8.293	4.3014
260	6.400	7.541	3.9124	6.677	8.841	4.3616	7.002	8.565	4.4032
270	6.479	7.784	4.0061	6.772	9.095	4.4491	7.106	8.831	4.5014
280	6.556	8.021	4.0958	6.869	9.343	4.5338	7.222	9.092	4.5964
290	6.629	8.252	4.1819	6.967	9.586	4.6160	7.368	9.348	4.6894
300	6.698	8.478	4.2646	7.062	9.823	4.6959	7.551	9.600	4.7816
350	6.997	9.534	4.6344	7.603	10.951	5.0713	6.897	10.697	5.1229
273.15	6.504	7.859	4.0348	6.814	9.174	4.4762	7.138	8.914	4.5318
298.15	6.685	8.437	4.2497	7.045	9.780	4.6814	7.515	9.554	4.7645

and Fe^{3+} ions in $Fe_{1-x}Se$ in the planes I and II normal to the c-axis of the NiAs-like structure is (when ϱ designates the fraction of Fe^{3+} ions in plane I, and all vacancies are in plane II):

Plane I	Plane II
$(\frac{1}{2}-2x\,\varrho)Fe^{2+} + 2x\varrho Fe^{3+}$	$(\frac{1}{2}-x-2x[1-\varrho])Fe^{2+} + 2x(1-\varrho)Fe^{3+}$

On the basis of spin-only magnetism the magnetic moment of Fe_7Se_8 resulting from antiparallel spin arrangement between lattice I and II is calculated to vary between 0.29 and 0.86 Bohr magnetons per iron atom as ϱ goes from 0 to 1, *i.e.*, as the number of Fe^{3+} ions in lattice I increases from zero to its maximum value. By assuming all Fe^{3+} ions and vacancies to be located in the same plane, the experimentally found moment of 0.2 Bohr magnetons is reasonably well explained.

For Fe_3Se_4 the same model predicts magnetic moments of 1.33 to 2.0 Bohr magnetons per iron atom as ϱ goes from its minimum value 0.5 (corresponding to equipartition of Fe^{3+} ions between lattice I and II) to its maximum value 1.0, when all Fe^{3+} ions are in lattice I. The magnetic measurements [6] are not in agreement with this, however, as the observed moment of Fe_3Se_4 is only 0.1 Bohr magneton. This shows that strong interactions are present between the paramagnetic ions and their neighbors so that the electrostatic description has to be modified.

Acknowledgment. The authors thank Bolidens Gruvaktiebolag for a generous supply of selenium and Roger Berg and Paul (Te Fu) Chang for assistance in the calorimetric measurements. One of us (F. Grønvold) expresses his appreciation to the *Nansen Foundation* for assistance. The partial support of the *Division of Research of the U. S. Atomic Energy Commission* is gratefully acknowledged.

REFERENCES

1. Hägg, G. and Kindström, A.-L. *Z. physik. Chem.* **B22** (1933) 453.
2. Haraldsen, H. and Grønvold, F. *Tidsskr. Kjemi, Bergvesen, Met.* **10** (1944) 98; *Structure Reports for 1942—1944*, **9** (1955) 97; and unpublished results.
3. Bertaut, F. *Acta Cryst.* **6** (1953) 557; *J. phys. radium* **15** (1954) 77S.
4. Okazaki, A. and Hirakawa, K. *J. Phys. Soc. Japan* **11** (1956) 930.
5. Tengnér, S. *Z. anorg. u. allgem. Chem.* **239** (1938) 126.
6. Hirone, T., Maeda, S. and Tsuya, N. *J. Phys. Soc. Japan* **9** (1954) 496.
7. Hirone, T. and Chiba, S. *J. Phys. Soc. Japan* **11** (1956) 666.
8. Hirakawa, K. *J. Phys. Soc. Japan* **12** (1957) 929.
9. Westrum, E. F. Jr. and Beale, A. F. Jr. *J. Am. Chem. Soc. (To be published).*
10. Jellinek, F. *Diss.*, Utrecht 1957; *Acta Cryst.* **10** (1957) 620.
11. Grønvold, F. *Paper presented at the 2. Nordiske Strukturmøte*, Oslo, 1955.
12. Yosida, K. *Progr. Theor. Phys. Japan* **6** (1951) 356.
13. Lotgering, F. K. *Z. physik. Chem.* **N.F. 4** (1955) 238; *Philips Research Reports* **11** (1956) 190.

Received September 30, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 249–267

Electroörganic Preparations

VI. Reduction of Compounds Containing the Azomethine group

HENNING LUND

Leo Pharmaceutical Products, Copenhagen, Denmark

The polarographic reduction in buffered medium of a number of ketimines, aniles, oximes, azines, phenylhydrazones, and semicarbazones have been investigated by use of controlled potential reductions. The number of electrons participating in the electrode reactions and the reduction products are reported and some indications of a probable reduction path are presented.

Compounds containing the azomethine group >C=N— have mostly been of interest for polarography as a means of determining carbonyl compounds by converting them to a suitable azomethine derivative. Thus aliphatic ketones not reducible as such in buffered solutions can be determined as the ketimines [1,2] or Girard hydrazones [3–5]. Other derivatives as hydrazones [6], semicarbazones [7], and oximes [8–11] have been investigated. Of these especially the oximes have recieved attention from various workers.

The polarographic behaviour of most of the compounds containing a carbon-nitrogen double bond have some features in common. Of the molecules in the equation

$$A + H^+ \rightleftharpoons (AH)^+ \quad (1)$$

the $(AH)^+$ is reducible at a less negative potential than A and when $(AH)^+$ is removed from the reaction mixture by the reduction the equilibrium is forced to the right. The height of the polarographic wave is, therefore, in a certain pH-region determined by the rate of the recombination (1) of the molecules with the hydrogen ions [12]. The phenomenon of recombination is found in the reduction of other compounds [13,14], but the special feature of many of the azomethine compounds is that in neutral and weakly alkaline solution the wave height at a certain pH is not constant but decreases at more negative potentials until another electrode reaction takes place. The phenomenon has been explained by the assumption that a change in the structure of the carbon-nitrogen double bond at the surface of the electrode caused by the lowering of the electrode potential lowers the rate of the recombination. As the subject

of the recombination in the polarography of oximes have been treated elsewhere [9,10] only these introductory remarks have seemed warranted.

The purpose of the present work has been to investigate the electrode reactions which occur during the polarographic reduction of compounds containing a carbon-nitrogen double bond, and to this end a polarographic investigation of some aniles, oximes, amidoximes, azines, phenylhydrazones, and semicarbazones has been made together with preparative reductions at controlled potential of representatives from each group.

As the polarographic behaviour, especially the half-wave potentials, of the compounds is dependent upon the medium used all investigations were performed in 40 % alcohol containing 1 M potassium chloride as supporting electrolyte and the reducible compound in a concentration of 20 μg/ml. In order to facilitate the comparison of the compounds the limiting currents are reported for the concentration 10^{-3} M, which is calculated from the experimental data by assuming a linear dependence of limiting current on concentration.

Ketimines

Ketones as acetone and *cyclo*hexanone give rise to a polarographic wave in a medium containing a primary amine or ammonia and a salt of the amine due to the reduction of the ketimine [1,2] formed in the equilibrium:

$$RR'C{=}O + H_2NR'' \rightleftharpoons RR'C{=}NR'' + H_2O$$

Controlled potential reduction of a solution of *cyclo*hexanone in a large excess of methylamine and methylamine hydrochloride showed that the reduction consumed two electrons per molecule *cyclo*hexanone and the product was the expected N-methyl *cyclo*hexyl amine.

$$RR'C{=}NCH_3 + 2e^- + 2H_2O \rightarrow RR'CH{-}NHCH_3 + 2OH^-$$

Aniles

Only a few compounds of this class were investigated. Benzalaniline and salicylaldehyde anile were found to be too unstable both in alkaline and acid solution for a polarographic investigation. Benzophenone anile is rather unstable below pH 5, but at higher pH-values the stability is sufficiently high to allow both a polarographic investigation and a preparative reduction.

Table 1. Limiting currents in μA and half-wave potentials *vs* S.C.E. of benzophenone anile at different pH-values. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	5.10		6.25		7.40		9.85		10.75		13	
	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
1. wave	2.2	0.82	2.4	0.89	2.4	0.96	4.8 [a]	1.10	4.8	1.13	2.4	1.21
2. wave	2.6	1.08	2.4	1.07	2.2	1.07					2.2	1.58

[a] slight tendency to minimum.

The polarographic behaviour of benzophenone anile is similar to that of benzophenone in that two waves are found at lower pH values and only one at higher values (Table 1). As for benzophenone the first wave is pH-dependent, whereas the second one is not, and the combined wave is less dependent upon pH than the first one. The half-wave potentials of the anile are, however, about 0.3 V more positive than those of benzophenone, and whereas the two waves of benzophenone merge about pH 5 the waves of the anile merge about pH 9. Furthermore, there are found two waves in strongly alkaline solution for the anile, which is not the case for benzophenone. In the pH-region 9—12 a small minimum is found about —1.5 V; this is similar to that found for oximes and hydrazones.

Controlled potential reduction at pH 10 showed that the electrode reaction was a two electron reduction and the isolated product was benzhydrylaniline.

$$(C_6H_5)_2C{=}NC_6H_5 + 2e^- + 2H_2O \rightarrow (C_6H_5)_2CH{-}NHC_6H_5 + 2OH^-$$

Attempts to reduce the anile at the plateau of the first wave at pH 6 were unsuccessful due to the difficulties of obtaining sufficient buffering in a medium in which the anile was appreciably soluble.

Oximes

Unconjugated oximes. The oximes of the following unconjugated carbonyl compounds were investigated polarographically: *Iso*butyraldehyde, phenylacetaldehyde, and benzylacetone (Table 2). They all show a single pH-dependent wave in acid solution and the height of the wave decreases at higher pH-values and disappears in alkaline solution. The polarographic wave shows, however, no minimum at higher pH-values.

Preparative reductions of *iso*butyraldoxime and phenylacetaldoxime showed that the electrode reaction was a four electron reduction, and from the latter reduction phenylethylamine was isolated.

$$C_6H_5CH_2CH{=}NOH + H^+ \rightleftharpoons (C_6H_5CH_2CH{=}NOH)H^+$$
$$+ 4e^- + 4H^+ \rightarrow C_6H_5CH_2CH_2NH_3^+ + H_2O$$

α,β-Unsaturated oximes. Oximes of the following α,β-unsaturated carbonyl compounds were investigated polarographically: Crotonaldehyde, mesityloxide, carvone, testosterone propionate, cinnamaldehyde, and benzalacetone. The polarographic behaviour of these oximes were rather different (Table 2).

Crotonaldoxime shows in acid solution a single pH-dependent wave with the height determined by the rate of the recombination. About pH 8 the first wave disappears without showing minima and a second wave of nearly the same height as the wave in strongly acid solution appears. The half-wave potentials of this wave is approximately independent of pH. The wave is probably due to the reduction of the unprotonated crotonaldoxime. This wave disappears in strongly alkaline solution which might be explained by the formation of the more difficultly reducible oxime anion R—NO^-. Controlled potential reduction in weakly acid solution showed the electrode reaction to be a four electron reduction.

Table 2. Limiting currents in μA and half-wave potentials *vs* S.C.E. of some oximes and N-benzylhydroxylamine. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	1.05		3.55		5.20		7.40		9.80		13	
Compound	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
*Iso*butyraldoxime	9.9	1.08	10.0	1.27	2.6	1.37	—		—		—	
Phenylacetaldoxime	9.8	0.92	9.3	1.08	2.8	1.28	—		—		—	
Benzylacetoneoxime	[d]		7.4	1.34	[d]		—		—		—	
Croton-	12.3	0.90	12.3	1.06	10.5	1.17	1.2	1.26	—		—	
aldoxime									10.3	1.72	—	
Mesityloxide												
oxime	10.3	1.01	10.3	1.16	8.3	1.25	0.9	1.33	—		—	
Carvone	5.5	0.84	10.5	1.05	9.5[a]	1.16	2.8[a]	1.25	—		—	
oxime	5.7	0.96										
Testosterone												
propionate	2.9	0.75	2.7	0.89	3.1	1.04	1.0	1.12	0.2	1.25	—	
oxime	3.1	0.99	2.9	1.09	3.9	1.22	4.0[a]	1.22				
anti-Cinnam-												
aldoxime	10.5	0.69	10.7	0.86	10.7	1.00	[b]		8.3	1.40	6.7	1.57
syn-Cinnam-	10.7	0.70	10.7	0.87	9.8	1.00	[b]		5.7	1.49	—	
aldoxime					1.9	1.40			3.8	1.76	0.6	1.60
Benzalacetone	9.5	0.75	9.3	0.92	9.0	1.03	[b]		1.7	1.42	—	
oxime			3.4	1.29	4.0	1.32			6.4	1.56	5.0	1.65
anti-Benz-	[c]	0.7	9.2	0.92	6.2	1.08	0.9	1.20	—		—	
aldoxime							7.6	1.63	8.0	1.62	6.7	1.72
syn-Benz-	[c]	0.7	9.2	0.92	5.3	1.08	0.2	1.15	—		—	
aldoxime							1.0	1.60	1.4	1.61	0.6	1.69
N-Benzyl												
hydroxylamine	—		—		[d]		[d]		−3.8	0.11	−4.0	0.47
p-Aminoaceto-												
phenone oxime	8.9	0.96	9.0	1.14	8.9	1.24	3.6[a]	1.34	—		—	
p-Dimethylamino-												
benzaldoxime	10.0	0.80	9.8	0.97	10.2	1.07	5.7[a]	1.19	—		—	

[a] Minimum.
[b] Drawn out wave
[c] The compound is hydrolyzed.
[d] Poorly defined wave which merges with the hydrogen wave.

Crotonaldoxime exists in a higher melting and a lower melting form. The higher melting isomer, which is investigated here, shows ressemblance with the *anti* cinnamaldoxime and the *anti* benzaldoxime in the behaviour in alkaline solution, where the higher melting form of crotonaldoxime and the mentioned *anti* oximes gives rise to a reduction wave due to the reduction of the unprotonated oxime of nearly the same heigt as that in acid solution, whereas the *syn* isomers give a smaller wave.

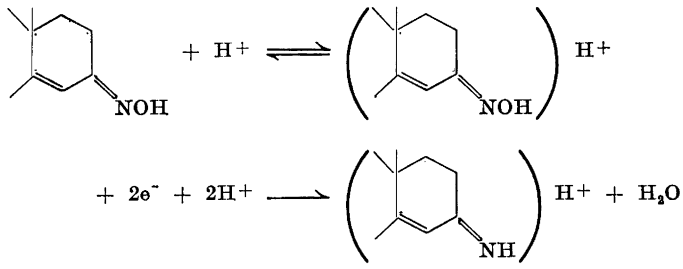
The polarographic wave of mesityloxide oxime is like the first wave of crotonaldoxime apart from a slight tendency to forming minimum. No wave appears in alkaline solution. The wave in acid solution was shown to be due to a four electron reduction.

Carvone oxime produces in mineral acid solution two two-electron waves which about pH 2 merge into one four-electron wave. This wave disappears

at higher pH-values and pronounced minima are found in this pH-interval. No reduction takes place in alkaline solution.

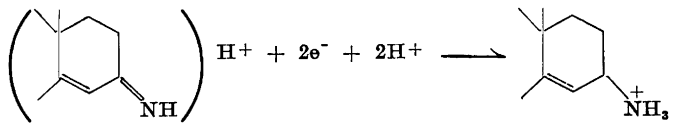
Testosterone propionate oxime is reduced in two two-electron reductions in acid solution until pH about 6, where the waves become deformed and diminish in height. The oxime is rather stable in acid solution at room temperature as the height of the waves at pH 1.0 is not diminished to any measurable degree during a period of 40 h.

Controlled potential reduction at pH 1 at —0.90 V *vs* S.C.E., which is the plateau of the first wave, showed that this wave was due to a two electron reduction. From the reaction mixture testosterone propionate could be isolated in good yield. The possibility that the oxime was hydrolyzed and the hydroxylammonium ion reduced must be excluded, as hydroxylamine is not reduced at that potential and the oxime was found to be stable at room temperature at pH 1. The reaction can thus be formulated:



followed by hydrolysis of the ketimine.

From controlled potential reduction at pH 1 at —1.2 V *vs* S.C.E., which is the plateau of the second wave, it was found that the total reduction of the oxime consumed four electrons and the second one thus two electrons. From this reduction was isolated an amine and the electrode reaction of the second wave, therefore, probably is the reduction of the formed ketimine to the amine:



The *anti* cinnamaldoxime shows a single pH-dependent wave at pH values lower than 6. At pH 1 the height of the wave varies with the height of the mercury reservoir as required for a diffusion controlled wave. About pH 6 the wave becomes drawn out and the wave diminishes somewhat. The height of the wave in strongly alkaline solution is, however, more than half of the height in acid solution. The height of the wave in strongly alkaline solution varies with the height of the mercury reservoir, but somewhat less than required for a diffusion controlled wave.

The *syn* cinnamaldoxime ressembles the *anti* isomer in acid solution but is different from it in alkaline medium. In strongly alkaline solution the wave disappears almost.

Controlled potential reduction in acid solution yielded some saturated aldehyde and the main product was cinnamylamine. The reaction consumed 3.8 electrons per molecule. In alkaline solution the *anti* cinnamaldoxime was reduced in a four electron reduction and hydrocinnamaldehyde was isolated in good yield.

Benzalacetone oxime shows in strongly acid solution a single wave the height of which varies linearly with the square root of the height of the mercury reservoir. The half-wave potentials of this wave vary linearly with pH from pH 0 to pH 6. At pH-values higher than 3 a second, smaller wave is found whose nature was not investigated further. In strongly alkaline solution a single wave is found, the height of which is somewhat bigger than half of the wave height in strongly acid solution. The height of the wave at pH 13 varies somewhat with the height of the mercury reservoir, but not as much as required by a diffusion controlled wave.

From controlled potential reduction in mineral acid solution of benzalacetone oxime it was found that the reaction consumed 3.5 electrons per molecule. The main product, 1-methyl-3-phenyl-allylamine, was accompanied by some benzylacetone and another saturated ketone. In view of the results obtained for testosterone propionate oxime the reaction might be formulated:

$$\begin{aligned} C_6H_5CH{=}CH{-}C(CH_3){=}NOH \; + \; H^+ \; &\rightleftharpoons \; (C_6H_5CH{=}CH{-}C(CH_3){=}NOH)H^+ \\ + \; 2e^- \; + \; 2H^+ \; &\rightarrow \; (C_6H_5CH{=}CH{-}C(CH_3){=}NH)H^+ \; + \; H_2O \\ +2e^- \; + \; 2H^+ \; &\rightarrow \; C_6H_5CH{=}CHCH(CH_3){-}NH_3{}^+ \end{aligned}$$

The formation of benzylacetone might be due to a reduction of the carbon-carbon double bond followed by hydrolysis of the benzylacetone oxime or to a reaction similar to that in alkaline solution.

In alkaline solution the benzalacetone oxime was reduced in a four electron reduction and besides the main product, benzylacetone, a very small amount of amine could be isolated. The reaction might be formulated to proceed in at least three ways:

A) $$R{-}CH{=}CH{-}C(CH_3){=}NOH \; + \; 2e^- \; + \; 2H_2O \; \rightarrow \; R{-}CH_2{-}CH_2{-}C(CH_3){=}NOH \; + \; 2 \; OH^-$$

followed by alkaline hydrolysis of the oxime and a two electron reduction of the formed hydroxylamine.

B) $$\begin{aligned} R{-}CH{=}CH{-}C(CH_3){=}NOH \\ + \; 2e^- \; + \; 2H_2O \; &\rightarrow \; R{-}CH_2{-}CH_2{-}C(CH_3){=}NOH \; + \; 2 \; OH^- \\ + \; 2e^- \; + \; 2H_2O \; &\rightarrow \; R{-}CH_2{-}CH_2{-}C(CH_3){=}NH \; + \; 2 \; OH^- \end{aligned}$$

followed by alkaline hydrolysis of the ketimine.

C) $$\begin{aligned} R{-}CH{=}CH{-}C(CH_3){=}NOH \\ + \; 2e^- \; + \; 2H_2O \; &\rightarrow \; R{-}CH{=}CH{-}C(CH_3){=}NH \; + \; 2 \; OH^- \\ + \; 2e^- \; + \; 2H_2O \; &\rightarrow \; R{-}CH_2{-}CH_2{-}C(CH_3){=}NH \; + \; 2 \; OH^- \end{aligned}$$

followed by hydrolysis of the ketimine to the ketone. It can not, however, be excluded that the hydrolysis precedes the last reduction which then would be the reduction of the benzalacetone to benzylacetone.

The first formulation is unlikely on the following grounds. Hydroxylamine is not reducible at the potential used in alkaline solution. Furthermore, benzylacetone oxime is not hydrolyzed easily in alkaline solution, which was shown by leaving a 10^{-3} M solution of benzylacetone oxime in 0.1 N sodium hydroxide for 18 h. The concentration of the oxime did not diminish to any appreciable degree which was found by polarography of the solution after lowering the pH to 3.

The second formulation (B) is unlikely, as benzylacetone oxime is not reduced in alkaline solution. No evidence has been found against the third formulation (C), why it must be regarded as the most likely approximation to the reaction path.

Aromatic oximes. The polarographic behaviour of oximes of simple aromatic carbonyl compounds have been thoroughly investigated elsewhere. Here the *syn* and *anti* benzaldoxime, which only seem to have been investigated in unbuffered systems [15,16], and oximes of some aminocarbonyl compounds have been included in the investigation.

The oximes of the aminocarbonyl compounds show a similar behaviour as, *e.g.*, acetophenone oxime and the electrode reaction was shown to be a four electron reduction to the amine.

The *syn* and *anti* benzaldoxime are very much alike in their polarographic behaviour below pH about 4, whereas at higher pH-values they behave differently from each other. The *syn* benzaldoxime produces in weakly alkaline solution only a small wave, whereas the reduction wave due to the reduction of the unprotonated oxime of the *anti* benzaldoxime is nearly as high as in weakly acid solution. In strongly alkaline medium the height of the wave of the *anti* benzaldoxime diminishes somewhat, probably due to the formation of the anion $R—CH=NO^-$.

Controlled potential reduction of benzophenone oxime at pH 3 yielded benzhydrylamine in a four electron reduction. *Anti* benzaldoxime was reduced at pH 9, where the unprotonated oxime is reduced, in a four electron reduction and benzylamine was the isolated product.

The reduction of aromatic oximes has been formulated [10] in the following way:

(A)
$$\begin{aligned} R'R''C{=}NOH + H^+ &\rightleftharpoons (R'R''C{=}NOH)H^+ \\ + 4e^- &\rightarrow (R'R''C{=}NOH)H^* \\ + 3H_2O &\rightarrow R'R''CH{-}NH_3^+ + 4\ OH^- \end{aligned}$$

where the symbol * represents an activation state or series of such states the charge type being unspecified.

An alternative formulation with two consecutive reductions occuring at the same potential (or at different potentials as in the cases of benzophenone oxime in strongly acid solution [10]), which evades the assumption of an electron uptake in aqueous solution of four electrons before an uptake of hydrogen ions, would be:

(B)
$$\begin{aligned} R'R''C{=}NOH + H^+ &\rightleftharpoons (R'R''C{=}NOH)H^+ \\ + 2e^- + 2H^+ &\rightarrow (R'R''CH{-}NHOH)H^+ \\ + 2e^- + 2H^+ &\rightarrow R'R''CH{-}NH_3^+ + H_2O \end{aligned}$$

$$\text{or (C)}\quad R'R''C=NOH + H^+ \rightleftharpoons (R'R''C=NOH)H^+$$
$$+ 2e^- + 2H^+ \rightarrow (R'R''C=NH)H^+ + H_2O$$
$$+ 2e^- + 2H^+ \rightarrow R'R''CH-NH_3^+$$

Of these (B) is unlikely as benzylhydroxylamine is not reduced in mineral acid solution (Table 2) and in slightly acid solution the reduction occurs at a potential appreciably more negative than the half-wave potential of benzaldoxime. Although the intermediate aldimine (C) is too unstable in acid solution to be investigated polarographically it seems not unlikely that the electron uptake is a faster reaction than the hydrolysis. In this connection it can be mentioned that benzylidene-methylamine in cold 50 % sulfuric acid has been reduced at a lead cathode in a two electron reduction to benzylmethylamine [17]. Although the postulated aldimine probably exists as an activated complex at the electrode surface it is believed that (C) gives a better approximation to the reaction path than (A) or (B).

N-benzylbenzaldoxime. The polarographic behaviour of this compound ressembles somewhat that of *anti* benzaldoxime (Table 3). In strongly acid solution, however, where the compound is hydrolyzed too rapidly for exact polarographic measurements, two waves are found, which merge into one wave about pH 3. About pH 8 the wave splits in two waves, but in more alkaline solution again one wave is found with a height somewhat lower than in weakly acidic solution.

Controlled potential reduction in neutral solution showed that the main reaction was a four electron reduction of the N-benzylbenzaldoxime to dibenzylamine. In mineral acid solution, where two waves are observed, the first wave is apparantly not corresponding to the reduction of the carbon-nitrogen double bond, as the expected product from such a reduction, dibenzylhydroxylamine, is not reducible in acid solution at the potential used. The reaction path in acid solution and probably also in neutral medium would thus be:

$$C_6H_5CH_2N(\rightarrow O)=CHC_6H_5 + H^+ \rightleftharpoons (C_6H_5CH_2N(\rightarrow O)=CHC_6H_5)H^+$$
$$+ 2e^- + 2H^+ \rightarrow (C_6H_5CH_2N=CHC_6H_5)H^+ + H_2O$$
$$+ 2e^- + 2H^+ \rightarrow (C_6H_5CH_2)_2NH_2^+$$

Table 3. Limiting currents in μA and half-wave potentials *vs* S.C.E. of N-benzylbenzaldoxime, N,N-dibenzylhydroxylamine, mesoxalic acid ester oxime, and benzamideoxime. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	1.05		3.55		5.20		7.40		9.80		13	
Compound	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
N-Benzyl-benzaldoxime	[a]	0.51	7.5	0.86	8.5	0.97	8.0	1.08	7.6[b]	1.40	5.1	1.57
N,N-Dibenzyl-hydroxylamine	[a]	0.71	4.8[b]	1.29	1.8	1.38	—		—		—	
Mesoxalic acid ester oxime	9.3	0.85	9.1	1.02	9.1	1.05	8.9	1.07	−4 8.6	0.15 1.31	−3.9 —	0.38
Benzamideoxime	—		4.3	1.32	4.5	1.36	2.9	1.51	1.2	1.71	5.1[a] —	1.55

[a] The compound is hydrolyzed.
[b] drawn out wave.

From a controlled potential oxidation at a mercury anode at —0.05 V *vs* S.C.E. in alkaline solution of dibenzylhydroxylamine was isolated N-benzylbenzaldoxime and benzaldehyde.

Ethyl ester of mesoxalic acid oxime. This compound has previously been investigated by Souchay and Ser [9] and the results obtained here are substantially in agreement with theirs. The half-wave potentials found in the medium used here are, however, somewhat more negative and the height of the wave corresponds nearly to a four electron reduction. The polarographic behaviour of the ester ressembles that of oximes containing two keto groups in the α-positions to the oxime group [9] and also that of N-benzylbenzaldoxime.

From the height of the wave of mesoxalic acid oxime Sartori *et al.*[18] concluded that the electrode reaction was a two electron reduction to the hydroxylamino malonic acid. Controlled potential reduction at pH 6 to the ester showed, however, that the electrode reaction in this case was a four electron reduction and aminomalonic acid ester was isolated from the reaction mixture. The electrode reaction is thus similar to that of other oximes.

Benzamideoxime. The polarographic behaviour of this compound resembles somewhat that of the unconjugated oximes. In strongly acid solution the wave is masked by the hydrogen wave but from pH about 2 a single wave is found. The height of this wave diminishes from pH about 6 and the wave disappears completely about pH 10. No minima has been observed in weakly alkaline solution. The height of the wave at pH 3 to 5 corresponds to a two electron reduction, whereas ordinary oximes are reduced by a four electron reduction.

Controlled potential reduction in aqueous solution at pH 4.8 of benzamideoxime confirmed that the electrode reaction consumed two electrons per molecule, and benzamidine, $C_6H_5C(NH_2) = NH$, was isolated in good yield as the picrate.

$$C_6H_5C(NH_2)=NOH + H^+ \rightleftharpoons (C_6H_5C(NH_2)=NOH)H^+$$
$$+ 2e^- + 2H^+ \rightarrow (C_6H_5C(NH_2)=NH)H^+ + H_2O$$

This reaction is similar to that postulated above as the first step of the reduction of ordinary oximes.

Benzalazine

Benzalazine has previously been investigated [6] in 70 % ethanol containing 0.5 M tetramethylammonium chloride. The results obtained in 40 % ethanol containing 1 M potassium chloride are somewhat different from those reported and are given in Table 4. The results of the polarographic investigation of benzaldehyde benzylhydrazone in the same medium are included in the table.

The benzalazine, which is rapidly hydrolyzed in mineral acid solution, shows a single pH-dependent wave at pH values lower than 7. Between 6 and 9 the height of the wave diminishes rapidly and shows a pronounced minimum. The height of the first wave is determined by the rate of some reaction which might be the recombination of the benzalazine and H^+ to the easier reducible protonated benzalazine or might rather be some reaction involving the primarily formed reaction product. The first wave degenerates to another pH-

Table 4. Limiting currents in μA and half-wave potentials *vs* S.C.E. at different pH-values of benzalazine and benzaldehyde benzylhydrazone. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	1.05		3.55		5.20		7.40		9.80		13	
	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
Benzalazine	[a]	0.73	14.7	0.91	15.2	1.05	13.1[b]	1.15	4.5 0.9	1.24 1.49	5.9	1.46
Benzaldehyde-benzyl-hydrazone	4.6 4.6	0.56 0.73	9.2	0.85	8.7	1.03	7.3[b]	1.19				

[a] The compound is hydrolyzed.
[b] Minimum.

dependent wave with a smaller wave height. This wave splits up in two waves in the pH-region 9.5—12. The wave with the most negative half-wave potential grows at the sacrifice of the other one in a manner, which resembles the reduction of *e.g.*, pyruvic acid.

The height of the wave about pH 4 is between 2.5 and 3 times the height of the wave in alkaline solution, where the wave is diffusion controlled. The height of the wave in alkaline solution corresponds to a two electron reduction, and a reduction requiring 5 to 6 electrons would then be expected in acid solution.

Benzaldehyde benzylhydrazone shows at about pH 1 two waves of equal height which merge to one wave about pH 3. Between pH 6 and 9 the height of the wave diminishes rapidly and disappears completely about pH 9. The height of the wave about pH 4 is between 1.5 and 2 times higher than the height of the wave of benzalazine in alkaline solution, and a reduction requiring 3 to 4 electrons per molecule would, therefore, be expected.

On comparing the half-wave potentials of benzalazine and benzaldehyde benzylhydrazone it is found that the half-wave potentials of the latter is equal to or less negative than the half-wave potentials of benzalazine, and in a mixture of the two compounds the benzaldehyde benzylhydrazone would thus be reduced before or simultaneously with benzalazine.

The polarographic behaviour of benzalazine could, therefore, be explained by assuming that the first wave was due to a reduction of benzalazine to benzaldehyde benzylhydrazone followed immediately by a reduction of this compound. If a two electron reduction wave is subtracted from the polarograms of benzalazine the resulting polarograms are very much like those of benzaldehyde benzylhydrazone.

Controlled potential reduction of benzalazine in alkaline solution showed that the electrode reaction consumed two electrons per molecule, and the isolated product was benzaldehyde benzylhydrazone

$$C_6H_5CH=N-N=CHC_6H_5 + 2e^- + 2H_2O \rightarrow C_6H_5CH_2NH-N=CHC_6H_5 + 2\ OH^-$$

Dibenzyl diimide, $C_6H_5CH_2—N = N—CH_2C_6H_5$ [6], has been suggested as the reduction product, but this compound is known to rearrange to benzaldehyde benzylhydrazone under the influence of alkali or acid. It cannot, however, be excluded that dibenzyl diimide is the primarily formed product, although in view of the reducibility of azobenzene it would not seem unlikely that dibenzyl diimide would be reduced further to dibenzylhydrazine.

At pH 5 benzaldehyde benzylhydrazone was reduced in a four electron reduction, and benzylamine was isolated from the reaction mixture. The reaction path is probably similar to that of benzaldehyde phenylhydrazone to be discussed later.

$$C_6H_5CH_2NH-N=CHC_6H_5 + 4e^- + 6H^+ \rightarrow 2\ C_6H_5CH_2NH_3^+$$

In weakly acid solution benzalazine was reduced consuming 5.5 electrons per molecule and benzylamine was isolated from the reaction. The main reaction is:

$$C_6H_5CH=N-N=CHC_6H_5 + 6e^- + 8H^+ \rightarrow 2\ C_6H_5CH_2NH_3^+$$

Phenylhydrazones

α,β-Unsaturated phenylhydrazones. Carvone phenylhydrazone produces in mineral acid solution two waves of equal height which merge into one wave about pH 3. This wave diminishes in height from pH about 5 and disappears completely about pH 8. Controlled potential reduction at pH 4 showed that the electrode reaction consumed four electrons per molecule and aniline was isolated from the reaction mixture indicating a reductive splitting of the nitrogen-nitrogen bond.

Aromatic phenylhydrazones. The phenylhydrazones of the following aromatic carbonyl compounds were investigated: benzaldehyde, benzophenone, N-*p*-dimethylamino benzaldehyde, and *p*-aminoacetophenone. As seen from Table 5 they all show a single, pH-dependent wave in acid solution, which diminishes in height at higher pH-values and disappears completely in alkaline solution. The half-wave potentials of the hydrazones were from 0.05 to 0.2 V more positive than those of the carbonyl compounds. In the table are included the data of N-benzyl-N′-phenyl-hydrazine.

In the pH-region where the wave height is approximately constant the half-wave potentials have a nearly linear dependence of pH. For the amino-compounds the influence of the amino group makes the pH-dependence more complicated in neutral solution. In alkaline medium are found pronounced minima as for the oximes. The explanation of these minima and the diminishing of the wave height at higher pH-values is probably the same as for the oximes [9].

Controlled potential reductions of benzaldehyde phenylhydrazone, benzophenone phenylhydrazone, and N-*p*-dimethylamino benzaldehyde phenylhydrazone in acid solution showed that the electrode reaction consumed four electrons per molecule and that the overall reaction could be formulated as follows:

$$RR'C=N-NHC_6H_5 + 4e^- + 6H^+ \rightarrow RR'CH-NH_3^+ + H_3N^+-C_6H_5$$

Table 5. Limiting currents in μA and half-wave potentials *vs* S.C.E. of some phenylhydrazones and N-benzyl-N′-phenylhydrazine at different pH-values. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	1.05		3.55		5.20		7.40		9.80		13	
Phenylhydrazone of	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
Benzaldehyde	10.2	0.75	11.4	1.00	11.0	1.11	1.4	1.24	—		—	
p-Dimethyl-aminobenz-aldehyde	8.8	0.73	8.8	0.94	8.8	1.08	7.6[a]	1.27	—		—	
p-Aminoaceto-phenone	6.7	0.87	7.9	1.08	7.9	1.21	5.9[a]	1.38	—		—	
Cinnamaldehyde	10.5	0.72	9.8	1.00	8.1[a]	1.12	4.3[a]	1.21	0.7	1.32	—	
									4[b]	1.75	4[b]	1.75
Carvone	5.0	0.78	9.1	1.09	8.5	1.20	2.6[a]	1.39	—		—	
	4.7	1.06			4.1[a]	1.44						
N-Benzyl-N′-phenylhydrazine	—		—		—		−5	0.13	−3.8	0.38	−3.6	0.61

[a] Minimum.
[b] Merge with the sodium wave.

The same results were obtained by Tafel and Pfeffermann [19] from the reduction of the phenylhydrazones of acetaldehyde, acetone, and benzaldehyde in 50 % sulfuric acid at a lead cathode. In the last case a small amount of benzylaniline was isolated besides the benzylamine and aniline.

The reduction of benzaldehyde phenylhydrazone might proceed by a reduction to N-benzyl-N′-phenyl-hydrazine followed by a reductive splitting of the nitrogen-nitrogen bond or a disproportionating to the amines and the hydrazone. This seems, however, unlikely as N-benzyl-N′-phenyl hydrazine is not reducible at the dropping mercury electrode and the compound seems perfectly stable in acid solution.

The cleavage of the protonated nitrogen-nitrogen bond seems, therefore, to be the initial step in the reaction and this is followed by a reduction of the formed aldimine:

$$\begin{aligned} C_6H_5CH{=}N{-}NH{-}C_6H_5 + H^+ &\rightleftharpoons (C_6H_5CH{=}N{-}NH{-}C_6H_5)H^+ \\ + 2e^- + 3H^+ &\rightarrow (C_6H_5CH{=}NH)H^+ + H_3N^+{-}C_6H_5 \\ + 2e^- + 2H^+ &\rightarrow C_6H_5CH_2NH_3^+ \end{aligned}$$

The side reaction, which occurs in 50 % sulfuric acid and leads to the formation of benzylaniline, is also better explained on the assumption that benzaldimine is the intermediate product than if the reduction of the carbon-nitrogen double bond is assumed to be the initial step.

A reductive cleavage of a nitrogen-nitrogen bond has previously been reported for the reduction of 3-hydroxy-1,3-diphenyl-triazene, which in a four electron reduction followed by a two electron reduction forms aniline and phenylhydrazine [20].

Semicarbazones

α,β-Unsaturated semicarbazones. Cinnamaldehyde semicarbazone produces in acid solution a single wave the height of which diminishes at higher pH-values and disappears about pH 10. From pH about 8 another wave appears, which is found in both weakly and strongly alkaline solution. The half-wave potentials of the first wave is pH-dependent, whereas the second one is not. The height of the first wave is determined by the rate of the recombination of the semicarbazone and the hydrogen ion, whereas the second one is diffusion controlled. This second wave is due to the reduction of the unprotonated semicarbazone.

Table 6. Limiting currents in μA and half-wave potentials *vs* S.C.E. of some semicarbazones at different pH-values. Concentration 10^{-3} M. 0.01 % gelatine added as maximum suppressor.

pH	1.05		3.55		5.20		7.40		9.80		13	
Semicarbazone of	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$	i_d	$-E_{\frac{1}{2}}$
Citral	9.4	0.87	9.7	1.08	8.0	1.24	2.1	1.40	—		—	
Cinnam-	8.6	0.73	5.0	0.94	4.7	1.10	3.6	1.22	0.5	1.29	—	
aldehyde			1.9	1.14			0.9	1.59	4.1	1.56	4.7	1.57
Benzal-	6.4	0.77	3.5	0.93	2.8	1.02	4.0	1.21	1.4*	1.27	—	
acetone	3.4	1.16	8.6	1.24	6.0	1.32			3.3	1.65	4.0	1.65
Benzaldehyde	10.3	0.80	9.1	1.08	4.7*	1.20	—		—		—	
p-Aminoaceto-	4.1	0.90	6.9	1.11	6.4*	1.24	3.8*	1.39	—		—	
phenone	1.9	1.03										

* Minimum.

Controlled potential reduction of cinnamaldehyde semicarbazone in alkaline solution showed that the electrode reaction consumed two electrons and the isolated product was hydrocinnamaldehyde semicarbazone:

$$C_6H_5CH=CH-CH=N-NHCONH_2 + 2e^- + 2H_2O \rightarrow C_6H_5CH_2CH_2CH=N-NHCONH_2 + 2\ OH^-$$

Attempts to reduce cinnamaldehyde semicarbazone in mineral acid solution was unsuccessful due to a partial hydrolysis of the semicarbazone.

Benzalacetone semicarbazone shows a similar behaviour as cinnamaldehyde semicarbazone. The benzalacetone semicarbazone is hydrolyzed in mineral acid solution, whereas it is stable in alkaline solution. Controlled potential reduction in alkaline medium showed that the electrode reaction was a two electron reduction to benzylacetone semicarbazone.

Aromatic semicarbazones. The semicarbazones of benzaldehyde and *p*-aminoacetophenone were investigated. Both showed a single pH-dependent wave in acid solution and the wave height diminished at higher pH-values. No wave was observed in alkaline solution. As in the cases of phenylhydrazones and oximes the semicarbazones showed pronounced minima in neutral and weakly alkaline solution.

Controlled potential reduction in mineral solution showed that a reductive splitting of the nitrogen-nitrogen bond took place in a reaction consuming four electrons per molecule. For benzaldehyde semicarbazone the overall electrode reaction thus is:

$$C_6H_5CH{=}N{-}NHCONH_2 + 4e^- + 5H^+ \rightarrow C_6H_5CH_2NH_3^+ + H_2NCONH_2$$

The reduction path is probably similar to the that of the reduction of benzaldehyde phenylhydrazone.

From the reduction of benzophenone semicarbazone, for which Souchay and Graizon [7] from the height of the polarographic wave suggested a 2 electron reduction to 1-benzhydryl semicarbazide, benzhydrylamine was obtained in good yield (70 %) in mineral acid solution. In an acetate buffer, pH 4, however, the reduction required less than 4 electrons and 1-benzhydryl semicarbazide was isolated in 30 % yield.

DISCUSSION

From the data presented it appears that in the polarographic reduction of RR'C = NYR'' the reaction depends on the nature of Y. Is Y a carbon atom, then the reduction consumes two electrons per molecule and the saturated compound is the reaction product. When, however, Y is nitrogen or oxygen the most common overall reaction in acid solution consumes four electrons per molecule and a reductive splitting of the N—Y bond takes place together with a saturation of the double bond. It also appears, that in general the splitting of the N—Y bond precedes the reduction of the carbon-nitrogen double bond thus giving the following reaction path:

$$\begin{aligned} RR'C{=}N{-}Y{-}R'' + H^+ &\rightleftharpoons (RR'C{=}N{-}Y{-}R'')H^+ \\ + 2e^- + 2H^+ &\rightarrow (RR'C{=}NH)H^+ + H{-}Y{-}R'' \\ + 2e^- + 2H^+ &\rightarrow RR'CH{-}NH_3^+ \end{aligned}$$

In a few cases, *e.g.* benzophenone semicarbazone at pH 4, however, the four electron reduction is accompanied by a two electron reduction of the carbon-nitrogen double bond and the compound formed by this two electron reduction is not reduced further. This might also be the case in the reduction of dimethylglycylhydrazones of α,β-unsaturated steroid ketones [5], where the wave height of the hydrazones were found to be twice the wave height of the uncondensated steroid ketones. As α,β-unsaturated steroid ketones are reduced by a one electron reduction [21], this would suggest a two electron reduction of the hydrazones. However, no controlled potential reduction of the dimethylglycylhydrazones were made due to their low stability in acid solution, and the nature of the electrode reaction is, therefore, still uncertain.

EXPERIMENTAL

The polarograph was a recording polarograph Radiometer Type PO 3a. The capillary delivered 2.27 mg of mercury per second at a corrected mercury column height of 48.5 cm. The drop time 3.90 sec.(H_2O, open circuit). The capillary constant $m^{2/3}t^{1/6} = 2.17$ $mg^{2/3}sec^{-1/2}$.

The apparatus used for the controlled potential reductions was an electromechanical potentiostat of the Lingane-Jones type. For the oxidations performed at an anode potential of −0.05 to −0.5 V *vs* S.C.E. the manually operated apparatus employed in Part I[21] was found suitable.

The buffer solutions used were from pH 2.5 to 8 a sodium phosphate-citric acid buffer and from pH 8 to 11 a sodium borate buffer. The medium employed was 40 % alcohol containing 1 M potassium chloride and 0.01 % gelatine.

The carbonyl derivatives were prepared in the conventional way. The only derivative whose data apparently not have been reported previously is testosterone propionate oxime, which was prepared from 5 g of testosterone propionate, 5 g of hydroxylamine hydrochloride and 4 ml pyridine in methanol solution. After 3 days at room temperature the mixture was diluted with water and the precipitate recrystallized from dilute alcohol and benzene-hexane. M. p. 184°. $(\alpha)_D = +132°$ (1 % in $CHCl_3$).

Reduction of cyclo*hexanone in methylamine solution.* 1.0 ml of freshly redestilled *cyclo*hexanone was reduced in a mixture of 40 ml 33 % aqueous methylamine solution, 50 ml 4 N hydrochloric acid, and 100 ml water at −1.65 V *vs* S.C.E. After completion of the reduction which required 2 electrons per molecule *cyclo*hexanone the reaction mixture was extracted three times with ether, which was washed three times with dilute sodium hydroxide. After drying, the ether was evaporated, together with the remaining traces of methylamine. The residue was dissolved in ether and on addition of dry hydrogen chloride a precipitate, 925 mg, was obtained, which after recrystallization from alcohol-ether had m. p. 175−176°. It was identified as N-methyl *cyclo*hexylamine hydrochloride (m. p.[22] 175−178°). (Found: C 55.95; H 10.76; N 8.90; Cl 23.56. Calc. for $C_7H_{16}NCl$: C 56.16; H 10.79; N 9.36; Cl 23.70.)

Reduction of benzophenone anile. 1.0 g of benzophenone anile was reduced in a medium of 40 % peroxide free tetrahydrofurane, 20 % alcohol and 40 % water. The buffer was a borate buffer pH 10, the cathode potential −1.70 V *vs* S.C.E. Small amounts of acid were added during the reduction to compensate for the hydrogen ions used; the consumption of hydrogen ions was calculated from the reading of the coulometer. — The yellow colour of the solution disappeared during the reduction, which required two electrons per molecule. After completion of the reaction the mixture was diluted with water, made a little more alkaline and extracted three times with ether. The combined extracts were dried with potassium carbonate, and on addition of dry hydrogen chloride a precipitate, 1.05 g, was obtained, m. p. 203°, which was identified as benzhydryl aniline hydrochloride,[1] m.p.[23] 201−203°. (Found: C 76.98; H 6.12; N 4.90; Cl 11.54. Calc. for $C_{19}H_{18}NCl$: C 77.16; H 6.13; N 4.74; Cl 11.97.)

Reduction of testosterone propionate oxime (1). A solution of 0.5 g testosterone propionate oxime was reduced in 180 ml 50 % alcohol containing 0.5 N HCl at −0.90 V *vs* S.C.E. until the first wave disappeared on the polarograms, which required 2.1 electrons. The reaction mixture was then extracted with chloroform, which was evaporated. The residue was dissolved in alcohol and added to an acid alcoholic solution of 2.4-dinitrophenylhydrazine. A precipitate, 0.7 g, was obtained, m. p. 190−195°; it was recrystallized from butanol and tetrahydrofurane-alcohol, m. p. 212−215° after sintering about 210°. (Testosterone propionate 2,4-dinitrophenylhydrazone m. p. 209.9−210.5°[24], 217−218°[25]). The IR-spectrum of the obtained hydrazone was found to be identical with that of actual testosterone propionate 2,4-dinitrophenylhydrazone.

Reduction of testosterone propionate oxime (2). A solution of 0.5 g of testosterone propionate oxime was reduced in 180 ml 50 % alcohol containing 0.5 N HCl at −1.20 V *vs* S.C.E. In this case a motor driven stirrer was found more suitable than a magnetic stirrer due to the formation of a surface-active reaction product. At higher concentrations of this compound the mercury emulgated thus making a further use of the electrode impossible. The reduction required a little less than four electrons. The reaction mixture was diluted with water, extracted with ether, made alkaline and again extracted with ether, which was dried. On addition of dry hydrogen chloride a precipitate, 275 mg, was obtained, m. p. 235−240° (decomp.). The amine hydrochloride was rather slightly soluble in water. The amine was set free with carbonate and extracted with ether, which was evaporated. The amine had m. p. 218−222°. (Found: C 75.73; H 10.16; N 4.13. Calc. for $C_{22}H_{35}NO_2$: C 76.49; H 10.21; N 4.06.)

Reduction of anti *cinnamaldoxime (1).* 1.0 g of *anti* cinnamaldoxime was reduced in a mixture of 80 ml N H_2SO_4, 80 ml alcohol, and 20 g of Na_2SO_4 at −0.8 V *vs* S.C.E. The

reduction consumed 3.8 electrons. After completion of the reduction the reaction mixture was extracted with ether, which was dried and evaporated leaving 225 mg of a compound with an IR-spectrum indicating the presence of an unconjugated aldehyde group. The aqueous phase was then made alkaline and extracted with ether, which was dried. On addition of dry hydrogen chloride a precipitate, 525 mg, was obtained, which was identified as cinnamyl amine hydrochloride through the benzoyl derivative, m. p. 93—94° (94—95°). (Found: C 80.73; H 6.37; N 5.75. Calc. for $C_{16}H_{15}NO$: C 80.98; H 6.37; N 5.91.)

Reduction of anti *cinnamaldoxime (2).* 1.0 g of *anti* cinnamaldoxime was reduced in 150 ml aqueous alkaline solution, to which benzene was added to dissolve the reaction product. The reduction required 4 electrons per molecule. After completion of the reaction the layers were separated and the aqueous phase extracted with ether. The combined organic solvents were washed with aqueous acid, from which a small amount of amine could be isolated. The organic solvents were evaporated leaving 800 mg of a compound which was identified as hydrocinnamaldehyde through the 2,4-dinitrophenylhydrazone, m. p. 149—151°. (Hydrocinnamaldehyde 2,4-dinitrophenylhydrazone, m. p. 147°[26].)

Reduction of benzalacetone oxime (1). A suspension of 1.0 g of benzalacetone oxime was reduced at pH about 0.2 in 50 % alcohol containing sulfuric acid and sodium sulfate at −0.85 V *vs* S.C.E. The reduction consumed 3.5 electrons. The reaction completed, the mixture was diluted with water and extracted several times with ether, which was evaporated. The IR-spectrum (in chloroform) of the residue indicated the presence of benzylacetone. The residue, however, also contained another unconjugated ketone forming a 2,4-dinitrophenylhydrazone with m. p. 30—40° higher than that of benzylacetone. The reaction medium was then made alkaline and extracted several times with ether. The ether was extracted with dilute hydrochloric acid, and the amine benzoylated in alkaline solution, yielding 825 mg benzoyl derivative. It was identified as N-(methyl-cinnamyl)-benzamide from its m. p. 135—137° (N-(methyl-cinnamyl)-benzamide m. p. 136—137°). (Found: C 81.06; H 6.78; N 5.77. Calc. for $C_{17}H_{17}NO$: C 81.30; H 6.82; N 5.58.)

Reduction of benzalacetone oxime (2). A solution of 1.0 g benzalacetone oxime was reduced in 150 ml aqueous sodium hydroxide containing potassium chloride. 40 ml benzene were added to extract the reduction product as soon as it was formed. The compound was reduced at −1.70 V *vs* S.C.E. and the reduction required 3.9 electrons. After separation of the layers, the aqueous layer was extracted with ether, and the organic phase washed with acid, from which a small amount of amine (75 mg) could be isolated. The organic solvents were evaporated leaving 820 mg of a compound with an IR-spectrum identical with that of benzylacetone. On reaction with 2,4-dinitrophenylhydrazine a precipitate, m. p. 126°, was obtained (benzylacetone 2,4-dinitrophenylhydrazone, m. p. 125—126.3°[27]).

Reduction of anti *benzaldoxime.* 1.0 g of *anti* benzaldoxime was reduced at pH 9 in a mixture of 30 % peroxide free tetrahydrofurane and 70 % aqueous buffer at −1.7 V *vs* S.C.E. The reduction required 4.0 electrons per molecule. After completion of the reaction the mixture was extracted three times with ether, which was dried. On addition of dry hydrogen chloride a precipitate, 700 mg, was obtained which was identified as benzylamine hydrochloride through the benzoyl derivative, m. p. 104—6° (N-benzyl-benzamide, m. p. 105—7°). (Found: C 79.65; H 5.99; N 6.70. Calc. for $C_{14}H_{13}NO$: C 79.57; H 6.21; N 6.64.)

Reduction of N-benzyl benzaldoxime. 1.0 g of N-benzyl benzaldoxime was reduced in aqueous medium of pH 7 at −1.2 V *vs* S.C.E. The reduction required 3.8 electrons per molecule. After completion of the reduction the reaction mixture was extracted with ether which was dried. On addition of dry hydrogen chloride a precipitate, 740 mg, was obtained, which after recrystallization from alcohol had m. p. 257—258°. (Dibenzylamine hydrochloride, m. p. 256°.) (Found: C 71.75; H 6.96; N 5.99; Cl 14.90. Calc. for $C_{14}H_{16}NCl$: C 71.93; H 6.90; N 6.00; Cl 15.17.)

Reduction of mesoxalic acid ester oxime. 1.0 g of mesoxalic acid ester oxime was reduced in a sodium bicarbonate solution that was saturated with carbon dioxide during the reduction, which required four electrons. pH was then raised to 9 and the mixture extracted with ether and ethyl acetate, which was dried. After addition of alcoholic hydrogen chloride the organic solvents were evaporated *in vacuo* and the residue dissolved in a small amount of alcohol. On addition of ether a precipitate was obtained with m. p. 159°, which was identified as aminomalonic acid ethylester hydrochloride (m. p. 162°). (Found:

C 39.76; H 6.76; N 6.81; Cl 16.70. Calc. for $C_7H_{14}NClO_4$: C 39.72; H 6.67; N 6.61; Cl 16.75.)

Reduction of benzamide oxime. 1.0 g of benzamide oxime was reduced in aqueous acetate buffer pH 4.7 at −1.3 V *vs* S.C.E. The reduction required two electrons per molecule. After completion of the reduction picric acid was added and a precipitate, 2.1 g, was obtained, which after recrystallization from alcohol had m. p. 231−233°. It was identified as benzamidine picrate (m. p. 233°) by its m. p. and analysis. (Found: C 44.72; H 3.07; N 20.10. Calc. for $C_{13}H_{11}N_5O_7$: C 44.71; H 3.17; N 20.05.)

Reduction of benzalazine. A suspension of 2.0 g of benzalazine in alkaline 50 % ethanol was reduced at −1.60 V *vs* S.C.E. The yellow colour of the solution disappeared as the reduction proceeded, and a white compound crystallized out. After consumption of 2 electrons per molecule the reduction was finished and the crystalline compound filtered off, m. p. 68−70°. It was identified as benzaldehyde benzylhydrazone (m. p. 65°, 69−70°). This compound is known to be unstable in contact with air, and the compound was, therefore, further characterized by its picrate, m. p. 130° (130°). (Found: C 54.25; H 3.95; N 15.91. Calc. for $C_{20}H_{17}N_5O_7$: C 54.66; H 3.90; N 15.94.)

Reduction of benzaldehyde benzylhydrazone. 1.5 g benzalazine was reduced in alkaline solution as described above (2 electrons per molecule). pH was then lowered to 5 by addition of glacial acetic acid and the reduction product from the alkaline reduction, benzaldehyde benzylhydrazone, reduced at −1.2 V *vs* S.C.E. After completion of the reduction which required 4 electrons hydrochloric acid was added and most of the solution evaporated *in vacuo*. The residue was made alkaline and extracted with ether which was dried. On addition of gaseous hydrogen chloride a precipitate, 1.08 g, was obtained which was identified as benzylamine hydrochloride from the m. p. 250−254° (250−256°) and the m. p. 104−106° of the benzoyl derivative (N-benzyl benzamide, m. p. 105−107°).

Reduction of benzaldehyde phenylhydrazone. A suspension of 1.0 g of benzaldehyde phenylhydrazone in a mixture of 90 ml peroxide free dioxane, 50 ml alcohol, and 50 ml N HCl was reduced at −0.80 V *vs* S.C.E. The reduction was completed after the consumption of 4.2 electrons. The reaction mixture was evaporated to dryness *in vacuo* and the residue dissolved in buffer pH 5. This was extracted with ether, which was shown to contain an amine, that could be diazotized. The aqueous phase was made alkaline and extracted with ether, which was dried. On addition of alcoholic hydrogen chloride a precipitate was obtained, m. p. 253−255°, which was recrystallized from alcohol-ether. The compound was identified as benzylamine hydrochloride from its analysis: (Found: C 58.37; H 7.08; N 9.74; Cl 24.75. Calc. for $C_7H_{10}NCl$: C 58.54; H 7.02; N 9.76; Cl 24.68.)

Reduction of benzophenone phenylhydrazone. A suspension of 1.0 g of benzophenone phenylhydrazone was reduced at −0.75 V *vs* S.C.E. in a mixture of 80 ml peroxide free dioxane, 80 ml alcohol, and 40 ml 2 N HCl. After completion of the reduction, which consumed four electrons per molecule, the reaction mixture was diluted with water and extracted with ether. pH was raised to 4 and the aqueous layer extracted with ether. The aqueous phase was then made alkaline and extracted with ether. After drying of this ether extract a precipitate, 460 mg, was obtained on addition of hydrogen chloride. It sublimed about 280°. It was identified as benzhydrylamine hydrochloride from its analysis. (Found: C 71.04; H 6.59; N 6.28; Cl 16.15. Calc. for $C_{13}H_{14}NCl$: C 71.07; H 6.42; N 6.37; Cl 16.14.)

Reduction of p-*dimethylaminobenzaldehyde phenylhydrazone.* 1.0 g of N-dimethylaminobenzaldehyde phenylhydrazone was reduced at pH 0.5 in 50 % alcohol. The cathode potential was −0.8 V *vs* S.C.E. The reduction was completed after the consumption of four electrons per molecule, and pH was then adjusted to 5. The reaction medium was extracted with ether, made alkaline and extracted several times with ether. The ether was dried and on addition of dry hydrogen chloride a precipitate, 480 mg, was obtained, which was identified as N^4,N^4-dimethyl-toluene-α,4-diamine dihydrochloride from its analysis. (Found: C 48.33; H 7.28; N 12.56; Cl 31.51. Calc. for $C_9H_{16}N_2Cl_2$: C 48.44; H 7.23; N 12.56; Cl 31.79.)

Reduction of cinnamaldehyde semicarbazone. 1.0 g of cinnamaldehyde semicarbazone was reduced in alkaline, 50 % alcoholic solution at a cathode potential of −1.70 V. After consumption of two electrons per molecule the reaction was completed and the reaction mixture diluted with water. It was extracted with ether which was evaporated. The residue was recrystallized from dilute alcohol and benzene. It was identified as hydro-

cinnamaldehyde semicarbazone from the m. p. 124—126° (127°), and its analysis. (Found: C 63.16; H 6.95; N 21.58. Calc. for $C_{10}H_{13}N_3O$: C 62.80; H 6.86; N 21.97.) On boiling with 2,4-dinitrophenylhydrazine in dilute alcohol a 2,4-dinitrophenylhydrazone, m. p. 149—151° was obtained (Hydrocinnamaldehyde 2,4-dinitrophenylhydrazone, m. p. 147° [26]).

Reduction of benzalacetone semicarbazone. A suspension of 1.0 g of benzalacetone semicarbazone was reduced at pH 13 in 50 % alcohol at a cathode potential of −1.70 V. The reduction required 2 electrons per molecule. The reaction mixture was then diluted with water and extracted with ether, which was evaporated. The residue was recrystallized from benzene-petrol ether, 0.65 g, m. p. 112°. The m. p. was not raised by recrystallization from dilute alcohol, but on recrystallization from benzene and seeding with a crystal of actual benzylacetone semicarbazone the m. p. was raised to 141—142° (benzylacetone semicarbazone m. p. 142°). (Found: C 64.19; H 7.36; N 20.48. Calc. for $C_{11}H_{15}N_3O$: C 64.37; H 7.37; N 20.47.) On boiling with 2,4-dinitrophenylhydrazine in dilute alcohol a 2,4-dinitrophenylhydrazone, m. p. 125—26°, was obtained. (Benzylacetone 2,4-dinitrophenylhydrazone, 125—126,3° [27]).

Reduction of benzaldehyde semicarbazone. A solution of 1.0 g of benzaldehyde semicarbazone was reduced in a mixture of 70 ml peroxide free dioxane, 70 ml alcohol and 50 ml N HCl at a cathode potential of −0.85 V *vs* S.C.E. The reduction required four electrons. After completion of the reaction the solvents were evaporated *in vacuo* and the residue dissolved in alkali, which was extracted with ether. After drying dry hydrogen chloride was added and a precipitate m. p. 252—255° was obtained. The amine was benzoylated yielding a derivative m. p. 104—106° (N-benzyl benzamide m. p. 105—107°). (Found: C 79.54; H 6.04; N 6.30. Calc. for $C_{14}H_{13}NO$: C 79.57; H 6.21; N 6.64.)

Reduction of benzophenone semicarbazone. 1.0 g of benzophenone semicarbazone was reduced in 200 ml 50 % ethanolic 0.5 N HCl containing 3 g of semicarbazide hydrochloride at −0.9 V *vs* S.C.E. The reduction required four electrons per molecule. The reaction medium was extracted with ether after completion of the reduction, and 0.1 g of an unidentified compound was isolated from the ether. The aqueous phase was made alkaline and extracted with ether, which was dried. On addition of alcoholic hydrogen chloride a precipitate, 620 mg, was obtained, which was identified as benzhydrylamine hydrochloride from its analysis. (Found: C 70.85; H 6.60; N 6.40; Cl 16.00. Calc. for $C_{13}H_{14}NCl$: C 71.07; H 6.42; N 6.37; Cl 16.14.)

From the reduction of benzophenone semicarbazone at pH 4 a hydrochloride was isolated, which on recrystallization from water yielded a compound, that was identified as 1-benzhydrylsemicarbazide from its m. p. 165° (164—165°) and analysis. (Found: C 69.43; H 6.32; N 17.59. Calc. for $C_{14}H_{15}N_3O$: C 69.68; H 6.27; N 17.42.)

REFERENCES

1. Zuman, P. *Nature* **165** (1950) 485.
2. Zuman, P. *Collection Czechoslov. Chem. Communs.* **15** (1950) 839.
3. Wolfe, J. K., Hershberg, E. B. and Fieser, L. F. *J. Biol. Chem.* **136** (1940) 653.
4. Prelog, V. and Häfliger, O. *Helv. Chim. Acta* **32** (1949) 2088.
5. Brezina, M., Volkova, V. and Volke, J. *Collection Czechoslov. Chem. Communs.* **19** (1954) 894.
6. Whitnack, G. C., Young, J. E., Sisler, H. H. and Gantz, E. St. C. *Anal. Chem.* **28** (1956) 833.
7. Souchay, P. and Graizon, M. *Chim. anal.* **36** (1954) 85.
8. Langer, A. *Ind. Eng. Chem., Anal. Ed.* **14** (1942) 283.
9. Souchay, P. and Ser, S. *J. chim. phys.* **49** (1952) C172.
10. Gardner, H. J. and Georgans, W. P. *J. Chem. Soc.* **1956** 4180.
11. Elofson, R. M. and Atkinson, J. G. *Can. J. Chem.* **34** (1956) 4.
12. Brdicka, R. and Wiesner, K. *Collection Czechoslov. Chem. Communs.* **12** (1947) 138.
13. Brdicka, R. *Collection Czechoslov. Chem. Communs.* **12** (1947) 212.
14. Zuman, P., Tengyl, J. and Brezina, M. *Collection Czechoslov. Chem. Communs.* **19** (1954) 46.
15. Tyutyulkov, I. N. and Buduvoff, S. *Compt. rend. acad. bulgare sci.* **6** (1953) 5; *Chem. Abstracts* **49** (1955) 5158.

16. Tyutyulkov, I. N. and Stefanova, L. *Compt. rend. acad. bulgare sci.* **9** (1956) 49; *Chem. Abstracts* **52** (1958) 4359.
17. Knudsen, P. *Ber.* **42** (1909) 3994.
18. Sartori, G. and Gaudiano, A. *Gazz. chim. ital.* **78** (1948) 77.
19. Tafel, J. and Pfeffermann, E. *Ber.* **35** (1902) 1510.
20. Calzolari, C. and Furlani, A. D. *Gazz. chim. ital.* **87** (1957) 862.
21. Lund, H. *Acta Chem. Scand.* **11** (1957) 283.
22. Blicke, F. F. and Lu, C. *J. Am. Chem. Soc.* **74** (1953) 3933.
23. Swain, C. G. and Dittmer, D. C. *J. Am. Chem. Soc.* **77** (1955) 3924.
24. Johnston, C. D. *Science* **106** (1947) 91.
25. Ercoli, A. and Giuseppe, L. *Farm. sci. e tec. Pavia* **6** (1951) 702.
26. Roduta, F. L. and Quibilan, G. *Rev. filipina med. Y farm.* **27** (1936) 123; *Chem. Abstracts* **31** (1937) 98.
27. Bunnett, J. F., Marks, J. L. and Moe, H. *J. Am. Chem. Soc.* **75** (1953) 985.

Received October 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 268—274

An X-Ray Analysis of the 1:1 Compound Trimethylamine-Iodine

KNUT O. STRØMME

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The crystal structure of the 1:1 molecular compound trimethylamine-iodine has been determined by X-ray methods. The nitrogen-halogen arrangement is linear. A short N—I distance (2.27 Å) suggests a strong interaction between nitrogen and halogen. The observed I—I distance (2.83 Å) is 0.17 Å longer than the covalent I—I bond length observed in the "free" I_2 molecule.

Structure determinations of addition compounds between weak organic amines (with dissociation constants in water solution of the order 10^{-9}) and halogens [1,2] show the nitrogen-halogen-halogen arrangement to be linear. The interaction between the donor (nitrogen) atom responsible for the Lewis base properties of the amine, and the acceptor (halogen) atom is strong, and the nitrogen-halogen distance slightly larger than that to be expected for a normal covalent bond. It might be questioned whether or not appreciably stronger organic bases behave in a similar way towards halogens. According to Hantzsch [3] the addition reaction between trimethylamine (diss. const. 7.4×10^{-5}) and bromine leads to the formation of bromotrimethylammonium bromide.

$$[(CH_3)_3NBr]^+Br^-$$

Attempts to obtain single crystals of this compound were not successful. Using iodine instead of bromine, however, single crystals of the corresponding addition compound suitable for X-ray analysis could be grown without too great difficulty.

EXPERIMENTAL

The compound trimethylamine-iodine was obtained as an orange coloured precipitate by adding a solution of potassium triiodide to a solution of the amine in water [4]. After filtration the precipitate was dried over phosphorus pentoxide in a desiccator. The composition was determined by titration of iodine against a standardized solution of sodium thiosulphate. Decomposition point 66°C. Recrystallisation was effected by cooling a

solution of the compound in methylene chloride to −50°C. The crystals show a marked tendency to twin.

The rather unstable crystals were mounted in boronlithium capillaries before exposure to X-rays. Their cross-sectional dimensions perpendicular to the rotation axis were about 0.15 × 0.20 mm².

Weissenberg and oscillation photopgraphs were taken with Cu-$K\alpha$ radiation at about −20°C, the cooling being achieved by an adjustable stream of cold air. Using the double film technique two series of Weissenberg photographs were given exposure times from 1 to 100 h. The intensities estimated visually by comparison with a standard scale, were corrected for Lorentz- and polarization effects. No correction for absorption and secondary extinction effects was applied. 66 $hk0$ and 125 $h0l$ reflections are attainable with Cu-$K\alpha$ radiation. Actually, 50 and 92 were observed, respectively. The atomic scattering factors employed in the calculations of the structure factors are given by the Hartree and Thomas-Fermi f-curves [5]. A correction of the form exp. $(-B \sin^2 \Theta/\lambda^2)$ was applied to the calculated structure factors. The temperature factor, B, was found by inspection to be 1.70 Å² and 1.20 Å² for the ($hk0$) and ($h0l$) zones, respectively. Absorption effects are probably mainly responsible for the smallness of these figures.

CRYSTAL DATA

The crystals are orthorhombic with axial dimensions: $a = 11.37$ Å, $b = 8.30$ Å, $c = 8.34$ Å. With four $(CH_3)_3N.I_2$ molecules per unit cell the calculated density is 2.64 g/cm³, as compared to the experimental value of 2.57 g/cm³.

When twinning occurs the individual crystals retain a common orientation of the a axis, whereas the orientations of the b and c axes interchange. Since these axes differ only slightly in length, superposition of X-ray reflections may occur.

Absence of reflections excludes all space groups but $Pnma$ and $Pna2_1$. The former is centrosymmetric and was assumed to be the correct one. It demands that the four amine groups be situated in mirror planes.

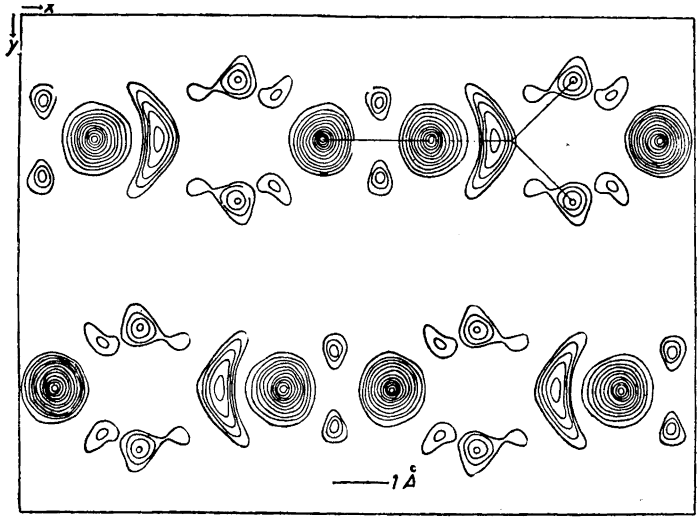


Fig. 1. Electron density projection along the c axis. Contour intervals: 10 e · Å⁻² for iodine atoms and 2 e · Å⁻² for nitrogen and carbon atoms. The first contour is the 6-electron line.

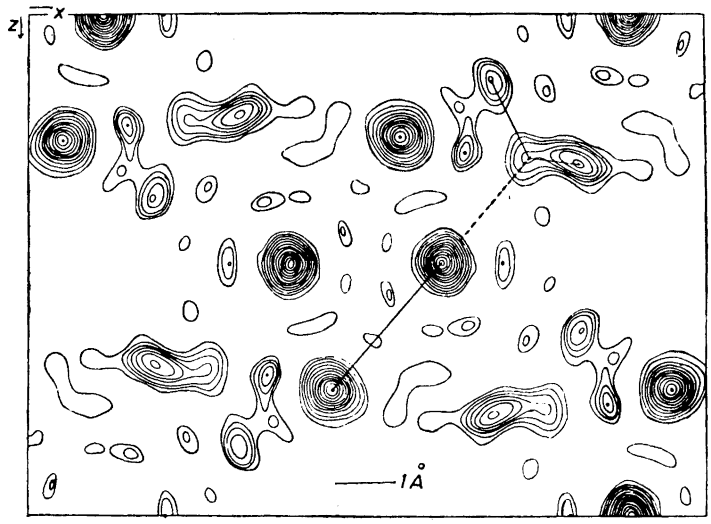


Fig. 2. Electron density projection along the *b* axis. Contours as in *Fig.* 1.

DETERMINATION OF THE STRUCTURE

The positions of the iodine atoms were found from two dimensional Patterson syntheses based on the (hk0) and (*h*0*l*) reflections, combined with trial and error computations. Using the heavy atom method for the sign determination, the electron density map, Fig. 1 was worked out. In this figure the full lines represent bonds within the amine and the halogen groups, whereas the broken line indicates the bond between the groups. The iodine atoms are seen to be situated in mirror planes, *i.e.* in fourfold positions.

For the (010) projection which is shown in Fig. 2, successive refinements had to be carried out. The small contributions of the heavy atoms to the structure factors with indices $h = 2n + 1$ and $l = 2n$ made the successive introduction of the light atoms necessary. Except for two crystallographically equivalent N—C distances the projection along the *b* axis on the mirror plane gives the intramolecular bond distances in their true lengths.

Table 1 contains a list of atomic parameters. The suffixes attached to the atomic symbols refer to the structure formula shown below. The *y* para-

Table 1. Atomic coordinates in fractions of the cell-edges. Origin at a center of symmetry.

Atom	*x*	*y*	*z*
I_1	0.613	0.250	0.498
I_2	0.448	0.250	0.752
N	0.741	0.250	0.289
C_1	0.812	0.101	0.302
C_2	0.812	0.399	0.302
C_3	0.686	0.250	0.133

Table 2. Standard deviations of peak positions [6].

I_1:	$\sigma(x) = 0.005$ Å;	$\sigma(z) = 0.006$ Å
I_2:	$\sigma(x) = 0.007$ Å;	$\sigma(z) = 0.007$ Å
N:	$\sigma(x) = 0.069$ Å;	$\sigma(z) = 0.073$ Å

meters of two carbon atoms situated outside the mirror plane are derived from a (F_{obs}—F_{hal}) synthesis using the ($hk0$) reflections. Almost identical values of the x parameters of the heavy atoms are obtained from the two projections.

The agreement factor, defined as $R = \Sigma \| F_{obs} | - | F_{calc} \| / \Sigma | F_{obs} |$, including not observed reflections when $| F_{calc} | > | F_{obs,\ min.} |$ is 0.13_7 for the ($hk0$) zone and 0.16_7 for the ($h0l$) zone. Observed and calculated F- values are listed in Table 3.

Table 3. Observed and calculated structure factors. The figures represent one fourth of the absolute values.

h	k	l	F_{obs}	F_{calc}	h	k	l	F_{obs}	F_{calc}
0	2	0	99.4	−102.6		1		31.7	− 29.6
	4		67.0	72.4		2		<5.4	0.0
	6		51.8	− 56.0		3		30.1	25.5
	8		35.2	42.6		4		<5.8	1.6
	10		16.1	− 29.1		5		26.6	− 20.7
2	0	0	26.8	32.1		6		<5.7	0.4
	1		19.2	18.7		7		18.9	19.0
	2		34.2	− 39.5		8		<4.2	− 2.1
	3		26.0	− 21.5	10	0	0	<5.9	− 5.8
	4		31.0	34.3		1		20.3	20.6
	5		19.2	15.9		2		<5.6	6.6
	6		22.3	− 24.2		3		17.6	− 15.0
	7		11.8	− 6.5		4		<5.7	− 6.7
	8		13.7	15.9		5		17.0	13.0
	9		<5.2	5.8		6		<4.8	5.0
4	0	0	26.6	− 28.8		7		13.3	− 13.6
	1		24.9	− 31.2	12	0	0	20.4	− 19.4
	2		28.8	27.5		1		26.6	26.0
	3		30.4	29.5		2		20.7	19.6
	4		21.8	− 21.2		3		23.3	− 23.1
	5		21.4	− 23.4		4		17.4	− 18.7
	6		21.9	16.7		5		16.6	20.2
	7		13.8	13.8		6		6.0	14.7
	8		11.7	− 12.7	14	0	0	17.7	− 13.2
	9		<4.0	− 9.5	0	1		<3.5	7.6
6	0	0	26.4	− 24.7		2		14.6	11.6
	1		53.8	− 59.8	0	0	2	25.4	− 11.9
	2		31.1	27.3			4	72.5	78.1
	3		56.9	50.4			6	10.1	1.8
	4		33.2	− 26.1			8	37.2	40.4
	5		46.9	− 40.4			10	6.9	2.0
	6		20.1	18.3	1	0	1	32.6	− 34.6
	7		36.9	32.4			2	<2.4	3.5
	8		11.5	− 11.9			3	42.9	− 35.8
	9		18.1	− 24.2			4	3.7	− 4.8
8	0	0	<5.2	1.0			5	19.8	− 21.5

h	k	l	F_{obs}	F_{calc}	h	k	l	F_{obs}	F_{calc}
		6	10.4	6.8			9	8.1	— 15.0
		7	24.1	— 21.3	7	0	1	9.8	— 9.7
		8	6.6	— 1.0			2	5.9	— 2.0
		9	10.1	— 9.0			3	7.3	— 6.0
		10	<3.6	— 0.1			4	<4.8	— 0.7
2	0	0	24.7	29.9			5	5.1	— 8.2
		1	20.7	— 25.5			6	<5.2	— 1.0
		2	20.6	— 20.5			7	5.7	— 4.2
		3	15.3	19.7			8	4.2	0.0
		4	29.5	30.2			9	7.9	— 5.5
		5	19.4	— 20.5	8	0	0	<4.5	— 3.5
		6	15.9	— 19.9			1	17.4	— 13.9
		7	11.0	14.2			2	48.1	47.1
		8	16.9	20.7			3	16.1	— 17.1
		9	14.3	— 14.8			4	<5.1	— 1.0
		10	6.6	— 11.7			5	10.2	— 10.6
3	0	1	16.4	20.5			6	29.0	32.2
		2	<2.8	— 2.8			7	10.9	10.3
		3	22.8	25.0			8	<3.7	0.4
		4	10.3	4.2	9	0	1	34.9	— 26.8
		5	19.7	15.7			2	<5.0	0.3
		6	4.8	— 4.4			3	25.0	— 20.1
		7	9.2	11.4			4	<5.2	1.6
		8	8.4	5.6			5	24.7	— 24.0
		9	11.3	10.6			6	<4.5	— 0.9
		10	<3.3	— 0.2			7	16.1	— 15.7
4	0	0	27.6	— 24.5			8	<3.9	1.0
		1	35.4	— 43.8	10	0	0	7.8	— 6.6
		2	43.6	— 52.0			1	<5.2	— 0.2
		3	33.0	37.5			2	40.6	36.5
		4	19.1	— 20.5			3	<5.2	— 0.4
		5	26.8	— 29.3			4	<5.0	— 6.4
		6	31.0	— 31.3			5	<4.7	0.2
		7	15.1	18.5			6	27.1	29.2
		8	15.9	— 13.9			7	<5.1	— 1.8
		9	10.3	— 13.8	11	0	1	<5.1	1.2
		10	14.5	— 17.4			2	<5.0	0.4
5	0	1	39.0	36.7			3	4.9	— 4.1
		2	<3.6	0.0			4	<4.6	0.4
		3	22.7	27.8			5	<4.2	0.3
		4	<4.3	1.3			6	<3.6	1.8
		5	28.8	26.5	12	0	0	24.3	— 28.4
		6	5.0	— 3.0			1	13.6	14.5
		7	15.6	19.3			2	4.9	0.8
		8	<4.8	1.3			3	13.9	— 14.6
		9	16.6	15.3			4	21.6	— 25.6
		10	<2.5	— 1.9			5	10.4	10.8
6	0	0	31.1	— 25.3	13	0	1	19.2	15.1
		1	30.5	— 31.0			2	<3.9	0.8
		2	6.6	— 2.0			3	21.4	18.8
		3	24.1	28.1			4	<3.0	— 2.0
		4	24.4	— 22.0			5	6.0	11.5
		5	17.7	— 21.9	14	0	0	14.4	— 18.3
		6	<5.2	— 0.1			1	10.5	12.7
		7	17.8	21.6			2	12.9	— 7.9
		8	13.6	— 14.3					

Table 4. Interatomic distances and angles in $(CH_3)_3N \cdot I_2$.

Bond type	Bond length (Å)	Sum of covalent radii (Å)	Angle type	Angle-dimension (degrees)
I_1-I_2	2.83	2.66	$N-I_1-I_2$	179
I_1-N	2.27	2.03	$I_1-N-C_{1\,(2)}$	107
$I_1-C_{1\,(2)}$	3.05		I_1-N-C_3	114
I_1-C_3	3.15		C_1-N-C_2	114
$N-C_{1\,(2)}$	1.48	1.47	$C_{1\,(2)}-N-C_3$	108
$N-C_3$	1.44	1.47		
C_1-C_2	2.36			
$C_{1\,(2)}-C_3$	2.47			

CONCLUSION

Table 4 contains calculated intramolecular distances and angles and some covalent bond lengths [7]. The suffixes attached to the atomic symbols refer to the structure formula

$$\begin{matrix} C_1 \diagdown \\ C_2 — N \ldots I_1 — I_2 \\ C_3 \diagup \end{matrix}$$

Intermolecular distances are found to be in satisfactory agreement with the sums of accepted van der Waals' radii [7]. Standard deviations in bond lengths [8] are as follows:

$$\sigma\ (I_1—I_2) = 0.009\ \text{Å}$$
$$\sigma\ (I — N) = 0.070\ \text{Å}$$

The structure of this compound is closely related to the structures of the 1:1 complexes between weak organic bases and halogen [1,2]. The nitrogen-halongen-halogen group is linear. The short N—I distance indicates a strong interaction between donor and acceptor atoms. At the same time the I—I bond is weakened, the bond being about 0.2 Å longer than in the "free" I_2 molecule. The values of the N—I bond lengths observed in the 1:1 compounds pyridine-iodomonochloride and trimethylamine-iodomonochloride are 2.26 Å [2] and 2.30 Å [10], respectively. A reinvestigation of the compound pyridine-iodomonochloride gave 2.30 Å for the N—I bond length [10].

The value of the dissociation constant of the amine or the nature of the second halogen atom appears to be of minor importance in determining the nitrogen-iodine bond length. The iodine atom directly linked to nitrogen is believed to possess in its valence shell five electron pairs in sp^3d_{z2} hybrid orbitals, whereas the second iodine atom retains its octet of electrons in its valence shell. The atomic arrangement to be expected is then a linear grouping of nitrogen-halogen-halogen. A more comprehensive discussion of these problems is given by Hassel [9,10].

Finally it should be stressed that the results of this investigation appear to contradict the assumption first expressed by Hantzsch that the addition compounds between trimethylamine or other tertiary amines and halongens [3,4] should be interpretated as salts of a halogen substituted ammonium cation.

The author is grateful to professor O. Hassel for his interest and advice throughout this work.

REFERENCES

1. Eia, G. and Hassel, O. *Acta Chem. Scand.* **10** (1956) 139.
2. Hassel, O. and Rømming, Chr. *Ibid.* **10** (1956) 696; **11** (1957) 195.
3. Hantzsch, A. *Ber.* **38** (1905) 2161.
4. Bøhme, H. and Krause, W. *Chem. Ber.* **84** (1951) 170.
5. *Internationale Tabellen zur Bestimmung von Kristallstrukturen*, Gebrüder Borntraeger, Berlin 1935.
6. Cruickshank, D. W. J. *Acta Cryst.* **2** (1949) 65.
7. Pauling, L. *The Nature of the Chemical Bond*, Cornell University Press, New York 1948.
8. Cruickshank, D. W. J. and Robertson, A. P. *Acta Cryst.* **6** (1953) 698.
9. Hassel, O. *Proc. Chem. Soc.* **1957** 250.
10. Hassel, O. *Molecular Physics* **1** (1958) 241.

Received October 25, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 275—280

Crystal Structure of the 1:1 Addition Compound Formed by Acetone and Bromine

O. HASSEL and KNUT O. STRØMME

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The crystal structure of the equimolecular compound containing acetone and bromine has been determined at temperatures near −30°C using Weissenberg technique (CuK-radiation) and precession technique (MoK-radiation). The space group is $C2/c$ and the lattice parameters: $a = 7.12$ Å; $b = 7.48$ Å; $c = 12.90$ Å; $\beta = 111.5°$. Endless chains are present in this structure, bromine atoms and the carbonyl carbon and oxygen atoms of each chain being coplanar. Each oxygen atom is linked to two neighbouring oxygen atoms of the same chain by halogen molecule bridges and the O—Br—Br—O arrangements are linear. The observed Br—Br distance was found equal to the distance observed in free bromine molecules (2.28 Å), the O—Br distance 2.82 Å. This structure affords the first example of two halogen molecule bridges starting from a common oxygen atom.

Results obtained in previous investigations of the crystal structure of addition compounds containing halogen molecules and electron donor molecules like ethers, amines and aromatic hydrocarbons made it appear of interest to include at least *one* addition compound between halogen and ketone in our research programme [1]. It would appear probable that in such compounds "halogen molecule bridges" are formed between keto oxygen atoms belonging to neighbouring ketone molecules. There is no doubt a marked tendency to establish endless chains of alternating halogen and donor molecules in addition compounds. In the case of ketones, however, such chains could not be formed unless both lone electron pairs of a particular keto oxygen atom are capable of forming electron transfer bonds at the same time.

We have chosen the 1:1 addition compound of acetone and bromine as an example. According to Mass and McIntosh [2] this compound has a melting point of —8°C and the X-ray analysis of single crystals should therefore not be more difficult than for several other substances which have already been successfully investigated.

On cooling equimolecular mixtures of the two components in thin capillary tubes it was found that single crystals could indeed be grown which appeared

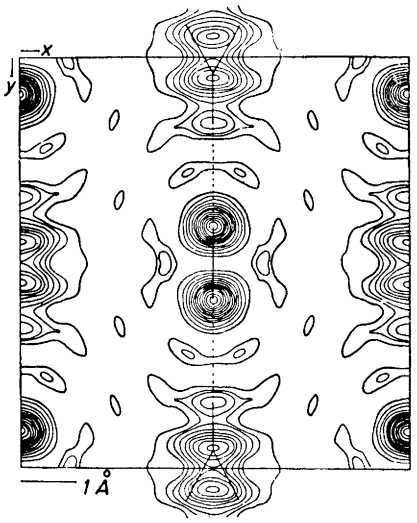


Fig. 1. Fourier projection along the *c* axis. First contour line represents 4 e · Å^{-2}. Contour intervals 2 e . Å^{-2} for light atoms, 10 e · Å^{-2} for bromine.

satisfactory for our purpose. When using Weissenberg technique, however, it is useful that crystals are available that have one of their crystallographic axes approximately parallel to the axis of the capillary tube. In our case we succeeded in producing crystals which fulfilled this requirement with respect to one crystallographic direction only, the *c*-axis. The material obtained from Weissenberg diagrams with Cu*K*-radiation had therefore to be supplemented by material obtained using a precession camera and Mo*K*-radiation.

No efforts were made in order to keep the crystal at a constant temperature during the exposure, but the temperature was never far from a mean value of about —30°C.

According to the X-ray data the addition compound is monoclinic and the space group either $C2/c$ or C/c. The lattice parameters are:

$$a = 7.12 \text{ Å}; \quad b = 7.48 \text{ Å}; \quad c = 12.90 \text{ Å}; \quad \beta = 111.5°$$

The density of the crystals was not determined experimentally, but a reasonable value is 2.1 which leads to a number of four molecules of each kind in the unit cell.

From a two dimensional Patterson synthesis preliminary x and y coordinates of the bromine atoms were determined and a Fourier map with projection along [001] was worked out. It was assumed that the space group is $C\ 2/c$ and the signs of the structure factors were determined from the coordinates of the bromine atoms alone, neglecting contributions from the lighter atoms. The resulting electron density map is reproduced in Fig. 1. No changes of structure factor signs occurred after introducing the contribution from carbon and oxygen atoms. Fig. 1 thus represents the final electron density projection along [001]. The R-factor of this projection is 0.13. The heavy peaks correspond to double bromine atoms, the overlapping of the atoms being complete or at least almost so. Approximate x and y coordinates of the lighter

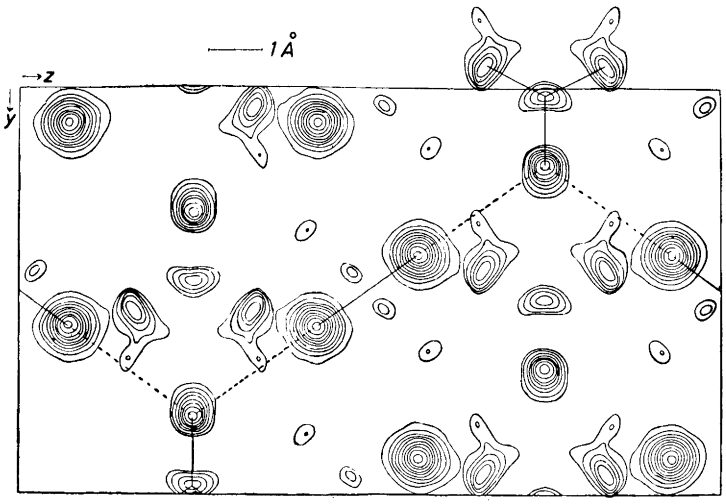


Fig. 2. Fourier projection along the *a* axis. First contour line represents 4 e · Å^{-2}. Contour intervals 1 e · Å^{-2} for light atoms, 6 e · Å^{-2} for bromine.

atoms may also be obtained from this projection. The C—O distance of the carbonyl group is in good agreement with the value deduced from the [100] projection (1.22 Å).

Before starting to work out the Fourier projection along [100] the corresponding Patterson map was computed in order to determine the *z* coordinates and verify the *y* coordinates already determined. Neglecting again the lighter atoms the Fourier chart reproduced in Fig. 2 was obtained. More accurate values for the coordinates of the light atoms were worked out by computing a difference synthesis with subtraction of the contribution from the bromine atoms. This procedure led to some modifications of the coordinates determined from the original map and also to minor corrections of the bromine parameters. With the new parameter values the *R* factor for the [100] projection dropped from 0.12 to 0.10. The final parameters for both the bromine and the light atoms are listed in Table 1.

The fall in observed X-ray intensities for increasing reflexion angles turned out to be smaller for the bromine acetone compound than for most addition

Table 1. Atomic coordinates in fractions of the cell-edges. Origin at a center of symmetry.

Atom	*x*	*y*	*z*
Br	0.000	0.091	0.071
O	0.000	0.307	0.250
C_1	0.000	0.469	0.250
C_2	0.062	0.577	0.169
C_3	−0.062	0.577	0.331

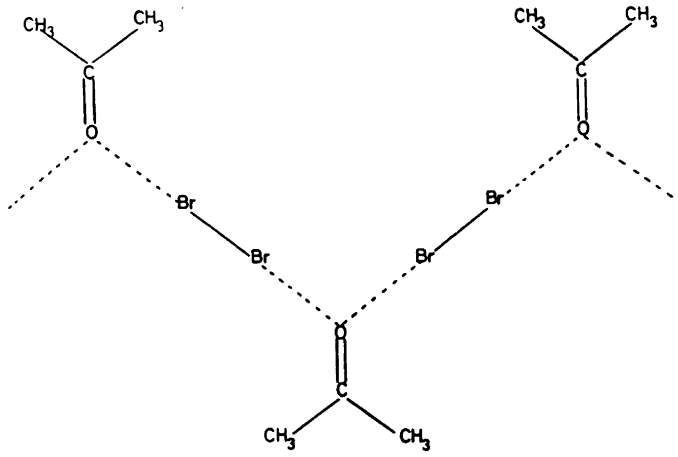


Fig. 3. The planar chains.

compounds with halogens previously investigated (*B* values 2.1 and 2.3 for the projection along [100] and [001]), respectively. This is probably due to the presence of the fairly rigid planar double chains present in the acetone-bromine structure (see below).

There is actually very strong evidence that not only are the bromine atoms lying in the (100) plane but also the carbon and oxygen atoms of keto groups belonging to a particular chain. The coordinate values of the latter atoms are listed in the upper part of Table 1. From these values follows a Br-Br bond distance of 2.28 Å. Allowing for probable errors in the bromine coordinates it may at least be stated that this distance cannot be substantially greater than the corresponding bond distance in the "free" molecule which is indeed 2.28 Å. The distance between a bromine atom and the nearest oxygen atom to which it is directly attached becomes 2.82 Å, a value somewhat larger than the O-Br distance observed in the addition compound between 1,4-dioxan and bromine namely 2.71 Å. The standard deviation in the Br-Br distance is 0.006 Å, in the O-Br distance 0.028 Å.

Table 2. Interatomic distances and angles.

Bond	Bond length (Å)	Sum of covalent radii (Å)	Angle	
Br—Br	2.28	2.28	Br—Br—O	180°
Br—O	2.82	1.80	Br—O—Br	110°
O—C_1	1.22		C_2—C_1—C_3	112°
C_1—$C_{2(3)}$	1.51	1.54		
C_2—C_3	2.56			
C_2—Br	3.77			
C_3—Br	3.73			

Table 3. Observed and calculated structure factors. The figures represent one fourth of the absolute values.

h k l	F_{obs}	F_{calc}
	(*hk*0) zone	
2 0 0	61.7	71.9
4 0 0	40.9	43.0
6 0 0	24.8	22.9
8 0 0	11.2	15.1
1 1 0	31.3	36.7
3 1 0	28.8	32.4
5 1 0	23.7	24.1
7 1 0	12.1	16.0
9 1 0	4.6	9.1
0 2 0	28.7	29.1
2 2 0	24.7	23.0
4 2 0	18.8	14.4
6 2 0	10.3	8.7
8 2 0	4.0	5.3
1 3 0	8.8	− 4.3
3 3 0	8.1	− 3.1
5 3 0	5.8	− 2.5
7 3 0	<2.1	− 1.7
0 4 0	27.6	−28.5
2 4 0	25.1	−25.1
4 4 0	20.8	−18.1
6 4 0	11.2	−11.8
8 4 0	5.7	− 7.3
1 5 0	33.2	−33.2
3 5 0	27.2	−26.6
5 5 0	20.3	−20.9
7 5 0	10.1	−14.3
0 6 0	26.8	−25.2
2 6 0	25.6	−22.5
4 6 0	19.1	−16.6
6 6 0	10.8	−10.8
1 7 0	14.5	−13.2
3 7 0	11.5	−12.4
5 7 0	7.6	−10.7
0 8 0	6.3	− 3.8
2 8 0	4.9	− 3.6
1 9 0	7.7	6.5
3 9 0	5.4	5.5

h k l	F_{obs}	F_{calc}
	(0*kl*) zone	
0 2 0	27.9	27.0
0 4 0	24.9	−25.9
0 6 0	22.2	−25.6
0 8 0	4.5	− 5.1
0 2 1	23.9	−22.5
0 4 1	19.5	−19.3
0 6 1	5.2	5.1
0 8 1	8.5	8.7
0 0 2	21.1	23.1
0 2 2	15.0	14.8
0 4 2	18.5	−17.9
0 6 2	15.6	−16.3
0 8 2	<4.9	− 0.1
0 2 3	41.0	−49.3
0 4 3	24.7	−25.9
0 6 3	<4.7	6.5
0 8 3	12.6	15.1
0 0 4	11.1	− 4.8
0 2 4	9.7	− 6.6
0 4 4	9.5	7.2
0 6 4	5.2	6.4
0 8 4	<4.7	0.4
0 2 5	24.3	−24.8
0 4 5	17.7	−19.3
0 6 5	9.0	7.8
0 8 5	12.8	11.5
0 0 6	37.9	−40.1
0 2 6	12.4	−11.2
0 4 6	16.9	12.8
0 6 6	15.0	14.5
0 8 6	<4.7	1.1
0 2 7	6.9	− 5.1
0 4 7	<4.4	− 0.5
0 6 7	<4.8	− 1.3
0 0 8	28.8	−28.0
0 2 8	11.7	−13.2
0 4 8	15.7	16.9
0 6 8	15.7	18.2
0 2 9	20.8	20.6
0 4 9	10.9	11.9
0 6 9	<4.9	− 1.9
0 0 10	13.9	−11.1
0 2 10	<4.6	− 2.8
0 4 10	<4.9	2.7
0 6 10	<4.9	3.4
0 2 11	19.6	18.9
0 4 11	14.0	15.0
0 6 11	<4.8	− 4.7
0 0 12	18.3	17.9
0 2 12	<4.9	5.3
0 4 12	<4.9	− 6.3
0 2 13	6.9	6.6
0 4 13	<4.7	3.2
0 0 14	13.4	11.8
0 2 14	<4.7	4.7

The O—Br—Br—O arrangement is linear. The angle between the two O—Br—Br—O lines meeting in a particular oxygen atom is 110°. The arrangement of the carbonyl and bromine atoms in the plane of the chains (*cf.* Fig. 3) thus appears to be determined in a very simple way by the position of the two lone electron pairs of the oxygen atom and the linearity of the O—Br—Br—O arrangement. The presence of halogen-molecule bridges between oxygen atoms of neighbouring acetone molecules was not unexpected; the new feature of this structure is the fact that a particular oxygen atom may indeed serve as a "bridge-head" for *two* such bridges.

The only atoms of the chains which do not appear to be situated in the (100) planes are those of the methyl groups. The angle between the C—C bonds and the plane of the chains is 16°. The reason for this tilting of the methyl groups is probably to be sought in the circumstance that only in this way the distance from the methyl carbon atom to the two nearest bromine atoms belonging to neighbouring chains will be of equal length and correspond to the accepted "van der Waals distance".

The relatively slow fall in X-ray intensities for increasing reflexion angles mentioned above indicates more moderate thermal vibrations than those met with in many other addition compounds. This finding must no doubt be a consequence of the particular form of chains present in the structure which should be expected to facilitate vibrations perpendicular to the plane. Actually, the observed anisotropy of the thermal vibrations is in accord with this view, the vibration parallel to the x-axis being more pronounced than in directions perpendicular to the x-axis.

Table 2 contains a list of observed interatomic distances and angles. In Table 3 observed and calculated structure factors are listed. The number of reflexions observed in the ($hk0$) zone is 37 (theor. 39), in the ($0kl$) zone 40 (theor. 60). The f-curves used in this calculations are those given in the *Internationale Tabellen* (1935).

This investigation has been sponsored by the United States Air Force Development Command under contract AF 61 (052)—71.

REFERENCES

1. Hassel, O. *Molecular Physics* **1** (1958) 241.
2. Maass, O. and McIntosh, D. *J. Am. Chem. Soc.* **34** (1912) 1273.

Received October 25, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 281—288

Aminosugars and Related Compounds

Part VI*. The Action of Alkali on Some Benzyloxycarbonylamino Derivatives

A. B. FOSTER, M. STACEY and S. V. VARDHEIM**

Chemistry Department, The University, Edgbaston, Birmingham 15, England

The action of aqueous ethanolic sodium hydroxide on methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (II) yields an unexpected product to which the structure methyl 2-amino-3:6-anhydro-2-*N*:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside (IV) has been allocated. The susceptibility of benzyloxycarbonylamino alkyl derivatives towards alkali appears to be influenced by the presence of hydroxyl groups in the same molecule. Benzyloxycarbonylamino*cyclo*hexane is converted to a mixture of di*cyclo*hexylurea and ethoxycarbonylamino*cyclo*hexane by the action of alkali. Several new derivatives of methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside are described.

The action of alkali on suitable 6-*O*-toluene-*p*-sulphonyl-D-glycopyranoside derivatives yields 3:6-anhydro-D-glucopyranoside compounds [1]. The reaction occurs in other hexopyranose derivatives and has been well studied [2]. 2-Amino-3:6-anhydro-2-deoxy-D-glucose and its derivatives are however unknown and an attempted synthesis of a derivative by the alkaline treatment of methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (II) gave unexpected results.

Treatment of methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (I) with one mole of toluene-*p*-sulphonyl chloride in pyridine gave, as anticipated [3], the 6-*O*-toluene-*p*-sulphonate (II). That the C_6 hydroxyl group had been substituted in this reaction was indicated by the ready exchange of the toluene-*p*-sulphonyloxy residue for iodine on treatment of (II) with sodium iodide in acetone at 100° for 2 h to yield methyl 2-benzyloxycarbonylamino-2:6-di-deoxy-6-iodo-α-D-glucopyranoside (III). This type of exchange

* Part V. *Acta Chem. Scand.* **12** (1958) 1605.

** Present address (S.V.V.) Papirindustriens Forskningsinstitutt, Boks 250, Vinderen Oslo, Norway.

reaction is generally characteristic of primary toluene-*p*-sulphonates [3,4]. The consumption of approximately 1 mole of lead tetra-acetate by the 6-*O*-toluene-*p*-sulphonate (II) indicated the presence of a vicinal glycol grouping in the molecule and confirmed the allocated structure. When methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (II) was treated with 0.33 N sodium hydroxide in boiling aqueous ethanol (water-ethanol 1:2 v/v)* a rapid reaction took place ($[M]_D$ + 445° → + 93° during 30 min). An almost identical change in $[M]_D$ occurred during 36 h when the reaction was carried out at room temperature. Only one compound, *A*, could be isolated from the reaction and it appeared to be the major if not the sole product. Compound *A* has been allocated the structure methyl 2-amino-3:6-anhydro-2-*N*:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside (IV) on the basis of the following arguments and observations.

Elemental analysis was consistent with the formula $C_8H_{10}O_5N$ and the molecular weight (196) obtained by cryoscopy in water indicated a monomeric structure.

Evidence, which may be interpreted as indicating the presence of a 3:6-anhydro ring in compound *A*, is provided by infra-red spectra measurements. The spectra of a series of 3:6-anhydrohexopyranosides have been studied by Barker and Stephens [5], who found that, in general the spectrum of a 3:6-anhydro compound in the region 700—900 cm^{-1} was more complex than that of the parent glycoside. Further, the 3:6-anhydrohexopyranosides showed apparently characteristic absorptions in the ranges 870 ± 7, 838 ± 16, 798 ± 21 and 747 ± 11 cm^{-1} (for a discussion of the assignment of these absorptions reference should be made to the original paper [5]). The absorptions at 878, 860, 820 and 755 cm^{-1} shown by compound *A* provide a reasonably close parallel with these findings and are suggestive of a 3:6-anhydro hexopyranoside structure.

Table 1 illustrates the similarities in the infra-red spectra in the region 700—900 cm^{-1} of methyl 3:6-anhydro-α-D-glucopyranoside, Compound *A* and the respective parent compounds.

Table 1.

Methyl α-D-gluco-pyranoside [a]	896 s		840 s		745 s
Methyl 3:6-anhydro-α-D-glucopyranoside [a]	909 vs 892 vs	868 s	842 s	815 vs	742 vs
Methyl 2-benzyloxycarbonyl-amino-2-deoxy-α-D-gluco-pyranoside [b,c]	902 w 910 w		850 w		740 m 764 m
Methyl 2-amino-3:6-anhydro-2-*N*:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside (Compound *A*) [b]	900 w 914 s	878 w	860 m	820 w	755 m 762 m

a Data taken from Ref.[5] and measured in "Nujol" mulls.
b Measured in KBr discs.
c Also showed absorption at 779 cm^{-1} (w).
Key: w week, m medium, s strong, v very.

* Unless otherwise stated, subsequent reference to alkali implies a reagent of this composition.

Further evidence suggesting the presence of a 3:6-anhydro-ring in compound *A* is the similar changes in optical rotation which occur when methyl 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside [1] and methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (II) are treated with alkali. The ready conversion, under these conditions, of the former compound to methyl 3:6-anhydro-α-D-glucopyranoside is well established [1].

Proof of the presence of a 3:6-anhydro ring in compound *A* followed when, on vigorous acidic hydrolysis a product *B*, presumably 2-amino-3:6-anhydro-2-deoxy-D-glucofuranose, was obtained which appeared homogeneous and displayed properties anticipated for an anhydro aminosugar on examination by paper chromatography and zone electrophoresis (ionophoresis). Reaction of *B* with phenylhydrazine yielded a compound identical with 3:6-anhydro-D-glucose phenylosazone obtained by treatment of authentic 3:6-anhydro-D-glucofuranose with phenylhydrazine.

Absorption in the range 1 700—1 736 cm^{-1} (amide I band) of a series of carbamates (R—O.CO.NH.R') has been associated with the carbonyl group [6]. Thus the strong absorption at 1 708 cm^{-1} in the spectrum of compound *A* is consistent with the presence of a carbamate grouping. However, the absence of absorption at *ca.* 1 540 cm^{-1} indicated that the carbamate grouping, if present, was part of a cyclic structure. Although an adequate explanation is lacking, it is well established [6] that the amide II band (N—H deformation) characteristic of acyclic amides is not shown when the NH—CO grouping is part of a cyclic system. This is true of tetrahydro-1:3-oxazine-2-one [7], the simplest cyclic carbamate analogue of compound *A*, and of a wide range of other related compounds [6,8]. The absorption at 3 280 cm^{-1} in the spectrum of compound *A* is therefore most likely due to an N—H bond and not to an O—H bond, an inference substantiated by the fact that compound *A* could not be converted to an *O*-acetate or *O*-toluene-*p*-sulphonate.

Since a 3:6-anhydro-ring is present in compound *A*, then, in a pyranoside structure, a cyclic carbamate grouping can involve only the amino and hydroxyl groups on C_2 and C_4 respectively. That the C_4-hydroxyl group is blocked is suggested by the resistance of compound *A* to cold dilute acid. Methyl 3:6-anhydro-α- and β-D-glucopyranoside [1] (and related compounds [2]) readily undergo an acid catalysed intramolecular rearrangement to yield the corresponding furanoside compounds. However, this rearrangement does not occur if the C_4-hydroxyl group is blocked, *e.g.* by methylation [1] (or in the galactose series [9] where the C_4-hydroxyl group is located in a sterically unfavourable position).

The presence of the carbamate grouping in compound *A* was confirmed when vigorous acidic hydrolysis liberated carbon dioxide.

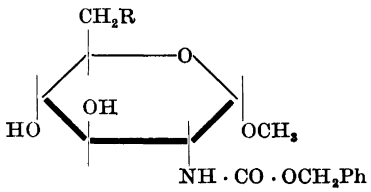
Formation of the 3:6-anhydro-ring during the action of alkali on methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (II) probably precedes the formation of the cyclic carbamate since, under similar alkaline reaction conditions, methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (I), in which 3:6-anhydro-ring formation is precluded, does not form a cyclic carbamate but undergoes rapid (*ca.* 30 min) decarboxylation (separation of sodium carbonate). Examination of accurate scale models shows that initial formation of the 3:6-anhydro-ring, following

the action of alkali on the 6-*O*-toluene-*p*-sulphonate (II), would bring the benzyloxycarbonylamino and hydroxyl groups at C_2 and C_4, respectively, into *meta* axial positions so that the hydroxyl group would be very favourably situated for nucleophilic attack on the carbonyl group of the benzyloxycarbonylamino residue with consequent expulsion of the benzyloxy group.

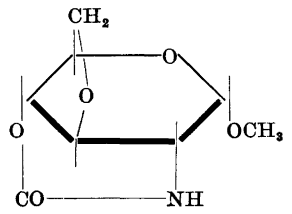
The stability of benzyloxycarbonylaminoalkyl groups towards alkali appears to be significantly influenced by the presence of hydroxyl groups in the same molecule. This is illustrated by the examples quoted above and by the facts that both 2-benzyloxycarbonylaminoethanol and 3-benzyloxycarbonylaminopropan-1-ol underwent rapid decarboxylation (30 min) on treatment with alkali. On the other hand benzyloxycarbonylamino*cyclo*hexane was much more resistant. Treatment of this compound (readily obtained from the reaction of *cyclo*hexylamine and benzylchloroformate) with alkali for 12 h did not cause complete reaction since 8 % of pure starting material could be recovered. The main product (59 %) formed together with benzyl alcohol was di*cyclo*hexylurea, identical with the compound obtained by the reaction of di*cyclo*hexyl carbodi-imide and phosphoric acid. Also produced in the reaction was ethoxycarbonylamino*cyclo*hexane (*N*-carbethoxy*cyclo*hexylamine) identical with the product obtained by the action of ethylchloroformate on *cyclo*hexylamine. No reaction occurred when benzyloxycarbonylamino*cyclo*hexane was treated with 0.33 N sodium hydroxide in aqueous dioxan (66 % dioxan).

The preceding observations indicate that care must be exercised in the selection of the amine protecting group in aminosugar syntheses where the use of alkaline reagents is contemplated.

Parallel with the above investigations a number of derivatives of methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside were prepared. Treatment [10] of methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside with benzaldehyde at 140° yielded methyl 4:6-*O*-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside which gave a crystalline 3-*O*-methyl ether, 3-*O*-benzoate, 3-*O*-methanesulphonate and 3-*O*-toluene-*p*-sulphonate. The location of the benzylidene residue across the C_4:C_6-hydroxyl groups in these compounds was shown on acidic hydrolysis of the 3-*O*-methyl derivative when known [11] 2-amino-2-deoxy 3-*O*-methyl-D-glucose hydrochloride was formed. This latter compound was readily converted to crystalline *N*-(2:4-dinitrophenyl) and *N*-(2-hydroxynaphthylidene-1) derivatives.



I, R = OH
II, R = $CH_3 \cdot C_6H_4 \cdot SO_2 \cdot O$
III, R = I



IV

EXPERIMENTAL

*Methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-*D*-glucopyranoside.* A solution of methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside[12] (1 g, m. p. 159°, $[\alpha]_D$ +89° in pyridine) in dry pyridine (10 ml) was cooled (0°) and treated gradually with a solution of freshly recrystallized toluene-*p*-sulphonyl chloride (0.58 g) in dry pyridine (2 ml). After storage at 0° for 2 h and then at room temperature overnight the reaction mixture was poured into ice water (150 ml). The oily compound which separated solidified after vigorous agitation of the suspension for 1 h. It was collected (0.73 g, 50 %) and recrystallized from aqueous-ethanol (50 %, v/v) to yield the product, m. p. 103°, $[\alpha]_D$ +96° (*c*, 1.0 in ethanol). Kyi[13] reports m. p. 103—104° for this product.

A solution of the above toluene-*p*-sulphonate (96 mg) in glacial acetic acid (20 ml) was treated with a 2.9 % solution (25 ml) of lead tetra-acetate in glacial acetic acid and the volume rapidly adjusted to 50 ml. Aliquots were withdrawn at suitable time intervals and the consumption of oxidant determined by a standard procedure[14]: the following results were obtained.

Time (h)	1	24	48	72	96	120
Oxidant consumed (mole)	0.096	0.29	0.483	0.725	0.918	0.918

Methyl 2-benzyloxycarbonylamino-2:6-dideoxy-6-iodo-α-D-glucopyranoside. A solution of methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (0.8 g) and sodium iodide (1.6 g) in acetone (16 ml) was heated in a sealed tube at 100° for 2 h. Sodium *p*-toluenesulphonate was precipitated. After filtration the reaction mixture was diluted to turbidity with water and stored at 0°. The *product* (0.7 g, 96 %) separated and after recrystallization from alcohol it had m. p. 156°, $[\alpha]_D$ +77° (*c*, 1.0 in acetone). (Found: C 41.5; H 4.7; N 3.1; I 29.0. $C_{15}H_{20}O_6NI$ requires C 41.2; H 4.6; N 3.2; I 29.1.)

*Action of alkali on methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside.* A solution of methyl 2-benzyloxycarbonylamino-2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (1 g) in ethanol (10 ml) and N sodium hydroxide (5 ml) was boiled under reflux. $[\alpha]_D$ changed +92.5 → +19.4° (based on the weight of the 6-*O*-toluene-*p*-sulphonate) during 30 min. After cooling and neutralisation with carbon dioxide the reaction solution was concentrated at 45° (bath)/15 mm, the residue extracted with boiling acetone (20 ml) for 15 min and the extract clarified by filtration through a carbon pad. Evaporation of the filtrate yielded a syrup which crystallized on trituration with ethyl acetate (3 ml) and heating. *Methyl 2-amino-3:6-anhydro-2-N:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside* (0.27 g, 65 %) obtained thus had m. p. 172°, $[\alpha]_D$ +46.4° (*c*, 1.0 in water). (Found: C 47.5; H 5.5; N 6.6. $C_8H_{11}O_5N$ requires C 47.8; H 5.5; N 7.0.)

When the reaction was allowed to proceed at room temperature $[\alpha]_D$ changed +92.5° → +19.2° (final constant value) during 36 h. From the solution a product (0.29 g, 69.5 %) was isolated and found to be identical with that described above.

A solution of methyl 2-amino-3:6-anhydro-2-*N*:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside (0.1304 g) in deionized water (4.8137 g) depressed the freezing point by 0.255°. Employing a freezing point constant of 18.5 (determined using 2-acetamido-2-deoxy-D-glucose as a standard) these results indicate a molecular weight of 196 (calc. 201).

*Acidic hydrolysis of methyl 2-amino-3:6-anhydro-2-N:4-*O*-carbonyl-2-deoxy-α-D-glucopyranoside.* A solution of (a) the cyclic carbamate (0.1 g) in 0.1 N sulphuric acid (5 ml) showed no change in $[\alpha]_D$ during 24 h storage at room temperature; neutralisation and evaporation of the solution gave unchanged starting material, (b) the cyclic carbamate (0.13 g) in 3 N hydrochloric acid (10 ml) was boiled under reflux in a stream of nitrogen for 3 h. The carbon dioxide evolved was collected as barium carbonate (105.4 mg, 83.5 %) which was formed on passage of the effluent gases through barium hydroxide solution.

The hydrolysate was freed from hydrochloric acid by prolonged codistillation with water at 40° (bath)/14—16 mm. Final evaporation yielded amorphous *B*, presumably 2-amino-3:6-anhydro-2-deoxy-D-glucofuranose hydrochloride (0.075 g). The infra red

spectrum (KBr disc) of the product showed absorption at 825 and 878 cm^{-1} indicative [5] of furanose configuration. The hydrolysate was examined by zone electrophoresis (ionophoresis) on Whatman No. 3 paper using the enclosed strip technique [15]. With a borate buffer [16] (pH 10) a single compact zone, M_G 0.9, was detected with aniline hydrogen phthalate. Under the same conditions 2-amino-2-deoxy-D-glucose affords a rather diffuse zone. In an acetate buffer (pH 5) a single zone of higher mobility than that of 2-amino-2-deoxy-D-glucose could be detected with ninhydrin.

Paper chromatographic analysis of the hydrolysate using the *tert*-butanol-acetic acid-water (2:1:1) solvent system [17] revealed a single component, detected with ninhydrin with R_F value (0.497) significantly higher than that of 2-amino-2-deoxy-D-glucose (0.327) but similar to that of 2-amino-1:6-anhydro-2-deoxy-D-gulopyranose [18] and the altrose analogue [18]. A solution of *B* (57.8 mg) in water (1 ml) was treated with acetic acid (0.1 ml), phenylhydrazine (0.12 g) and sodium acetate (0.18 g) and the mixture maintained at 95° for 1.5 h. The solid which separated on cooling was dissolved in methanol (2 ml) and diluted with water (20 ml). 3:6-Anhydro-D-glucose phenylosazone (5.9 mg) separated and after collection and washing with water it gave an infra-red spectrum (KBr disc) identical with that of authentic material described below.

Under identical reaction conditions 2-amino-2-deoxy-D-glucose hydrochloride (50 mg) yielded D-glucosephenylosazone (15.6 mg).

3:6-Anhydro-D-glucosephenylosazone. Crystalline 3:6-anhydro-D-glucose (50 mg) was treated at 75—80° for 2 h with a solution (3 ml) prepared from water (25 ml), sodium acetate (2 g), acetic acid (1 ml) and phenylhydrazine (1 g). On cooling a mixture of crystals and oil separated. The crystals were freed from oil by trituration with aqueous methanol yielding 3:6-anhydro-D-glucose phenylosazone (27.4 mg), m. p. 185—187°. A further amount (13.5 mg) of product was obtained on dilution of the combined mother liquors with water.

Benzyloxycarbonylaminocyclohexane. Sodium hydrogen carbonate (21 g) was added to a solution of *cyclo*hexylamine (25 g) in water (150 ml) followed by benzyl chloroformate (43 g) gradually during 1 h with continuous vigorous stirring. The crude product was collected, washed successively with light petroleum (60—80°) and water, then dissolved in ether and the solution dried ($MgSO_4$). Addition of light petroleum (60—80°) gave *benzyloxycarbonylaminocyclohexane.* (39 g, 66 %), m. p. 93° (Found: C 72.4; H 8.0; N 6.0. $C_{14}H_{19}O_2N$ requires C 72.1; H 8.15; N 6.0). Infra-red spectrum (KBr disc) showed absorptions at 1 545 cm^{-1} (amide II band) and 1 688 cm^{-1} (amide carbonyl).

Action of alkali on benzyloxycarbonylaminocyclohexane. (a) A solution of benzyloxycarbonylamino*cyclo*hexane (25 g) in N sodium hydroxide (125 ml) and ethanol (250 ml) was boiled under reflux for 12 h. On cooling sodium carbonate (2.4 g) separated. The solution was neutralised with carbon dioxide, freed from insoluble material and fractionally distilled using a 12″ column packed with nickel gauze. Ethyl alcohol was collected at 78° followed by a fraction at 98° which after condensation separated into three phases *B*, *C* and *D* of increasing density, respectively. *N:N'*-Di*cyclo*hexyl urea (7.1 g) separated after dissolution of the still residue in aqueous alcohol; it had, after recrystallization from alcohol, m. p. 236° alone or in admixture with an authentic specimen. (Found: C 69.4; H 10.75; Calc. for $C_{13}H_{24}N_2O$: C 69.5; H 10.7.) The identity of the compound was confirmed by infra-red spectroscopy. From the mother liquors by dilution with water unchanged benzyloxycarbonylamino*cyclo*hexane (1.6 g, 8 %) separated with m. p. 92° alone or in admixture with authentic material.

Examination of fractions *B* and *D* by gas phase chromatography (Perkin-Elmer apparatus with packing A at 155° with a flow rate of 15 ml/min) revealed a single component with properties closely similar to those of benzyl alcohol.

The infra-red spectra (liquid film) of fractions *B* and *D* revealed a very close similarity indeed and both showed strong absorptions at 1 692 (amide carbonyl [6]), 1 572 (amide II band, characteristic of acyclic amides) and 697 and 738 cm^{-1} (aromatic group).

Portions of fractions *B* and *D* were combined (2 ml total volume), diluted with 50 % aqueous ethanol (3 ml) and stored at 0°. Ethoxycarbonylamino*cyclo*hexane separated and after recrystallization from ethanol-ether it had m. p. 56° alone or in admixture with an authentic sample. Identity was confirmed by infra-red spectroscopy.

The infra-red spectrum (liquid film) of a solution of ethoxycarbonylamino*cyclo*hexane (1 g) in benzyl alcohol (2 ml) was virtually identical with the spectra of fractions *B* and *D*.

Ethoxycarbonylaminocyclohexane. To a solution of *cyclo*hexylamine (20 g) and sodium hydrogen carbonate (18 g) in water (100 ml), ethyl chloroformate (22 g) was gradually added during 1 h with constant vigorous stirring of the mixture. The crude product was collected, washed with water and a small volume of cold ethanol. Recrystallization from ethanol-ether afforded ethoxycarbonylamino*cyclo*hexane (23 g, 66.5 %) m. p. 55—56°. (Gillibrand and Lamberton [19] record m. p. 56° for this compound prepared by a different procedure.) The infra-red spectrum (KBr disc) showed absorptions at 1 537 cm^{-1} (amide II absorption) and 1 700 cm^{-1} (amide carbonyl).

N:N'-Dicyclohexylurea. A solution of di*cyclo*hexylcarbodi-imide (1 g) in ether (4 ml) was added to a solution of phosphoric acid (0.86 g) in ether (20 ml). Recrystallization, of the product which separated, after 30 min from ethanol yielded N:N'-di*cyclo*hexyl urea (0.7 g, 64 %), m. p. 235°. (Skita and Rolfes [20] record m. p. 230° for this compound prepared by a different method.) The infra-red spectrum (nujol mull) showed absorptions at 1 541 cm^{-1} (amide II absorption) and 1 633 cm^{-1} (amide carbonyl).

Methyl 4:6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside. Methyl 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (2 g) was heated with freshly distilled benzaldehyde at 140—145° for 5 h whilst a stream of carbon dioxide was passed through the reaction mixture. The cooled mixture was then diluted with light petroleum (10 vol, 60—80°) and the precipitate (2.0 g) collected, washed with light petroleum and recrystallized from chloroform-acetone. *Methyl 4:6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside* (1.82 g, 71.5 %) was obtained, m. p. 209—210° $[\alpha]_D$ +51° (*c*, 1.0 in chloroform). (Found: C 63.3; H 5.8; N 3.7. $C_{22}H_{25}O_7N$ requires C 63.6; H 6.0; N 3.4.)

By the usual methods the benzylidene compound was converted to a 3-*O*-toluene-*p*-sulphonate, m. p. 116°, after softening at 60—65°, $[\alpha]_D$ +8° (*c*, 1.0 in $CHCl_3$). (Found: C 61.1; H 5.5; N 2.2; S 5.9. $C_{29}H_{31}O_9NS$ requires C 61.2; H 5.45; N 2.5; S 5.6); 3-*O*-benzoate, m. p. 165—166°, $[\alpha]_D$ +43° (*c*, 1.0 in $CHCl_3$). (Found: C 67.3; H 5.6; N 2.4. $C_{29}H_{29}O_8N$ requires C 67.1; H 5.6; N 2.7); 3-*O*-methanesulphonate, m. p. 138—140°, $[\alpha]_D$ +38° (*c*, 1.0 in $CHCl_3$). (Found: C 55.7; H 5.3; N 2.8; S 6.7. $C_{23}H_{27}O_9NS$ requires C 56.0; H 5.5; N 2.8; S 6.5.)

Methylation of methyl 4:6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside. A solution of methyl 4:6-*O*-benzylidene 2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (0.5 g) in methyl iodide (6 ml) was boiled under reflux in the presence of dry silver oxide (1 g) for 2 h. During this period two additional amounts (1 g) of silver oxide were added. After filtration through a carbon pad the reaction mixture was evaporated and the residue recrystallized from a methanol-chloroform mixture. *Methyl 4:6-O-benzylidene-2-benzyloxycarbonylamino-2-deoxy-3-O-methyl-α-D-glucopyranoside* (0.32 g) was obtained, m. p. 223—224°. (Found: C 64.3; H 6.2; N 3.1. $C_{23}H_{27}O_7N$ requires C 64.3; H 6.3; N 3.3.)

A solution of the above methyl ether (1 g) in 2.5 N hydrochloric acid (15 ml) was boiled under reflux for 4 h. After filtration through a carbon pad the hydrolysate was diluted with water and twice extracted with light petroleum (60—80°). Subsequent evaporation of the aqueous solution gave a syrupy residue which crystallized from methanol-acetone to yield 2-amino-2-deoxy-3-*O*-methyl-D-glucose hydrochloride (0.25 g, 47 %), m. p. 210—215° (decomp.) with discoloration at *ca.* 175°. (Neuberger [11] records m. p. 215° for this compound prepared by a different method.) Examination of the crystalline methyl ether by paper chromatography using the organic phase of a butanol-ethanol-water (4:1:5) system revealed that it contained a trace of the parent amino sugar.

2-Amino-2-deoxy-3-*O*-methyl-D-glucose hydrochloride was readily converted to an *N*-(2:4-dinitrophenyl) derivative which darkened at *ca.* 174° and had m. p. 184°. (Found: C 42.6; H 4.5; N 11.7. $C_{13}H_{17}O_9N_3$ requires C 43.45; H 4.7; N 11.7) and an *N*-(2-hydroxynaphthylidene-1) derivative, m. p. 184—185° (decomp.). (Found: C 62.6; H 6.0; N 4.1. $C_{18}H_{21}O_6N$ requires C 62.3; H 6.05; N 4.0.)

The authors thank Dr. D. H. Whiffen and Dr. K. J. Morgan for the infra-red spectra measurements and for valuable discussion, Mr. G. Durant for samples of 2-benzyloxycarbonylaminoethanol and 3-benzyloxycarbonylaminopropan-1-ol, and Miss B. Parkinson for experimental assistance. One of them (S.V.V.) thanks *Papirindustriens Forskningsinstitutt,* Oslo, Norway for a grant.

REFERENCES

1. Haworth, W. N., Owen, L. N. and Smith, F. *J. Chem. Soc.* **1941** 88.
2. Foster, A. B., Overend, W. G., Stacey, M. and Vaughan, G. *Ibid.* **1954** 3367; Peat, S. *Advances Carbohydrate Chem.* **2** (1946) 38.
3. Tipson, R. S. *Ibid.*, **8** (1953) 107.
4. Oldham, J. W. H. and Rutherford, J. K. *J. Am. Chem. Soc.* **54** (1932) **366.**
5. Barker, S. A. and Stephens, R. *J. Chem. Soc.* **1954** 4550.
6. Bellamy, L. J. *Infra-red spectra of complex molecules.* Methuen, London 1956.
7. Delaby, R., Damiens, R. and d'Huyteza, G. *Compt. rend.* **239** (1954) 674.
8. Randall, H. M., Fowler, R. G., Fuson, N. and Dangl, J. R. *Infra-red determination of organic structures.* van Nostrand, New York 1949.
9. Haworth, W. N., Jackson, J. and Smith, F. *J. Chem. Soc.* **1940** 620.
10. Oldham, M. A. and Honeyman, J. *Ibid.* **1946** 986.
11. Neuberger, A. *Ibid.* **1941** 47, 50.
12. Foster, A. B., Horton, D. and Stacey, M. *Ibid.* **1957** 81.
13. Kyi, Z-Y. *Ph.D. Thesis,* University of Birmingham 1952.
14. McClenahan, W. S. and Hockett, R. C. *J. Am. Chem. Soc.* **60** (1938) 2061.
15. Foster, A. B. *Chem. & Ind. London* **1952** 1050.
16. Foster, A. B., Newton-Hearn, P. A. and Stacey, M. *J. Chem. Soc.* **1956** 30.
17. van Tamelen, E. E. *Personal communication.*
18. Foster, A. B., Stacey, M. and Vardheim, S. V. *Nature* **180** (1957) 247; *Acta Chem. Scand.* **12** (1958) 1605.
19. Gillibrand, M. I. and Lamberton, A. H. *J. Chem. Soc.* **1949** 1883.
20. Skita, A. and Rolfes, H. *Chem. Ber.* **53** (1920) 1242.

Received August 8, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 289—300

On the Kinetics of the Reaction of Cyanic Acid with Amines

MOGENS BALLUND JENSEN

The Royal Danish School of Pharmacy, Copenhagen, Denmark

The velocity constants, k_u, for the reactions: $RR'NH_2^+ + OCN^- \rightarrow RR'NCONH_2$ were determined for ammonium ion and a number of aminium ions. Assuming that the formation of urea or substituted urea is a result of a reaction between free ammonia or amine and free cyanic acid the velocity constants, $k_{OCNH\cdot Am}$, for the reactions: $RR'NH + OCNH \rightarrow RR'NCONH_2$ were calculated from $k_{OCNH\cdot Am} = k_u \cdot K_{Am} \cdot K_{OCNH}/K_{H_2O} \cdot f^2$ where K_{Am}, K_{OCNH} and K_{H_2O} are the dissociation constants for the amine, for cyanic acid and for water, respectively. f is an activity coefficient. The values of $k_{OCNH\cdot Am}$ show a simple relationship to the velocity constants, $k_{CO_2\cdot Am}$, for the analogous reactions between amines and carbon dioxide: $RR'NH + OCO \rightarrow RR'NCOOH$, *viz.* $\log k' = \log k'' - 1.08$ (18°C), where k' stands for $k_{OCNH\cdot Am}$ and k'' for $k_{CO_2\cdot Am}$.

The same relation is observed for the velocity constants for the reactions: $OH^- + OCNH \rightarrow NH_2COO^-$ and $OH^- + OCO \rightarrow HCO_3^-$ and for the velocity constants for the reactions: $H_2O + OCNH \rightarrow NH_3 + CO_2$ and $H_2O + OCO \rightarrow H_2CO_3$.

The present investigation is based on the assumption that the rate determining step for the reaction between ammonium ion and cyanate ion leading to the formation of urea in aqueous solution is a reaction between free ammonia and free cyanic acid. In the case of the analogous reactions between aminium ion and cyanate ion yielding substituted urea, the rate determining step is correspondingly assumed to be a reaction between free amine and free cyanic acid.

The purpose of the investigation has been to demonstrate that the velocity constants for the reaction of cyanic acid with amines (*incl.* ammonia) calculated from the velocity constants for the reaction between aminium ion and cyanate ion show a simple relationship to the velocity constants for the reaction of carbon dioxide with amines.

The reactions to compare are

$$RR'NH + OCNH \rightarrow RR'NCONH_2 \quad (I)$$

and

$$RR'NH + OCO \rightarrow RR'NCOOH \quad (II)$$

The reaction products are urea or substituted urea and carbamic acid or substituted carbamic acid, respectively.

For reaction I the velocity constants, $k_{OCNH\cdot Am}$, were obtained from the velocity constants, k_u, for the gross reaction

$$RR'NH_2^+ + OCN^- \rightarrow RR'NCONH_2 \qquad (III)$$

upon considering the equilibrium

$$RR'NH_2^+ + OCN^- \rightleftharpoons RR'NH + OCNH \qquad (IV).$$

The following relations will hold:

$$\text{rate} = k_{OCNH\cdot Am} \cdot c_{RR'NH} \cdot c_{OCNH} =$$

$$k_{OCNH\cdot Am} \cdot (K_{H_2O}/K_{Am} \cdot K_{OCNH}) \cdot f^2 \cdot c_{RR'NH_2^+} \cdot c_{OCN^-} = k_u \cdot c_{RR'NH_2^+} \cdot c_{OCN^-}$$

K_{H_2O} is the dissociation constant of water, K_{Am} is the basic dissociation constant of the amine, and K_{OCNH} is the acidic dissociation constant of cyanic acid. The activity coefficient of the aminium ion is set equal to that of the cyanate ion and is denoted f. The activity coefficients of the free amine and the free cyanic acid are put equal to unity.

From the above relations the following expression is obtained for $k_{OCNH\cdot Am}$:

$$k_{OCNH\cdot Am} = k_u \cdot K_{Am} \cdot K_{OCNH}/K_{H_2O} \cdot f^2$$

The values of k_u were determined for ammonia and some primary and secondary amines and the values of $k_{OCNH\cdot Am}$ were calculated from the above expression.

The experiments on the determination of k_u are given in the following section.

The velocity constants for reaction II, $k_{CO_2\cdot Am}$, have been determined previously for ammonia and a number of amines including the amines [1-7] here investigated.

The final section gives the results and a discussion.

The experiments were carried out at 18°C. The initial ionic strength was 0.2 and decreased only slightly during an experiment. The velocity constants were calculated on the basis of Napierian logarithms and refer to molar concentration, the unit of time being the minute.

ON THE REACTION: $RR'NH_2^+ + OCN^- \rightarrow RR'NCONH_2$

The formation of urea in solutions containing aminium ion and cyanate ion was followed by determing the content of cyanate at different times. The samples to be analyzed were brought to pH = approx. 5.5 by the addition of acetic acid. The cyanate was precipitated as silver cyanate by the addition of a relatively great excess of silver nitrate and back-titration with ammonium thiocyanate was carried out in a quota part of the filtrate from the silver cyanate [8].

Table 1. The velocity constant for the reaction: $NH_4^+ + OCN^- \rightarrow NH_2CONH_2$. 18°C.

Initial solution					t min.	% cyanate left	% carbonate	k	k mean	k_u
$c_{NH_4^+}$	c_{NH_3}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}						
0.18	0.05	0.02	0.18	0.02	1 501	84.6	1.7	0.000112	0.000101	0.000588
					4 638	62.3		0.000103		
					8 326	43.7	1.9	0.000100		
					12 821	28.8		0.000097		
					21 312	14.2		0.000092		
0.18	0.15	0.02	0.18	0.02	1 694	83.1		0.000109	0.000103	0.000595
					4 520	64.4	0	0.000098		
					6 902	49.8		0.000101		
					12 788	27.4	1.9	0.000102		
0.075	0.095	0.02	0.18	0.125	3 204	88.8	0	0.0000375	0.000040	0.000574
					6 902	75.1		0.0000416		
					12 789	58.3	0.2	0.0000422		
					19 877	45.7		0.0000394		
0.135	0.095	0.02	0.18	0.065	3 203	79.4	0	0.0000720	0.000071	0.000562
					5 990	64.9		0.0000727		
					12 794	39.7	0.1	0.0000725		
					19 874	25.1		0.0000698		
0.18	*	0.02	0.18	0.02	1 778	77.5	5.7	0.000144	0.000119	0.000696
					3 267	65.8	6.9	0.000128		
					5 762	49.8	9.8	0.000121		
					8 648	36.6	12.6	0.000116		
					14 264	23.4	12.3	0.000102		
					17 152	16.6		0.000105		

* No ammonia added.

k_u (mean): 0.000577

The experiments with a few exceptions were carried out with solutions containing both aminium ion and free amine, *i.e.* with solutions being in most cases moderately basic. Under these conditions it was found that the concurrent formation of carbonate was only slight.

The experiments which were carried out in the case of the ammonium ion are listed in Table 1. In all the runs there was a relatively great concentration of ammonium ion compared to that of the cyanate ion, *i.e.* the concentration of ammonium ion varied only slightly during a run meaning that the reaction is approximately of the first order with respect to cyanate ion. The velocity constants, k, calculated from $k \cdot t = \ln a/a\text{-}x$ are also listed in Table 1. The value of k_u was obtained dividing the mean value of k with the mean value of the concentration of ammonium ion. The values of k_u calculated in this way are also given in Table 1 together with the mean value of the values of k_u obtained from the first four experiments.

Table 2. The velocity constant for the reaction:
$CH_3NH_3^+ + OCN^- \rightarrow CH_3NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.18	0.04	0.02	0.18	0.02	903	84.5	0.000186	0.000176	0.00102
					1 255	80.0	0.000178		
					2 260	67.5	0.000174		
					4 003	50.2	0.000172		
					6 988	30.3	0.000171		
0.12	0.10	0.02	0.18	0.08	996	88.1	0.000127	0.000117	0.00104
					2 247	77.0	0.000117		
					3 993	63.6	0.000113		
					6 975	45.5	0.000114		
					11 231	27.6	0.000115		
					4.3×10^4	0.1			
0.18	0.15	0.02	0.18	0.02	954	84.1	0.000182	0.000176	0.00102
					1 225	80.5	0.000177		
					2 434	65.2	0.000176		
					3 986	49.6	0.000176		
					8 008	26.2	0.000167		

k_u (mean): 0.00103

Table 3. The velocity constant for the reaction:
$(CH_3)_2NH_2^+ + OCN^- \rightarrow (CH_3)_2N \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.18	0.04	0.02	0.18	0.02	1 701	37.7	0.000573	0.000556	0.00325
					2 600	23.8	0.000552		
					2 977	19.9	0.000542		
					3 924	11.3	0.000555		
					5 476	4.7	0.000559		
					3.6×10^4	0.3			
0.18	0.04	0.04	0.16	0.02	1 124	53.3	0.000566	0.000547	0.00340
					2 440	24.5	0.000576		
					4 517	9.7	0.000517		
					7 120	2.3	0.000530		
0.18	0.04	0.02	0 *	0.02	1 061	54.1	0.000578	0.000575	0.00336
					2 367	24.6	0.000591		
					3 984	10.9	0.000556		
					7 200	0.7			
0.10	0.10	0.02	0.18	0.10	345	89.2	0.000330	0.000318	0.00349
					1 362	64.1	0.000326		
					2 940	39.2	0.000319		
					4 246	26.5	0.000313		
					5 856	15.8	0.000315		
					8 641	7.4	0.000302		

* $c_{ClO_4^-} = 0.18$ k_u (mean): 0.00338

Table 4. The velocity constant for the reaction:
$C_2H_5NH_3^+ + OCN^- \rightarrow C_2H_5NH \cdot CO \cdot NH_2$. 18°C.

Initial solution				t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$					
0.18	0.04	0.02	0.18*	1 428	86.7	0.0001000	0.0000998	0.000582
				2 936	74.3	0.0001012		
				5 954	55.0	0.0001004		
				10 079	36.8	0.0000992		
				18 562	16.1	0.0000984		
				4.1×10^4	0.2			
0.132	0.03	0.02	0.18**	1 251	90.8	0.0000775	0.0000725	0.000583
				2 736	82.5	0.0000705		
				6 799	60.8	0.0000731		
				12 730	39.8	0.0000723		
				16 911	29.9	0.0000714		
				22 643	20.3	0.0000702		

* $c_{Na^+} = 0.02$
** $c_{K^+} = 0.02$; $c_{Na^+} = 0.05$

k_u (mean): 0.000583

Furthermore, from Table 1 can be seen that with exception of the last mentioned experiment only small amounts of carbonate were formed. In the case of the last mentioned experiment the solution contained only ammo-

Table 5. The velocity constant for the reaction:
$(C_2H_5)_2NH_2^+ + OCN^- \rightarrow (C_2H_5)_2N \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.18	0.04	0.02	0.18	0.02	410	94.2	0.000146	0.000154	0.000890
					1 438	79.7	0.000158		
					2 866	63.2	0.000160		
					4 597	48.7	0.000157		
					7 294	33.3	0.000151		
0.18	0.18	0.02	0.18	0.02	1 428	79.5	0.000160	0.000160	0.000934
					2 857	63.2	0.000160		
					4 400	49.3	0.000161		
					7 404	30.3	0.000161		
					13 042	12.5	0.000160		
0.10	0.10	0.02	0.18	0.10	1 421	87.9	0.0000908	0.0000884	0.000946
					2 834	76.7	0.0000936		
					4 564	67.4	0.0000864		
					7 587	50.9	0.0000869		
					13 077	33.2	0.0000844		

k_u (mean): 0.000923

Table 6. The velocity constant for the reaction:
$C_6H_5CH_2NH_3^+ + OCN^- \rightarrow C_6H_5CH_2NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k_u	k_u mean
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}				
0.04	0.04	0.02	0.18	0.16	218	94.7	0.00691	0.00666
					1 038	77.5	0.00670	
					1 370	72.0	0.00660	
					1 768	65.7	0.00662	
					2 498	56.1	0.00665	
					6 882	25.7	0.00648	
0.02	0.04	0.019	0.18	0.18	458	94.2	0.00674	0.00678
					1 190	86.8	0.00640	
					5 524	57.2	0.00677	
					9 815	42.9	0.00674	
					19 887	27.0	0.00679	
					27 622	20.0	0.00724	

k_u (mean): 0.00672

nium ions and cyanate ions without addition of free ammonia, *i.e.* the pH of this solution was about 6 whereas the pH of the other solutions was about 9.

The formation of considerable amounts of carbonate in more acid solution can be explained through the more rapid decomposition of cyanate into carbon dioxide and ammonia at the lower pH [8].

The contents of carbonate were determined by acidimetric titration of the barium carbonate precipitated from the solutions upon addition of sodium hydroxide and barium chloride.

Table 7. The velocity constant for the reaction:
$CH_2 = CH \cdot CH_2 \cdot NH_3^+ + OCN^- \rightarrow CH_2 = CH \cdot CH_2 \cdot NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.181	0.04	0.02	0.18	0.02	141	91.1	0.000666	0.000618	0.00359
					278	83.2	0.000663		
					1 340	45.0	0.000596		
					2 557	22.3	0.000586		
					2 969	17.6	0.000585		
					4 377	6.8	0.000614		
0.10	0.10	0.02	0.18	0.10	282	90.2	0.000367	0.000332	0.00355
					1 314	64.5	0.000335		
					2 531	49.4	0.000320		
					2 947	40.8	0.000305		
					2×10^4	−0.2			

k_u (mean): 0.00357

Table 8. The velocity constant for the reaction:
$^-OOCCH_2NH_3^+ + OCN^- \rightarrow {}^-OOCCH_2NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	$c_{Na^+}{}_i$					
0.14	0.04	0.02	0	0.06	129	89.7	0.000842	0.000833	0.00630
					308	77.3	0.000836		
					438	69.1	0.000846		
					1 425	30.8	0.000825		
					1 792	23.2	0.000814		
0.10	0.04	0.02	0.04	0.10	843	60.3	0.000600	0.000574	0.00630
					1 184	50.2	0.000582		
					1 483	42.4	0.000579		
					2 417	25.8	0.000560		
					3 774	12.5	0.000550		
					2.2×10^4	0.7			
0.14		0.02	0.04	0.06	217	82.7	0.000876	0.000851	0.00650
					359	73.6	0.000854		
					657	56.7	0.000864		
					1 384	31.1	0.000858		
					3 089	8.4	0.000801		

k_u (mean): 0.00637

The back-reaction, *viz.* the decomposition of urea into ammonium ion and cyanate ion, was neglected. The equilibrium constant for this reaction is reported [9] to be $10^{-4.52}$ (25°C).

From the first four experiments the mean value 0.000577 for k_u was obtained. Walker and Hambly [10] have determined k_u for temperatures between 25°C and 80°C. By extrapolation to 18°C the value 0.00085 is obtained at an ionic strength of 0.1 corresponding to approx. 0.00072 at the ionic strength 0.2. Wyatt and Kornberg [11], however, report the values of Walker and Hambly to be about 20 % too large (at 50°C) because of the concurrent formation of carbonate.

Table 9. The velocity constant for the reaction:
tert.-$C_4H_9NH_3^+ + OCN^- \rightarrow$ *tert.*-$C_4H_9NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.10	0.02	0.02	0.18	0.10	4 018	96.7	(0.00000791)	0.0000102	0.000102
					10 095	89.7	0.0000108		
					21 615	81.1	0.0000096		

Table 10. The velocity constant for the reaction:
$C_6H_5NH_3^+ + OCN^- \rightarrow C_6H_5NH \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k_u	k_u mean
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}				
0.021	0.10	0.017	0.18	0.18	2.0	87.5	3.49	3.63
					5.0	71.9	3.68	
					8.0	60.9	3.68	
					15.0	43.3	3.78	
					22.1	34.9	3.50	
0.044	0.05	0.016	0.18	0.15	1.5	79.9	3.61	3.67
					4.0	55.4	3.71	
					8.0	32.8	3.73	
					14.0	16.7	3.63	
					1 h	0.8		
0.043	0.10	0.016	0.18	0.15	1.9	72.7	4.10	4.04
					3.9	53.7	4.07	
					6.9	36.6	3.92	
					10.9	21.2	4.06	

k_u (mean): 3.8

In the Tables 2—11 are listed the experiments which were carried out with different aminium ions. In these tables are also given the values of k_u. In those experiments where a relatively great excess of aminium ion was applied, the values of k_u were obtained dividing the mean value of k with the mean value of the aminium ion concentration. The values of k were calculated from the expression for an unimolecular reaction. In those experiments where the

Table 11. The velocity constant for the reaction:
$CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2^+ + OCN^- \rightarrow CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot N \cdot CO \cdot NH_2$. 18°C.

Initial solution					t min.	% cyanate left	k	k mean	k_u
c_{AmH^+}	c_{Am}	c_{OCN^-}	$c_{NO_3^-}$	c_{Na^+}					
0.18	0.12	0.02	0.18	0.02	1 102	82.9	0.000170	0.000173	0.000989
					2 530	64.2	0.000176		
					4 192	48.3	0.000174		
0.095	0.10	0.02	0.18	0.09	1 532	87.0	0.0000913	0.0000867	0.000993
					2 976	76.4	0.0000905		
					5 411	61.5	0.0000898		
					9 746	43.7	0.0000851		
					15 452	27.7	0.0000830		
					22 724	16.1	0.0000803		
					3 months	<0.5			

k_u (mean): 0.000992

concentration of aminium ion and the concentration of cyanate ion were of the same order of magnitude the values of k_u were calculated from the expressions for bimolecular reactions.

In the experiments with anilinium ion (Table 10) a few percent of the cyanate was present as free cyanic acid but this was not taken into consideration in the calculation of k_u.

Determinations of the contents of carbonate carried out in some of the experiments showed that only small amounts of carbonate were formed.

From the experiments with methylaminium ion (Table 2) the value 0.00103 was obtained for k_u. This value agrees well with the value 0.00109 calculated by extrapolation from the data of Miller [12] at an ionic strength of 0.082.

RESULTS AND DISCUSSION

In column 1 of Table 12 is given a survey on the values of k_u determined in the preceding section. Column 1 also gives the value of k_u for the reaction between hydrazinium ion and cyanate ion yielding semicarbazide, $H_2N{\cdot}NH{\cdot}CO{\cdot}NH_2$. The value of k_u for this reaction was calculated from the data of Baker and Gilbert [13].

Column 2 of Table 12 contains the pK_{Am} values for the amines. From the columns 1 and 2 can be seen that some correlation appears to exist between the values of k_u and pK_{Am}. The value of k_u for anilinium ion is for instance about 7×10^3 times greater than the value of k_u for ammonium ion. There are, however, large deviations from linearity in a plot of log k_u against pK_{Am}.

Column 3 of Table 12 presents the values of $k_{OCNH{\cdot}Am}$ calculated from

$$k_{OCNH{\cdot}Am} = k_u \cdot K_{Am} \cdot K_{OCNH}/K_{H_2O} \cdot f^2 = k_u \cdot K_{Am} \times 10^{11.03}.$$

The values $10^{-3.54}$ (Ref.[8]) and $10^{-14.22}$ have been used for K_{OCNH} and K_{H_2O}, respectively. From the expression of Svirbely and Warner [14] the value $10^{-0.35}$ is obtained for f^2 in the case of the ammonium ion. This value has been used for the aminium ions as well.

Column 3 also contains the velocity constants [8] at 18°C for the reactions

$$H_2O + OCNH \rightarrow NH_3 + CO_2 \qquad (V)$$

and

$$OH^- + OCNH \rightarrow NH_2COO^- \qquad (VI)$$

The velocity constants for the reactions V and VI are denoted k_{OCNH} and $k_{OCNH{\cdot}OH^-}$, respectively. The value of $k_{OCNH{\cdot}OH^-}$ has not been determined directly but was calculated from the previously determined velocity constant, k_{OCN^-}, for the gross reaction: $OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^-$. ($k_{OCN^-}$ is $10^{-6.48}$ at 18°C). Assuming reaction VI to be the rate determining step for this reaction [15] the following equation will hold: $k_{OCNH{\cdot}OH^-} = k_{OCN^-} \cdot K_{OCNH}/K_{H_2O}$

For $k_{OCNH{\cdot}Am}$, $k_{OCNH{\cdot}OH^-}$ and k_{OCNH} the joint designation k' is used.

Column 4 of Table 12 presents the values of $k_{CO_2{\cdot}Am}$ at 18°C.

Table 12. Velocity constants for the reactions: $RR'NH^+ + OCN^- \rightarrow RR'NCONH_2$ (k_u), $RR'NH + OCNH \rightarrow RR'NCONH_2$($k_{OCNH\cdot Am}$) and $RR'NH + OCO \rightarrow RR'NCOOH$($k_{CO_2\cdot Am}$). 18°C.

Amine	1 k_u	2 pK_{Am}[a]	3 $k_{OCNH\cdot Am}$	4 $k_{CO_2\cdot Am}$
Ammonia	0.000577	4.76	$10^{3.03}$	$10^{4.02}$
Methylamine	0.00103	3.38	$10^{4.66}$	$10^{5.77}$ b
Dimethylamine	0.00338	3.22	$10^{5.34}$	$10^{6.18}$
Ethylamine	0.000583	3.34	$10^{4.46}$	$10^{5.55}$
Diethylamine	0.000923	3.00	$10^{5.00}$	$10^{5.82}$
Benzylamine	0.00672	4.74	$10^{4.12}$	$10^{5.42}$
Allylamine	0.00357	4.38	$10^{4.20}$	$10^{5.42}$
Glycinate ion	0.00637	4.34	$10^{4.32}$ c	$10^{5.41}$
tert.-Butylamine	0.000102	3.55	$10^{3.49}$	$10^{4.64}$
Aniline	3.8	9.46 d	$10^{2.15}$	$10^{3.2}$ e
Hydrazine	0.749 f	6.06	$10^{4.84}$	$10^{5.87}$
Piperidine	0.000992	2.95	$10^{5.08}$	$10^{6.25}$
Hydroxyl ion			$10^{4.20}$	$10^{5.38}$
Water			0.047	1.52 g 0.87 h

a The values of pK_{Am} are, with exception of the pK_{Am}-value for aniline, the same as have been used in the publications on the carbamates.

b Redetermined, not previously published value.

c In the case of glycine the value of $k_{OCNH\cdot Am}$ was calculated from $k_{OCNH\cdot Am} = k_u \cdot K_{OCNH} / K'_{AmH^+} \cdot f$ where $a_{H^+} \cdot c_{^-OOC\cdot CH_2NH_2} / c_{^-OOC\cdot CH_2NH_3^+} = K'_{AmH^+} = 10^{-9.88}$ and f was put equal to $10^{0.18}$.

d Value of Lundén [19].

e Only small amounts of phenyl carbamate are formed upon addition of carbon dioxide to solutions containing aniline. In a previous paper [5] values of $k_{CO_2\cdot Am}$ from $10^{2.64}$ to $10^{3.78}$ were reported. The listed value of $k_{CO_2\cdot Am}$ is the mean value of these values.

f Calculated from the data of Baker and Gilbert [13].

g Value of Faurholt [1]. h Value of Pinsent, Pearson and Roughton [16].

Column 4 also contains the velocity constants at 18°C for the reactions [1,16]

$$H_2O + OCO \rightarrow H_2CO_3 \qquad \text{(VII)}$$

and

$$OH^- + OCO \rightarrow HCO_3^- \qquad \text{(VIII)}$$

The velocity constants for the reactions VII and VIII are denoted k_{CO_2} and $k_{CO_2\cdot OH^-}$, respectively.

For $k_{CO_2\cdot Am}$, $k_{CO_2\cdot OH^-}$ and k_{CO_2} the joint designation k'' is used.

From the columns 3 and 4 can be seen that the values of k' are about a twelfth of the values of k''. Roughly, one can still say that the two set of velocity constants are of the same order of magnitude.

Fig. 1 shows that a straight line is obtained if log k' is plotted against log k''. The slope of the line is approx. 1 and the intersection with the ordinate axis is at — 1.08. This indicates the following relation between k' and k''

$$\log k' = \log k'' - 1.08$$

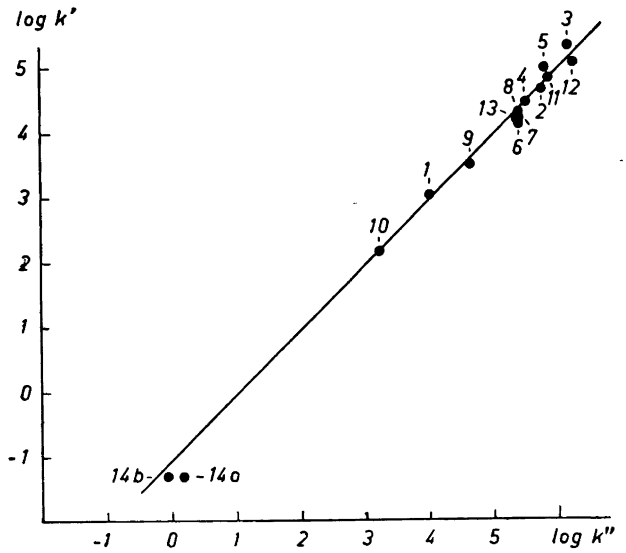


Fig. 1. Plot of log k' against log k''. k' and k'' are the velocity constants (18°C, min.) for the reactions between cyanic acid or carbon dioxide, respectively, and the following molecules and ions: 1) ammonia, 2) methylamine, 3) dimethylamine, 4) ethylamine, 5) diethylamine, 6) benzylamine, 7) allylamine, 8) glycinate ion, 9) *tert.*-butylamine, 10) aniline, 11) hydrazine, 12) piperidine, 13) hydroxyl ion, 14a) water (k'': value of Faurholt [1]) and 14b) water (k'': value of Pinsent, Pearson and Roughton [16]).

The largest deviations from the straight line are found in the case of the two secondary amines dimethylamine and diethylamine where the deviations amount to approx. + 0.2 units in the logarithm. In the case of the secondary amine piperidine the above stated expression fits well, however. In the case of benzylamine the deviation amounts to approx. — 0.2 units in the logarithm.

It should be noted that the reactions I and III are kinetically indistinguishable, because of the mobile equilibrium indicated in IV. Also the theoretical effect of changing ionic strength and dielectric constant will be the same for both mecanisms. As pointed out by Frost and Pearson [17] reaction I is, however, the most probable, seen from a stereochemical point of wiev. The reason is supposed to be that this reaction easily can take place by addition of the nitrogen atom's unshared pair of electrons to the positively polarized carbon atom of cyanic acid. In the case of ammonia the reaction is

$$\begin{array}{ccccc} HN{=}C{=}O & \rightarrow & HN{=}C{-}O^- & \rightarrow & HN{-}C{=}O \\ & & | & & H \quad | \\ H\ddot{N}H & & HNH^+ & & NH \\ H & & H & & H \end{array}$$

A simple proton shift completes the transformation to urea. This is similar to a host of reactions involving addition of basic reagents to carbonyl groups.

Reaction II between amines and carbon dioxide is an example of such a reaction.

The above stated analogy between cyanic acid and carbon dioxide requires that at least a part of the cyanic acid exists as the imide form corresponding to the formula OCNH in aqueous medium. Electron diffraction appears to show that the acid has the imide structure (at least mainly) [18]. If only a minor part of the cyanic acid is present as OCNH in aqueous solution and if only the OCNH form is the one that reacts the values of $k_{OCNH \cdot Am}$ have to be multiplied with a common factor greater than 1 in order to give the true values of the velocity constants for reaction I. This is one possible reason which could explain the fact that the velocity constants for the reactions with cyanic acid are smaller than the velocity constants for the reactions with carbon dioxide. Another possibility, or at least a contributory cause, could be that the carbon atom in OCNH is less positively polarized than the carbon atom in OCO, *i.e.* a somewhat weaker attraction exists between the carbon atom and the nitrogen atom in the cyanic acid-amine reactions than is to be found in the carbon dioxide-amine reactions.

It should be added that a detailed discussion of the mecanism of the addition of amines to cyanic acid recently has been given by Johncock, Kohnstam and Speight [20].

Acknowledgements. The author wishes to express his gratitude to Professor Carl Faurholt for his kind interest in the investigation.

REFERENCES

1. Faurholt, C. *J. Chim. phys.* **22** (1925) 1.
2. Lund, V. and Faurholt, C. *Dansk Tidskr. Farm.* **22** (1948) 109.
3. Staal, E. and Faurholt, C. *Dansk Tidskr. Farm.* **25** (1951) 1.
4. Jensen, A., Jensen, J. B. and Faurholt, C. *Acta Chem. Scand.* **6** (1952) 395.
5. Jensen, A., Jensen, M. B. and Faurholt, C. *Acta Chem. Scand.* **6** (1952) 1073.
6. Jensen, A., Christensen, R. and Faurholt, C. *Acta Chem. Scand.* **6** (1952) 1086.
7. Jensen, M. B. *Acta Chem. Scand.* **11** (1957) 499.
8. Jensen, M. B. *Acta Chem. Scand.* **12** (1958) 1657.
9. Lewis, G. N. and Randall, M. *Thermodynamics.* McGraw-Hill Book Company, Inc. 1923, p. 587.
10. Walker, J. and Hambly, F. J. *J. Chem. Soc.* **67** (1895) 746.
11. Wyatt, P. A. H. and Kornberg, H. L. *Trans. Faraday Soc.* **48** (1952) 454.
12. Miller, C. C. *Proc. Roy. Soc.* **A 145** (1934) 288.
13. Baker, E. M. and Gilbert, E. C. *J. Am. Chem. Soc.* **64** (1942) 2777.
14. Svirbely, W. J. and Warner, J. C. *J. Am. Chem. Soc.* **57** (1935) 1883.
15. Kemp, I. A. and Kohnstam, G. *J. Chem. Soc.* **1956** 900.
16. Pinsent, B. R. W., Pearson, L. and Roughton, F. J. W. *Trans. Faraday Soc.* **52** (1956) 1512.
17. Frost, A. A. and Pearson, R. G. *Kinetics and Mecanism.* John Wiley and Sons, Inc. 1953, p. 257.
18. Eyster, E. H., Gilette, R. H. and Brockway, L. O *J. Am. Chem. Soc.* **62** (1940) 3236.
19. Lundén, H. *J. Chim. phys.* **5** (1907) 145.
20. Johncock, P., Kohnstam, G. and Speight, D. *J. Chem. Soc.* **1958** 2544.

Received October 9, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 301—311

The Oxidation of Emulsified 1-*p*-Menthene with Molecular Oxygen

VÄINÖ E. VEIJOLA and ATSO O. ILVESPÄÄ

Department of Chemistry, Finland Institute of Technology, Helsinki, Finland

1-*p*-Menthene prepared by hydrogenating purified *d*-limonene has been emulsified in water and oxidized at temperatures between 65 and 80° without any catalyst and at 10° with catalyst present.

Under the former conditions the oxidation of 1-*p*-menthene took place at position 6 while under the latter conditions it took place at positions 3 and 6. The hydroperoxides formed, 1-*p*-menthene-3-hydroperoxide and 1-*p*-menthene-6-hydroperoxide, were found to have the *trans* configuration.

The refractive index n_D^{25} of *trans*-carvotanacetol was found to be 1.4736. This alcohol underwent rearrangement to the *cis* form in both acid and alkaline media. The refractive index n_D^{25} of the resulting *cis*-carvotanacetol was 1.4772.

On chromic acid oxidation, both the *trans* and *cis* forms of carvotanacetol were converted into carvotanacetone ($n_D^{25} = 1.4785$). The infrared absorption spectrum of carvotanacetone was recorded. The infrared spectrum of the piperitone ($n_D^{25} = 1.4825$) derived from 1-*p*-menthene-3-hydroperoxide was identical with that reported by Le Fèvre *et al.* for the compound.

In all stages of the investigation the micro sorption method developed by Blohm proved very useful. With this method it was possible to separate, for example, the *trans* and *cis* forms of carvotanacetol from each other.

When unsaturated cyclic hydrocarbons are oxidized with molecular oxygen, the oxygen usually attacks the carbon atom or atoms in the alpha positions relative to the double bond.[1] When the oxidation is photosensibilized, the double bond is shifted at the same time [2]. In both cases the compounds first formed are hydroperoxides. The photosensibilized oxidations will not be discussed in this publication.

Because the oxidation of 1-*p*-menthene with molecular oxygen has not been reported before, the oxidation of a number of compounds similar in structure to 1-*p*-menthene will be first briefly examined. In the oxidation of limonene [3–7] the point of attack is primarily position 6 although according

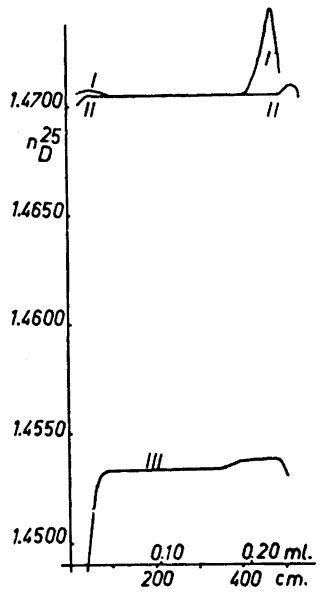


Fig. 1. Sorptograms. I, pomegranate oil; II, product isolated by steam distillation; III, hydrogenated product.

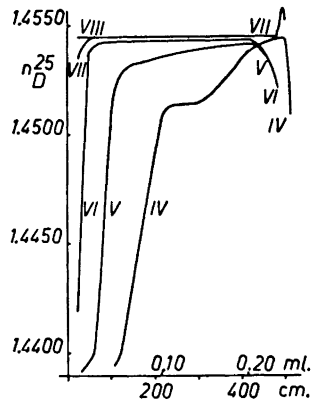


Fig. 2. Sorptograms. IV, initial fraction 1; V—VIII, fractions 2—5.

to Eschinazi [6] addition to position 3 also occurs to a slight extent. Fushizaki *et al.*[8] found that carbon 3 is attacked in the oxidation of terpinolene. The products of oxidation of 1-*p*-methyl*cyclo*hexene have indicated that both carbons 3 and 6 are attacked [9–12]. However, Cook [13] has reported that the oxidation occurs almost solely at carbon 3. He performed the oxidation at 70° with iron phthalocyanine as catalyst.

The results reported in the literature lead one to conclude that the oxidation of 1-*p*-menthene by molecular oxygen will involve the methylene groups in the alpha positions relative to the double bond, *i.e.* positions 3 and 6. The main purpose of the present study was to determine whether the oxidation takes place at one or both of these positions. To decide this, 1-*p*-menthene was emulsified in water and oxidized with molecular oxygen both with and without catalyst present and the reaction products or their derivatives identified. In addition to customary methods, the micro sorption method of Blohm [14] was used to separate the terpene alcohols and ketones from one another before identification.

Preparation of 1-p-*menthene.* 1-*p*-Menthene was prepared by hydrogenating *d*-limonene isolated from French pomegranate oil (Roure-Bertrand Fils, Grasse). According to Igolen *et al.*[15], pomegranate oil contains over 90 % *d*-limonene. The high *d*-limonene content of the oil used is shown by the sorptogram I (Fig. 1). The oil was first subjected to steam distillation. A part of the minor components passed over in the first fractions and a part remained in the distillation residue. In this and in subsequent stages the products

were protected by a carbon dioxide atmosphere. The distillate was separated from the condensed water and dried with sodium wire at room temperature. The refractive index n_D^{25} of the dried distillate was 1.47021, and its specific rotation $[a]_D^{25} = 97°5'$. The refractive indices of the fractions on the plateau of its sorptogram II (Fig. 1) were the same, $n_D^{25} = 1.47056$, as those of the fractions on the plateau of sorptogram I. Widmark [16] has reported for pure *d*-limonene a refractive index n_D^{25} of 1.4703.

The *d*-limonene fractions were hydrogenated [17,18] in absolute alcohol with Raney nickel as catalyst. The hydrogenation of *d*-limonene to 1-*p*-methene proceeded fairly rapidly, but the hydrogenation of the latter to *p*-menthane took place much more slowly. Since it is difficult to separate *d*-limonene and 1-*p*-methene by fractional distillation, whereas 1-*p*-menthene and *p*-menthane are readily separated, the hydrogenation was continued until the *d*-limonene had been completely hydrogenated. The progress of the reaction could be easily followed by recording sorptograms of samples taken during the reaction. As the unsaturation of *d*-limonene decreased, the refractive index of the reaction mixture decreased rapidly and at the same time the adsorption on the sorptogram column. Owing to this, the first sections of the sorptograms gave the refractive index of *p*-menthane, then that of 1-*p*-menthene, and finally that of *d*-limonene. The hydrogenation was terminated when the peak for *d*-limonene had disappeared from the sorptogram. Water, one tenth of the volume of the hydrogenated mixture, was added, whereupon the hydrogenation products rose to the surface and the product containing oxygen remained in solution[19]. The upper layer was washed with water and its sorptogram (Fig. 1, III, $n_D^{25} = 1.4533$) recorded.

The products of hydrogenation were fractionally distilled in a packed column with 10 theoretical plates. The distillate was separated into the fractions shown in Table 1. From the sorptograms IV—VIII for these fractions the 1-*p*-menthene contents were estimated by assuming the fractions to contain only 1-*p*-menthene, $n_D^{25} = 1.45445$, and *p*-menthane, $n_D^{25} = 1.4372$ ($n_D^{20} = 1.4395$ [18]).

Table 1. Fractional distillation of hydrogenated product.

Fraction No.	B.p. (°C) at 13 mm Hg	Weight in grams	n_D^{25}	% 1-*p*-menthene
1	—60.5	53	1.44696	57
2	60.5—61	59	1.45123	81
3	61 —61.5	205	1.45355	95
4	61.5—62	212	1.45441	100
5	62	78	1.45442	100
residue		15		

Specific rotation of fraction 4: $[a]_D^{25} = 98°5'$.

The oxidation of 1-p-menthene with molecular oxygen. The oxidations with and without catalyst were conducted by passing oxygen into an emulsion of 1-*p*-menthene in water. The results are presented in Fig. 3 and Table 2.

The previously employed emulsion oxidation procedure [21] was slightly modified. To effect a vigorous mixing a vibrator mixer (Vibro-Mischer, A. G. für Chemie Apparatebau, Zürich) was employed. This made possible the use of a thermostat. As in the earlier experiments [21], a 0.5 % aqueous solution of sodium stearate, to which sodium carbonate had been added to give the pH indicated, was first used as emulsifying medium (Expts. Nos. 1—6). A 0.3 % sodium stearate solution proved to be more effective than the 0.5 % solution at the temperatures employed (*cf.* expts. Nos. 2 and 5, Fig. 3).

As shown by the curves for expts. Nos. 1, 2 and 3 in Fig. 3, the rate of oxygen absorption rose rapidly as the temperature was increased. The oxidation was interrupted in these experiments as soon as 10 % of the oxygen required

Table 2. Oxidation of 1-*p*-menthene.

Expt. No.	t°C	Emulsion 1-*p*-menthene	Emulsion Water ml	Emulsifier in water % [b]	Time min.	Oxygen ml [a]	Peroxide formed, %	% of oxygen consumed in peroxide	Initial pH	Final pH
1	65	20	40	0.5	325	270	8.7	72	10.0	10.0
2	70	20	40	0.5	250	270	8.6	70	10.0	10.0
3	80	20	40	0.5	70	270	8.8	72	10.0	7.9
4 [c]	70	20	40	0.5	320	510	14.5	64	10.0	7.1
5	70	20	40	0.3	285	510	15.0	66	10.1	7.8
6	80	20	40	0.5	175	520	15.0	65	10.0	6.7
7	70	60	60	0.3	440		14.4		10.1	7.9
8	10	50	100	[d]	1 165	2 235	13.7	36	9.2	7.6

[a] Oxygen (N.T.P.) introduced.
[b] Emulsifier sodium stearate except in Expt. No. 8.
[c] 1-*p*-Menthene previously treated with 8 % sodium hydroxide solution [20].
[d] Emulsifier 1 % Teepol (Shell Chem. Corp.; dry matter 40 %).

to oxidize 1-*p*-menthene to 1-*p*-menthene-hydroperoxide had been consumed. In these experiments the hydroperoxide values of the reaction products were equal, which indicates that the rate of oxidation and the rate of hydroperoxide decomposition increased in the same degree with increasing temperature. About 70 % of the oxygen added was found in the hydroperoxides formed in these experiments. When the reaction time was prolonged, the hydroperoxide content of the product increased, but at the same time the proportion of added oxygen that was bound as hydroperoxide decreased.

When the product of expt. No. 2 was stored at room temperature for four months, the hydroperoxide content fell gradually from 8.6 to 5.6 %.

In expt. No. 8 cobalt resinate was employed as catalyst and the temperature was only 10 °. Since even a dilute sodium stearate solution becomes viscous at this temperature, sodium secondary alkyl sulphate (Teepol 710) was

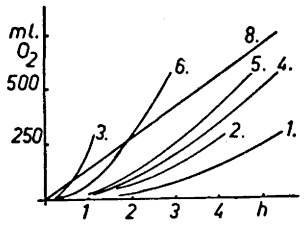


Fig. 3. Oxygen consumption as a function of time.

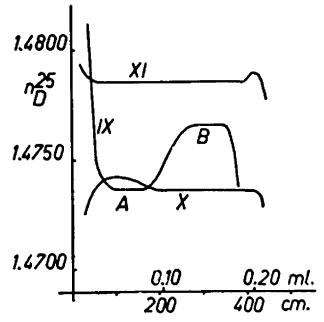


Fig. 4. Sorptograms. IX, expts. Nos. 1, 3—6, "ketone mixture"; X, expt. No. 7, "alcohol mixture"; XI, synthetic carvotan-acetone.

used as emulsifier. The rate of oxidation was nearly as great in this experiment as in expt. No. 2 at 70°. No induction period was observed as in the other experiments. Despite the low temperature, the hydroperoxide decomposed more rapidly than in the other experiments in which the temperatures were much higher. The higher rate of decomposition was evidently due to the presence of the catalyst.

Identification of oxidation products

The oxygen compounds formed in the emulsion oxidation were for the most part hydroperoxides in all experiments except No. 8. The other products formed were mainly alcohols, ketones and polymers.

The hydroperoxides in the reaction products were reduced in sodium sulphide[22] and sodium sulphite solutions [23]. The reduced oxidation products were separated from 1-*p*-menthene by passing their solution in petroleum ether through a column packed with silica gel (BDH, for chromatographic analysis) as proposed by Kirchner *et al.*[24] When this separation was performed on the oxidation product of expt. No. 8, a part of the oxidized material passed through the silica gel layer; this fraction was recovered subsequently by passing it a second time through a silica gel column. The oxygen-containing compounds were retained quantitatively by the silica gel and the sorptogram of the product that passed through the column revealed it to be pure 1-*p*-menthene. The oxidation products were eluted from the silica gel with anhydrous ether. The results are shown in Table 3.

Table 3. Reduced oxidation products after separation from 1-*p*-menthene.

Expt. No.	1, 3—6	7	8	
Oxidation products	8.0 g	8.3 g	8.9 g	(2.8 g[a])
Ketone content		12 %	34 %	

[a] Oxidation product recovered in the second elution.

The reduced oxidation products, which mainly consisted of alcohols, were oxidized to the corresponding ketones with chromic acid in acetic acid solution [25,26] (Table 4). The ketones in the resulting mixtures were determined gravimetrically as their dinitrophenylhydrazones according to Scholtens [27] (Tables 3 and 4).

Table 4. Ketone mixtures.

Expt. No.	1, 3—6	7	8	
Weight of mixture	8.0 g	1.4 g	6.4 g	(2.2 g[a])
Steam distilled product[b]	5.7 g	0.8 g	4.1 g	(1.1 g)
Ketone content	60 %	*ca.* 80 %	*ca.* 90 %	

[a] See subnote to Table 3.
[b] Amount removed by steam distillation from products of chromic acid oxidation.

Oxidations without added catalyst. Expts. Nos. 1, 3—7. The ketones obtained in expts. 1 and 3—6 were combined and converted into a dinitrophenylhydrazone which melted at 187—190° after three crystallizations from ethanol. With synthesized carvotanacetone-2,4-dinitrophenylhydrazone (m.p. 191—192° [28,29]), this derivative gave a mixed melting point of 187—191°. Since the

solubility of piperitone dinitrophenylhydrazone in ethanol was much greater than that of the corresponding derivative of carvotanacetone, the original dinitrophenylhydrazone precipitate was fractionated by chromatography according to Montes [30]. The dinitrophenylhydrazone passed through the adsorbent as one zone, from which it was concluded that no appreciable amounts of 1-*p*-menthene-3-hydroperoxide corresponding to piperitone, but only 1-*p*-menthene-6-hydroperoxide corresponding to carvotanacetone, had been formed in the oxidation.

The sorptogram IX of the same ketone mixture is shown in Fig. 4. Two different sections, one (A) showing a minimum and the other (B) a maximum, are evident in the sorptogram.

The fractions of section A gave a copious dinitrophenylhydrazone precipitate which, after two crystallizations from ethanol, melted at 189—191°. The refractive index, $n_D^{25} = 1.4736$, of section A is much lower than the refractive index, $n_D^{25} = 1.47855$, of pure carvotanacetone (sorptogram XI, Fig. 4). Despite this large difference, the fractions giving section A can be concluded to have been impure carvotanacetone.

The fractions of section B, which yielded only a negligible amount of a dinitrophenylhydrazone, were concluded to be carvotanacetol that had not been oxidized to carvotanacetone in the chromic acid treatment [25]. On the other hand, the reduced oxidation product of expt. No. 7 gave the sorptogram X (Fig. 4), the refractive index of which was, however, much lower than the refractive index of section B in sorptogram IX. The only explanation for the difference between the sorptograms appeared to be that the alcohol (carvotanacetol) that had not been converted into ketone by chromic acid had undergone isomerization to another alcohol which had an appreciably higher refractive index than the alcohol formed in the reduction of the original hydroperoxide.

To decide this, the reduced oxidation product of expt. No. 7 was isomerized by boiling in glacial acetic acid. The resulting product gave the sorptogram XII in Fig. 5. This sorptogram consists of three separate sections. The refractive index of the first highest plateau was almost the same as that of section B in sorptogram IX. The refractive index of the third section or the second highest plateau was identical with that of sorptogram X for the reduced product of expt. No. 7. It is hence obvious that only a part of the alcohols had been isomerized. The section of the sorptogram between the two plateaus was evidently due to the acetate of one of the alcohols, as indicated by the strong odour of carvotanacetol acetate given off by the fractions.

All of the isomerized product was saponified with ethanolic potassium hydroxide, and the product liberated distilled with steam. The distilled alcohol gave the sorptogram XIII of Fig. 5. After a gradual rise the sorptogram levelled off, and the refractive index of this section was close to the refractive index of the isomerized carvotanacetol that was assumed to be present. It is thus obvious that not only were the esters saponified but also the unisomerized alcohol underwent isomerization. The refractive index in the main section of the sorptogram XIII for the distilled and isomerized alcohol was close to that for synthesized carvotanacetol [31], $n_D^{25} = 1.4772$ (Sorptogram XIV, Fig. 6).

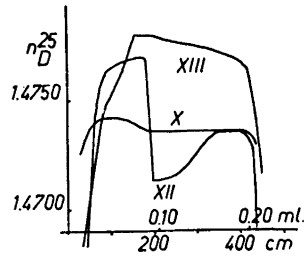


Fig. 5. X, expt. No. 7, "alcohol mixture"; XII, expt. No. 7, isomerized product; XIII, expt. No. 7, saponified product.

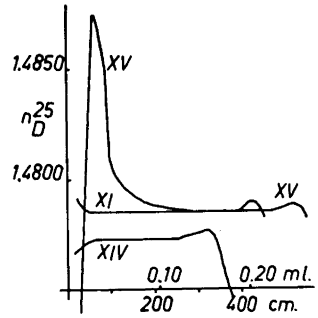


Fig. 6. XI, synthetic carvotanacetone; XIV, synthetic carvotanacetol; XV, expt. No. 7, "ketone mixture" prepared after saponification.

The saponified product contained 10 % ketone. This ketone evidently increased the refractive index of carvotanacetol, and since the ketone precedes the alcohol, as in sorptogram IX, the highest part in the sorptogram XIII is due to carvotanacetone.

Under the conditions where the isomerization was carried out [29], carvotanacetol is dehydrated to some extent and forms menthadienes. These hydrocarbons appear in the first fractions of the sorptograms (*e.g.* XII and XIII) and lower the refractive index. After the isomerized and saponified product was oxidized with chromic acid and isolated by steam distillation, it gave the sorptogram XV (Fig. 6) which shows first a high peak and then falls close to the refractive index of carvotanacetone (n_D^{25} for synthesized carvotanacetone [31] was 1.4785; sorptogram XI, Fig. 6). The refractive index, $n_D^{25} = 1.4875$, at the peak in sorptogram XV is very close to the refractive index, $n_D^{25} = 1.4883$, of *p*-cymene [32]. The fractions giving the peak had the odour of *p*-cymene. The *p*-cymene may have resulted from the oxidation of the menthadienes by chromic acid. It is obvious that for this reason there is a gradual decrease before the refractive index of the ketone is reached in all the sorptograms (IX, XV, XVII and XIX) of the products of the chromic acid oxidations.

According to the above, the alcohol with the lower refractive index is formed when the hydroperoxide formed in the emulsion oxidation is reduced, while the alcohol with the higher refractive index results from the isomerization of this alcohol. Synthesized carvotanacetol has the same refractive index as the isomerized alcohol. Hence, since the alcohols both yield carvotanacetone on oxidation with chromic acid, they are carvotanacetols.

According to the rule of Auwers-Skita [33], the alcohol with the higher refractive index must be the *cis* form. On the other hand, Gastambide [34] has stated that the sterically more hindered form, in this case the *cis* form, is less strongly adsorbed. Consequently the alcohol with the higher refractive index first appearing in the sorptogram XII was *cis*-carvotanacetol. The synthesized carvotanacetol is hence also the *cis* form. However, the carvotanacetol obtained by reducing the oxidation product was more strongly adsorbed than the

preceding and had a lower refractive index and was hence *trans*-carvotanacetol. From this it may be deduced that the main product of the oxidation under the chosen conditions was *trans*-1-*p*-menthene-6-hydroperoxide. This conclusion is confirmed by the nature of the oxidation products of certain terpenes, which products have been shown to have the *trans* configuration [22,35].

Oxidation at low temperature in the presence of catalyst. Expt. No. 8. The main part of the reduced oxidation products of this experiment contained 34 % ketone (Table 3). The dinitrophenylhydrazone of the ketone melted at 190—192° after two crystallizations from ethanol, which indicated that the ketone was carvotanacetone. The reduced product of expt. No. 8 gave the sorptogram XVI, which differed completely from the sorptogram X of the corresponding product of expt. No. 7. Certain observations at the time of the experiment and later led to the conclusion that the orptograms XVI had been formed by partly separated fractions consisting of carvotanacetone, *trans*-piperitol, *trans*-carvotanacetol and piperitone, in this order.

The product of the chromic acid oxidation of the main product of expt. No. 8 (Table 4) gave the sorptogram XVII. In this sorptogram a very high initial peak is followed by a sharp minimum and a fairly high, broad maximum. The minimum is evidently due to carvotanacetone. The mean refractive index of the fractions contributing to the initial peak, $n_D^{25} = 1.4818$, is close to the refractive index of piperitone ($n_D^{20} = 1.4848$ [36]; $n_D^{25} = 1.4823$). In addition, these fractions smelled strongly of piperitone.

This product of chromic acid oxidation of the main fraction was distilled in a spinning band column with 25 theoretical plates. The fractions shown in Table 5 were obtained.

Table 5. The distillation of the main product of expt. No. 8 after chromic acid oxidation.

Fraction	t°C at 3.4 mm Hg	ml	n_D^{25}
1	78.5—79.5	1.0	1.47787
2	—80.5	0.8	1.48082
3	—82.5	0.7	1.48190
4	82.5	0.1	1.48212
residue		0.8	

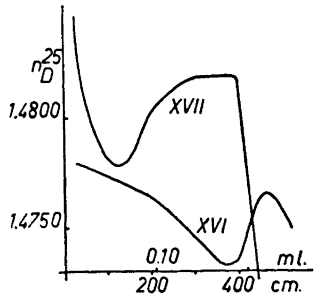


Fig. 7. XVI, expt. No. 8, "alcohol mixture"; XVII, expt. No. 8, "ketone mixture".

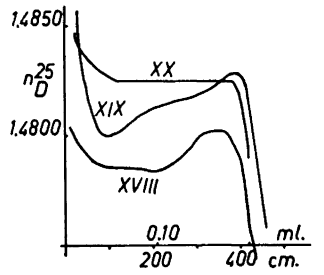


Fig. 8. XVIII, expt. No. 8, ketone fraction 1; XIX—XX, expt. No. 8, ketone fractions 2 and 3.

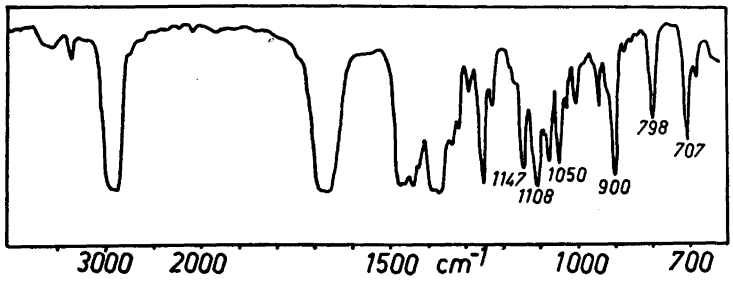


Fig. 9. Infrared spectrum of synthesized carvotanacetone.

The fractions 1, 2 and 3 yielded the sorptograms XVIII, XIX and XX (Fig. 8). The refractive index n_D^{25} of the horizontal section of the sorptogram XVIII was 1.4785, from which it was concluded that fraction 1 consisted mainly of carvotanacetone. The refractive index, $n_D^{25} = 1.4825$, of sorptogram XX suggested that the fraction 3 was almost pure piperitone. Fraction 2 evidently contained both these compounds.

The 2,4-dinitrophenylhydrazone prepared from fraction 3 melted at 113—116° after crystallization from an ethanol-ethyl acetate mixture. The corresponding derivative of piperitone has been reported [37] to melt at 116°.

The high yield of piperitone suggests that the oxidation at a low temperature with catalyst present involves addition to position 6, but also to position 3 to a considerable extent.

Infrared spectra

A 30-μl sample was taken from the middle fractions of sorptogram XX and its infrared spectrum recorded. This spectrum was identical with the spectrum recorded for piperitone by Le Fèvre *et al.*[38]

An infrared spectrum was also recorded for a sample from the middle fractions of synthetic carvotanacetone giving the sorptogram X. Since the infrared spectrum for carvotanacetone was not found in the literature, the recorded spectrum (Fig. 9) was compared with the Raman spectrum for the compound [39]. The two spectra for carvotanacetone were found to conform well with each other.

The infrared spectrum obtained for piperitone was also found to agree well with the Raman spectrum of this compound [40].

Micro sorption method

Blohm's micro sorption method [14] was employed in a slightly modified form. Whereas Blohm and Widmark employed sorptograms only in the analysis of hydrocarbons [14,16,32,41], the method was found in this study very suitable also for liquid alcohols and ketones. After the present work had been completed, Widmark published sorptograms for various liquid compounds including carvotanacetone [42]. The refractive index he obtained for

carvotanacetone, $n_D^{25} = 1.4782$, deviates slightly from the value, $n_D^{25} = 1.47855$, obtained in this work.

Commercially available silica gel (BDH, for chromatographic analysis) proved to be a suitable adsorbent. The sieve fraction passing through 200 mesh was employed. The sorptogram tube chosen after some experimentation was a capillary tube 150 cm long with an inner diameter of 1.5 mm. The fractions were collected into capillary tubes 0.76 mm in inner diameter (15—20 tubes). The volumes of the fractions were obtained by measuring the lengths of the liquid columns in the calibrated capillary tubes. In the sorptogram plots the abscissa is the cumulative length of the liquid column of the fractions (10 cm corresponds to 46.5 μl).

For the recording of each sorptogram an accurately measured 0.20-ml or 0.25-ml volume of the sample was taken. The sample volume was transferred to the top of the column with an injection syringe of 0.5-ml capacity with a scale graduated in 0.01-ml divisions. By using a needle of suitable length the sample could be added so that none of it adhered to the upper walls of the column. After the sample had penetrated into the column packing, alcohol was added from a side tube.

The pressure applied to the column varied from 1.5 to 4.5 atmospheres. Depending on the pressure and the nature of the sample, the passage of the sample through the column took from 3 to 5 h. The oxygen-containing terpenes are more viscous than the hydrocarbons and hence required higher pressures. The passage of synthetic carvotanacetol through the column took over 10 h under a pressure of 4 atm.

The refractive indices of the fractions were measured to the fifth decimal with an immersion refractometer (Eintauch-Refraktometer Nr. 67130, Carl Zeiss, Jena). The light source was a sodium lamp. When the refractive indices were measured the temperature was maintained at 25 ± 0.05°C with a thermostat (Colora, Ultrathermostat NB-26093).

REFERENCES

1. Frank, C. E. *Chem. Revs.* **46** (1950) 155.
2. Schenk, G. O. *Angew. Chem.* **64** (1952) 12.
3. Blumann, A. and Zeitschel, O. *Ber.* **47** (1914) 2623.
4. Dupont, R. *Ind. chim. belge* **11** (1940) 3.
5. Erofeev, B. V. and Chirko, A. I. Uchenye Zapiski Belorus. *Gosudarst. Univ. im. V. I. Lenina Ser. Khim.* **1955**, No. 24, 16; Ref. *Chem. Abstracts* **51** (1957) 5731.
6. Eschinazi, H. E. *Bull. Research Council Israel* **1** (1951) 109, 2 (1952) 73, *Perfumery Essent. Oil Record* **44** (1953) 242.
7. Royals, E. E. and Horne, S. E. *J. Am. Chem. Soc.* **77** (1955) 187.
8. Fushizaki, Y. and Saito, M. *J. Chem. Soc. Japan, Ind. Chem. Sect.* **57** (1954) 897; Ref. *Chem. Abstracts* **50** (1956) 886; Saito, M., Chijimatsu, Y., Matsuo, T. and Fushizaki, Y. *Ibid.* **59** (1956) 130; Ref. *Chem. Abstracts* **51** (1957) 1097. Fushizaki, Y. and Saito, M. *Bull. Univ. Osaka Prefect.* Ser. **A4** (1956) 145; Ref. *Chem. Abstracts* **51** (1957) 5732.
9. Criegee, R., Pilz, H. and Flygare, H. *Ber.* **72** (1939) 1799.
10. Dupont, R. *Bull. soc. chim. Belges* **45** (1936) 57.
11. Farmer, E. H. and Sundralingam, A. *J. Chem. Soc.* **1942** 121.
12. Schmidt, H. *Diss.*, Göttingen 1925.
13. Cook, A. H. *J. Chem. Soc.* **1938** 1774.
14. Blohm, S. G. *Mikrochemie ver. Mikrochim. Acta* **36/37** (1951) 322; *Arkiv Kemi* **6** (1954) 317.
15. Igolen, G. and Sontag, D. *Chim. & ind. Paris* **45** (1941) 157. (Suppl. to No. 3); Ref. *Chem. Abstracts* **38** (1944) 4381.
16. Widmark, G. *Acta Chem. Scand.* **9** (1955) 938.
17. Colonge, J. and Duroux, E. *Bull. soc. chim. France* (5) **7** (1940) 459.
18. Smith, A. H., Fuzek, J. F. and Meriwether, H. T. *J. Am. Chem. Soc.* **71** (1949) 3765.
19. Othmer, D. F., Jacobs, M. B. and Wishnefsky, N. U.S.P. 2.631.145 (1953).
20. Joris, G. G. U.S.P. 2.577.768 (1951), 2.619.509 and 2.621.213 (1952).
21. Veijola, V. E., Kirjakka, P. and Heliö, K. *Suomen Kemistilehti* **A26** (1953) 281.
22. Moore, R. N., Golumbic, C. and Fisher, S. S. *J. Am. Chem. Soc.* **78** (1956) 1173.

23. Hock, H. and Lang, S. *Ber.* **75** (1942) 313.
24. Kirchner, J. G. and Miller, J. M. *Ind. Eng. Chem.* **44** (1952) 318; Eidem, U.S.P. 2.712.008 (1955).
25. Treibs, W. and Bast, H. *Ann.* **561** (1949) 165.
26. Hock, H. and Lang, S. *Ber.* **75** (1942) 300.
27. Scholtens, C. *Perfumery Essent. Oil Record* **38** (1947) 235.
28. Fujita, K. and Matsuura, T. *J. Sci. Hiroshima Univ.* **18A** (1955) 455.
29. Read, J. and Swann, G. *J. Chem. Soc.* **1937** 239.
30. Montes, A. L. *Anales asoc. quim. argentina* **40** (1952) 273.
31. Treibs, W., Lucius, G., Kögler, H. and Breslauer, H. *Ann.* **581** (1953) 59.
32. Widmark, G. *Acta Chem. Scand.* **9** (1955) 1296.
33. v. Auwers, K. *Ann.* **410** (1915) 287; **420** (1920) 91.
34. Gastambide, B. *Diss.*, Paris 1954.
35. Bluman, A. and Wood, W. R. *J. Chem. Soc.* **1952** 4420.
36. Read, J. and Smith, H. G. *J. Chem. Soc.* **123** (1923) 2268.
37. Birch, A. J. and Mucherji, S. M. *J. Chem. Soc.* **1949** 2531.
38. Le Fèvre, R. J. M., Maramba, F. and Werner, R. L. *J. Chem. Soc.* **1953** 2497.
39. Naves, Y. R. and Papazian, G. *Helv. Chim. Acta* **25** (1942) 1052.
40. Dulou, R., *Diss.* Bordeaux 1934, p. 98.
41. Widmark, G. *Acta Chem. Scand.* **9** (1955) 925.
42. Widmark, G. and Holm, B. *Arkiv Kemi* **11** (1957) 227.

Received October 18, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 312—322

A Comparative Study on the Chelate Formation between Germanic Acid and Some Glycols and Polyalcohols in Aqueous Solutions

P. J. ANTIKAINEN

Institute of Chemistry, University of Helsinki, Helsinki, Finland

The influence of a series of glycols and polyalcohols (hexoses) on the ionization of germanic acid has been studied potentiometrically in aqueous solution at 25°C. Germanic acid displays a tendency to form one-to-one and one-to-two chelate compounds with glycols, while the polyalcohols and their derivatives (aldoses and ketoses) seem to yield solely one-to-two chelate acids (one molecule of germanic acid combined with two molecules of polyol). It thus appears that the number of hydroxyl groups present in the organic ligand plays a very important part in determining the structure of the chelates formed. On the other hand, an increase in the number of carbon atoms in the ligand chain seems to increase the values of the chelate formation constants but not to affect the structure of the chelate. Furthermore, an increase in the number of hydroxyl groups in the ligand (*e. g.* propylene glycol and glycerol) seems to lead to increased values for the chelate formation constants. Additional functional groups as well as different configurations of the hydroxyl groups in the ligands apparently affect the values of the chelate formation constants. Lactol forms may be partly responsible for the observed differences in the chelating ability of germanic acid with carbohydrate substances.

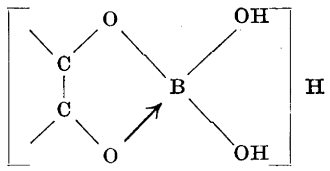
Analogously with boric acid [1] and telluric acid [2], germanic acid [3–7] forms chelate acids with di- or polyhydroxy compounds containing at least two hydroxyl groups in the *cis*-position. The structure and the formation constants of most of such chelate compounds of germanic acid seem to be still unknown. The object of the present investigation was to determine potentiometrically the structure and formation constants of the chelate acids formed by the interaction of germanic acid and the following alcohols in aqueous solutions: ethylene glycol, propylene glycol, 3-methoxy-1,2-propylene glycol, D-mannose, D-glucose and D-galactose. Furthermore an endeavour was made to establish the chelate-forming ability of germanic acid with these alcohols as related to that with glycerol, mannitol and fructose which has been studied previously by the author.

THEORY AND METHOD USED

The author [6] has previously observed that germanic acid is capable of forming one-to-one and one-to-two anionic chelate compounds with some polyalcohols:

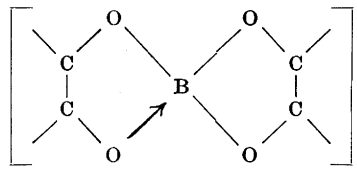
$$\underset{\text{one-to-one}}{HGeD} \qquad \underset{\text{one-to-two}}{HGeD_2} \tag{1}$$

These chelate compounds of germanic acid are generally strong mono-basic acids. However, germanium is also known to exhibit a cordination number six in complex formation, thus resembling boron, which has a tendency to form chelate acids with the co-ordination number four under corresponding conditions:



HBD

one-to-one



HBD_2

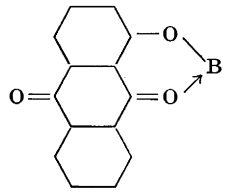
one-to-two

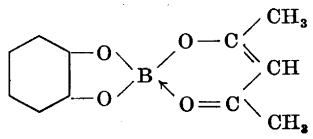
(2)

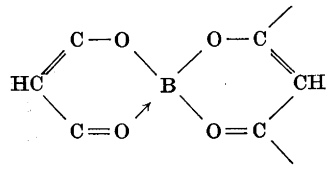
These configurations are in good agreement with the concepts regarding the structure of the acid anions of boron and germanium in which these elements exhibit tetra- and hexacovalency, respectively [8,9]:

$$B(OH)_4^- \qquad Ge(OH)_6^{--} \tag{3}$$

and also with the general tendency of these elements to form complex compounds with the co-ordination number four or six, *e. g.* the fluoborate and fluogermanate anions, BF_4^- and GeF_6^{--}. Chelates of type (2) require the hydroxyl groups to be of *cis*-configuration and attached to adjacent carbon atoms. Carbonyl groups may at times participate in chelate bonding as has been demonstrated for the following chelates of boric acid [10–13]

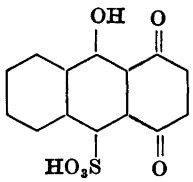




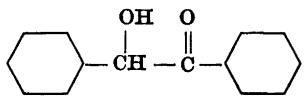


(4)

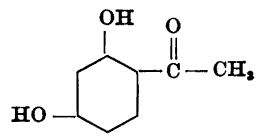
Germanic acid appears to behave in a similar manner as is evidenced by its reaction with the following compounds:



9-Hydroxynaphthacene-quinonesulphonic acid



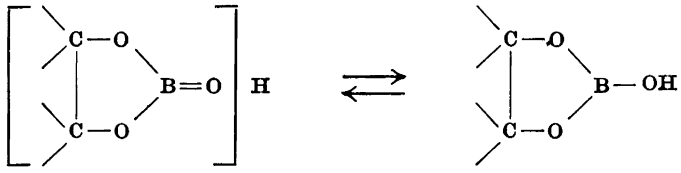
Benzoin



Resacetophenone

(5)

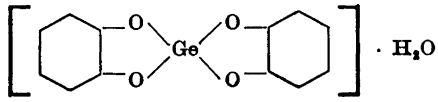
pH and conductivity measurements in general show the germanic acid-polyhydroxy chelates to be strong acids in aqueous solution. It has been noted that boric acid is also capable of forming neutral 1:1 chelates with certain glycols. In that case, according to Böeseken [14], a so-called pseudo acid is formed, which is in equilibrium with the strong chelate acid, the equilibrium being almost completely on the side of the pseudo acid



chelate acid — pseudo acid

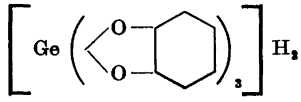
(6)

According to Bevillard [4] and others the existence of *o*-diphenol chelates of germanic acid, which possess the following structure



(7)

may be interpreted as indicating that similar neutral chelates may also be formed by this element. It has, however, not been possible to demonstrate the presence of such chelates in solution. It is thus obvious that germanic acid in solutions with polyhydroxy compounds generally forms strong chelate acids, the presence of which can be demonstrated, for instance, by pH measurements. Further, it is apparent from the above examples that the chelate acids formed are mono-basic acids. On the other hand, it is interesting to find that the germanic acid chelates which are formed with certain *o*-diphenols are of the following structure [4]



(8)

and act as strong di-basic acids. Summing up it may be said that the known polyhydroxy chelates of germanic acid are comprised of strong mono-basic acids of the one-to-one or one-to-two type and strong di-basic acids of the one-to-three type.

The author has previously used the so-called buffer capacity method [15,1,2] for the investigation of some polyhydroxy chelates of boric, telluric and germanic acid in aqueous solution. This method enables the stoichiometry of the reactions in question as well as the formation constants of the chelates formed to be determined.

When it is assumed that the germanic acid chelates formed are mono-basic acids, the reactions may be expressed as follows

$$H_2GeO_3 + nD \rightarrow HGeD_n$$

In this equation $HGeD_n$ designates the mono-basic acid formed and D the polyhydroxy compound; n represents the number of ligand molecules which combine with one molecule of germanic acid. If several different chelates are formed simultaneously, n designates the average number of ligand molecules per molecule of germanic acid. In such cases n is generally not a whole number. When the chelate formation reaction is expressed according to eqn. (9) the equilibrium constant for the reaction can be written as follows

$$K_k = \frac{[HGeD_n]}{[HGe] \cdot [D]^n} \tag{10}$$

When the real first-ionization constant of germanic acid is designated by K_1 and the corresponding constant for the chelate acid by K_1', the germanic acid solution containing chelate acids can be treated as an aqueous solution of an apparently mono-basic strong acid. The ionization constant, K^*, of this acid will consequently take the following form

$$K^* = K_k \cdot K_1' \, [D]^n + K_1 = K_n \cdot [D]^n + K_1 \tag{11}$$

It is therefore possible to determine the constants of this equation by measuring the apparent value of the ionization constant of germanic acid for various concentrations of the polyhydroxy compound. By using large concentrations of the ligand and small concentrations of germanic acid, it may be assumed that [D] is equal to the corresponding stoichiometric concentration, C_D, of the ligand and, eqn. (11) becomes

$$p(K^*—K_1) = pK_n—n \, . \log C_D \tag{12}$$

The expression $p(K^*—K_1)$ should, according to the above equation, be a linear function of $-\log C_D$, the slope of the curve giving the value of n, while the value of pK_n may be obtained from the point of intersection of this curve and the line obtained when $-\log C_D = 0$. The following assumptions have been made in the derivation of eqn. (12)

1. Both the germanic acid and its chelate acids are mono-basic acids.
2. $[D] = C_D$
3. Changes in the dielectric constants of the solutions as may possibly be caused by the ligand are too small to have any effect on the results. (High concentrations of the ligand have been avoided.)

In the event that these assumptions prove to be invalid eqn. (12) will, of course, no longer represent a straight line. This is especially true for assumption 2 (*cf.* Fig. 1).

If the chelate formation is almost complete, the apparent ionization constant, K^*, will approach the real ionization constant [1,2] of the chelate acid and the following equation is obtained

$$\lim K^* \rightarrow K_1' \qquad (13)$$

$$C_D \rightarrow >> C_{H_4GeO_4}$$

The graph of eqn. (12) may be curved for other reasons also. If, for instance, two chelates are formed simultaneously (one-to-one and one-to-two, for example) the graph takes a shape, in which the slopes of the ends of the curve approach the whole numbers. These slopes, n_1 and n_2, indicate the structures of the chelates formed in each reaction. Hence, eqn. (11) becomes

$$K^* = k_1 \cdot C_D^{n_1} + k_2 \cdot C_D^{n_2} + K_1 \qquad (14)$$

by the aid of which both formation constants of the apparent chelate formation reactions may be determined in the manner described above. In this equation k_1 and k_2 correspond to the constant K_n in eqn. (11). For the determination of these constants n_1 and n_2 and, in addition, the apparent ionization constant, K^*, must be known for at least two different concentrations of the ligand. In practice, however, it is best to determine several points along the curve corresponding to eqn. (14) as well as its asymptotes which have the slopes n_1 and n_2, and then to calculate the constants k_1 and k_2 with these values for several pairs of K^*'s.

According to eqn. (11) the chelate formation constant, K_n, is equal to the product of K_1' and K_k. Hence,

$$K_n = \frac{[H^+] \cdot [GeD_n^-]}{[HGe] \cdot [D]^n} \qquad (15)$$

The constants k_1 and k_2 of eqn. (14) also correspond to this equation. If the above equation is divided by the equation

$$K_1 = \frac{[H^+] \cdot [Ge^-]}{[HGe]} \qquad (16)$$

which represents the first ionization constant of germanic acid, the following new expression is obtained for the chelate formation constant

$$K_n' = \frac{[GeD_n^-]}{[Ge^-] \cdot [D]^n} = K_n/K_1 \qquad (17)$$

In eqns. (16) and (17) Ge^- designates the germanic acid anion ($H_2GeO_3^-$) and GeD_n^- the chelate acid anion formed. Again, both the germanic acid and its chelate acid are considered to be mono-basic acid. According to eqn. (17) the chelate formation reactions under investigation may be considered to take place between the germanic acid anion and the organic ligand as follows

$$Ge^- + nD \rightleftharpoons GeD_n^- \qquad (18)$$

This equation corresponds to eqn. (9). The constant, K_n', gives a good indication of the extent of the chelate formation reaction, and is therefore employed in the final considerations. It should also be noted that, according to eqn. (18), the value of the constant, K_n', is not dependent upon the ionic strength. The constants obtained are therefore comparable to the corresponding thermodynamic constants.

EXPERIMENTAL

All measurements were made by potentiometric titration using a quinhydrone electrode. A saturated calomel electrode served as reference standard. The temperature was regulated at 25 ± 0.05°C by means of a thermostat.

Carbon dioxide was removed from the test solutions effected by bubbling a stream of purified nitrogen through them for as many as 20 h previous to the titration. A slow, continuous flow of nitrogen was maintained during the titration. These precautions proved essential when working in ranges of small buffer capacity where the presence of even exceedingly low concentrations of carbon dioxide can lead to unreliable results.

The sodium hydroxide solution was added by means of a calibrated micro syringe designed by Ellilä [16]. With this syringe it was possible to attain an accuracy for small additions (0.0002—0.5 ml) of hydroxide solution that completely corresponded to the accuracy of the potentiometer. Addition of 0.003—0.08 ml of an approx. 0.1 N sodium hydroxide solution to the test solutions generally corresponded to a max. potential jump of 10—15 mV when the concentrations of germanic acid and polyhydroxy compounds were chosen to be within the molar ranges of 0.008—0.012 and 0.05—1.0, respectively.

The test solutions were prepared in 50 ml volumetric flasks by measuring with pipettes the appropriate amounts of germanic acid, polyhydroxylic compounds and potassium chloride from corresponding stock solutions. Small quantities of hydrochloric acid were also added before filling up the flasks. About 50 ml of these solutions were used for the titrations by emptying the calibrated volumetric flasks into the titration vessels. Whenever possible the reagents used were the best *pro analysi* products available. If these could not be obtained inferior products were purified.

The aqueous stock solution of germanic acid was prepared from Merck's germanium dioxide. Its concentration was determined by titration [3]. The stock solutions of the glycols were prepared by weighing out the appropriate quantities of dried glycols. The glycols were dried in small portions by being stirred on an oil bath at a temperature near the boiling point of each glycol (100—130°C) for about one hour and were then kept for a week in vacuum desiccators containing sulphuric acid and phosphorus pentoxide as drying agents. The molarities of the aqueous solutions prepared from these dried glycols were as follows:

ethylene glycol 2.290 (1.0160), propylene glycol 1.239 (1.0044),
butylene glycol 1.566 (1.0051), 3-methoxy-1,2-propylene glycol 2.422 (1.0347). (The numbers in brackets indicate the corresponding densities of the solutions.)

Because of the slow mutarotation of hexoses in aqueous solution, their solutions had to be prepared about 24 h before the measurements were made in order to permit the attainment of equilibrium.

Table 1. Values of the apparent ionization constant of germanic acid in approximately 0.1 M aqueous potassium chloride solutions with various concentrations of some di- and polyhydroxy compounds at 25°C. (In the calculations the value $K_1 = 2.63 \times 10^{-9}$ has been used [19].)

$C_{H_4GeO_4} \times 10^3$	C_D	$P_m \times 10^4$	$p(K^* - K_1)$
	Ethylene glycol		
11.96	0.1142	0.2882	9.194
11.93	0.2282	0.2973	9.076
11.87	0.3431	0.3129	8.900
11.90	0.5720	0.3402	8.528
11.88	0.9153	0.4111	8.395
	Propylene glycol		
11.91	0.1236	0.3005	9.032
11.93	0.2473	0.3158	8.889
11.95	0.3710	0.3300	8.604
11.89	0.6177	0.4099	8.398
11.98	0.9058	0.4545	8.177
	Butylene glycol		
11.97	0.09998	0.3255	8.817
11.91	0.1248	0.3373	8.728
11.87	0.2992	0.3920	8.463
11.93	0.4988	0.4810	8.188
11.89	0.6253	0.5144	8.107
11.99	0.7828	0.7636	7.834
	3-Methoxy-1,2-propylene glycol		
11.98	0.0960	0.3340	8.757
11.93	0.1814	0.3731	8.542
11.94	0.2723	0.4638	8.236
11.89	0.3630	0.5032	8.133
11.99	0.5456	0.5973	7.945
11.99	0.7275	0.7481	7.713
	D-Galactose		
3.996	0.09965	0.6535	7.300
3.994	0.1498	1.028	6.906
3.988	0.3990	2.050	6.305
3.986	0.5974	3.420	5.861
3.987	0.7976	3.830	5.762
	D-Glucose		
3.980	0.04978	0.4521	7.618
7.996	0.1998	0.7275	6.506
7.982	0.4496	1.530	5.860
7.975	0.6971	2.400	5.468
3.998	0.7992	2.240	5.228
	D-Mannose		
7.982	0.04995	1.209	7.065
7.963	0.09962	2.464	6.451
7.976	0.1496	3.720	6.090
7.950	0.1981	4.428	5.932
7.958	0.2984	5.901	5.678
7.948	0.3963	7.725	5.430

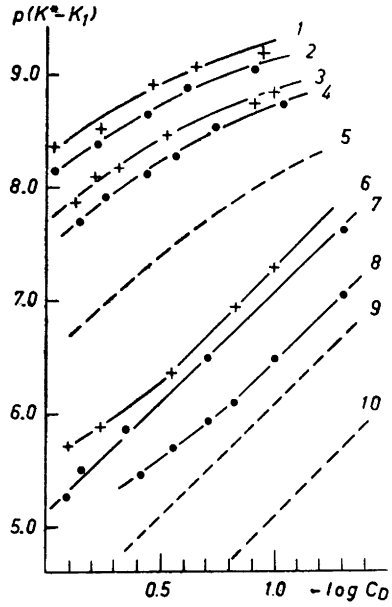


Fig. 1. The dependence of the apparent ionization constant of germanic acid on the concentrations of 1) ethylene glycol, 2) propylene glycol, 3) butylene glycol, 4) 3-methoxy-2,3-propylene glycol, 5) glycerol, 6) D-galactose, 7) D-glucose, 8) D-mannose, 9) D-mannitol, 10) D-fructose (*cf.* Table 1 and Ref.[6]).

RESULTS AND DISCUSSION

Table 1. gives the values of the apparent ionization constant of germanic acid in approx. 0.1 M potassium chloride solutions at different concentrations of various glycols and polyalcohols. These values are in good agreement with eqn. (14) as is illustrated by Fig. 1. Furthermore, it appears that the glycols have a weak tendency to form one-to-one and one-to-two chelates, while the polyalcohols (hexoses) seem to possess a strong tendency to form one-to-two chelates only, as has been expressed previously by the author [6] (*cf.* D-galactose and D-mannose and eqn. 13). Thus it appears that the chelate-forming abilities of the glycols and polyalcohols investigated increase in the order

ethylene glycol, propylene glycol, butylene glycol, 3-methoxy-1,2-propylene glycol, glycerol, D-galactose, D-glucose, D-mannose, D-mannitol, D-fructose. (19)

The different chelating abilities of these ligands with germanic acid may also be seen from Table 2 which lists the calculated values of pk_1, pk_2, $\log k_1'$ and $\log k_2'$ (employing eqn. 14). Here k_1 and k_2 correspond to the constants in eqn. (17). Thus it becomes apparent that the number of hydroxyl groups in the organic ligand has a very important influence on the structure of the chelates formed. In the case of aliphatic alcohols an increase in the number of hydroxyl groups in the ligand molecule apparently favours the formation of one-to-two chelates and also tends to increase the chelate formation constants (*cf.* propylene glycol, 3-methoxy-1,2-propylene glycol and glycerol). It is interesting to note here that according to Bevillard [4] the aromatic dihydroxy compounds

Table 2. Chelate formation constants of chelates formed by germanic acid and some di- and polyhydroxy compounds. The values in Table 1 and eqns. (12), (14) and (17) have been used in the calculations, the values of n, n_1 and n_2 being 2, 1 and 2, respectively, *c. f.* Ref.[6].

Chelating agent	pk_1	pk_2	log k_1'	log k_2'
Ethylene glycol	8.410	8.954	0.170	−0.374
Propylene glycol	8.298	8.521	0.282	0.059
Butylene glycol	7.943	8.187	0.637	0.040
3-Methoxy-2,3-propylene glycol	7.745	8.000	0.835	0.580
Glycerol	7.320	6.636	1.212	1.944
D-Galactose	—	5.295	—	3.285
D-Glucose	—	5.120	—	3.460
D-Mannose	—	4.453	—	4.127
D-Mannitol	—	4.050	—	4.530
D-Fructose	—	3.100	—	5.480

(*o*-diphenols) form solely one-to-three chelates in aqueous solutions. The occurrence of such one-to-three chelates has also been observed by the author and his co-worker [17] through potentiometric measurements.

Apart from the number of hydroxyl groups in the ligand molecule, the size of the carbon chain also seems to be an important factor in the stability of the chelates. An increase in the number of carbon atoms appears only to increase the stability of chelates but not to affect the structure of the chelates, as may be seen from the fact that all glycols as well as glycerol, independent of the number of carbon atoms, form both one-to-one and one-to-two chelates. On the other hand, an increase in the number of hydroxyl groups seems to favour the formation of one-to-two chelates, as has been found earlier [18].

In a more critical study of the observed differences in the stabilities of the chelates formed it must be born in mind that the hexoses will occur in aqueous solutions mainly in their lactol forms (*cf.* Ref.[6]). Thus, the real ligands in the case of hexoses consist of heterocyclic molecules. Such structural differences have naturally considerable influence on the stability of the chelates formed. In some cases the presence of the necessary hydroxyl groups in the *cis*-position depends on lactol formation, *e. g.* for D-glucose; of its two anomeric forms only the *α*-form possesses two hydroxyl groups in the *cis*-position. It is therefore apparent that glycosidic hydroxyl groups may also participate in chelate formation. Mutarotation alters the space configuration of the glycosidic hydroxyl groups and thus affects chelate formation. Since the mutarotation is dependent on pH, the chelate formation of hexoses will also be affected by pH and it is therefore important to mention the conditions under which the measurements were performed. In this investigation the measurements were made in acidic solutions, only exceedingly small quantities of base being added to germanic acid-ligand solutions.

From Fig. 1 and Table 2 it can be seen that of all the chelate formers investigated fructose showed the greatest chelating effect on germanic acid. This may be attributed to the fact that fructose, in contrast to the other hexoses, occurs in aqueous solution almost entirely in the open-chain form.

It may therefore be concluded that lactol formation diminishes the ability of the ligand to form chelates with germanic acid. This is supported by the fact that although D-mannitol and D-mannose have very similar space configurations with respect to the hydroxyl groups, mannose possesses a lower chelate-forming ability than mannitol. A similar reduction in chelate formation owing to lactol configuration has been observed earlier by the author [6] in the hexose chelates of boric acid. Boric acid and germanic acid display similar behaviour in chelate formation in other respects also. Thus, for instance, the chelating effect of the chelate formers seems to increase according to series (19) for both acids. It must, however, be noted that, as may be seen from the chelate formation constants obtained, the germanic acid chelates are more stable than the corresponding chelates of boric acid.

According to Saito [5], the hydroxyl groups in positions 1 and 2 play a very important part in the chelate formation between sugars and germanic acid. This becomes evident from the chelating ability of fructose as compared with its two glycosidic derivatives inuline and sucrose, which display a noticeably smaller chelating ability than does the parent sugar. Accordingly it is also possible that fructose undergoes chelation by virtue of its lactol form, even though this form may be present to only a minor extent in aqueous solution. The fact that the aldohexoses display a considerably smaller chelating ability than does fructose may possibly be explained that in the case of the aldoses C-atoms 1 and 2 are both incorporated in the rigid lactol ring, while C-atom 1 of fructose is outside the ring. The hydroxyl groups of C-atom 1 of the ketoses may therefore, owing to their less restricted state, assume a sterically more favourable position for chelate formation than is possible for the corresponding hydroxyl groups of the aldoses. That flexibility in structure plays an important part in chelate formation is furthermore evidenced from the chelate formation constants of D-mannitol and D-mannose. The great importance of the hydroxyl groups in positions 1 and 2 for the formation of germanic acid chelates of ketoses has also been observed polarographically by Saito [5].

This investigation has been supported by a grant from the *State Commission of Natural Sciences (Valtion Luonnontieteellinen Toimikunta)*, which is gratefully acknowledged.

REFERENCES

1. Antikainen, P. J. *Ann. Acad. Sci. Fennicae*, Ser **A II** (1954) 56 (and the literature cited there); *Acta Chem. Scand.* **9** (1955) 1008; *Suomen Kemistilehti* **B 29** (1956) 179; **B 30** (1957) 185.
2. Antikainen, P. J. *Suomen Kemistilehti* **B 29** (1956) 14, 123, 135; **B 39** (1957) 45 (and the literature cited there).
3. Tchakirian, A. *Compt. rend.* **185** (1928) 229; Poluektoff, N. S. *Mikrochemie* **18** (1935) 48.
4. Bévillard, P. *Compt. rend.* **234** (1952) 2606; **235** (1952) 880.
5. Saito, K. *J. Chem. Soc. Japan, Pure Chem. Sec.* **73** (1952) 519, 573.
6. Antikainen, P. J. *Suomen Kemistilehti* **B 30** (1957) 147; **B 31** (1958) 255.
7. Pflugmacher, A. and Rohrmann, I. *Angew. Chem.* **69** (1957) 778.
8. Sidqwick, N. V. *The Chemical Elements and their Compounds.* Vol. I. Oxford 1950.
9. Edwards, J. O., Morrison, G. C., Ross, V. F. and Schultz, J. W. *J. Am. Chem. Soc.* **77** (1955) 266.
10. Morgan, G. T. and Tunstall, R. B. *J. Chem. Soc.* **125** (1924) 1963.

11. Dimroth, O. and Faust, T. *Ber.* **54** (1921) 3020; *Ann.* **446** (1925) 97.
12. Maunsell, P. W. *N. Zealand J. Sci. Techn.* **22 B** (1940) 100.
13. Poluektoff, N. S. *Chem. J. Ser. B, J. appl. Chem.* (Russ.) **9** (1936) 2302; *Chem. Abstracts* **31** (1937) 4615; Raju, N. A. and Rao, G. G. *Nature* **174** (1954) 400; **175** (1955) 167.
14. Böeseken, J. *et. al. Rec. trav. chim.* **45** (1926) 406 (and later papers in that journal).
15. Kilpi, S. *J. Am. Chem. Soc.* **74** (1952) 5296.
16. Ellilä, A. *Ann. Acad. Sci. Fennicae*, Ser. **A II** (1953) 51.
17. Antikainen, P. J. and Mälkönen, P. *Unpublished.*
18. Antikainen, P. J. *Suomen Kemistilehti* **B 31** (1958) 291.
19. Antikainen, P. J. *Ibid.* **B 30** (1957) 123.

Received September 29, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 323—328

Some Features of the Salts of Deoxyribonucleic Acid with Bivalent Metals in Relation to Deoxyribonuclease Activity

JORMA ERKAMA and PIRJO SUUTARINEN

Institute of Biochemistry, University of Helsinki, Finland

Mg++, Mn++, and Zn++ salts of deoxyribonucleic acid (DNA) were prepared from the sodium salt of calf thymus DNA. The products were characterized by ultraviolet absorption spectra, viscosity measurements and titration curves, in addition to nitrogen, phosphorus and metal analyses. From the pH titration curves it is apparent that bivalent metals may interact with the titratable groups of DNA to produce structures similar to the chelated complexes. Pancreas deoxyribonuclease I (DNase I) showed a pH optimum at 6.0 for ZnDNA and at 6.5 for NaDNA, MgDNA and MnDNA when tested without addition of bivalent ions. Mg++ at 0.003 M concentration (a considerable excess over the amount of stoichiometrically bound metal) increased the rate of hydrolysis of MgDNA and MnDNA but was without appreciable effect on the action of DNase I when ZnDNA was used as substrate. It can be assumed that complex formation between the metal and the enzyme may also be essential for the activity of DNase I.

Previous studies on the activation of pancreas deoxyribonuclease I (DNase I) indicate a correlation between metal-binding of deoxyribonucleic acid (DNA) and its rate of hydrolysis [1–4]. It has been suggested that magnesium and similar ions may form chelate bridges between an amino group and a phosphate group of DNA [5].

Jungner [6] has obtained large molecular aggregates on precipitation of sodium thymonucleate from aqueous solution with ethanol in the presence of some bivalent ions. It has been found in this laboratory that the content of sodium in these aggregates is rather low (< 0.1 %) when the precipitation procedure has been repeated several times. Thus, the aggregates, albeit artificial products, may also be considered as salts of DNA with bivalent metals.

In order to gain some qualitative information on the degree of chelation of the metals, potentiometric titration of the aggregate compounds was undertaken. In addition, a study was made of the action of DNase I on these "metallosubstrates".

MATERIALS AND METHODS

Salts of DNA. DNA was isolated from calf thymus as the sodium salt by the method of Kay, Simmons and Dounce [7]. The product was stored as a solution in the frozen state. Analysis of two different preparations: I, N 14.0; P 8.0; N/P 3.87; η_{rel} (0.1 %) 4.4; ε(P) 7 500. II, N 12.0; P 7.0; N/P 3.79; η_{rel} (0.1 %) 4.7; ε(P) 8 350.

Mg^{++}, Mn^{++}, and Zn^{++} salts of DNA were prepared mainly by Jungner's method [6]. To 300 ml of sodium deoxyribonucleate solution containing 0.50 g of NaDNA, NaCl was added to bring the NaCl concentration up to 1 M. The nucleate was precipitated with ethanol and dissolved with stirring in 50 ml of distilled water in the refrigerator. The solution was dialyzed for 9 h against ion exchange water and 18 h against re-distilled water. The chloride of the bivalent metal in 10 ml of water was added to the concentration of approximately 0.05 M and the mixture was shaken for several minutes and placed in the refrigerator. The nucleate was precipitated with 5 volumes of ethanol, washed with ethanol and dissolved in water. The precipitation process was repeated twice.

DNase I was the once crystallized preparation D537 from the Worthington Biochemical Corp. It was found that in the cold a stock solution, containing 0.14 mg of enzyme per ml, remained stable for several days.

Spectral measurements were carried out with a Unicam spectrophotometer, model S.P. 500, 1 cm silica cells being employed. The nucleate concentration was 0.03 %.

Viscosity measurements were made at 25 ± 0.05° in an Ubbelohde viscometer with distilled water as solvent. The data were plotted as relative viscosity.

Potentiometric titration. A sample containing 20 mg of nucleate in 12 ml of water was used in the titration experiments, carried out mainly by following the directions of Gulland, Jordan and Taylor [8]. No attempt was made to maintain the ionic strength constant during the titrations. All pH measurements were made with a Beckman Glass Electrode pH Meter.

Measurements of DNase I activity. The enzyme activity was measured by Kunitz's spectrophotometric procedure [9]. One ml of enzyme solution, containing 5 μg of DNase I, and 3 ml of substrate solution (2 mg of nucleate and 5 ml of phosphate buffer in 50 ml of H_2O or 0.005 M $MgSO_4$) was kept 10—15 min at 25°, then mixed and placed in a 1-cm quartz cell with mixing. The linear change in the optical density at 260 mμ was followed during 5—10 min against a substrate-water control. The DNase activity was expressed as the increase in absorption per min.

Table 1. Composition and properties of Mg, Mn, and Zn salts of deoxyribonucleic acid.

		MgDNA	MnDNA	ZnDNA
N, %	found	13.7	12.4	12.5
	calc. *	16.3	15.6	15.4
P, %	found	8.0	7.0	7.3
	calc.	9.7	9.3	9.2
N/P,	found	3.80	3.93	3.79
	calc.	3.72	3.71	3.70
Mg, %	found	4.0		
	calc.	3.80		
Mn, %	found		7.54	
	calc.		8.19	
Zn, %	found			10.7
	calc.			9.6
Metal/2 P		1.28	1.22	1.38
$\eta_{rel.}$(0.1 % solution)		4.7	3.6	3.9
ε(P)		7 350	7 100	7 350

* Calculated for $(C_{39}H_{45}O_{24}N_{15}P_4Me_2)_x$.

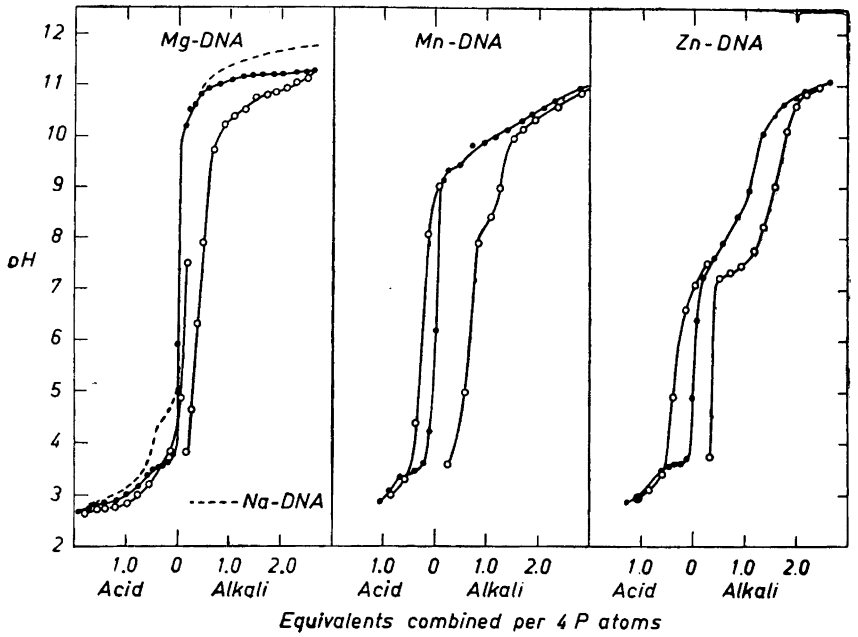


Fig. 1. Titration curves of the salts of DNA with bivalent metals. ● Forward-titration; O Back-titration.

RESULTS AND DISCUSSION

General properties of the preparations. The low nitrogen values of the preparations (Table 1) are probably due to the Kjeldahl procedure having been used. The atomic N/P ratio is in good agreement with the calculated figures. The protein content of the aggregates was not estimated, but in an earlier preparation of MgDNA, precipitated in a similar way, a protein content of about 3 % was found.

It can be seen from the analytical data that the molar ratio of metal to phosphorus is considerably greater than the theoretical value. One explanation, proposed by Jungner, is the possibility that the metal can also enter less acid radicals and not only the phosphoric acid groups. However, Shack, Jenkins and Thompsett [10] have reported data which suggest that there is no binding of metal on DNA with groups other than phosphorus. Cavalieri, Angelos and Balis [11] suggest that at least two types of sites, one with monovalent and one with divalent phosphate, may be involved. In our view, the excess of metal can also be interpreted as due to the retention of chloride ions.

A partial denaturation of the nucleate in dilute solutions during preservation and prolonged dialysis [12] may contribute to the rather high values of ε(P).

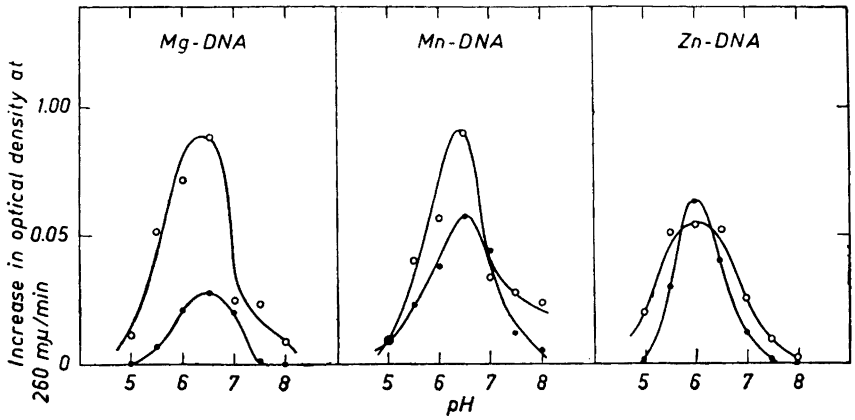


Fig. 2. Effect of pH on the rate of enzymatic digestion of salts of DNA. ● Without added Mg^{++}; ○ With 3.75×10^{-3} mmoles of Mg^{++}. Test solution: 0.03 mg DNA/ml; 5 μg DNase I; phosphate buffer; total volume 4 ml; 25°C.

Titration curves. Fig. 1 summarizes the changes in pH on forward- and back-titration of preparations. The titration curve for NaDNA is also included. All the preparations show a clear-cut pH drop in the acid range as compared with the titration curve of NaDNA. It can be concluded that some acidic protons of DNA, titrating between pH 5 and 3.5, have been displaced by the chelating bivalent metals, and that the amino groups of DNA are involved. This conforms with the views of Brown and Watson [5] who suggest the possibility that magnesium and similar ions may form chelate bridges between the amino group of guanine and a neighbouring phosphate group.

Neither in the sodium nucleate nor in the magnesium nucleate are any groups titrated in the alkaline region below pH 10. On the other hand, the titration curves of MnDNA and ZnDNA indicate that there is a very distinct tendency for Mn^{++} ions and especially for Zn^{++} ions to form chelates with the groups dissociating in the alkaline range. The different titration steps with distinct inflection points are most clearly separated in the titration curve of ZnDNA. The liberation of the titrating groups in the alkaline region begins at pH 7, and at approximately pH 7.3 the back-titration curve of ZnDNA shows a very steep bend. All this evidence indicates linkages of a different kind in ZnDNA as compared with the other preparations investigated.

Experiments with DNase I. Parallel studies (Fig. 2) were made without added Mg^{++} and with addition of 0.003 M Mg^{++}. The concentration of metal in the substrate solution without added Mg^{++} was 4.95×10^{-5} M for MgDNA, 4.12×10^{-5} M for MnDNA and 4.93×10^{-5} M for ZnDNA.

It will be noted that the optimal pH for the digestion of MgDNA and MnDNA is 6.5, as shown by Kunitz [9]. The same optimal pH was also obtained with NaDNA. With ZnDNA, however, the optimal pH for the action of DNase I is about 6.0. When no Mg^{++} is added to the digestion mixture, the reaction at the optimal pH range is most rapid with ZnDNA as substrate.

On the other hand, addition of Mg^{++} at 0.003 M concentration (a considerable excess over the amount of stoichiometrically bound metal in the substrate) increases the rate of hydrolysis of MgDNA and MnDNA but has no activating effect on the action of DNase I with ZnDNA at pH 6.0.

The activating effect of added Zn^{++} was not studied at the pH optimum of the DNase because free zinc ions cause precipitation of phosphate above pH 5.5. In an experiment made at pH 5.0 and with NaDNA as substrate, 0.003 M $ZnSO_4$ accelerated the reaction rate to approximately 200 % of that with 0.003 M $MgSO_4$ activation.

Our results are not in agreement with the observation of Gilbert, Overland, and Webb [13] that 0.01 M of zinc sulphate completely inhibited the action of deoxyribonuclease. These workers followed the course of the hydrolysis by measuring the increase in acid-soluble phosphorus. It seems possible that the increase in ultraviolet absorption, catalyzed by DNase I during the initial stage of the degradation, and the cleavage of the phosphate ester bonds are not simultaneous changes.

The formation of complexes of DNA with Mg^{++}, Ca^{++}, and Mn^{++} and the respective dissociation constants have been reported in the work of Wiberg and Neuman [3]. In a recent paper [4], Wiberg has studied the mechanism of metal activation of DNase I. He found that Ca^{++} is a potent synergist in the Mg-activated reaction.

The data reported here and the evidence presented by Wiberg indicate that the mechanism of the metal activation of DNase I cannot be explained satisfactorily by the concept of metallosubstrate alone.

DNase I is found to excercise its catalytic power within the pH range where no substrate groups are dissociated and where the hydrogen bonds of the Watson-Crick double-helical model are intact. The pH of optimum stability of the enzyme — from pH 5 to 6 — lies within the same pH range. Since the isoelectric point of DNase is in the region of pH 4.7 to 5.0 [9], the active form of the enzyme has a negative charge. It can be assumed that complex formation between the metal and the enzyme may also be essential for the activity of DNase I.

The chelation of the substrate with the bivalent metals may alter the substrate either in such a way that the enzyme system can function, as postulated by Weissman and Fisher [1], or also, we would add, in such a way that the combination of the metallosubstrate with the metalloenzyme is no longer possible. In this way the observations, to some extent contradictory, regarding the effect of the metals and the observed synergistic or antagonistic behaviour of the pairs of metals could be satisfactorily explained.

REFERENCES

1. Weissman, N. and Fisher, J. *J. Biol. Chem.* **178** (1949) 1007.
2. Cavalieri, L. F. *J. Am. Chem. Soc.* **74** (1952) 1242.
3. Wiberg, J. S. and Neuman, W. F. *Arch. Biochem. Biophys.* **72** (1957) 66.
4. Wiberg, J. S. *Arch. Biochem. Biophys.* **73** (1958) 337.
5. Brown, G. L. and Watson, M. *Nature* **172** (1953) 339.

6. Jungner, G. *Acta Chem. Scand.* **5** (1950) 168.
7. Kay, E. R. M., Simmons, N. E. and Dounce, A. L. *J. Am. Chem. Soc.* **74** (1952) 1724.
8. Gulland, J. M., Jordan, D. O. and Taylor, H. F. W. *J. Chem. Soc.* **1947** 1131.
9. Kunitz, M. *J. Gen. Physiol.* **33** (1950) 349, 363.
10. Shack, J., Jenkins, R. J. and Thompsett, J. M. *J. Biol. Chem.* **198** (1952) 85.
11. Cavalieri, L. F., Angelos, A. and Balis, M. E. *J. Am. Chem. Soc.* **73** (1951) 4902.
12. Thomas, R. *Trans. Faraday Soc.* **50** (1954) 290.
13. Gilbert, L. M., Overland, W. G. and Webb, M. *Exptl. Cell Research* **2** (1951) 349.

Received October 30, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 329—333

Synthesis of Phenolic Alkamine Ethers of Adrenaline and Some Related Compounds

JAAKKO HUKKI and ERKKI HONKANEN

Lääketehdas Orion Oy, Helsinki, Finland

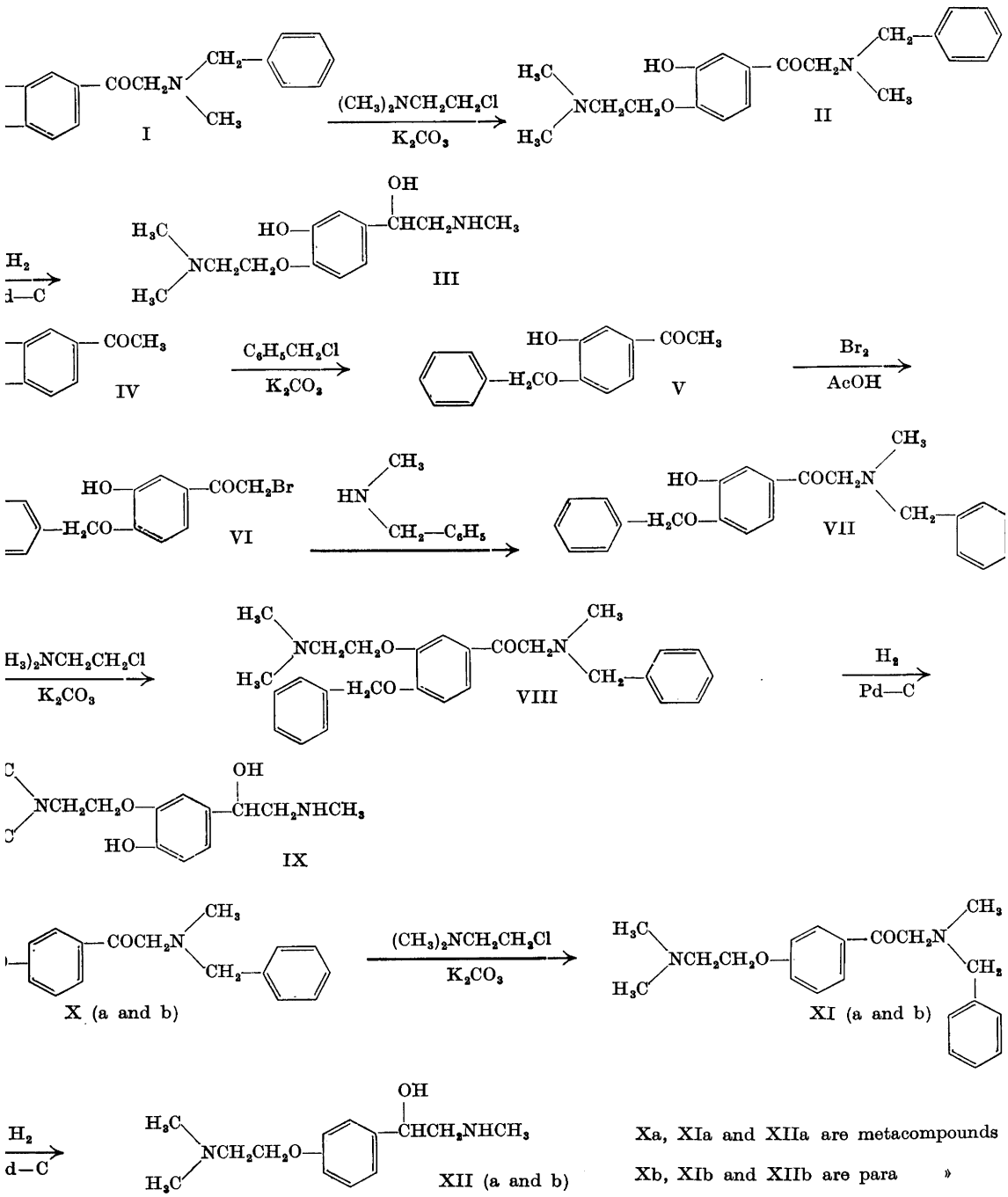
Both of the possible isomers of the phenolic dimethylaminoethyl monoethers of adrenaline (III and IX) were synthesized. The corresponding ethers of *meta* and *para*-synephrine (XIIa and XIIb) were also prepared.

In the hope of obtaining adrenaline-like substances possessing antihistamine activity the synthesis of phenolic dimethylaminoethyl ethers of adrenaline was attempted. Compounds of this type are not reported in the literature.

In the case of adrenaline, there are two possible isomeric phenolic monoethers (III and IX). To obtain each of these, the preferential reactivity of the hydroxyl group in the 4-position in 3,4-dihydroxyacetophenones was made use of. N-Benzyladrenalone (I), when allowed to react with one equivalent of dimethylaminoethylchloride in dry acetone in the presence of anhydrous potassium carbonate, was converted to the 4-dimethylaminoethyl ether (II). This, after subjection to simultaneous catalytic hydrogenation and hydrogenolysis, afforded the desired 4-dimethylaminoethyl ether of adrenaline (III).

In order to prepare the other isomer, an attempt was made to protect the hydroxyl group in the 4-position in N-benzyladrenalone by benzylation, after which the hydroxyl group in the 3-position would be amenable to selective etherification with dimethylaminoethylchloride. Unfortunately, the reaction of N-benzyladrenalone with benzyl chloride resulted in the formation of a dark resin, from which no individual compound could be isolated. Thus, a more complicated method of synthesis had to be devised:

From the reaction of 3,4-dihydroxyacetophenone (IV) with one equivalent of benzylchloride the 4-benzyl ether (V) was obtained. To confirm the position of the benzyl group this compound was methylated with diazomethane. A methyl ether was obtained which proved identical with one prepared by benzylation of acetovanillone (3-methoxy-4-hydroxyacetophenone). 3-Hydroxy-4-benzyloxyacetophenone was brominated to give the ω-bromoderivative (VI), which on condensation with methylbenzylamine formed the very compound (VII), which could not be obtained from N-benzyladrenalone by the direct



Xa, XIa and XIIa are metacompounds

Xb, XIb and XIIb are para »

procedure described above. Etherification of the 4-benzyl ether (VII) with dimethylaminoethylchloride and hydrogenation and hydrogenolysis of the resulting compound (VIII) afforded the desired 3-dimethylaminoethyl ether of adrenalone (IX). The above mode of synthesis being unequivocal at every step, the constitution of the final product (IX) is established. Hence the other monoether prepared earlier, being distinctly different, must have the alternative constitution III.

For comparative pharmacologic evaluation, the corresponding phenolic alkamine ethers of *meta*- and *para*-synephrine (XII) were also prepared. The principle in both syntheses was the same: *m*- or *p*-hydroxy-ω-methylbenzylaminoacetophenone (X) was etherified with dimethylaminoethylchloride and the resulting alkamine ether ketone (XI) subjected to simultaneous catalytic hydrogenation and hydrogenolysis.

As salts, these dimethylaminoethyl ethers of adrenaline, *m*- and *p*-synephrine, are stable, colorless, crystalline compounds, readily soluble in water.

EXPERIMENTAL

All melting points are uncorrected. Microanalyses were performed by the Microanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany.

N-Benzyladrenalone (I). This was prepared from 3,4-dihydroxy-ω-chloroacetophenone and methylbenzylamine according to Dalgliesh [1]. It was isolated as the monoethanolate.

3-Hydroxy-4-dimethylaminoethoxy-ω-methylbenzylaminoacetophenone (II). A mixture of 15.9 g (0.05 mole) of N-benzyladrenalone monoethanolate, 7.3 g (0.05 mole) of dimethylaminoethylchloride hydrochloride and 18.0 g (0.13 mole) of anhydrous potassium carbonate in 150 ml of dry acetone was refluxed for 24 h. The inorganic salts were filtered off and the solvent evaporated. The residue, upon admixture of ether saturated with hydrogen chloride, afforded the desired product as the dihydrochloride. The yield was 5.0 g (24 %), m. p. 235—236° (decomp.) (from dilute ethanol). (Found: C 57.48; H 6.95; N 6.51; Cl 16.74. Calc. for $C_{20}H_{28}O_3N_2$, 2HCl: C 57.80; H 6.78; N 6.74; Cl 17.08.)

α-(3-Hydroxy-4-dimethylaminoethoxyphenyl)-β-methylaminoethanol dihydrochloride (III). A solution of 1.04 g (0.0025 mole) of 3-hydroxy-4-dimethylaminoethoxy-ω-methylbenzylaminoacetophenone dihydrochloride in 20 ml of water was hydrogenated with 0.5 g of 10 % palladium-on-carbon catalyst at room temperature and atmospheric pressure. Two molar equivalents of hydrogen were absorbed in about 2 h. After removal of the catalyst by filtration, the solvent was evaporated under reduced pressure. The residue, after crystallization from acetone-methanol, afforded the dihydrochloride of the 4-dimethylaminoethyl ether of adrenaline, m. p. 225—226°. (Found: C 47.89; H 7.63; N 8.31; Cl 21.66. Calc. for $C_{13}H_{22}O_3N_2$, 2HCl: C 47.75; H 7.40; N 8.57; Cl 21.69.)

3,4-Dihydroxyacetophenone (IV). This was prepared from catechol diacetate by the method of Rosenmund and Lohfert [2].

3-Hydroxy-4-benzyloxyacetophenone (V). To a boiling solution of 15.2 g (0.1 mole) of 3,4-dihydroxyacetophenone and 12.7 g (0.1 mole) of benzylchloride in 250 ml of absolute ethanol, 2.3 g (0.1 mole) of sodium in 100 ml of absolute ethanol was added gradually during 1 h, after which time boiling was continued for a further 4 h. After filtration the solvent was evaporated. The residue was dissolved in 80 ml of 2 N sodium hydroxide solution and extracted with chloroform to remove the dibenzyl ether formed (about 7.5 g). Upon neutralization of the aqueous layer with carbon dioxide, 3-hydroxy-4-benzyloxyacetophenone was precipitated. The yield, after crystallization from ethanol, was 12.7 g (52.5 %), m. p. 119—120°. (Found: C 74.10; H 5.94. Calc. for $C_{15}H_{14}O_3$: C 74.36; H 5.83.)

From the above compound the following derivatives were prepared:

The *benzoate*, m. p. 76—77°. (Found: C 76.59; H 5.42. Calc. for $C_{22}H_{18}O_4$: C 76.28; H 5.24.)

The *methyl ether*, m. p. 83—84° (from methanol), was obtained with diazomethane. (Found: C 74.38; H 6.25. Calc. for $C_{16}H_{16}O_3$: C 74.98; H 6.29.) It was shown by mixed melting point determination to be identical with 3-methoxy-4-benzyloxyacetophenone. This was prepared by benzylation of acetovanillone obtained from veratrole by the method of Pratt and Robinson [3].

3-Hydroxy-4-benzyloxy-ω-bromoacetophenone (VI). A solution of 12.1 g (0.05 mole) of 3-hydroxy-4-benzyloxyacetophenone in 250 ml of glacial acetic acid (99—100 %) was cooled below +10°. To this solution 8.0 g (0.05 mole) of bromine in 50 ml of glacial acetic acid was added in the course of half an hour with stirring. The solvent was evaporated under reduced pressure. The residue, upon trituration with water, deposited crystals, which were recrystallized first from benzene, then from methanol. The yield was 8.0 g (50 %), m. p. 143—144°. (Found: C 55.84; H 4.13; Br 25.09. Calc. for $C_{15}H_{13}O_3Br$: C 56.09; H 4.08; Br 24.90.)

From the above compound the *benzoate*, m. p. 104—106°, was also prepared.

3-Hydroxy-4-benzyloxy-ω-methylbenzylaminoacetophenone (VII). A mixture of 3.21 g (0.01 mole) of 3-hydroxy-4-benzyloxy-ω-bromoacetophenone and 2.42 g (0.02 mole) of methylbenzylamine in 50 ml of benzene was shaken for 4 h and allowed to stand at room temperature overnight. The separated methylbenzylamine hydrochloride (1.55 g = 98 %) was filtered off and the solvent evaporated. The residue was a reddish brown oil, which did not crystallize. It was used without further purification for the following reaction.

3-Dimethylaminoethoxy-4-benzyloxy-ω-methylbenzylaminoacetophenone dihydrochloride (VIII). A mixture of 3.6 g (0.01 mole) of the crude 3-hydroxy-4-benzyloxy-ω-methylbenzylaminoacetophenone, 2.16 g (0.015 mole) of dimethylaminoethylchloride hydrochloride and 5.6 g (0.04 mole) of anhydrous potassium carbonate in 100 ml of acetone was refluxed for 48 h. After filtration the solvent was evaporated. The residue, upon addition of absolute ethanol and ether saturated with hydrogen chloride, afforded the desired product as the dihydrochloride. The overall yield from 3-hydroxy-4-benzyloxy-ω-bromoacetophenone (VI → VIII), after recrystallization from acetone-methanol, was 2.0 g (40 %), m. p. 202—204°. (Found: N 5.86; Cl 14.77. Calc. for $C_{27}H_{32}O_3N_2$, 2HCl: N 5.53; Cl 14.10.)

α-(3-Dimethylaminoethoxy-4-hydroxyphenyl)-β-methylaminoethanol dihydrochloride (IX). A solution of 1.0 g (0.002 mole) of 3-dimethylaminoethoxy-4-benzyloxy-ω-methylbenzylaminoacetophenone dihydrochloride in 20 ml of water was hydrogenated at room temperature and atmospheric pressure in the presence of 0.5 g of 10 % palladium-on-carbon catalyst. Three molar equivalents of hydrogen were absorbed in 2 h. After filtration, the solvent was evaporated under diminished pressure. The residue, upon trituration with acetone-methanol, yielded the desired product as the crystalline dihydrochloride, m. p. 248—249° (decomp.). (Found: C 47.50; H 7.46; N 8.59; Cl 21.92. Calc. for $C_{13}H_{22}O_3N_2$, 2HCl: C 47.75; H 7.40; N 8.57; Cl 21.62.)

3-Hydroxy-ω-methylbenzylaminoacetophenone hydrochloride (Xa). The synthesis of this compound was carried out by the sequence of reactions devised by Sergievskaya and Ravdel [4]. The starting material, 3-hydroxyacetophenone (a product of Winthrop Products Co.), was brominated after protection of the phenolic hydroxyl group by benzoylation, to give 3-benzoyloxy-ω-bromoacetophenone. This, by condensation with methylbenzylamine and subsequent debenzoylation, afforded 3-hydroxy-ω-methylbenzylaminoacetophenone hydrochloride, m. p. 202—203°, in 46 % overall yield (based on 3-hydroxyacetophenone).

3-Dimethylaminoethoxy-ω-methylbenzylaminoacetophenone hydrochloride (XIa). A mixture of 2.92 g (0.01 mole) of 3-hydroxy-ω-methylbenzylaminoacetophenone hydrochloride, 2.16 g (0.015 mole) of dimethylaminoethylchloride hydrochloride and 6.9 g (0.05 mole) of anhydrous potassium carbonate in 100 ml of dry acetone was refluxed for 48 h. After filtration the solvent was evaporated under reduced pressure. The residue was dissolved in ethanol. This solution, upon addition of ether saturated with hydrogen chloride, deposited crystals of dihydrochloride, which were recrystallized from acetone-methanol. The yield was 3.5 g (88 %), m. p. 198—200°. (Found: C 59.59; H 7.46; N 6.88; Cl 17.87. Calc. for $C_{20}H_{26}O_2N_2$, 2HCl: C 60.00; H 7.07; N 7.02; Cl 17.77.)

α-(3-Dimethylaminoethoxyphenyl)-β-methylaminoethanol dioxalate (XIIa). A solution of 2.00 g (0.005 mole) of 3-dimethylaminoethoxy-ω-methylbenzylaminoacetophenone dihydrochloride in 30 ml of water was hydrogenated with 0.5 g of 10 % palladium-on-carbon catalyst at room temperature and atmospheric pressure. Two molar equivalents of hydrogen were absorbed in 2 h. After removal of the catalyst and evaporation of the solvent under reduced pressure, the remainder, a viscous, colorless, non-crystallizable oil, was dissolved in 5 ml of 2 N aqueous sodium hydroxide solution and extracted with ether. The ethereal layer was dried over anhydrous sodium sulfate. After evaporation of the solvent the residue was dissolved in methanol. This solution, after addition of 0.9 g (0.01 mole) of anhydrous oxalic acid, was heated to boiling. On cooling, the dioxalate of the desired amine was obtained as colorless crystals, m. p. 156–158°. (Found: C 48.40; H 6.31; N 6.59. Calc. for $C_{13}H_{22}O_2N_2$, 2(COOH): C 48.79; H 6.27; N 6.70.)

4-Hydroxy-ω-methylbenzylaminoacetophenone monohydrate (Xb). A mixture of 3.41 g (0.02 mole) of 4-hydroxy-ω-chloroacetophenone (prepared by the method of Tutin *et al.*[5]) and 4.84 g (0.04 mole) of methylbenzylamine in 20 ml of benzene was shaken for 4 h and allowed to stand overnight at room temperature. After removal of the methylbenzylamine hydrochloride formed and evaporation of the solvent, the residue was crystallized from dilute ethanol. The yield was 5.2 g (95 %) and m. p. 106–109°. This same compound has been prepared previously by Asscher [6] from phenol and methylbenzylaminoacetonitrile. The yield and melting point recorded by this author are 16.5 % and 102–110°, respectively.

4-Dimethylaminoethoxy-ω-methylbenzylaminoacetophenone dioxalate (XIb). A mixture of 2.73 g (0.01 mole) of 4-hydroxy-ω-methylbenzylaminoacetophenone monohydrate 2.16 g (0.015 mole) of dimethylaminoethylchloride hydrochloride and 5.60 g (0.04 mole) of anhydrous potassium carbonate in 100 ml of acetone was refluxed for 48 h. The inorganic salts were filtered off, the solvent evaporated and the residue dissolved in ethanol. After addition of 1.80 g (0.02 mole) of anhydrous oxalic acid, the alkamine ether separated as the dioxalate. The yield was 2.7 g (52 %), m. p., after recrystallization from ethanol, 148–150°. (Found: N 5.42. Calc. for $C_{20}H_{26}O_2N_2$, 2(COOH): N 5.54.)

α-(4-Dimethylaminoethoxy)-β-methylaminoethanol dihydrochloride (XIIb). A solution of 2.53 g (0.005 mole) of 4-dimethylaminoethoxy-ω-methylbenzylaminoacetophenone dioxalate in 30 ml of water was hydrogenated at room temperature and atmospheric pressure in the presence of 0.5 g of 10 % palladium-on-carbon catalyst. Two molar equivalents of hydrogen were consumed in 2 h. After removal of the catalyst and evaporation of the solvent the hydrogenation product was obtained as crystalline dioxalate, m. p. 153–154°. By treatment with ether saturated with hydrogen chloride, this was converted to the corresponding dihydrochloride, m. p., after recrystallization from acetone-methanol, 200–201°. (Found: C 50.02; H 7.86; N 9.04; Cl 22.49. Calc. for $C_{13}H_{22}O_2N_2$, 2HCl: C 50.16; H 7.78; N 9.04; Cl 22.78.)

REFERENCES

1. Dalgliesh, C. *J. Chem. Soc.* **1949** 90.
2. Rosenmund, K. and Lohfert, H. *Ber.* **61** (1928) 2601.
3. Pratt, D. and Robinson, R. *J. Chem. Soc.* **123** (1923) 753.
4. Sergievskaya, S. and Ravdel, G. *Zhur. Obshcheĭ Khim* **22** (1952) 496.
5. Tutin, F., Caton, F. and Hann, A. *J. Chem. Soc.* **95** (1909) 2113.
6. Asscher, M. *Rec. trav. chim.* **68** (1949) 965.

Received November 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 334—336

Calculated Mean Amplitudes of Vibration in Boron Trihalides

S. J. CYVIN

Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The mean amplitudes of vibration from spectroscopic data in boron trifluoride, trichloride and tribromide are recalculated. The results are presented together with new calculations for boron triiodide.

Bakken [1] has presented a list of mean amplitude values for a number of four-atomic molecules including boron trifluoride, trichloride and tribromide. Professor Morino during his stay in Trondheim pointed out a discrepancy between Bakken's values and unpublished values obtained by T. Iijima for the non-bonded distances in boron trifluoride and trichloride. In the present work a recalculation of the mean amplitudes of the three mentioned

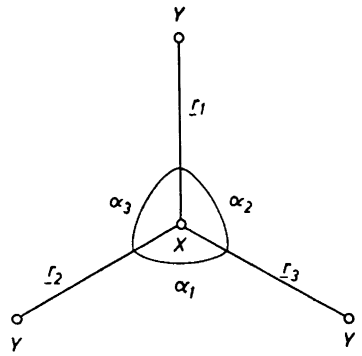


Fig. 1. Internal coordinates for plane symmetrical XY_3 molecules. The symbols denote displacements from the equilibrium distances and angles. Symmetry coordinates:

$$\text{Symm.species } A'_1: \quad S_1 = 3^{-\frac{1}{2}}(r_1 + r_2 + r_3)$$

$$\text{Symm.species } E': \begin{cases} S_3 = 6^{-\frac{1}{2}}(2r_1 - r_2 - r_3) \\ S_4 = 6^{-\frac{1}{2}}(2\alpha_1 - \alpha_2 - \alpha_3) \end{cases}$$

R denotes the equilibrium X—Y distance.

molecules has been performed together with the calculations for boron triiodide. These calculations revealed an error in the numerical calculations of Bakken.

General treatment [2-5]. The molecular model (XY_3) is that of D_{3h} symmetry in the equilibrium position. Small harmonic vibrations are assumed. It is found that only 3 symmetry coordinates (see Fig. 1) are needed for setting up the energy matrices for the in-plane vibrations. The adopted symbols for the force constants of the symmetrized potential energy matrix are given by

$$K_1 \text{ (symmetry species A'}_1\text{)}; \quad \begin{bmatrix} K_2 & \Gamma \\ \Gamma & \Phi \end{bmatrix} \text{ (symmetry species E')}.$$

The secular equation yields the following equations for the vibrational frequencies ($\lambda = 4\pi^2\nu^2$),

$$\lambda_1 = K_1\mu_Y$$

$$\lambda_3 + \lambda_4 = \tfrac{1}{2}(K_2 + 3\Phi)\ (3\mu_X + 2\mu_Y) + 3\tfrac{3}{2}\Gamma\mu_X$$

$$\lambda_3\lambda_4 = 3(K_2\Phi - \Gamma^2)\ (3\mu_X + \mu_Y)\ \mu_Y$$

where μ_X and μ_Y denote the inverse masses of X and Y, respectively. The two types of interatomic distance deviations will be represented by $r_{XY} = r_1$

Table 1. Experimental fundamental frequencies and calculated force constants of boron trihalides.

Fundamentals (gaseous state) in cm^{-1} units [a]				
	$^{11}BF_3$	$^{11}BCl_3$	$^{11}BBr_3$	$^{11}BI_3$
ν_1	888	471	278	190
ν_3	1 453.5	954.2	819.8	704.1
ν_4	480.4	243	148	100
	$^{10}BF_3$	$^{10}BCl_3$	$^{10}BBr_3$	$^{10}BI_3$
ν_3	1 504.7	993.7	856.1	737.0

Force constants in mdyne/Å units [b]				
	BF_3	BCl_3	BBr_3	BI_3
K_1	8.8236	4.6324	3.6374	2.6984
K_2	7.4068	3.3424	3.3021	1.7292
Φ	0.50013	0.23082	0.18071	0.15079
Γ	−0.63980	−0.19575	−0.34545	−0.03493

[a] The data for BF_3 and BCl_3 are from Ref.[6]; those of BBr_3 and BI_3 from Ref.[7].

[b] The additional solutions arising from the quadratic secular equation yield unreliable force constant values which have been rejected.

Table 2. Mean amplitudes of vibration in boron trihalides.

Mean amplitude (Å)	Abs. temp. °K	Molecule $^{11}BF_3$	$^{11}BCl_3$	$^{11}BBr_3$	$^{11}BI_3$
u_{XY}	0	0.0424	0.0478	0.0479	0.0508
	298	0.0425	0.0491	0.0506	0.0570
u_{YY}	0	0.0517	0.0536	0.0461	0.0449
	298	0.0552	0.0689	0.0726	0.0848

and $r_{YY} = \frac{1}{2}\,3^{\frac{1}{2}}\,(r_2 + r_3) + \frac{1}{2}Ra_1$. They are connected with the symmetry coordinates by the following linear combinations.

$$r_{XY} = 3^{-\frac{1}{2}}S_1 + 6^{-\frac{1}{2}}2S_3$$

$$r_{YY} = S_1 - 2^{-\frac{1}{2}}S_3 + 6^{-\frac{1}{2}}S_4$$

To compute the corresponding mean amplitudes, *viz.* $u_{XY} = < r_{XY}^2 >_{Av}^{1/2}$ and $u_{YY} = < r_{YY}^2 >_{Av}^{1/2}$, the interatomic distance deviations need be expressed in terms of the normal coordinates. Consequently the transformation between the symmetry coordinates and the normal coordinates is required. (For a detailed theoretical treatment, see especially Refs. [4,5]).

Numerical computations. The applied experimental values of vibrational frequencies are given in Table 1. Calculated force constants which are consistent with these frequency values are also included in the table.

The data from Table 1 have been used for computations of the mean amplitudes of vibration (u) for the boron halides of the ^{11}B isotope. The resulting values of u at the absolute zero point and the temperature 298 °K are given in Table 2.

Acknowledgement. I wish to express my best thanks to Professor Yonezo Morino, who pointed out the error in Bakken's paper, for interesting discussions during his visit in Trondheim.

REFERENCES

1. Bakken, J. *Acta Chem. Scand.* **12** (1958) 594.
2. Wilson, E. B. Jr., Decius, J. C. and Cross, P. C. *Molecular Vibrations*, McGraw-Hill, N.Y. 1955.
3. Morino, Y., Kuchitsu, K. and Shimanouchi, T. *J. Chem. Phys.* **20** (1952) 726.
4. Cyvin, S. J. *Acta Chem. Scand.* **11** (1957) 1499.
5. Cyvin, S. J. *J. Chem. Phys.* **29** (1958) 583.
6. Lindeman, L. P. and Wilson, M. K. *J. Chem. Phys.* **24** (1956) 242.
7. Wentink, T. Jr. and Tiensuu, V. H. *J. Chem. Phys.* **28** (1958) 826.

Received November 6, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 337—341

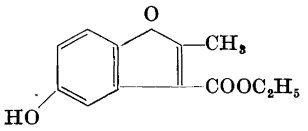
Ozonolysis and Structure of Some Benzofurans

ERLING BERNATEK and INGE BØ

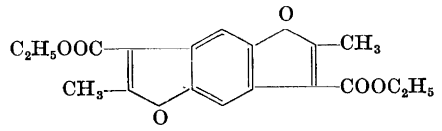
Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

Stable, crystalline ozonides have been obtained by ozonisation of 2-methyl-3-carbethoxy-5-acetoxybenzofuran and 2,7-dimethyl-3-carbethoxy-5-acetoxybenzofuran. The structure of reaction products from toluquinone and ethyl acetoacetate and acetylacetone, respectively, has been determined. Further the ozonolysis of 2-methyl-3-carbethoxy-5-hydroxybenzofuran, 2-methyl-3-acetyl-5-hydroxybenzofuran and 2-methyl-3-acetyl-5-acetoxybenzofuran is described.

By the reaction between ethyl acetoacetate and *p*-benzoquinone in the presence of anhydrous zinc chloride there is formed a mixture of a benzofuran and a benzodifuran. The synthesis was first described by v. Pechmann [1] and Ikuta [2], the latter author providing the proof for the structures (I and II) of the products.



I



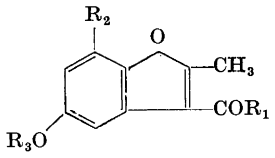
II

Some years later Graebe and Levy [3] extended the synthesis to include toluquinone and proposed a revision of some of Ikuta's structural formulae. These have, however, been corroborated by more recent work by Bernatek [4]. Further extensions of the synthesis have been made through the investigations of the latter [4–6] and by Grinev and his collaborators [7,8].

Ozonolysis of benzofurans was originally established by v. Wacek, Eppinger and v. Bezard [9] but the reaction seems not to have been exploited until some benzodifurans were ozonised by Bernatek and Thoresen [10] in 1955. That investigation was from the outset aimed at structural problems but as an interesting sideline it was found that when suitably substituted a benzodifuran would yield very stable and excellently crystallising ozonides.

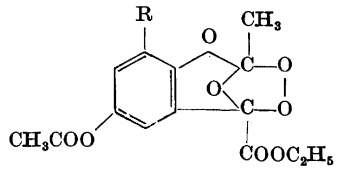
The present paper deals with the ozonolysis of I and III (a product from benzoquinone and acetylacetone) as well as their homologs IV and V contain-

ing a methyl group in the benzene ring. IV was prepared by Graebe and Levy but no attempts to determine the position of the methyl group were made at that time. Our interest is therefore in part directed towards structural problems and in part towards the possible formation of stable ozonides.



IA: $R_1 = OC_2H_5$, $R_2 = H$, $R_3 = CH_3CO$
III: $R_1 = CH_3$, $R_2 = R_3 = H$
IIIA: $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3CO$
IV: $R_1 = OC_2H_5$, $R_2 = CH_3$, $R_3 = H$
IVA: $R_1 = OC_2H_5$, $R_2 = CH_3$, $R_3 = CH_3CO$
V: $R_1 = R_2 = CH_3$, $R_3 = H$
VA: $R_1 = R_2 = CH_3$, $R_3 = CH_3CO$

The ozonolyses were performed in ethyl acetate at 0°. In order to isolate stable ozonides it was found necessary to ozonise the acetates rather than the free hydroxybenzofurans. Thus substance I gave no ozonide when concentrating the reaction mixture, but a yellow, peroxidic syrup which decomposed violently at room temperature. No definite decomposition products were isolated. It is believed that the presence of a free hydroxyl group enhances combustion of the molecule. IA on the other hand yielded a stable, monomeric ozonide $C_{14}H_{14}O_8$ (VI).

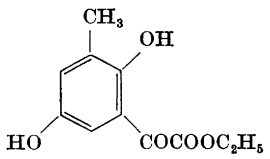


VI: R = H
VII: R = CH_3

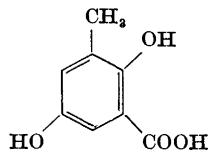
Ozonolysis of III gave a light brown syrup which was decomposed with aqueous sodium carbonate and acidified with hydrochloric acid. After several days in a refrigerator a small amount of crystals appeared and were identified as gentisic acid (hydroquinone carboxylic acid).

The immediate ozonisation product of IIIA was also a brownish, peroxidic syrup which was rather stable and may have contained an ozonide, but no such was isolated. After some time the syrup deposited white crystals of gentisic acid diacetate. From these results it is clear that the hydroxyl in III must be in *para*-position to the oxygen bridge of the furan ring. We have thus another support for the original formula of Ikuta in contrast to the proposals of Graebe and Levy where the hydroxyl and the oxygen bridge would have been in *meta*-position to each other.

IV gave after ozonisation a colourless syrup which decomposed slowly with evolution of gas. After some days white crystals separated which were identified as oxalic acid. The degradation had in this case evidently gone very far. IVA yielded as expected a stable ozonide $C_{15}H_{16}O_8$ (VII). On treatment with sodium iodide in glacial acetic acid it was reduced to a monoacetate of ethyl 2,5-dihydroxy-3-methylphenylglyoxalate (VIII).



VIII



IX

Whether the acetoxy group is attached to the 2- or 5-position cannot be proved conclusively, but the latter position is considered the more likely one. If the ozonide (VII) had been reduced without complications, the diacetate of VIII would have formed. During the reaction the new acetoxy group probably has been split off *in statu nascendi*. The acetate of VIII was boiled with glacial acetic acid and sodium acetate to give the free ketoacid. This was in turn oxidised with hydrogen peroxide and hydrolysed with hydrochloric acid to the methyl gentisic acid IX (2,5-dihydroxy-3-methyl-benzoic acid). Proof is hereby secured for the position of the methyl group *ortho* to the oxygen bridge in the reaction product from toluquinone and ethyl acetoacetate (IV).

Of V (the product from toluquinone and acetylacetone) only its acetate VA was ozonised. No ozonide was obtained from this compound but a peroxidic syrup which deposited crystals of 2,5-diacetoxy-3-methylbenzoic acid (diacetate of IX). The position of the methyl group in the benzene ring of V must consequently be the same as in IV.

Regarding the course of ozonolysis it is obvious that IA and IVA mainly undergo the normal reaction. The same probably holds true also for I and IV, but here the free hydroxyl group is responsible for a secondary and far-reaching decomposition. III, IIIA and VA all lose their 3-acetyl groups which is consistent with an anomalous ozonolysis. The fact that the peroxidic syrups resulting from the ozonisation had any stability at all at room temperature, however, makes it more probable that the ozonisation proper was a normal one and followed by a rearrangement.

EXPERIMENTAL

(Melting points not corrected)

Ozonisation procedure. The substance was usually dissolved in about 50 times its weight of pure ethyl acetate and the solution cooled in an ice-bath. A current of oxygen containing 4.5 % (wt) of ozone was passed through the solution at a rate of 30 l/h. Some ozone always escaped from the reaction mixture and the reagent was therefore passed through in excess of the calculated amount. The ozonised solution was concentrated *in vacuo* and worked up as described below.

2-Methyl-3-carbethoxy-5-hydroxybenzofuran (I) and its acetate (IA). I (3.0 g) in ethyl acetate (150 ml) was ozonised for 60 min. During the reaction a strong yellow colour appeared but vanished towards the end. After concentration *in vacuo* at room temperature a yellow syrup remained which almost immediately decomposed violently.

IA (3.0 g) in ethyl acetate (150 ml) was ozonised for 60 min. Transitory yellow colour. After concentration a yellow syrup remained which crystallised after two days in a refrigerator. Recrystallised from ethanol, m. p. 105° (1.2 g). (Found: C 54.2; H 4.7; act. O 5.2; M (benzene) 290. Calc. for $C_{14}H_{14}O_8$: C 54.2; H 4.6; act. O 5.2; M 310.)

2-Methyl-3-acetyl-5-hydroxybenzofuran (II) and its acetate (IIA). II (2.5 g) in ethyl acetate (100 ml) was ozonised for 30 min. Transitory yellow colour. After concentration the brownish syrup did not crystallise after one week in a refrigerator. The syrup was treated with aqueous sodium carbonate until a clear solution remained. Upon acidification white crystals slowly began to separate. Recrystallised from a small amount of water, m. p. 199°. Mixed m. p. with an authentic sample of gentisic acid showed no depression. IIA (3 g) in ethyl acetate (150 ml) was ozonised for 60 min. Concentration gave a brownish syrup which after being kept in a refrigerator overnight deposited white crystals. Recrystallised from ethyl acetate-petrol ether m. p. 122° (0.35 g). Mixed m. p. with an authentic sample of diacetylgentisic acid (m. p. 119°) showed no depression. (Found: C 55.5; H 4.1; E 237. Calc. for $C_{11}H_{10}O_6$: C 55.5; H 4.2; E 238.)

2,7-Dimethyl-3-carbethoxy-5-hydroxybenzofuran (IV) and its acetate (IVA). IV (1.5 g) in ethyl acetate (75 ml) was ozonised for 30 min. Concentration *in vacuo* afforded a syrup which at room temperature underwent a slow decomposition with evolution of gas. After two days in a refrigerator white crystals separated. Recrystallised from water, m. p. *ca.* 102° (0.05 g). Mixed m. p. with oxalic acid was undepressed. (Found: C 19.2; H 4.9. Calc. for $C_2H_2O_4 \cdot 2H_2O$: C 19.1; H 4.8).

IVA (2.5 g) in ethyl acetate (150 ml) was ozonised for 60 min. During concentration a yellowish substance separated. M. p. 109—111° (1.7 g). Recrystallised from ethanol, m. p. 112°. (Found: C 55.6; H 5.0; act. O 4.9. Calc. fo · $C_{15}H_{16}O_8$: C 55.6; H 5.0; act. O 5.0.) The ozonide (VII) (0.8 g) was treated overnight with sodium iodide (0.75 g) in glacial acetic acid and the liberated iodine removed with aqueous sodium sulphite. A yellow oil separated and crystallised on scratching. Recrystallised from dilute ethanol, yellowish needles of m. p. 69°, gave an orange-coloured precipitate with 2,4-dinitrophenylhydrazine. (Found: C 58.6; H 5.3. Calc. for $C_{13}H_{14}O_6$: C 58.6; H 5.3.)

$C_{13}H_{14}O_6$ (monoacetate of VIII) (0.20 g), anhydrous sodium acetate (0.25 g) and glacial acetic acid (5 ml) were refluxed for one hour and evaporated to dryness *in vacuo*. The yellow residue (sodium salt) was taken up in water and acidified with hydrochloric acid when a yellow substance separated. Recrystallised from benzene, yellow-orange needles, m. p. 132° (0.15 g). Red precipitate with 2,4-dinitrophenylhydrazine. (Found: C 55.4; H 4.6. Calc. for $C_{11}H_{10}O_6$: C 55.5; H 4.2.)

The ketoacid $C_{11}H_{10}O_6$ (0.15 g) and hydrogen peroxide (5 ml, 1 %) were stirred at room temperature for 2 h and left for another 17 h. The yellow colour had disappeared and a white substance separated. Recrystallised from ethyl acetate-ligroin and from dilute ethanol, m. p. 146°. This substance was refluxed with hydrochloric acid (5 ml, 1 N) for 30 min. On cooling yellowish needles separated. Recrystallised from a small amount of water. White needles, m. p. 216°. Mixed m. p. with an authentic sample of 2,5-dihydroxy-3-methylbenzoic acid (prepared from *o*-cresotic acid by persulphate oxidation [11]) showed no depression. Also the I.R.-spectra of the two samples were identical.

2,7-Dimethyl-3-acetyl-5-hydroxybenzofuran (V) and its acetate (VA). To a mixture of acetylacetone (8 ml), acetone (30 ml) and anhydrous zinc chloride (11 g) was added toluquinone (4 g) in small portions. The mixture was refluxed for 30 min and on cooling a white substance separated. Recrystallized from acetone, m. p. 235° (4 g). The red colour characteristic for benzofurans was produced by heating with concentrated sulphuric acid. With 2,4-dinitrophenylhydrazine a red substance was formed, m. p. 255°. (Found: C 70.7; H 5.9. Calc. for $C_{12}H_{12}O_3$: C 70.6; H 5.9.)

V (3 g) in acetyl chloride (15 ml) was refluxed for 15 min. The mixture was poured into 600 ml of ice-water and stirred vigorously. The separated substance was washed thoroughly and recrystallised from 50 % ethanol, 2.7 g, m. p. 97°. (Found: C 68.2; H 5.6. Calc. for $C_{14}H_{14}O_4$: C 68.3; H 5.7.)

VA (2.7 g) in ethyl acetate (150 ml) was ozonised for 60 min. On concentration a light yellow syrup remained which after some days in a refrigerator deposited a white substance. Recrystallised from ethyl acetate-ligroin and from dilute ethanol, m. p. 148°. (Found: C 57.0; H 4.7; E 252. Calc. for $C_{12}H_{12}O_6$: C 57.2; H 4.8; E 251.) Mixed m. p. with an authentic sample of 2,5-diacetoxy-3-methylbenzoic acid was undepressed. Also the I.R.-spectra of the two compounds were identical.

REFERENCES

1. v. Pechmann, H. *Ber.* **21** (1888) 3005.
2. Ikuta, M. *J. Prakt. Chem.* [2] **45** (1892) 65.
3. Graebe, C. and Levy, S. *Ann.* **283** (1894) 245.
4. Bernatek, E. *Acta Chem. Scand.* **7** (1953) 677.
5. Bernatek, E. and Ramstad, S. *Acta Chem. Scand.* **7** (1953) 1351.
6. Bernatek, E. *Acta Chem. Scand.* **10** (1956) 273.
7. Grinev, A. N., Bon-Khvar, P. and Terent'ev, A. P. *Zhur. Obshchei Khim.* **26** (1956) 2928.
8. Grinev, A. N., Bon-Khvar, P. and Terent'ev, A. P. *Zhur. Obshchei Khim.* **27** (1957) 1087.
9. v. Wacek, A., Eppinger, H. O. and v. Bézard, A. *Ber.* **73** (1940) 521.
10. Bernatek, E. and Thoresen, F. *Acta Chem. Scand.* **9** (1955) 743.
11. Chem. Fabr. auf Actien German Patent 81297 (1894).

Received October 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 342—344

On the Acid-Catalysed Rearrangement of Ozonides *

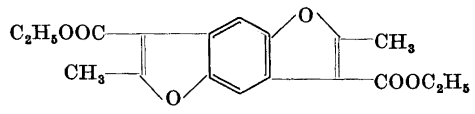
ERLING BERNATEK and FREDRIK THORESEN

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

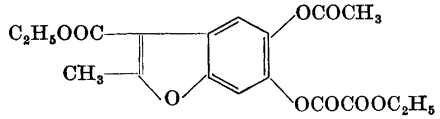
An acid-catalysed rearrangement of two stable, crystalline ozonides is described. The reaction is intramolecular and the reacting entities are probably the conjugate acids of the ozonides.

It has been reported [1] that suitably substituted benzo [1,2—b:4,5—b'] difurans yield stable, crystalline ozonides. This is also the case with some benzofurans [2]. Criegee's statement [3] that stable ozonides often can be obtained from unsaturated five-membered rings is consequently further amplified. Ozonides can, like other peroxy compounds, undergo rearrangements where one of the "peroxidic" oxygen atoms is interposed between two carbon atoms. Undoubtedly such rearrangements often have been confused with anomalous ozonolysis because the net results of the reactions generally are identical. Only in the later years when really pure, stable and monomeric ozonides have become available, such rearrangements have been studied in some detail. Thus, Criegee [4] has reported on the rearrangement of ozonides from 2,3-disubstituted indenones, while Witkop [5] described a rearrangement of 2-phenylskatole ozonide.

We wish to describe the acid-catalysed rearrangement of the monozonide and diozonide of diethyl 2,6-dimethylbenzo [1,2—b:4,5—b'] difuran-3,7-dicarboxylate (I).



I



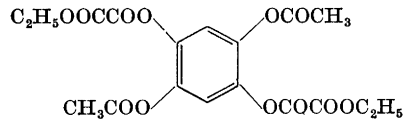
II

When the ozonides were dissolved in acetic anhydride containing a small amount of perchloric acid, the content of active oxygen disappeared rapidly. From the monozonide ($C_{18}H_{18}O_9$) two products could be isolated. A very small amount of an acid which could not be identified and a substance $C_{18}H_{18}O_9$.

* Presented at the 9. *Nordiske Kemikermöde*, Aarhus 1956.

The latter contained one O-acetyl group, split off one mole of oxalic acid on alkaline hydrolysis and could be alcoholysed to diethyl oxalate and a substance $C_{12}H_{12}O_5$. The latter could in turn be acetylated to a diacetate $C_{16}H_{16}O_7$. These results are only compatible with the structure II for the rearrangement product $C_{18}H_{18}O_9$.

Also the diozonide ($C_{18}H_{18}O_{12}$) yielded two products; an acid $C_{12}H_{10}O_8$ and a substance $C_{18}H_{18}O_{12}$. The acid gave a dimethyl ester and was identified as 2.5-diacetoxyterephthalic acid. $C_{18}H_{18}O_{12}$ contained two O-acetyl groups and by alkaline hydrolysis split off two moles of oxalic acid. Treatment with sodium acetate in acetic anhydride gave 1,2,4,5-tetracetoxybenzene. Evidently $C_{18}H_{18}O_{12}$ has the structure III.



III

The rearrangements are isomeric and most probably intramolecular since the same products in approximately the same yields were obtained by rearrangement in propionic anhydride. Further it is likely that the first step in the reaction is an addition of a proton, *i.e.* the reacting entities are the conjugate acids of the ozonides. A semiquantitative experiment shows that that with perchloric acid the reaction is very fast at 0°. With sulphuric acid it is somewhat slower but still fast and with hydrogen chloride very slow. The rate of the reaction is apparently dependent on the proton-donating strength of the catalyst. Witkop [5] has outlined a probable mechanism for the rearrangement of 2-phenylskatole ozonide but did not support it with any special experimental evidence. Especially is the point of protonation arbitrarily chosen. No mechanism will be propounded here (several can be written), but it ought to be mentioned that the point of attack may as well be the ether bridge in the trioxolane ring of an ozonide as the peroxy bridge.

The formation of 2,5-diacetoxyterephthalic acid may be a result of a different course of the rearrangement. In this case the primary product is not an ester but a mixed anhydride of diacetoxyterephthalic acid and ethyl carbonate which is hydrolysed during isolation.

In the experimental part is further described the reduction of the ozonides to the corresponding α-ketoesters.

EXPERIMENTAL

(Melting points are not corrected)

Rearrangement of monozonide. Monozonide (0.90 g) was added in small portions to an ice-cooled mixture of perchloric acid (3 drops, 70 %) and acetic anhydride (10 ml). After 30 min the content of active oxygen had disappeared and the mixture was poured into ice-water. A white substance (0.82 g) separated and was recrystallised from petrol ether, m. p. 89—90°. (Found: C 57.4; H 4.7; O-acetyl 11.5. Calc. for $C_{18}H_{18}O_9$ (II): C 57.2; H 4.8; O-acetyl 11.4.) A small amount of the raw product did not dissolve in petrol ether. It was an acid and decomposed at 197—198°.

II (86.7 mg) was boiled with sodium carbonate (10 ml, 2 N) for 60 min in a hydrogen atmosphere. The solution was analysed for oxalic acid. Found: Calcium oxalate dihydrate 30.0 mg. Calc. for $C_{14}H_{13}O_5(OCOCOOC_2H_5)$: 33.5 mg. The calcium oxalate was dissolved in dilute hydrochloric acid, extracted with ether and the isolated oxalic acid identified by its X-ray powder diagram.

II (0.98 g) was refluxed with anhydrous ethanol (50 ml) containing hydrogen chloride (3 %) for 60 min). The mixture was evaporated to dryness and extracted with several portions of petrol ether and the extracts concentrated on a water-bath. The residue smelled like an ester and by means of its I.R.-spectrum was identified as diethyl oxalate. From the petrol ether extractions a white substance remained (0.60 g, 98 %). Recrystallised from dilute ethanol, colourless crystals m. p. 182°. (Found: C 61.2; H 5.1. Calc. for $C_{12}H_{12}O_5$: C 61.0; H 5.1.)

$C_{12}H_{12}O_5$ (0.24 g) and acetic anhydride (10 ml) were refluxed for 4 h and poured into ice-water. The separated substance (0.3 g) was recrystallised from benzene. Yellowish crystals, m. p. 158°. (Found: C 60.1; H 5.1; O-acetyl 27.0. Calc. for $C_{16}H_{16}O_7$: C 60.0; H 5.0; two O-acetyl 26.9.)

Rearrangement of diozonide. Diozonide (2.0 g) was rearranged as described for the monozonide. The product was an oil which solidified after two days in a refrigerator. The product (1.2 g) was extracted with hot benzene and the undissolved part (0.4 g) recrystallised from dilute ethanol. Decomp. $>300°$. (Found: C 50.9; H 3.9. Calc. for $C_{12}H_{10}O_8$: C 51.1; H 3.6.) This substance (0.28 g) was methylated with diazomethane and the product recrystallised from ethanol, m. p. 164—165°. Mixed m. p. with an authentic sample of 2,5-diacetoxyterephthalic acid dimethyl ester was undepressed.

The benzene extract was evaporated and washed with aqueous sodium bicarbonate, the residue being recrystallised from benzene, m. p. 138° (0.6 g). (Found: C 50.7; H 4.3; O-acetyl 20.5. Calc. for $C_{18}H_{18}O_{12}$ (III): C 50.7; H 4.3; two O-acetyl 20.2.)

III (0.23 g), acetic anhydride (10 ml) and sodium acetate (0.5 g) were refluxed for 3 h. Poured into ice-water when a brownish substance separated (0.17 g). Recrystallised from glacial acetic acid (charcoal), m. p. 227—228°. Mixed m. p. with an authentic sample of 1,2,4,5-tetracetoxybenzene was undepressed.

III (268 mg) was hydrolysed with sodium hydroxide and the solution analysed for oxalic acid. Found: calcium oxalate dihydrate 188 mg. Calc for $C_{10}H_8O_4(OCOCOOC_2H_5)_2$ 184 mg.

The rearrangements were performed in propionic anhydride and the results were found to be essentially the same as described above.

Reduction of the ozonides. Monozonide (1.8 g) was dissolved in ethyl acetate (100 ml) and a solution of sodium iodide (3 g) in glacial acetic acid (15 ml) was added and the mixture shaken well. After 10 min the liberated iodine was removed with aqueous sodium sulphite. The ethyl acetate was washed with water, dried and concentrated when a dark oil (1.6 g) remained. This crystallised after some time and was recrystallised from dilute ethanol. White crystals, m. p. 99°. Gives a precipitate with 2,4-dinitrophenylhydrazine. (Found: C 59.5; H 5.0. Calc. for $C_{18}H_{18}O_8$: C 59.7; H 5.0.) The substance is regarded as ethyl 2-methyl-3-carbethoxy-5-acetoxybenzofuranyl-6-glyoxalate (normal reduction product of the monozonide).

Diozonide (1.9 g) in chloroform (100 ml) was chilled to $-23°$ and mixed with sodium iodide (6 g) in glacial acetic acid (30 ml). After 10 min the iodine was removed with aqueous sodium sulphite and the chloroform washed with aqueous sodium bicarbonate, dried and concentrated. A brown oil remained and crystallised in needles (1.4 g). Recrystallised from benzine, m. p. 135°. Positive reaction on carbonyl with 2,4-dinitrophenylhydrazine. (Found: C 54.9; H 4.6; O-acetyl 22.2. Calc. for $C_{18}H_{18}O_{10}$: C 54.8; H 4.6; two O-acetyl 21.8.) The substance is the expected reduction product of the diozonide: Diethyl 1,4-diacetoxybenzene-2,5-diglyoxalate.

One of the authors (E.B.) wishes to express his gratitude to *Fridtjof Nansens Fond* for a grant.

REFERENCES

1. Bernatek, E. and Thoresen, F. *Acta Chem. Scand.* **9** (1955) 743.
2. Bernatek, E. and Bø, I. *Acta Chem. Scand.* **13** (1959) 337.
3. Criegee, R., Kerckow, A. and Zinke, H. *Ber.* **88** (1955) 1878.
4. Criegee, R., de Bruyn, P. and Lohaus, G. *Ann.* **583** (1953) 19.
5. Witkop, B. and Patrick, J. B. *J. Am. Chem. Soc.* **74** (1952) 3861.

Received October 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 345—359

The Crystal and Molecular Structure of Acetyl Choline Bromide

HARALD SÖRUM

Forsvarets Forskningsinstitutt, Kjeller, Norway
and
Fysisk Institutt, Norges Tekniske Högskole, Trondheim, Norway

The crystal and molecular structure of acetyl choline bromide has been determined by the aid of X-ray methods. The crystals are monoclinic with $a = 11.10$ Å, $b = 13.67$ Å, $c = 7.18$ Å and $\beta = 110°$. The space group is $P2_1$ and there are four molecules per unit cell. It is shown that the acetyl choline ion is present in the structure in two distinct structural forms, one of which is the previously assumed extended form, the other being a sort of "ring" structure, where the choline radical, including the oxygen, forms a roughly planar "ring" with a distance from the ester oxygen to the methyl group in the "ring" plane of ~3 Å. Shortened intermolecular distances of ~3 Å link the molecules together to form double strings of molecules running in the direction of the crystallographic a-axis. Atomic coordinates, interatomic distances and bond angles are given. It is suggested that the ability of acetyl choline to exist in two different structural forms may be of primary importance for its behaviour at lipoid-water interfaces, possibly also for its specific action in the transfer of nerve impulses.

Choline, $(HO{\cdot}CH_2{\cdot}CH_2{\cdot}N(CH_3)_3)^+OH^-$, is the parent compound of a series of physiologically active derivatives, widely distributed in plants and animals. The most important one of these derivatives may be acetyl choline, to which is ascribed the formula $(CH_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(CH_3)_3)^+OH^-$, and which is known to play a specific role in the cholinergic nerve system, besides other physiological activities.

The choline derivatives or "cholines" form, along with the acid and dipolar derivatives named "betaines", an interesting type of compounds characterized by the tetra-substituted ammonium group. Accurate determinations of the structures of such compounds are rare in the literature. X-Ray investigations on the crystal structures of the iodides and bromides of palmityl and stearyl cholines have been reported by Stora [1], but accurate atomic positions were not derived in these works. Jellinek [2] has recently reported a structure determination of muscarine iodide, a substance which is related to acetyl choline bromide.

The understanding of the specific actions of acetyl choline is apparently closely connected with a detailed knowledge of the structure of the acetyl choline ion. The present structure determination was therefore undertaken to provide as complete a description of the acetyl choline ion and of the molecular packing as possible, and it is intended to carry out structure determinations for several characteristic compounds of related type.

EXPERIMENTAL

Crystallographic data of acetyl choline bromide have been given in a preliminary account of this work [3]. Repeating here the values of the lattice constants, correcting a misprint in a and choosing the obtuse angle we obtain: $a = 11.10$ Å, $b = 13.67$ Å, $c = 7.18$ Å, $\beta = 110.0°$. There are four molecules per unit cell. The space group is $P2_1$.

Relative intensities of the $hk0$-reflexions were obtained by visual estimation from multiple film Weissenberg photographs, taken with Cu$K\alpha$-radiation. As the specimen used was very small and of fairly spherical form, no correction has been applied for absorption. After corrections for Lorentz and polarization factors the intensities were brought to an approximately absolute scale by the method of Wilson [4]. The scale factor thus found was later on by comparison with calculated intensities found to be correct within 4 %. Intensities of the reflexions on the equatorial layer lines with rotation around the [010]- and the [100]-axis, respectively, and on the first seven layer lines around the [010]-axis were recorded in the same way. These intensities were transferred directly to an absolute scale by comparison with calculated intensities.

The X-ray photographs show subsidiary reflexions [3], indicating some sort of long range modulation of the structure amplitude of the pseudo-cell. It has, however, been assumed that the structure of the pseudo-cell, representing an average structure, could be derived from the intensities of the main reflexions, disregarding those of the subsidiary reflexions. This "anomaly" has greatly complicated the derivation of the structure, and also impeded an ultimate refinement of the atomic coordinates. On the other hand, it may well be that the actual structure found in this work may reveal characteristic and important features of the acetyl choline ion, which might have been lost if a "normal" crystal, showing no subsidiary X-ray reflexions, had been obtained by crystallization under different conditions. In the continued work on this problem it is intended to investigate if this is possible.

The extrapolation graph for determination of the scale factor indicates rather strong thermal vibrations, the derived constant in the temperature factor being about 5 Å^2. This temperature factor has not been used in preparing the data for the Fourier syntheses, but has been introduced in comparing observed and calculated structure factors and amplitudes. Atomic scattering factors are taken from *Internationale Tabellen* 1935 and from Viervoll and Ögrim [5].

DETERMINATION OF THE STRUCTURE PROJECTED ON (001)

A Patterson projection on (001) is shown in Fig. 1. There is only one high and sharp maximum with coordinates u, $b/2$ and this maximum must therefore correspond to interaction between bromine atoms connected by the screw axis. Since there are four molecules in the unit cell, and the space group has twofold general positions, there must be two independent molecules in the asymmetric unit. The only maximum which may be assumed to correspond to interactions between independent bromine atoms is the apparent double maximum around the position $a/2,b/4$ in the Patterson projection. These

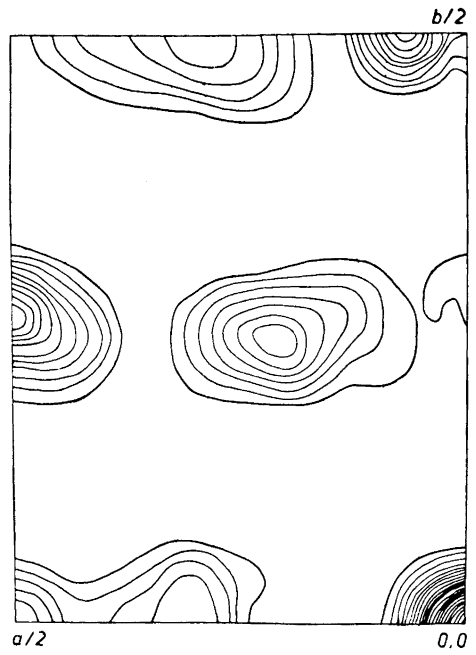


Fig. 1. Asymmetric part of the Patterson projection on (001) of acetyl choline bromide.

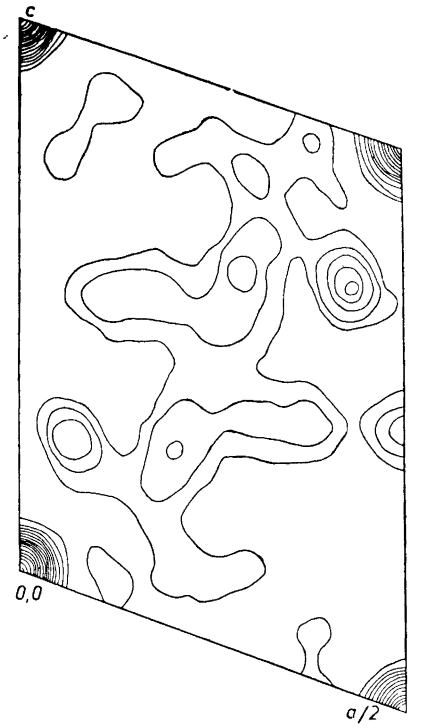


Fig. 2. Patterson projection on (010) of acetyl choline bromide.

maxima indicate the following approximate coordinates for the bromine atoms: $x_1 = \pm 0.036$, $y_1 = 0$ and $x_2 = 0.5 \pm 0.036$, $y_2 = 0.25 \pm 0.015$. The number of possible sign combinations is reduced by the choice of origin, and calculation of some structure amplitudes enabled a choice between the remaining possibilities; thus, the coordinates $x_1 = 0.036$, $y_1 = 0$ and $x_2 = 0.536$, $y_2 = 0.265$ were derived for the independent bromine atoms.

Derivation of coordinates of the light atoms from the Patterson projection would be rather fortuitous, but the projection clearly suggests that the molecules are arranged in layers parallel to (010) and interspaced approximately $b/4$. The square of the bromine scattering factor is about 1.6 times the sum of the squares of the scattering factors of the lighter atoms, and it might thus be expected that the knowledge of the bromine positions would lead to a recognizable structure of the acetyl choline ion in a Fourier synthesis calculated on this basis, although in this case the heavy atom may have a somewhat too strong influence. Only the (010)-projection, however, is centrosymmetric, and in this projection the overlapping of four molecules would be a serious obstacle for a straigt-forward derivation of the structure.

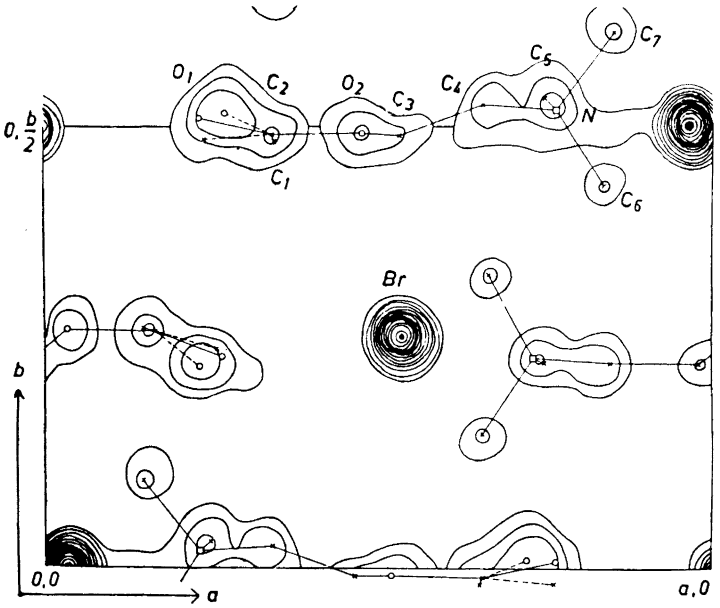


Fig. 3. Projection of electron density on (001) of acetyl choline bromide. Contour lines are drawn at equal but arbitrary intervals. Full circles, open circles, squares and crosses indicate bromine, oxygen, nitrogen and carbon atoms, respectively. Dashed lines indicate an alternative orientation of the acetyl groups.

The proposed layer structure and spatial considerations suggest that the independent acetyl choline ions were to be placed between bromine atoms in the direction of the a-axis, and having their long directions running in the same direction. Tentative coordinates of the lighter atoms, except hydrogen, were derived in this way and were included in the calculation of the first complete set of structure amplitudes and phases of the $hk0$-reflexions, leading to a value of R of 0.32, which is only slightly lower than the value of R (0.35) when the bromine atoms only are included in the calculations. Two ambiguities had to be reckoned with in the derivation of the tentative coordinates, *first*, the direction of the molecules with respect to the positive a-axis direction, and *secondly*, whether independent molecules should run in the same or in opposite direction.

The first Fourier projection gave a fairly clear picture of the structure, but maxima corresponding to the two methyl groups, lying outside the approximate plane of the rest of the molecule, indicated that the directions of both molecules should be reversed. This feature is noteworthy as it indicates that the influence of the bromine atoms is strong enough to lead to a correct structure, even if some of the lighter atoms were included with wrong positions. The coordinates derived from the first Fourier projection led to a marked drop in the value of R, from 0.32 to 0.23, and the second Fourier projection gave a

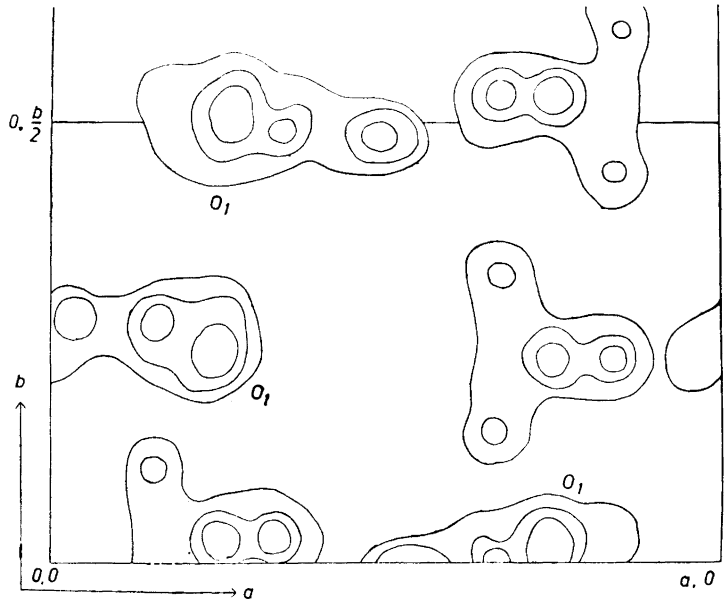


Fig. 4. Projection of electron density on (001) of acetyl choline bromide after subtraction of the bromine peaks. Contour lines at arbitrary intervals. The maxima corresponding to the O_1-positions are broadened considerably in the direction of the *b*-axis.

substantially better picture of the molecular structure. In solving this projection the independent acetyl choline ions were, in the first stage, assumed to have approximately similar structures. This assumption had later on to be abandoned, though the corrections for this projection proved to be rather small (see next section). A map of electron density after subtraction of the bromine peaks has been calculated in order to correct for diffraction effects of the heavy bromine atoms. Four cycles of Fourier synthesis — structure factor calculation were carried out, and the resulting values of R are given in Table 1. Non-observed reflexions were included in evaluating R as far as the calculated structure amplitude exceeds the minimum value observed.

The projection of electron density on (001) is shown in Fig. 3, and the same projection, after subtraction of the bromine peaks, is shown in Fig. 4. Calculated structure amplitudes are compared with the observed ones in Table 4.

DETERMINATION OF THE STRUCTURE PROJECTED ON (010)

The projection of electron density on (001) in Fig. 3 shows that very pronounced overlapping of atoms of the individual molecules must be expected in a projection on (100), and overlapping of four different molecules in a projection on (010). The latter one, has, however, the advantage of being centrosymmetrical, and offers, furthermore, the possibility of improving the resolu-

tion by sectional syntheses along the b-axis, such that the projections of single molecules could be obtained. The projection on (010) was therefore chosen for the next stage in deriving the structure. The strong overlapping of the molecules in this projection impedes taking the full advantage of the heavy atom method. On the other hand the sectional syntheses involve too many tedious calculations to be used as a method of refinement. This projection was therefore, in the first stage, solved from the Patterson projection on (010) together with trial and error methods, using structure factor graphs, refined in the next stage as far as possible by the method of least squares, and finally three different sectional syntheses were computed as a check on the proposed structure. The Patterson projection on (010) is shown in Fig. 2. Since the x-coordinates of the bromine atoms were known from the (010)-projection, the maxima corresponding to Br-Br interactions were easily located, indicating z-coordinates of approximately 0.14 for both of the independent bromine atoms. The maximum at 1/2, 0, 0 is only slightly lower than the origin peak as (010) is an *approximate* glide plane with translation $a/2$. The rest of the Patterson projection shows strongly overlapping maxima, and could not give any detailed informations as to the position of the acetyl choline ion. In the first attempts to derive the structure in this projection an extended model of the molecule was assumed, in accordance with the generally accepted point of view, the direction of the molecules being approximately parallel to the [101]- direction (see next section). This assumption could in effect lead to a reasonable packing of the molecules. Calculation of structure factors revealed, however, very serious contradictions for some low order reflexions. By turning the part of the molecule containing the acetyl group about 180° around the C_4—C_3-bond, such that $C_5 \cdot N \cdot C_4 \cdot C_3 \cdot O_2$ form an approximately planar ring with a distance of about 3 Å from C_5 to O_2, reasonably good agreement for all $h0l$-reflexions could be obtained. A difficulty in deriving the structure lies in the small but significant difference between the two independent molecules, which are to a rough approximation related by a glide-plane of symmetry. The intensities of, *e.g.* the $h0l$-reflexions with h odd (the values of h deviate slightly from odd integral values [3]), show that such differences exist, but do not give very much clue to the real nature of these differences.

THE SECTIONAL SYNTHESES

The projection of the structure on (001) clearly shows that a projected section between the limits $-b/8$ and $b/8$ could be expected to yield the structure of one of the molecules without serious overlapping. Similarly, a section between the limits $b/8$ and $3b/8$ would give the structure of the other molecule. Because of the screw axis, however, a calculation of, *e.g.*, the first of these sections, would also include the section between the limits $3b/8$ to $5b/8$, whereas the latter would also comprise the section between $5b/8$ to $7b/8$. This makes, however, no difference as the first molecule in either case is placed at such a distance from the screw axis that it does not overlap with the equivalent molecule in the projected section, which comes out as centrosymmetrical. In order to reduce the overlapping of the methyl groups

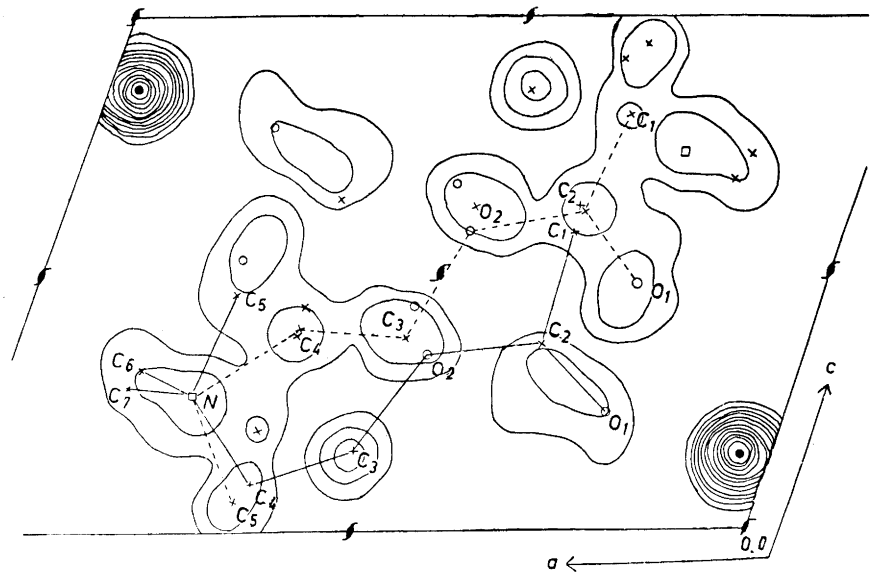


Fig. 5. Projected section of electron density on (010) between the limits $-b/16 - +b/16$ with the equivalent section between the limits $7b/16 - 9b/16$. The calculation of this section is based exclusively on the bromine positions. Filled circles, open circles, squares and crosses indicate bromine, oxygen, nitrogen and carbon atoms respectively. An acetyl choline ion, lying roughly in the plane at $y = 0$, is indicated by dashed lines for the extended form, and with full lines for the "ring" form. The maxima of both forms will appear in this projection (see text). The maxima corresponding to C_3-positions are too high, probably because of bromine atoms lying just above in the adjacent section. Atomic positions for the equivalent section are shown, but are not connected by lines.

a section was also calculated for the limits $-b/16$ to $b/16$ with its equivalent section form $7b/16$ to $9b/16$. The reflexions on the equatorial layer line and on the seven lowest layer lines around the [010]-axis were used for these sectional syntheses, comprising about 1 200 reflexions.

These sections, which appeared as very similar except for a translation of $a/2$ of the second section, showed maxima as expected for the assumed structure, but in addition there appeared some maxima which could not be very well accounted for. Some improvement could be obtained by assuming two possible orientations of the acetyl groups. More serious difficulties were encountered in calculation of interatomic distances between atoms of adjacent molecules, showing that a methyl group of one molecule would approach the acetyl group of another molecule to a distance of about 2.5 Å, which seems to be unreasonably short. To check the proposed structure once more, the section between the limits $-b/16$ to $b/16$ with the equivalent section from $7b/16$ to $9b/16$ was recalculated with amplitudes based exclusively on the bromine positions. This new section is closely similar to the section previously calculated, but it might be noticed that the heights of the additional maxima were slightly increased when using Br-positions only. This similarity is not surprising,

considering the strong influence of the bromine atoms, for example, the bromine atoms determine the signs of all the *h*0*l*-reflexions (with *h* even) except for one extremely weak term.

It may be seen from this section, which is reproduced in Fig. 5, that the presence of two different structures of the acetyl choline ion, from consideration of peak heights both being of the same probability, would be necessary to account satisfactorily for the maxima in this section, *i.e.* the "ring" form, as previously assumed, and an extended form having its long direction approximately parallel to the crystallographic [101]-direction. The arguments for assuming such a mixed structure may be summarized as follows:

1. *h*0*l*-reflexions with *h* close to an odd integral value are present in the X-ray photographs, but these reflexions are rather weak. This shows that the actual structure deviates from an "ideal" structure with a glide-plane parallel to (010) and translation $a/2$, the observed intensities of these reflexions being a measure of this deviation. $F(h0l)$'s with *h* odd, calculated on the assumption of "ring" form only, or extended form only, show in both cases calculated values which, on the average, are only about one tenth of the observed average value. If a structure with the independent molecules being of the "ring" form and the extended form, respectively, is assumed, calculated $F(h0l)$'s with *h* odd are of the same magnitude (or somewhat higher) as the observed ones. Some agreement between observed and calculated values is also obtained, the *R*-factor being 0.42 for these weak reflexions, which is not too bad, considering that the bromine atoms contribute practically nothing to these reflexions, and that their intensities may be more or less influenced by the apparent long range modulation of the structure.

2. As mentioned above the maxima of both forms show up in a section along the [010]-axis based exclusively on the bromine positions. Both forms are likely to appear in this section, since weak reflexions such as the *h*0*l*-reflexions with *h* close to an odd integral value, and which would differentiate between the two forms, were not included in the calculation of the section.

3. The agreement between observed and calculated structure factors and amplitudes is considerably improved by assuming the presence of the two forms, both for an ordered structure of the two forms and for a disordered structure of the same forms.

4. All the interatomic distances lie within reasonable limits for a structure consisting of the two forms of the acetyl choline ion, whereas some unreasonably short distances are found in the case of "ring" form only or extended form only.

The coordinates of the atoms for an ordered structure of the two forms of the acetyl choline ion are given in Table 2, and interatomic distances within each ion may be found in Fig. 6 together with the bond angles. Observed and calculated structure factors of the *h*0*l*-reflexions are compared in Table 3. The reliability index *R* is surveyed in Table 1.

Table 1. Survey of the values of the reliability index R for different sets of reflexions and for different assumed structures of acetyl choline bromide. The structure with the acetyl choline ion of the "ring" form is designated by I, and the structure with the extended form of the acetyl choline ion is designated by II. The value of R, when the Br-atoms only are included in the calculations, is included for comparison.

	I "ring" form	II extended form	I + II disordered	I + II ordered	Br only
*h*0*l*	0.187	0.212	0.146	0.135	0.353
*hk*0	0.169	0.173	0.151	0.163	0.344
hkl	0.223	0.204	0.182		0.303

DESCRIPTION AND DISCUSSION OF THE STRUCTURE

i) *The acetyl choline ion.* A drawing of the acetyl choline ion, as resulting from the present determination, is shown in Figs. 6 *a* and 6 *b* for the "ring" form and the extended form, respectively. The atoms of the ion lie in both cases approximately in a plane, except for those of the methyl groups C_6 and C_7. In contradiction to the previously accepted point of view of an extended form only, the acetyl choline ion is consequently able to exist also in a sort of "ring" structure with the atoms C_5, N, C_4, C_3 and O_2 lying approximately in a roughly planar ring, the distance between C_5 and O_3 being close to 3 Å. It is then likely that both of these forms exist in solutions in polar solvents. While preparing this article a paper appeared on the structure of the related molecule of muscarine iodide [2], for which a closely similar "ring" structure was found. The deviation from a tetrahedral arrangement around the nitrogen

Table 2. Atomic coordinates of the two independent molecules of acetyl choline bromide, assuming that the first molecule corresponds to the extended form and the second molecule corresponds to the "ring" form, *i. e.* for an ordered structure.

First molecule				Second molecule			
Atom	*x/a*	*y/b*	*z/c*	Atom	*x/a*	*y/b*	*z/c*
Br_1	0.0367	0	0.142	Br_2	0.535	0.264	0.142
N	0.250	0.014	0.731	N	0.733	0.239	0.742
O_1	0.750	0.008	0.533	O_1	0.269	0.283	0.792
O_2	0.517	−0.008	0.417	O_2	0.025	0.283	0.667
C_1	0.672	−0.017	0.194	C_1	0.150	0.261	0.431
C_2	0.653	−0.017	0.394	C_2	0.164	0.294	0.644
C_3	0.478	−0.014	0.611	C_3	0.978	0.244	0.842
C_4	0.342	0.025	0.611	C_4	0.856	0.228	0.906
C_5	0.339	0.017	0.939	C_5	0.744	0.222	0.536
C_6	0.167	−0.081	0.700	C_6	0.658	0.153	0.739
C_7	0.150	0.089	0.733	C_7	0.667	0.333	0.722

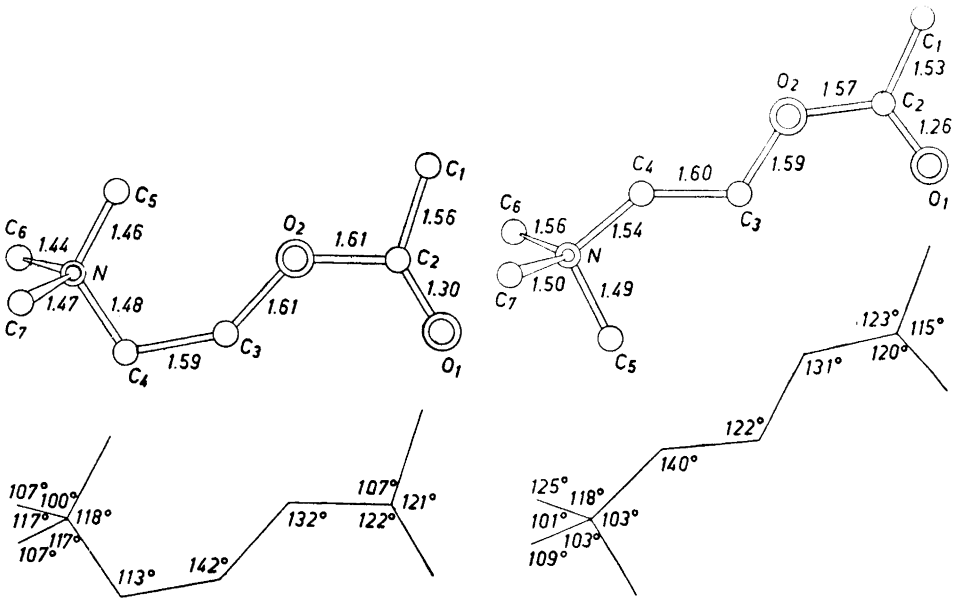


Fig. 6. a. Schematic drawings of the "ring" form of the acetyl choline ion showing interatomic distances and bond angles. Interatomic distances are given in Å.

b. Schematic drawings of the extended form of the acetyl choline ion with interatomic distances in Å and bond angles.

atom may be slightly more pronounced in the structure of the acetyl choline bromide than in muscarine iodide, and this may possibly be a consequence of a tighter packing due to the smaller size of the bromine atom. The distance in muscarine iodide corresponding to the C_5—O_2 distance in acetyl choline is reported by Jellinek to be 2.87 Å, a value close to that quoted above. The interatomic distances and bond angles within the "onium" group and the first bond in the chain show normal values within the limits of accuracy, though the values of the bond angles are spred from about 100° to about 120°. The shortest distances between non-bonded atoms belonging to the same molecule lie in the range 2.3—2.5 Å.

The bond lengths from the O_2-atoms are considerably longer than usually found for C—O single bonds, thus C_3—O_2 is 1.59 Å and 1.61 Å, C_2—O_2 1.57 Å and 1.61 Å for the extended form and the "ring" form, respectively. The anomaly in these bond lengths may to some extent be due to inaccuracies in the derivation of the atomic coordinates, but it seems rather unlikely that the anomaly should be solely due to inaccuracies. The observed bond lengths from the ester oxygens would therefore suggest that these bonds were in some way affected by the neighbouring charged groups and ions. The bond angles also show some anomalous values, thus in the extended form the angle C_3C_4N is 140° and the angle $C_3O_2C_2$ is 131°, in the "ring" form the angle $C_4C_3O_2$ is

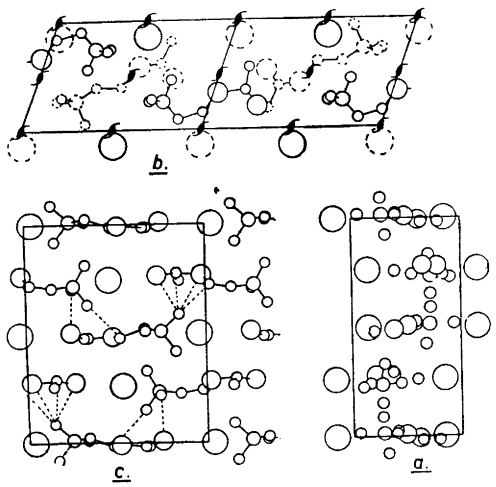


Fig. 7. The packing of the molecules of acetyl choline bromide as viewed along the *a*-axis direction (*a*), the *b*-axis direction (*b*) and along the *c*-axis direction (*c*). Bromine, oxygen, nitrogen and carbon atoms are represented by circles, the radius of which decreases from bromine to carbon, and in *b* dashed circles are used to indicate atoms of a molecule lying close $y = 0$ or $y = 1/2$ (in the unit cell on the right side) whereas circles in full lines indicate the atoms of molecules close to the plane through $y = 1/4$ or $y = 3/4$ (in the top left corner). Bonded atoms are connected by full lines. Some relatively short interatomic distances between neighbouring molecules, the lengths of which are about 3 Å, are indicated by dashed lines in *c*. These shortened interatomic distances link the molecules together to form endless double strings running in the direction of the *a*-axis.

142° and angle $C_3O_2C_2$ is 132°. It is likely that the high values of these bond angles originate from the same reasons as the large values of the bond lengths. It may be noted that bond angles of approximately the same magnitude have been found in other molecules showing Zwitter-ion character, *e.g.*, in cysteylglycine [6]. The structures of the acetyl groups are normal within the limits of accuracy.

ii) *The packing of the molecules.* The packing of the molecules in the crystal lattice is indicated in Figs. 7 *a*, *b* and *c* as viewed in the direction of the *a*-, *b*- and the *c*-axis, respectively. The Br_1-ion has 9 nearest neighbours within a distance below 4 Å, these are two O_2'-atoms at distances 3.36 and 3.43, two C_3'-atoms at 3.51 and 3.92, one C_4'-atom at 3.96, one C_7'-atom at 3.85, one C_4-atom at 3.91, one C_6-atom at 3.94 and one C_7-atom at 3.76, a prime denoting that the atom belongs to an adjacent molecule. Two N-atoms are at distances 4.11 and 4.37 and two N'-atoms at distances 4.30 and 4.88. There are five more nearest neighbours in the range from 4 to 4.5 Å, thus C_1, C_5, C_6, C_7 and O_1'.

The Br_2-ion has eight nearest neighbours within a distance of 4 Å, thus C_3' at 3.54, C_4' at 4.00, C_5' at 3.83, C_6' at 3.76, O_1 at 3.22, C_7 at 3.88, O_6 at 3.89 and C_5 at a distance of 3.05. Two N-atoms are found at a distance of 4.17 and 4.14, and two more N'-atoms at distances of 4.08 and 4,89. The

Table 3. Observed and calculated structure factors $F(h0l)$ for acetyl choline bromide, the "ring" form being designated by I and the extended form by II. I + II is the ordered structure of the two forms.

hkl	$F(hkl)$ obs.	$F(hkl)$ calculated			*hkl*	$F(hkl)$ obs.	$F(hkl)$ calculated		
		I	II	I + II			I	II	I + II
001	22	45	3	25	1004	22	3	−7	16
002	81	−88	−36	−61	1204	48	57	47	46
003	74	−89	−86	−89	$20\bar{4}$	82	−73	−77	−75
004	84	−84	−87	−84	$40\bar{4}$	24	−16	−18	−16
005	13	−31	−7	−19	$60\bar{4}$	—	6	7	−2
006	23	36	9	27	$80\bar{4}$	64	63	63	68
007	85	79	77	80	205	62	−59	−44	−46
008	52	47	51	46	405	75	−77	−72	−77
201	124	113	133	134	605	96	−99	−51	−78
401	108	106	75	96	805	63	−67	−83	−66
601	96	85	99	91	1005	54	−37	−28	−45
801	48	30	40	36	1205	—	−12	1	−5
1001	30	36	28	32	$20\bar{5}$	36	13	47	34
1201	19	−34	−20	−24	$40\bar{5}$	46	54	40	41
$20\bar{1}$	44	64	12	57	$60\bar{5}$	90	59	83	78
$40\bar{1}$	55	−43	−61	−65	$80\bar{5}$	68	58	68	57
$60\bar{1}$	51	−64	−74	−52	206	71	48	47	47
$80\bar{1}$	96	−63	−108	−94	406	62	−18	−57	−32
$100\bar{1}$	55	−73	−66	−62	606	34	−40	−71	−54
$120\bar{1}$	74	−74	−73	−72	806	51	−84	−46	−68
202	85	88	47	83	1006	63	−50	−75	−59
402	54	45	81	74	1206	57	−73	−75	−70
602	87	104	73	81	$20\bar{6}$	51	91	28	60
802	99	104	83	103	$40\bar{6}$	98	97	89	94
1002	72	89	60	68	$60\bar{6}$	68	57	46	51
1202	36	36	27	35	207	44	63	68	57
$20\bar{2}$	43	−82	−43	−79	407	37	34	47	46
$40\bar{2}$	75	−107	−59	−71	607	—	19	3	7
$60\bar{2}$	110	−108	−88	−108	807	25	−30	−21	−19
$80\bar{2}$	73	−93	−49	−68	1007	60	−53	−55	−59
$100\bar{2}$	44	−51	−20	−40	1207	43	−56	−62	−55
203	—	−16	−12	−14	$20\bar{7}$	65	65	71	62
403	69	−57	−41	−59	$40\bar{7}$	53	39	58	53
603	67	41	51	50	208	60	53	57	61
803	76	72	98	78	408	84	80	82	78
1003	60	68	68	71	608	58	65	63	66
1203	65	61	71	67	808	39	50	51	44
$20\bar{3}$	121	−118	−131	−123	2010	30	−25	−30	−24
$40\bar{3}$	88	−80	−86	−84	4010	44	−43	−16	−29
$60\bar{3}$	31	−18	−34	−30	200	24	58	64	62
$80\bar{3}$	55	−50	−56	−54	400	98	102	95	99
204	153	−165	−156	−159	600	48	46	71	52
404	48	−60	−72	−71	800	9	8	1	7
604	96	−104	−95	−93	1000	93	−92	−91	−90
804	25	−25	−24	−38	1200	34	−35	−14	−26

Table 4. Observed and calculated structure amplitudes and phases for the *hk*0-reflexions of acetyl choline bromide. 1/2(I + II) is the average of structure amplitudes calculated for the "ring" form (I) and the extended form (II). Both structure amplitudes and phases are given for the ordered structure of the two forms of acetyl choline bromide.

hkl	*F*(*hkl*) obs.	*F*(*hkl*) calc.		*a*(*hkl*)	*hkl*	*F*(*hkl*) obs.	*F*(*hkl*) calc.		*a*(*hkl*)
		½ (I + II)	ord. str.				½ (I + II)	ord. str.	
020	15	5	19	−74.0	110	24	31	27	39.2
040	209	235	234	10.9	130	17	26	36	127.2
060	31	27	48	−60.5	150	24	13	22	256.5
080	143	150	139	19.1	170	11	14	29	138.3
0100	—	24	45	−39.8	190	14	8	11	−56.6
0120	59	97	89	21.1	1110	29	31	40	144.8
0140	35	43	49	−32.0	1130	18	10	10	150.8
0160	32	39	37	41.5	1150	9	8	12	175.1
120	66	61	63	7.2	210	62	55	62	141.8
140	19	17	9	212.4	230	63	62	60	49.1
160	57	57	57	23.6	250	15	9	7	256.1
180	26	25	20	267.2	270	66	58	50	58.6
1100	71	67	72	27.8	290	8	8	5	145.1
1120	19	24	26	−63.2	2110	39	56	50	60.4
1140	60	58	63	42.2	2130	—	4	7	70.8
1160	33	35	40	−51.4	2150	47	48	39	71.2
220	34	26	20	−84.2	310	55	57	57	57.2
240	59	81	84	8.7	330	51	47	41	138.4
260	67	47	41	−88.0	350	49	56	59	67.6
280	61	69	71	17.8	370	26	30	21	161.8
2100	72	62	54	−79.5	390	51	53	59	73.0
2120	61	55	55	34.7	3110	31	22	15	167.7
2140	52	52	53	−72.7	3130	40	45	49	81.8
2160	51	48	49	29.0	3150	19	10	6	180.0
320	94	92	89	6.8	3170	37	42	40	87.1
340	16	10	14	−36.0	410	50	48	50	137.9
360	91	94	94	12.5	430	61	60	65	59.7
380	14	21	16	−44.6	450	62	59	59	151.0
3100	51	49	51	34.6	470	58	63	69	71.4
3120	41	39	27	−66.4	490	26	29	27	170.6
3140	27	35	41	43.6	4110	55	53	56	91.2
3160	23	24	21	−51.0	4130	17	17	15	201.4
420	10	6	12	250.0	4150	29	42	49	99.0
440	105	108	107	5.5	510	45	65	60	39.4
460	19	6	20	−68.4	530	45	57	56	140.5
480	73	78	73	17.9	550	74	86	83	43.4
4100	16	16	28	−45.4	570	49	45	49	138.4
4120	37	48	42	30.8	590	70	75	70	52.5
4140	16	19	30	−35.2	5110	—	4	7	159.2
520	19	25	26	3.8	5130	73	69	66	59.3
540	28	4	16	196.1	5150	—	2	9	139.2
560	17	21	28	20.8	610	67	66	66	138.0
580	19	5	11	165.8	630	84	80	80	51.9
5100	22	25	28	33.4	650	59	52	57	142.3
5120	16	4	8	−83.5	670	70	73	71	64 3
5140	26	27	26	46.9	690	29	32	35	150 4
5160	17	11	17	45.0	6110	63	67	63	77.0
620	9	9	10	−48.6	6130	—	15	13	154.0

Table 4 (continued).

hkl	*F(hkl)* obs.	*F(hkl)* calc.		*a(hkl)*	*hkl*	*F(hkl)* obs.	*F(hkl)* calc.		*a(hkl)*
		½ (I + II)	ord. str.				½ (I + II)	ord. str.	
640	14	21	22	18.6	6150	55	61	57	85.8
660	35	28	24	−74.5	710	58	65	67	52.1
680	10	17	17	16.3	730	52	51	50	135.0
6100	24	21	19	81.1	750	57	57	62	65.6
6120	—	18	22	0	770	46	38	40	154.9
6140	33	27	20	−81.2	790	52	57	67	70.7
720	—	42	39	184.0	7110	42	37	40	165.5
740	—	10	10	94.8	7130	57	58	69	77.9
760	28	40	35	184.6	7150	18	16	17	198.6
780	8	7	8	167.5	810	59	65	64	143.0
7100	14	11	5	180.0	830	73	78	77	53.9
7120	—	6	7	143.9	850	—	20	17	180.0
7140	21	7	10	90.0	870	87	87	83	67.2
820	—	9	8	57.9	890	—	20	10	204.3
840	12	7	8	−20.5	8110	83	87	83	72.8
860	—	15	14	51.6	910	55	55	52	44.3
920	24	21	21	5.0	930	48	50	54	139.4
940	—	8	9	151.3	950	50	46	45	46.5
960	15	9	12	190.3	970	23	35	40	143.8
980	—	12	9	101.7	990	47	47	47	46.8
9100	34	28	34	191.1	1010	49	35	39	137.2
1020	—	4	4	189.7	1030	46	42	41	56.1
1040	67	68	67	−12.6	1050	48	38	45	148.6
1060	13	8	11	138.4	1070	50	48	49	61.8
1080	63	58	56	202.0	1090	25	21	25	161.6
10100	9	8	14	166.4	10110	46	45	44	80.9
1120	55	63	62	192.2	1110	26	26	26	43.6
1140	7	7	7	114.1	1130	13	20	19	152.8
1160	58	59	57	194.7	1150	47	47	49	50.2
1180	—	17	26	112.6	1170	—	13	14	151.4
11100	55	50	46	202.8	1190	55	49	50	54.1
1220	—	9	10	19.5	1210	—	13	13	140.2
1240	52	44	47	189.6	1230	33	27	28	60.2
1260	22	17	20	62.3	1250	—	20	13	157.3
1280	52	42	45	197.7	1270	29	24	27	90.0
1320	67	66	66	185.2	1310	26	14	15	21.4
					1330	17	9	10	133.2

Br_2-ion has seven neighbours within distances lying in the range from 4.0 to 4.5 Å, thus C_4, C_6, C_7, O_2', C_5', $2C_1'$. There are thus four "onium"-groups around each Br-atom, and the arrangement of these groups are approximately tetrahedral with the Br-atoms in the space center of the tetrahedra.

The methyl group C_7 of one molecule and the methyl group C_6 of the other molecule approach the acetyl group, C_3 and O_2 of an adjacent molecule to distances, which are somewhat below the normal van der Waals distances. The shortest of these distances lie between 2.9 and 3.0 Å, and it may be seen from Fig. 7 *c*, where they are indicated by dashed lines, that these short intermolecular distances connect the molecules to form double strings of

molecules in the direction of the a-axis. The methyl group C_5 of the extended form is also at a distance of 3 Å from the O_1-atom of the "ring" form.

All the distances between atoms or groups of molecules belonging to different double strings are about or above the normal van der Waals distances, thus, for example, the distance $O_1—O_1'$ is 3.8 Å.

The origin of the subsidiary reflexions, with non-integral values of the indices, has not as yet been investigated in detail. The existence of the acetyl choline ion in two different forms in the structure may perhaps offer an explanation, which might serve as a possible working hypothesis. In the calculation of the structure factors for an ordered structure of the two forms, it was assumed that the extended form is located about the position x from 0 to 0.7, $y \simeq 0$, whereas the "ring" form is located about the position x from 0.5 to 1.2 and $y \simeq 0.25$. The y-coordinates of the C_3-groups give a slight preference for this arrangement as it leads to slightly more reasonable interatomic distances, else the positions of the different forms could just as well be interchanged. It is then reasonable to suggest that stacking faults in the sequence extended form — "ring" form produce a long range modulation of the structure, which gives rise to the observed subsidiary reflexions. Such a partially disordered structure would be in accordance with the observation that the averages of the structure factors for the two forms agree just as well with the observed values as those calculated for the ordered structure, and appreciably better than for either of the forms separately. Another observation pointing in the same direction is the marked broadening of the maxima of the O_1-atoms in the direction of the b-axis. This broadening is especially pronounced in the difference map, reproduced in Fig. 4. Such a broadening might be expected for a disordered structure as the y-coordinates for the O_1-atom would differ considerably for the two forms of the acetyl choline ion.

It is not unlikely that the ability of the acetyl choline ion to exist in two distinct structural forms may account for some of its specific activities. The extended form would be likely to prevail in solution in polar media, whereas the "ring" form might be the prevalent form in unpolar media. Such an ability of the acetyl choline ion would evidently have a strong bearing on its characteristic behaviour at lipoid-water interfaces, and possibly also on its specific action in the transfer of nerve impulses.

The present work was begun in the Department of Chemistry at *Forsvarets Forskningsinstitutt*, Kjeller, where the experimental part and the first stages of the structure determination were carried out. The work has been continued at *Fysisk Institutt, Norges Tekniske Högskole*, with financial support from *Forsvarets Forskningsinstitutt*, which is gratefully acknowledged.

REFERENCES

1. Stora, C. *Bull. Soc. Chim.* **199** (1949) 867 and **200** (1949) 874.
2. Jellinek, F. *Acta Cryst.* **10** (1957) 277.
3. Sörum, H. *Acta Chem. Scand.* **10** (1956) 1669.
4. Wilson, A. J. C. *Nature* **150** (1942) 152.
5. Viervoll, H. and Ögrim, K. *Acta Cryst.* **2** (1949) 277.
6. Dyer, H. B. *Acta Cryst.* **4** (1951) 42.

Received November 3, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 360—364

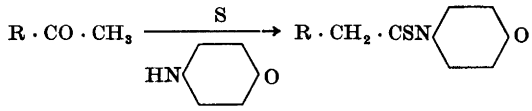
Preparation of 3-Reteneacetic Acid by the Willgerodt-Kindler Reaction

EYLIF DYNESEN

Chemical Institute, Aarhus University, Denmark

3-Reteneacetic acid has been prepared through the Willgerodt-Kindler reaction. By hydrolysis of the crude thiomorpholide two by-products, retene-3-carboxylic acid and retene-3-glyoxylic acid were isolated. The precursor of these byproducts, 3-reteneglyoxylmorpholide has been isolated by chromatography of the crude thiomorpholide. 3-Reteneacetomorpholide can be obtained by partial hydrolysis of the thiomorpholide.

In a synthetic program 3-reteneacetic acid was required as starting material and as 3-acetylretene can be prepared in good yield [1,2], the Willgerodt reaction seemed promising. The Kindler procedure [3,4] in the Schwenk-Bloch modification [5] using morpholine as the amine was selected. The main reaction product then is the thiomorpholide:



The mole ratios applied were 1:2.5:2 for ketone, sulphur and morpholine, respectively, in accordance with King and McMillan [6].

In a preliminary experiment the crude reaction product was hydrolyzed directly in a mixture of acetic acid and sulfuric acid. The purification of the reteneacetic acid met, however, with great difficulties. Next, a recrystallization of the thiomorpholide from alcohol was tried. When cooling the hot solution an oil separated at first, but by careful work a 75—80 % yield of light yellow crystals could be obtained. The melting point of this material was, however, far from sharp, even after several recrystallizations.

Next, hydrolysis of the thiomorpholide by means of alcoholic potassium hydroxide was tried. After boiling for a short time much crystalline material separated in the reaction mixture. The substance was colourless and contained no sulphur. It could be hydrolyzed to 3-reteneacetic acid and was thus shown to be the morpholide of this acid.

Under suitable conditions the partial alkaline hydrolysis gave a satisfactory yield of almost pure morpholide, and for the purpose of preparation of pure 3-reteneacetic acid it is advantageous to isolate the morpholide and afterwards bring the hydrolysis to completion. It turned out, that it was unnecessary to recrystallize the crude Willgerodt reaction product. Partial alkaline hydrolysis of this material gave an about 70 % yield of almost pure morpholide. The second step in the hydrolysis was most advantageously carried out by means of a solution of potassium hydroxide in *n*-propanol. By this procedure an overall yield of 60 % of 3-reteneacetic acid could be obtained.

By examination of the alkaline mother liquor from partial hydrolysis of the crude thiomorpholide two by-products were isolated and identified, *viz.* 3-retenecarboxylic acid and 3-reteneglyoxylic acid. The latter acid could not be obtained in quite pure condition but could be identified by conversion to the phenylhydrazone and the methyl ester, which was characterized through the dinitrophenylhydrazone.

In order to isolate the precursors of these by-products in the Willgerodt reaction and to isolate the thiomorpholide in pure condition, the crystalline product from the Willgerodt reaction was chromatographed on alumina. In this way 3-retenethioacetomorpholide could be isolated as a pure white compound composing the bulk of the crystalline product, but two other substances which were stronger adsorbed than the thiomorpholide were isolated as white compounds constituting about 10 % of the impure thiomorpholide. One of these compounds could be identified as 3-reteneacetomorpholide. The other substance was a neutral compound, which contained no sulphur.

The ultraviolet absorption spectrum was quite different from the spectra of 3-retenethioacetomorpholide and 3-reteneacetomorpholide, which were very similar and showed a typical retene structure, while the spectrum of the by-product showed a broad absorption band at 320—340 mμ. Hydrolysis of this product with alcoholic potassium hydroxide gave 3-retenecarboxylic acid and 3-reteneglyoxylic acid. As the spectrum of methyl-3-reteneglyoxylate is almost identical with that of the by-product, it is reasonable to assume, that the by-product is 3-reteneglyoxylmorpholide. The elementary analysis supports this assumption.

EXPERIMENTAL

3-Acetylretene was prepared by the method of Dodd, Schramm and Elderfield [2] with the modification, that acetic anhydride was used instead of acetyl chloride in the Friedel-Crafts reaction. The yield of pure 3-acetylretene was 61 %.

Willgerodt-Kindler reaction. A mixture of 3-acetylretene (24.0 g, 0.087 mole), sulphur (7.05 g, 0.22 g-atom) and morpholine (15.2 ml, 0.174 mole) was heated under gentle reflux for 5 h, during which time a considerable amount of hydrogen sulphide was evolved. The dark coloured viscous product was poured in hot alcohol (200 ml) and left standing overnight. Crystals and oily material had now separated in the solution. The solution and the main part of the crystalline material was decanted from the oil, which was redissolved in alcohol. On cooling, some substance at first separated as an oil but later crystalline material separated. By warming the solution gently, more of the oil crystallized. The solution with the crystalline material was again decanted from residual oil and by repeating this process twice, 23 g of crystalline light yellow material was isolated, melting point 110—114°, yield 75 % calculated as pure 3-retenethioacetomorpholide.

3-Reteneacetomorpholide. The crystalline thiomorpholide (23 g) was dissolved in hot alcohol (100 ml), and a solution of potassium hydroxide (17.1 g) in water (17 ml) was added. The solution turned immediately black and subsequently red. The solution was refluxed. After 4 min boiling much precipitate suddenly separated in the reaction mixture. The precipitate was filtered by suction and washed with alcohol. Yield of 3-reteneacetomorpholide 17.1 g (78 %, calc. from the thiomorpholide), m. p. 184.5—185.5°. Recrystallization from *n*-propanol gave white crystals, m. p. 187—188.5°. (Found: C 79.5; H 7.55; N 3.90. Calc. for $C_{24}H_{27}NO_2$ (361.5): C 79.7; H 7.53; N 3.88.) U.V. absorption (in ethanol): λ_{max} 260.5 (log ε 4.81); 282 (4.13); 291 (4.01); 303.5 (4.13); 329 (2.41); 335.5 (2.51); 352 mμ (2.19). λ_{min} 234; 278; 287.5; 297; 325; 331.5; 341—345 (shoulder); 349.5 mμ.

Alkaline hydrolysis of 3-reteneacetomorpholide. The morpholide (1.8 g, 0.005 mole) was dissolved in *n*-propanol (10 ml). To the warm solution was added a solution of potassium hydroxide (1.4 g, 0.025 mole) in water (1—2 ml). The mixture was refluxed for 5.5 h. After dilution with water (200 ml) the clear solution was acidified with hydrochloric acid. A white precipitate separated (1.4 g, m. p. 209—213°). The product was recrystallized from *n*-propanol (15 ml) giving 3-reteneacetic acid 1.15 g (78.8 %), m. p. 210—212.5° (subl.). (Found: Equiv.wt.: 293.3; C 82.1; H 6.84. Calc. for $C_{20}H_{20}O_2$ (292.4): C 82.2; H 6.90.)

Synthesis of 3-reteneacetic acid. A mixture of 3-acetylretene (41.4 g, 0.15 mole), sulphur (12.03 g, 0.375 g-atom) and morpholine (26.1 ml, 0.3 mole) was heated under gentle reflux for 5 h. The dark reaction mixture was dissolved in alcohol (150 ml). To the warm mixture was added a solution of potassium hydroxide (20 g) in water (10 ml) and alcohol (20 ml). The solution was refluxed for 8 min and then cooled. The precipitate was filtered by suction and washed with alcohol. The alkaline filtrate was further boiled for 15 min and left overnight. A second crop of morpholide was obtained. The crude morpholide was treated twice with boiling water. Yield of crude morpholide 38.1 g (70.2 % calc. from 3-acetylretene), m. p. 177—184°.

The morpholide (38.1 g) was dissolved in hot *n*-propanol (200 ml), and potassium hydroxide (17.7 g) in water (12 ml) was added. The mixture was boiled under reflux for 4 h. The slightly soluble potassium salt separated in the mixture. Water (300 ml) was added, and propanol was removed by distillation. The aqueous solution was boiled with charcoal, filtered and acidified. 3-Reteneacetic acid separated as colourless crystals (30.3 g). The crude product was recrystallized from a mixture of alcohol and acetone. Yield 26.8 g (61.2 % calc. from 3-acetylretene), m. p. 209—212.5° (subl.).

3-Retenecarboxylic acid. To the dark brown alkaline mother liquor from the partial hydrolysis of the crude Willgerodt reaction product was added potassium hydroxide (10 g), and the solution was refluxed for 2 h. The main part of the alcohol was removed by distillation *in vacuo*. To the residue was added water, and the solution was acidified with hydrochloric acid. A brown precipitate separated (7.5 g) and was dissolved in hot benzene. On cooling crystalline material separated (1 g). Two further recrystallizations from benzene gave 3-retenecarboxylic acid (0.3 g), m. p. 242—244° (subl.); gives no depression when mixed with an authentic specimen. (Found: C 82.1; H 6.45. Calc. for $C_{19}H_{18}O_2$: C 82.0; H 6.52.)

3-Reteneglyoxylic acid. The aqueous filtrate, resulting from digestion of the crude morpholide with hot water, was yellowish and gave on cooling a precipitate of a potassium salt. Acidification gave a yellow precipitate (2 g), which was recrystallized from a mixture of benzene and hexane and gave yellow crystals (1 g), m. p. 144—148.5°. One further recrystallization from benzene-hexane gave 3-reteneglyoxylic acid (0.8 g), m. p. 147—150°. (Found: C 78.8; H 6.15. Calc. for $C_{20}H_{18}O_3$ (306.3): C 78.4; H 5.92.)

Phenylhydrazone of 3-reteneglyoxylic acid. The keto acid (0.4 g) was dissolved in alcohol (7 ml). To the solution was added phenylhydrazine hydrochloride (0.3 g) and potassium acetate (0.2 g). The mixture was refluxed for one hour and cooled. The precipitate was washed several times with hydrochloric acid, recrystallized from glacial acetic acid. The phenylhydrazone (0.1 g) formed yellow crystals m. p. 166—168.5°. (Found: Equiv.wt. 403; N 6.9. Calc. for $C_{26}H_{24}N_2O_2$ (396.5): N 7.1.)

Methyl ester of 3-reteneglyoxylic acid. 3-Reteneglyoxylic acid (0.6 g) in methanol (25 ml) and concentrated sulfuric acid (0.1 ml) was refluxed for 8 h. The excess of methanol was distilled off and the residue dissolved in ether-benzene. The solution was washed with water, potassium bicarbonate and saturated sodium chloride. After drying with anhydrous calcium chloride the solvent was distilled off and the residue dissolved in hot

alcohol. After some time crystalline light yellow material had separated. Recrystallized from alcohol the methyl-3-reteneglyoxylate (0.08 g) had m. p. 101—102°. (Found: C 78.7; H 6.12. Calc. for $C_{21}H_{20}O_3$ (320.4): C 78.7; H 6.29.) U.V. absorption (in cyclohexane): λ_{max} 248.5 (log ε 4.61); 264 (4.58); 289—92 (shoulder) (3.82); 336 mμ (4.18); λ_{min} 223 (4.13); 256 (4.56); 299 mμ (3.74).

Dinitrophenylhydrazone of methyl-3-reteneglyoxylate. 2,4-Dinitrophenylhydrazine (0.2 g) and alcohol (10 ml) was heated to boiling. To the hot solution was gradually added concentrated sulfuric acid until clear solution. To this solution was added a solution of the keto ester (0.2 g) in alcohol (5 ml). A reddish orange precipitate at once separated. The dinitrophenylhydrazone (0.2 g) was recrystallized from xylene. Yield 0.1 g, m. p. 293—294.5°. (Found: C 64.3; H 4.85; N 10.9. Calc. for $C_{27}H_{24}N_4O_6$ (500.5): C 64.8; H 4.83; N 11.2.)

Chromatography of the crude thiomorpholide. From the Willgerodt-Kindler reaction of 3-acetylretene (16.6 g, 0.06 mole) 18.4 g of crystalline light yellow material was isolated, consisting of several fractions with the main fraction melting at 110—114°. As purification through fractional crystallization was unsuccessful, a fraction (11 g, m. p. 105—116°) was chromatographed on a column (21 × 6.5 cm) of alumina, solvent petroleum ether (600 ml) + carbon tetrachloride (400 ml).

The chromatogram was developed by the same solvent containing increasing amounts of carbon tetrachloride, and subsequently with carbon tetrachloride.

The surface of the column became strongly coloured. Next came a light yellow zone gradually changing to light grey. Next followed a broad pure white zone containing the thiomorpholide and finally a narrow yellow layer.

The column was fractionally eluted by washing with benzene. In the filtrate 3-retenethioacetomorpholide could be isolated by evaporation of the solvent and recrystallization of the residue. Yield: 7.2 g, m. p. 119.5—122°. Two further recrystallizations gave white crystals, m. p. 120—121.5°. (Found: C 76.6; H 7.18; N 3.70; S 8.44. Calc. for $C_{24}H_{27}NSO$ (377.5): C 76.4; H 7.21; N 3.71; S 8.49.) U.V. absorption (in ethanol): λ_{max} 261.5 (log ε 4.83); 282 (4.46); 303.5 (4.19); 329 (2.51); 335.5 (2.60); 352.5 mμ (2.37) λ_{min} 233.5; 278; 299; 325.5; 331.5; 341—345 (shoulder); 350 mμ.

From the column the surface layer was discarded, and the rest of the column was divided into two nearly equal parts, which were eluted separately with boiling alcohol. The eluate from the upper half was stronger yellow than that from the lower half of the column. From these two eluates two white crystalline neutral substances could be isolated. One of these, isolated from the lower half of the column, has been identified as 3-reteneacetomorpholide, yield 0.2 g, m. p. 186.5—188.5°; gives no depression, when mixed with an authentic specimen, prepared by alkaline desulphurization of 3-retenethioacetomorpholide.

The eluate from the upper half of the column was concentrated. From the yellow solution crystalline material separated (1 g), m. p. 147.5—152°. Fractional crystallization of this fraction gave a white compound (0.4 g), m. p. 175—178°. One further recrystallization from alcohol gave 3-reteneglyoxylmorpholide, m. p. 176—178°. (Found: C 76.6; H 6.79; N 3.77. Calc. for $C_{24}H_{25}NO_3$ (375.5): C 76.8; H 6.71; N 3.73.) U.V. absorption (in cyclohexane): λ_{max} 248 (log ε 4.59); 264.5 (4.60); 289—92 (shoulder) (3.93); 334 mμ (4.18). λ_{min} 223 (4.19); 254 (4.57); 300 mμ (3.84).

Alkaline hydrolysis of 3-reteneglyoxylmorpholide. A mixture of 3-reteneglyoxylmorpholide (0.3 g) potassium hydroxide (0.2 g) and alcohol (15 ml) was refluxed for about one hour. The potassium salt of 3-reteneglyoxylic acid partly separated in the boiling liquid and more was obtained on cooling. The salt was filtered off and washed with ether. On acidification of an aqueous solution of the salt, a yellow product was obtained, which after recrystallization from benzene-hexane had m. p. 148—151°, yield 0.1 g. (Found: Equiv.wt. 301.5. Calc. for $C_{20}H_{18}O_3$: 306.3.) The acid, which was not quite pure, was shown to be 3-reteneglyoxylic acid by conversion to the methyl ester.

After filtration of the potassium salt the alkaline filtrate was evaporated to dryness. The residue was dissolved in boiling water and the solution acidified. The brownish precipitate was dissolved in benzene. On cooling a small white precipitate separated. One further recrystallization from benzene gave 3-retenecarboxylic acid, m. p. 239.5—242°; gives no depression in m. p. when mixed with an authentic specimen.

Analyses and U.V. spectra were performed in this laboratory by miss B. Møller.

REFERENCES

1. Campbell, W. P. and Todd, D. *J. Am. Chem. Soc.* **62** (1940) 1287.
2. Dodd, T. N., Schramm, C. H. and Elderfield, R. C. *J. Org. Chem.* **11** (1946) 253.
3. Kindler, K. *Ann.* **431** (1923) 187.
4. Kindler, K. *Arch. Pharm.* **265** (1927) 389.
5. Schwenk, E. and Bloch, E. *J. Am. Chem. Soc.* **64** (1942) 3051.
6. King, J. A. and McMillan, F. H. *J. Am. Chem. Soc.* **68** (1946) 2335.

Received December 2, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 365—376

On the Defect Structure of Rutile

TOR HURLEN

Central Institute for Industrial Research, Blindern, Oslo, Norway

From the point of view of its importance to the understanding of the oxidation behaviour of titanium and titanium alloys, a theoretical reinvestigation is made of the defect structure of rutile with special emphasis on what is to be expected from the ideal rutile structure itself.

A discussion is moreover given of previous experimental results on the subject, and it is concluded that most of the available data are possibly best explained by assuming the defect structure to be a combination of a non-stoichiometric and a stoichiometric one, both involving titanium interstitial ions.

1.0 INTRODUCTION

It is well known that the oxidation of a metal usually leads to the formation of a more or less compact oxide film on the metal surface, and that the further oxidation requires passage through this film either of metal outwards or of oxygen inwards. Very often, the rate of transport of material through the oxide lattice itself (volume diffusion) is found to be determining for the oxidation resistance of the metal (Wagner [1] mechanism of oxidation).

It is further known that the volume diffusion in ionic crystals depends on the defect structure of the crystals, and that the defect structure in many cases may be partly controlled by the incorporation of foreign ions in the lattice (Wagner-Hauffe [2] approach to alloy oxidation).

The application of this basic knowledge to the oxidation resistance of a special metal and to its improvement by alloying, is greatly facilitated by the understanding of the defect structure of the pure oxide(s) in question.

Rutile has been shown [3,4] to be the main oxide formed on titanium under the most actual oxidation conditions. This oxide is of an *n*-conducting [5], oxygen deficient [5–7] type. It does not seem well recognized, however, whether the latter is due to the presence of oxygen vacancies or of titanium interstitials in the rutile lattice.

The purpose of the present work is to elucidate this matter and to gain the best possible understanding of the defect structure of rutile from already known experimental data. Our hope is that this might facilitate the interpretation and understanding of the oxidation behaviour of titanium and titanium alloys.

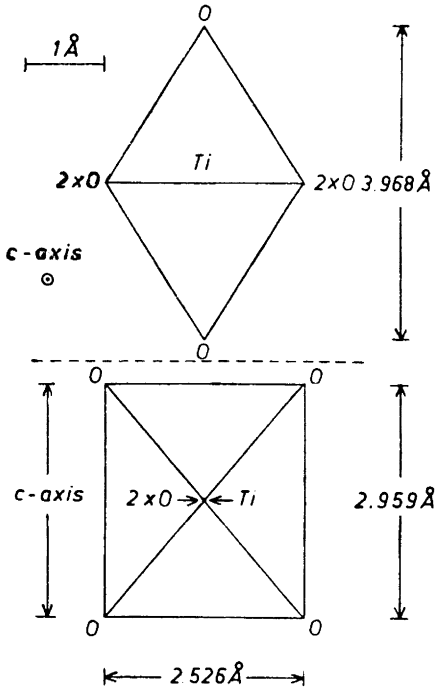


Fig. 1. The shape and size of the TiO_6-octahedra in rutile.

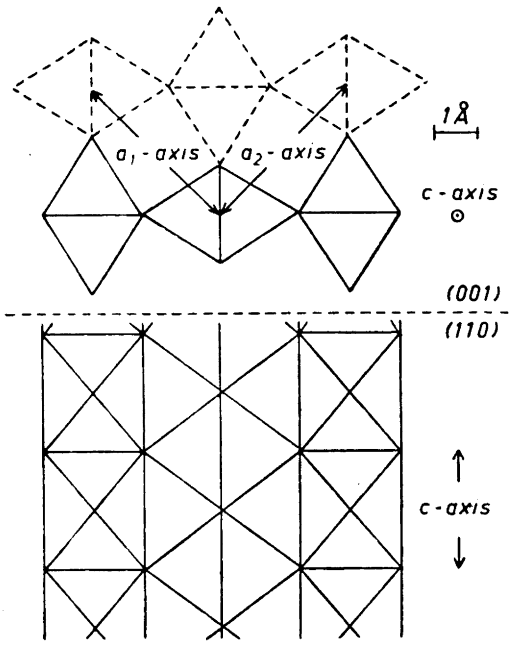


Fig. 2. The octahedron model of rutile.

2.0 THE RUTILE STRUCTURE

Titanium forms a number of different oxides, and so far the following ones have been identified [7]: Ti_2O, Ti_3O_2, TiO, Ti_2O_3, Ti_3O_5, Ti_nO_{2n-1} (n = 4, 5, 6, 7, 8, 9 and 10), and TiO_2. The lowest of these oxides, Ti_2O, has a structure which may be represented by OTi_6-octahedra sharing corners and edges. The other titanium oxides may be considered to consist of TiO_6-octahedra sharing corners, edges, and possibly faces in various ways.

From X-ray data on TiO_2 (rutile) is found that its TiO_6-octahedra are not exactly regular. Fig. 1 shows a rutile octahedron in two projections, and the dimensions given are those determined by Magnéli and co-workers [7], which are in close agreement with those determined by Cromer and Herrington [8]. The octahedra are characterized by:

4 Ti—O distances of 1.946 Å
2 Ti—O distances of 1.984 Å
8 O—O distances of 2.779 Å
2 O—O distances of 2.959 Å
2 O—O distances of 2.526 Å

In rutile, these octahedra are joined by edges and corners in such a way as to make each oxygen ion belong to three neighbouring octahedra. This type of octahedron coupling is shown in Fig. 2, where also the direction and length of the crystallographic axes in relation to the octahedra are indicated.

From Fig. 2 is easily seen that this type of coupling between the octahedra results in a kind of parallel octahedron chains in which neighbouring octahedra have an edge in common. These chains are further joined to each other so that the side corners in one chain's octahedra become top corners in a neighbouring chain's octahedra and *vice versa*. In rutile, the *c*-axis becomes parallel to the octahedron chains.

From Fig. 2 is further seen that this type of coupling between the chains results in the existence of just as many "open" channels parallel to the *c*-axis as there are octahedron chains, and in Fig. 3 is shown that such a channel contains a row of octahedral interstitial positions for titanium. The distance between these positions (in direction of the *c*-axis) is half only of that between normal titanium lattice positions in an octahedron chain. The "interstitial" oxygen octahedra are all of the same shape and size, and along the channels every second one is turned 90° around the *c*-axis in relation to the rest of them, and they are placed half way into each other. From Fig. 3 is further seen that an "interstitial" O_6-octahedron shares two faces and four edges with neighbouring TiO_6-octahedra.

The shape and size of the "interstitial" O_6-octahedra are shown in Fig. 4. By comparing with Fig. 1, it is seen that these O_6-octahedra differ distinctly from the normal TiO_6-octahedra in rutile, especially in being larger. The volume of a TiO_6-octahedron is about 9.89 Å^3, whereas the volume of an "interstitial" O_6-octahedron is about 10.91 Å^3.

The rutile structure also contains tetrahedral interstitial positions, and it is easily seen that there are just as many of these as there are octahedral interstitial positions (twice that of normal titanium lattice positions). The tetrahedral positions are not arranged along channels of any kind, and as titanium is not known to occur with the coordination number *four*, these positions seem to be of minor interest.

3.0 THE DEFECT STRUCTURE OF RUTILE

The defects or imperfections which can exist in crystal lattices can be classified as reversible (ions in interstitial positions and ion vacancies connected with an equivalent number of quasi-free electrons and electron holes) or irreversible (flaws, cracks, dislocations). The present discussion is confined to the former class of defects.

Ehrlich [6] reported that rutile exists over a rather wide oxygen concentration range ($TiO_{1.90}$—$TiO_{2.00}$), whereas Magnéli and coworkers [7] found that the minimum content of oxygen is higher than that corresponding to $TiO_{1.96}$. These investigators seem to agree, however, that the higher oxygen limit corresponds to the stoichiometric composition $TiO_{2.00}$.

The possible *non-stoichiometric* defect equilibria in rutile may be represented by the following equations:

$$\text{Nil} \rightleftharpoons O_v^{\cdot\cdot} + 2e^- + \tfrac{1}{2}O_2(g) \quad (1a)$$
$$\text{Nil} \rightleftharpoons O_v^{\cdot\cdot} e^- + e^- + \tfrac{1}{2}O_2\ (g) \quad (1b)$$
$$\text{Nil} \rightleftharpoons O_v^{\cdot\cdot} 2e^- + \tfrac{1}{2}O_2\ (g) \quad (1c)$$

for anion vacancies ($O_v^{\cdot\cdot}$) with or without trapped electrons (*AV-type* defects), and by

$$TiO_2 \rightleftharpoons Ti_i^{n+} + ne^- + O_2\ (g) \quad (2)$$

for cation interstitials (Ti_i^{n+}) with n positive charges (*CI-type* defects). As to n-values other than four, see under section 3.4 below.

In addition, there is also a hypothetical possibility of a *stoichiometric* defect equilibrium:

$$\text{Nil} \rightleftharpoons Ti_v^{''''} + Ti_i^{++++} \quad (3)$$

involving titanium vacancies ($Ti_v^{''''}$).

The symbols $'$ and $\cdot$ mean a negative and a positive surplus charge, respectively. e^- means a quasi-free electron, and "Nil" designates an ideal stoichiometric crystal.

In the following, we shall discuss the above possibilities on the basis of the ideal rutile structure itself and of other available data pertinent to the subject.

A critical discussion of results obtained up to 1954 has previously been given by Hauffe [5], who concludes that the defect structure most likely is of the AV-type.

3.1 Structural considerations

Under section 2.0 is shown that the rutile structure contains favourable octahedral interstitial positions for titanium, and on this basis it seems likely that rutile should be an oxide of the metal excess type (CI-type) rather than of the oxygen deficient type (AV-type), and also that stoichiometric defects (Eqn. 3) could be present in rutile.

An interesting fact in this connection is that a Ti_nO_{2n-1}-crystal structurally may be described [7,9] as being composed of parallel sheet crystals of rutile, the "grain boundaries" between which are free of misfit and differ from the ideal rutile structure mainly in containing titanium in octahedral interstitial positions. At these "grain boundaries", the rutile layers are slightly displaced in relation to each other (see below). Were it not so, the Ti_nO_{2n-1}-structures would have been identical to rutile with an ordered arrangement of titanium interstitials. It seems thus the most natural that in removing oxygen from rutile, one should go directly towards the Ti_nO_{2n-1}-structures by forming titanium interstitials, and not make a roundabout way by first forming oxygen vacancies which then have to be eliminated (at least to a large extent) when the oxygen content of the highest member of the Ti_nO_{2n-1}-series is reached (see under section 2.0).

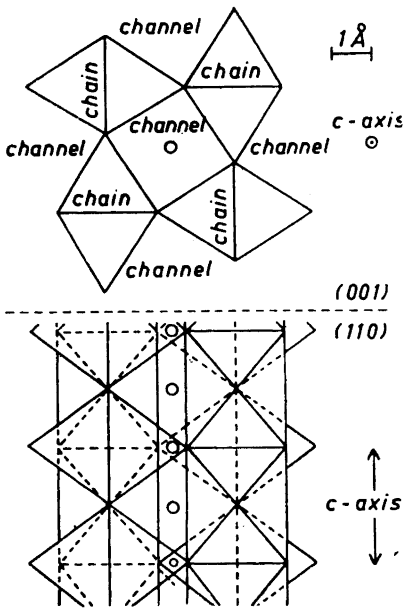


Fig. 3. Octahedral interstitial positions for Ti-ions in rutile.

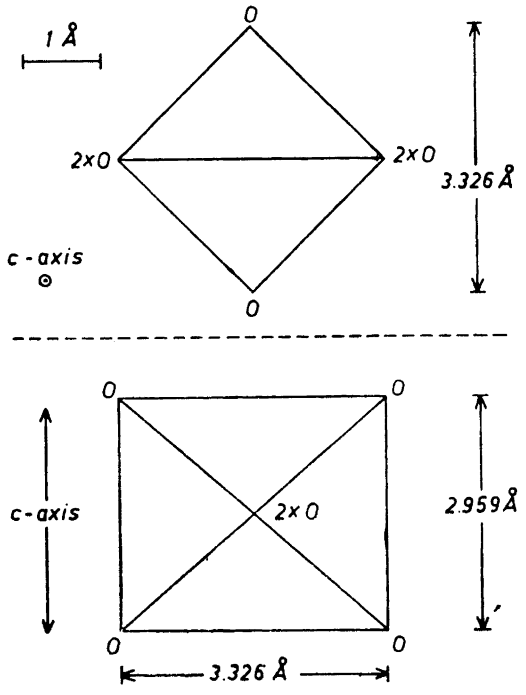


Fig. 4. The shape and size of the O_6-octahedra surrounding interstitial positions in rutile.

Magnéli and coworkers [7] have further found that the rutile lattice is slightly expanded, especially normal to the *c*-axis, with decreasing oxygen content:

$a = 4.584$ Å,	$c = 2.959$ Å	for maximum O-content
$a = 4.603$ Å,	$c = 2.960$ Å	for minimum O-content

It is difficult to judge what this expansion might really mean. If we assume, however, that rutile is a normal ionic crystal in which the lattice dimensions are mainly determined by the size of the ions, it is difficult to understand how the formation of oxygen vacancies may give such an effect. It is easier to imagine that the formation of titanium interstitials might cause a lattice expansion. The radius of the fourvalent titanium ion is namely about 20 % too large to allow six surrounding oxygen ions to come into contact with each other. From Figs. 3 and 4, it is further seen that a titanium interstitial should be expected to expand the lattice mainly in directions normal to the *c*-axis (just as observed).

It may seem that the above-mentioned displacement of the rutile layers at the "grain boundaries" or Magnéli-Andersson planes — as they better are

called — in the Ti_nO_{2n-1}-structures, is merely so that the octahedron chains on one side of such a plane fit into the open octahedron channels on the other side of the plane and *vice versa* (see Fig. 3). The oxygen framework of the rutile structure is thus maintained essentially unaltered all through a Ti_nO_{2n-1}-lattice. It is mainly the relative positions of the titanium ions in this framework that are changed at a Magnéli-Andersson plane. The titanium ions on one side of this plane are normally in positions which may be regarded as interstitial in relation to the rutile lattice on the other side of the plane and *vice versa*. Just at the plane, both the normal and the interstitial octahedron positions are occupied.

On this basis, the Ti_nO_{2n-1}-structures could possibly be described as a rutile structure with ordered arrangements of titanium vacancies and titanium interstitials, the latter in excess. This may suggest that rutile itself also could have defects of this kind more or less randomly distributed in the lattice (see eqns. 2 and 3).

3.2 Density measurements

Ehrlich [6] reports to have found a slightly lower density for $TiO_{1.97}$ than for TiO_2 by pycnometric measurements on powders. These results are used by Hauffe [5] as one of the main arguments for the existence of AV-defects in rutile.

Ehrlich himself pointed out that his $TiO_{1.97}$-sample was not quite a sound one. Pycnometric density measurements on powders are moreover easily affected with both systematic and accidental errors. As a comparison, we shall here give some density values determined by Magnéli and coworkers [7]:

$$d(TiO) = 4.95, \quad d(TiO_{1.67}) = 4.20, \quad d(TiO_{1.80}) = 4.29 \text{ gcm}^{-3}$$

and by Ehrlich [6]:

$$d(TiO) = 4.89, \quad d(TiO_{1.70}) = 4.21 \quad d(TiO_{1.80}) = 4.17 \text{ gcm}^{-3}$$

all of them on powders.

The inconsistence of these results should make it clear that, so far, one has to be careful in using the density data in elucidating the defect structure of rutile.

3.3 Colour observations

It is well known (see, *e.g.*, Ref.[5]) that rutile acquires a dark grey-blue colour when kept in a reducing atmosphere at high temperatures, and that it is slightly coloured in this way also by the incorporation of a higher-valent metal oxide (Ta_2O_5, Nb_2O_5, Sb_2O_5 or WO_3).

Hauffe [5] has suggested that this may be due to a formation of Pohl-Schottky colour centres, the so-called F-centres. Such a centre is equivalent to an oxygen vacancy with a trapped electron ($O_v^{\cdot\cdot}e^-$). The colouring of rutile, therefore, has naturally been used as another argument in favour of a defect structure of the AV-type.

A threevalent titanium ion in rutile may also give rise to a blue colour, however, and — in our opinion — the significance of the colour change is not understood well enough for the present to be used as an argument in this discussion.

3.4 Conductivity measurements

According to the Schottky-Frenkel theory of reversible defects [10,11], a defect equilibrium is given by the mass action law in quite an ordinary way. The equilibria of the defect systems (1), (2), and (3) above, should thus be represented by the following equations:

$$(O_v^{\cdot\cdot})(e^-)^2 p^{1/2} = \text{const.} \tag{4a}$$

$$(O_v^{\cdot\cdot} e^-)(e^-) p^{1/2} = \text{const.} \tag{4b}$$

$$(O_v^{\cdot\cdot} 2e^-) p^{1/2} = \text{const.} \tag{4c}$$

and

$$(Ti_i^{n+})(e^-)^n p = \text{const.} \tag{5}$$

and

$$(Ti_i^{++++})(Ti_v^{\prime\prime\prime\prime}) = \text{const.} \tag{6}$$

where p means the partial pressure of O_2 in the gas phase, and the brackets indicate that it is the concentration of the defect species we are dealing with in these equations. The constants are of course temperature dependent.

If we now assume that the conductivity (q) of rutile is proportional to the concentration of quasi-free electrons, we easily have by combining the eqns. (1) and (4) that

$$q = \text{const. } p^{-1/6} \tag{7a}$$

$$q = \text{const. } p^{-1/4} \tag{7b}$$

$$q = \text{const. (independent of } p) \tag{7c}$$

in the case of oxygen vacancies with none, one, or two trapped electrons, respectively.

In the case of a defect structure of the CI-type, we have

$$q = \text{const. } p^{-1/n+1} \tag{8}$$

This is easily seen by combining the eqns. (2) and (5).

Hauffe and coworkers [5,12] have found that the conductivity of rutile is proportional to $p^{-1/4.5}$ to $p^{-1/5}$ in the temperature range 800—1 000°C. This may be explained on the assumption of three- and fourvalent titanium ions in interstitial positions ($n = 3$ and 4 in eqn. 8). The results are not compatible with a defect structure of the AV-type.

It could here possibly be argued that a threevalent titanium ion is equivalent to a quasi-free electron and that it has no sense to use other n-values than four in eqns. (2), (5), and (8). This cannot be quite correct, however.

Cronemeyer [13] has namely shown that the electronic conductivity of rutile is distinctly higher in direction of the *c*-axis than in a direction normal to this axis, and as there is practically no possibility for interstitial titanium ions to take part in an electron exchange in the *c*-direction, whereas this may seem a preferred exchange direction for titanium ions in normal lattice positions when judging from interatomic distances (see Fig. 3), a threevalent titanium interstitial ion cannot be regarded synonymous with a quasi-free electron. There is at least a distinct difference between interstitially and normally placed titanium ions in this respect. This may easily be seen from the description of the rutile structure under section 2.0 above.

A combined stoichiometric and non-stoichiometric defect equilibrium in rutile should be defined by the following simultaneous equations:

$$\begin{aligned} (\mathrm{Ti_i^{++++}})(\mathrm{Ti_v^{''''}}) &= k_1 \\ \mathrm{Ti_i^{++++}})(e^-)^4 p &= k_2 \\ 4(\mathrm{Ti_i^{++++}}) &= 4(\mathrm{Ti_v^{''''}}) + (e^-) \end{aligned} \tag{9}$$

The two first equations are the same as (5) and (6) above. The last equation is required by the electroneutrality principle, and the two last equations imply the simplifying assumption that $n = 4$ in eqn. (2).

By solving this equation system, we easily have:

$$(e^-)^5(4k_1(e^-)^3 p + k_2) = 4k_2^2 p^{-1} \tag{10}$$

which should represent the conductivity/oxygen pressure relationship in this case, when the conductivity is assumed proportional to the concentration of quasi-free electrons.

In case $4k_1(e^-)^3 p \ll k_2$, this equation reduces to:

$$(e^-) = (4k_2)^{1/5} p^{-1/5} \tag{11}$$

which is equivalent with the previous eqn. (8), showing the conductivity to vary inversely with the 1/5-power of the oxygen partial pressure when $n = 4$.

In case $4k_1(e^-)^3 p \gg k_2$, eqn. (10) reduces to:

$$(e^-) = (k_2^2/k_1)^{1/8} p^{-1/4} \tag{12}$$

whereby it is seen that the presence of a stoichiometric defect equilibrium in addition to a non-stoichiometric one should make the conductivity q to be relatively more sensitive ($d \log q$) to relative changes ($d \log p$) in the oxygen pressure p.

The conductivity/pressure relationships found by Hauffe and coworkers [12] could possibly be interpreted also along this line of thought.

The conductivity measurements referred to, have been made on powders. The above discussion does show, however, that even single crystal data of this kind may not be quite conclusive in determining the defect structure of rutile.

3.5 Weight change measurements

From eqns. (1) and (2) is seen that a rutile crystal should loose some oxygen and, thereby, decrease in weight on the formation of defects. By also taking eqns. (4) and (5) into consideration, we easily have for the equilibrium weight:

$$w = w_0 - \text{const. } p^{-1/6} \quad (13a)$$

$$w = w_0 - \text{const. } p^{-1/4} \quad (13b)$$

$$w = w_0 - \text{const. } p^{-1/2} \quad (13c)$$

in the case of AV-defects with none, one, and two trapped electrons, respectively, and

$$w = w_0 - \text{const. } p^{-1/n+1} \quad (14)$$

in the case of CI-defects with n positive charges.

w_0 means here the weight of the crystal when without defects. Buessem, Förland, T., Förland, K.S., and Marshall [14] have studied the equilibrium weight of a rutile sample (powder) as a function of the oxygen pressure (from about 10 to 760 torr) at temperatures from 860 to 1 050°C. Their results are apparently best in agreement with (13a) above, thereby indicating a defect structure of the AV-type.

As their rutile sample was not quite pure, and, considering the very small difference between a 1/6- and a 1/5-power relationship, we don't feel quite convinced that the former and not the latter is the correct one. The latter relationship is more consistent with the results of the conductivity measurements (see above), and is the one to be expected if the defect structure is of the CI-type ($n = 4$ in eqn. (14) above).

Förland [15] is now making renewed and extended weight change measurements on a high purity rutile sample, and it shall be interesting to see the results of these measurements.

3.6 Incorporation experiments

It is well known that an incorporation of even small amounts of foreign ions into a host lattice may drastically change its defect equilibrium, and that a knowledge of the effect of such incorporations on various properties of a crystal may assist in elucidating its original defect structure (see, *e.g.*, Ref.[5]).

In the case of rutile, we are only aware of data on the effect of incorporations on its electronic conductivity and on its oxidation protective properties when present as a film on titanium. The latter effect will be discussed under a later section (3.7), and we shall here only deal with the former effect.

If rutile should have a defect structure purely of the AV-type, it seems natural to assume that the cation interstitial positions in this lattice — for one reason or another — must be so unfavourable that not even incorporated cations will make use of them. An incorporation of a foreign metal oxide must then take place in a way as here exemplified by Cr_2O_3:

$$Cr_2O_3 + 2e^- + \tfrac{1}{2}O_2 = 2Cr_S'(Ti) + 2TiO_2 \quad (13)$$

$Cr_S'(Ti)$ means here a threevalent chromium ion substituting a fourvalent titanium ion, and this represents one negative surplus charge in the lattice.

From this equation is seen that the incorporation of a cation of lower valence than four should lead to a consumption of free electrons and, thereby, to a *decrease* in electronic conductivity in the case of AV-defects in rutile. Small amounts of Ga_2O_3, Al_2O_3, and NiO have been found [5], however, to have almost no effect on the conductivity, and Cr_2O_3 even to increase the conductivity at temperatures below 900°C.

These observations are perhaps easier explained on the assumption of a defect structure of the CI-type in rutile. In this case we should be allowed to assume that also foreign cations could enter into interstitial positions in the rutile lattice. Thus, by once more taking Cr_2O_3 additions as an example, and by assuming this oxide to be built into the rutile lattice in such a simple way as to give a fraction x of the Cr^{+++}-ions as substitutes for Ti^{++++}-ions in normal lattice positions:

$$x\,Cr_2O_3 + 2x\,e^- + \tfrac{x}{2}O_2 = 2x\,Cr_S'(Ti) + 2x\,TiO_2 \qquad (14)$$

and the remaining fraction $(1-x)$ of the Cr^{+++}-ions, in interstitial positions:

$$(1-x)Cr_2O_3 = 2(1-x)Cr_i^{+++} + 6(1-x)\,e^- + \frac{3(1-x)}{2}O_2 \qquad (15)$$

the total incorporation is represented by:

$$Cr_2O_3 = 2x\,Cr_S'(Ti) + 2(1-x)\,Cr_i^{+++} + 2(3-4x)\,e^- + 2x\,TiO_2 + \frac{3-4x}{2}O_2 \qquad (16)$$

From this equation is seen that the effect of Cr_2O_3-additions on the conductivity of rutile in this case should depend on the fraction factor x (for $x \gtreqless \frac{3}{4}$ we will have for the conductivity $\Delta q \lesseqgtr 0$). This factor may very well be a function of both Cr_2O_3-concentration, temperature, and oxygen pressure, and eqn. (16) could thus possibly explain the observed effects of these variables on the conductivity of the TiO_2/Cr_2O_3 mixed oxides (see Ref.[5]).

A similar treatment could be given also to other rutile/foreign oxide systems.

3.7 Oxidation studies

Through studies of the oxidation of titanium under conditions at which rutile is formed on the metal surface, it should also be possible to gain some information as to the defect structure of rutile.

Most of the previous work on the oxidation of titanium is reviewed by Kofstad and Hauffe [3] and by Kofstad, Hauffe, and Kjöllesdal [4]. Some further work is now being prepared for publication by Hurlen, Kjöllesdal, Markali and Norman.

In this latter work, rather strong evidence is found that the oxidation, at temperatures from 300 to 500°C, is governed by a cavity formation at the metal oxide interface. Such a cavity formation is probably most easily understood by assuming the occurrence and growth of cavities to be due to a reten-

tion at the metal/oxide interface of inward diffusing metal ion vacancies or of vacancies left behind in the metal when metal ions enter into interstitial positions in the oxide and migrate outwards.

In the same work is also found that rutile whiskers sometimes are formed on top of the rutile film, and Kinna and Knorr [16] have found that platinum markers placed on a titanium surface, are deeply buried by the rutile layer formed during oxidation at 1 000 and 1 200°C. All these observations are difficult to understand without assuming a preferential titanium diffusion in rutile.

Also Gulbransen and Andrew [17] assume mainly a titanium diffusion in the oxide film all over the temperature region (250—600°C) covered by their oxidation experiments, and, in the relatively high negative activation entropy of oxidation calculated from the results of these authors, Moore [18] finds theoretical evidence for the validity of this assumption.

Most of the workers within this field seem to assume a preferential oxygen diffusion in rutile, however, and, from marker studies with radioactive silver at 900°C, Davies and Birchenall [19] got some results which have been taken as being in favour of this assumption even though they are not quite conclusive.

As the diffusion in solids may be regarded a diffusion of defects, it seems that the results of the oxidation studies are more in accordance with a defect structure of the CI-type (or a combined CI/CV-type) than with one of the AV-type.

The fact that the Wagner-Hauffe [2] approach to alloy oxidation has proved unsuccessful in the case of titanium [20] may also have some explanation [21] on the assumption of a combined CI/CV defect structure in rutile. The Wagner-Hauffe theory does not take such a combined defect structure into account. It only applies to cases in which the oxide has a single defect system.

4.0 CONCLUSION

The defect structure of rutile has here been discussed on the basis of structural considerations, density measurements, colour observations, conductivity measurements, weight change measurements, incorporation experiments, and oxidation studies.

It has been shown that some of the available data are inconclusive, that some data (especially those by Marshall and Förland [14] on the equilibrium weight of a rutile sample as a function of the oxygen pressure) may indicate a defect structure of the anion vacancy type (AV-type), but that the majority of the data is most easily interpreted on the assumption of a defect structure of the CI-type (or a combined CI/CV-type).

The strongest evidence in favour of this assumption is that the rutile structure contains apparently very favourable octahedral interstitial positions for titanium ions, and that positions of this kind actually are being occupied in an ordered way in the rutile-like oxides belonging to the homologous series of formula Ti_nO_{2n-1} (see Ref.[7]). This series of oxides extends directly up to the lower O-limit of the homogeneity range of the TiO_2 (rutile), and it seems logical and natural that we here should have just a transition from an ordered to a random occupancy of such sites.

Acknowledgement. This work forms part of a research project sponsored by the *Air Research and Development Command, US Air Force*, through its European Office under Contract AF 61(514)-892.

The author is much indebted to Dr. A. Magnéli and Dr. S. Andersson at the University of Stockholm, to Siv. ing. K. Seip Förland and Dr. T. Förland at the Technical University of Norway, Trondheim, and to Dr. N. Norman, Cand. real. J. Markali, Siv. ing. H. Kjöllesdal and other members of the staff at the Central Institute for Industrial Research, Blindern, Oslo, Norway, for helpful discussions.

REFERENCES

1. Wagner, C. *Z. physik. Chem.* **(B) 21** (1933) 25.
2. Hauffe, K. *Oxydation von Metallen und Metallegierungen*, Springer Verlag, Berlin 1956.
3. Kofstad, P. and Hauffe, K. *Werkstoffe u. Korrosion* **7** (1956) 642.
4. Kofstad, P., Hauffe, K. and Kjöllesdal, H. *Acta Chem. Scand.* **12** (1958) 239.
5. Hauffe, K. *Reaktionen in und an Festen Stoffen*, Springer Verlag, Berlin 1955.
6. Ehrlich, P. *Z. Elektrochem.* **45** (1939) 362; *Z. anorg. allgem. Chem.* **247** (1941) 53.
7. Andersson, S., Collén, B., Kuylenstierna, U. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1641.
8. Cromer, D. T. and Herrington, K. *J. Am. Chem. Soc.* **77** (1955) 4708.
9. Andersson, S. *Private communication.*
10. Shottky, W. *Z. physik. Chem.* **(B) 11** (1930) 163.
11. Frenkel, J. *Z. Physik.* **35** (1926) 652.
12. Hauffe, K., Grunewald, H. and Tränkler-Greese, R. *Z. Elektrochem.* **56** (1952) 937.
13. Cronemeyer, D. C. *Electrical and Optical Properties of Rutile Single Crystals, Technical Report 46, Laboratory for Insulation Research, M.I.T.*, August 1951.
14. Buessem, W., Förland, T., Förland, K. S. and Marshall, P. A. Linden Laboratories, State College, Pa., U.S.A. *Private communication* with Mrs. K. Seip Förland.
15. Förland, K. S. *Private communication.*
16. Kinna, W. and Knorr, W. *Z. Metallkunde* **47** (1956) 594.
17. Gulbransen, E. A. and Andrew, K. F. *Met. Trans.* **185** (1949) 741.
18. Moore, W. J. *J. Elektrochem. Soc.* **100** (1953) 302 (appendix).
19. Davies, M. H. and Birchenall, C. E. *J. Metals* **3** (1951) 877.
20. Kofstad, P. *Investigation of the Mechanism of Oxidation of Titanium and Titanium Alloys at High Temperatures, ARDC-Report*, Central Institute for Industrial Research, Blindern, Oslo, Norway, April 1957.
21. Hurlen, T., Kjöllesdal, H., Markali, J. and Norman, N. *Oxidation of Titanium, ARDC-Report*, Central Institute for Industrial Research, Blindern, Oslo, Norway, April 1958.

Received November 18, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 377—394

Short Communications

The Crystal Structure of $K_3V_5O_{14}$ *

ANN MARIE BYSTROM and
HOWARD T. EVANS, Jr.

Geological Survey of Sweden, Stockholm 50, Sweden, and
U. S. Geological Survey, Washington 25, D. C., U.S.A.

As a part of a study of the system $K_2O-V_2O_5-H_2O$ at the U.S. Geological Survey, Washington, D.C., the crystal structure of $K_3V_5O_{14}$ has been worked out. $K_3V_5O_{14}$ crystallizes in red hexagonal prisms by rapid evaporation of dilute solutions of V_2O_5 and K_2O above 40°C. (R. Marvin, U.S. Geological Survey, written communication.) The crystals, which are often twinned, show strong dichroism, brown to dark red, with absorption $\varepsilon < \omega$; ω = dark red and ε = greenish yellow. They are optically uniaxial, negative, with refractive indices $\omega = 2.42$ and $\varepsilon = 1.748$. The habit clearly indicates that the point group symmetry is ditrigonal pyramidal, C_{3v}.

From photographs of the (*hk*0), (*hhl*), and (*h*0*l*) zones, made with a Buerger precession camera and MoKα radiation, and the habit of the crystal, the space group was established as $P31m$ (C_{3v}^2). The cell dimensions were determined with an x-ray single crystal goniometer using a scintillation counter. The following values were obtained:

$a = 8.6796 \pm 0.0020$ Å MoKα_1 = 0.70926 Å
$c = 4.9914 \pm 0.0008$ MoKα_2 = 0.71354
$V = 325.65$ Å^3 MoKβ = 0.63225

Density (observed) = 3.01 g cm^{-3}
Density (calculated) = 3.03 g cm^{-3}.

Intensities were recorded for (*hk*0), (*hk*1), and (*h*0*l*) zones on a Weissenberg camera using unfiltered MoK radiation. The multiple film technique was used and intensities were measured by visual comparison with a calibrated strip.

* Publication authorized by the Director, U.S. Geological Survey.

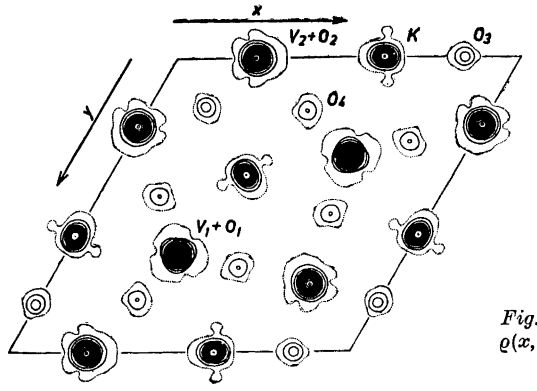


Fig. 1. Electron density projection of $K_3V_5O_{14}$, $\varrho(x, y)$. Dotted contour, 4 electrons/Å^2; contour interval 4 electrons/Å^2.

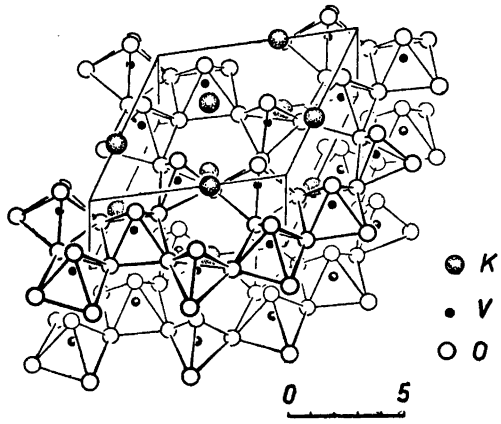


Fig. 2. View of the crystal structure of $K_3V_5O_{14}$, showing triangular and square pyramids linked into a sheet. Scale in Ångström units.

Owing to difficulties involved in getting a pure sample for chemical analysis, the cell content was uncertain at the outset. Thus, all attempts to interpret the Patterson function $P(u,v)$ in terms of 3K and 6V atoms failed. A solution was readily found for 3K and 5V atoms, however, and the resulting model was confirmed by the Patterson $P(u,w)$. The oxygen locations were then found mainly by space reasoning. From the preliminary positions of the atoms, electron density maps of the functions $\varrho(x,y)$ and $\varrho(x,z)$ were prepared, and by successive steps of refinement, the following coordinates were determined:

Atom	x	y	z
3 K in (*c*)	0.605	0	0
2 V_1 in (*b*)	1/3	2/3	0.471
3 V_2 in (*c*)	0.231	0	0.472
2 O_1 in (*b*)	1/3	2/3	0.796
3 O_2 in (*c*)	0.240	0	0.782
3 O_3 in (*c*)	0.838	0	0.367
6 O_4 in (*d*)	0.469	0.177	0.366

For these coordinates the reliability factors and temperature coefficients have been found as follows:

(*hk*0) (63 obs. data) $R = 0.116$, $B = 1.59$ Å^2;
(*hk*1) (51 obs. data) $R = 0.145$, $B = 0.77$;
(*h*0*l*) (93 obs. data) $R = 0.164$, $B = 0.90$.

In Fig. 1 the electron density projection $\varrho(x,y)$ is illustrated.

The five vanadium atoms lie very nearly in the same *z*-plane forming almost regular pentagons. The fourteen oxygen atoms are bound to the vanadium atoms in rather an unusual way, reminiscent of the bonding in V_2O_5 (Byström *et al.*[1]) and $KVO_3 \cdot H_2O$ (Christ *et al.*[2]). They form nearly square pyramids around the V_2 atoms and trigonal pyramids around the V_1 atoms, the pyramids all with apices pointing in the same direction along the *c*-axis and joined by sharing neighboring basal corners. Each hollow space resulting from the five-ring configuration is occupied by a potassium atom, placed about half way between the layers. Each potassium is surrounded by 10 oxygens, five from the apex oxygens of the polyhedra in the lower layer and five from basal corners of polyhedra in the layer above.

The V—O bonds in the *c*-direction of $K_3V_5O_{14}$ may be compared with the situation along the *b*-axis in V_2O_5 (Byström *et al.*[1]). In both cases there is a short V—O distance of 1.5—1.6 Å within the layer and then a long distance to the neighboring layer. In V_2O_5 the long distance is still short enough, V—O = 2.81 Å, to act as a weak bond, giving the structure a three dimensional network character. In $K_3V_5O_{14}$ the corresponding distance is considerably prolonged, of the order of 3.4 Å, too long for a bond. The vanadium-oxygen polyhedra thus form a two dimensional network, now held together by the interfoliated potassium ions. The nine V—O distances range from 1.55 to 1.87 Å and the K—O distances from 2.72 to 3.36 Å.

A pictorial view of the structure is given in Fig. 2, where the V—O polyhedra have been especially emphasized.

The work on the structure is being continued and a full report will appear at a later date.

1. Byström, A., Wilhelmi, K.-A. and Brotzen, O. *Acta Chem. Scand.* **4** (1950) 1119.
2. Christ, C. L., Clark, J. R. and Evans, H. T., Jr. *Acta Cryst.* **7** (1954) 801.

Received January 14, 1959.

An Infra-red Spectroscopic Study of the Structural Relationships of Dextropimaric, *Iso*dextropimaric, and Cryptopimaric Acids

HENRIK H. BRUUN

Institute of Wood Chemistry, Åbo Akademi, Åbo, Finland

INGRID FISCHMEISTER and EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, University of Upsala, Upsala, Sweden

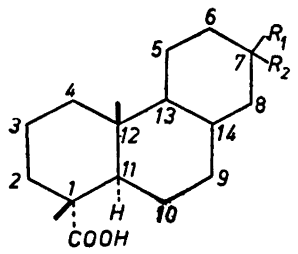


Fig. 1. Ring system numbering of rosin acids. Stereochemical configuration at the carbon atoms 1, 11, and 12 indicated.

Recent mass spectrometric investigations[1] of the structural relations between dextropimaric, *iso*dextropimaric, and cryptopimaric acid have led to the conclusion that dextropimaric acid and cryptopimaric acid can be structurally different only with regard to the orientation of the methyl and vinyl group attached to C_7 (Fig. 1; R_1 = vinyl, R_2 = methyl), whereas the difference between dextropimaric acid and *iso*dextropimaric acid must also involve the geometry of the ring system. In the present study the infra-red spectra of these compounds and their methyl esters have been recorded, and it will be shown that the vinyl group absorption and a skeletal vibration at 8.4 μ exhibit evidence of differences which are in accordance with the above conclusions. Moreover, they show that dextropimaric and *iso*dextropimaric acid are epimeric not only at C_{13} but also at C_7.

The absorption band due to the $=C\begin{matrix}H\\H\end{matrix}$ out-of-plane deformation vibration is found at 10.90 μ for dextropimaric acid and at 10.94 μ for both *iso*dextro- and cryptopimaric acid. In the spectra of the methyl esters the same band occurs at 10.92 μ for dextropimaric acid and at 10.98 μ for the other acids. In both cases the band for dextropimaric acid is shifted *ca.* 0.05 μ towards shorter wavelengths as compared with the (coincident) bands of *iso*dextro- and cryptopimaric acid.

The slightly weaker band at 10 μ due to the $-CH=$ out-of-plane deformation of the vinyl group only shows differences within the experimental error of the apparatus.

For the acids in the solid state (in KBr) the position of the vinyl group absorption deviates somewhat from that shown by the acids in solution and by the methyl esters. Again, dextropimaric acid differs from the two others with regard to the $=CH_2$ out-of-plane vibration, but here the shift is towards longer wavelengths (*cf.* Table 1). Cryptopimaric acid shows a double band, and the others single maxima with inflexions. The 10 μ band, again, exhibits only differences lying within the experimental error of the apparatus.

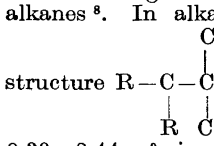
These spectral differences indicate that the vinyl group in dextropimaric acid has a different molecular environment, *i. e.* a different stereochemical position, than in *iso*dextro- and cryptopimaric acid. Since surface-film measurements[2] suggest that the position is quasi-axial for dextropimaric acid and quasi-equatorial for *iso*dextropimaric acid, these absorption differences seem to correspond to axial and equatorial positions of the vinyl group, respectively (see also an earlier infra-red investigation of the solid state spectra of dextro- and *iso*dextropimaric acid[3]). Spectral differences for substituents in axial and equatorial position are well known in steroid chemistry. For instance, hydroxyl groups[4], as well as carbonyl groups in the neighbourhood of halogen atoms[5], exhibit such shifts. Also, the acetate band at 8 μ [6,7] is single when the group is in the equatorial position, but splits into several peaks when it is in the axial position.

The medium-intensity band at about 8.4 μ is common to the spectra of the CCl_4 solutions of dextro-, *iso*dextro-, and cryptopimaric acids, to the spectra of the esters, and to those of the solid acids in KBr. In

Table 1.

Acid	Skeletal vibration, λ (μ)			RHC=C(H)(H) out-of-plane deformation vibration λ (μ)		
	Acid, in CCl_4-soln.	Ester, liquid	Acid, in KBr	Acid, in CCl_4-soln.	Ester, liquid	Acid, in KBr
Dextropimaric	8.44	8.40; 8.50	8.42	10.90	10.92	— (11.02)* 11.09
*Iso*dextropimaric	8.38	8.39; 8.45	8.39	10.94	11.00	10.88 (11.00) —
Cryptopimaric	8.44	8.40; 8.51	8.45; 8.49	10.94	10.97	10.88 11.02 (11.09)

* Values in parentheses signify inflexions.

each case this band has the same position for dextro- and cryptopimaric acid, but appears at a shorter wavelength in the spectrum of *iso*dextropimaric acid. The numerical values are listed in Table 1. A skeletal vibration in this region occurs for branched-chain alkanes [8]. In alkanes with the general structure  the band is at 8.39—8.44 μ [9], *i. e.* it is in the same position as found in our spectra, and it is therefore possible that one of the methyl-substituted carbon atoms (see Fig. 1) in our substances is involved in this vibration. Skeletal vibrations are known to be very sensitive to changes in the immediate environment of the vibrating group. We should therefore have a methyl substituted carbon atom whose environment is the same in dextro- and cryptopimaric acid but different in *iso*dextropimaric acid. This is the case for C_{12}, taking into account the mass spectrometric evidence of different ring fusion between rings B and C in dextropimaric and *iso*dextropimaric acid.

In summary, we can state that the spectral shifts discussed above suggest that dextropimaric and *iso*dextropimaric acid are epimeric at C_7 and C_{13}, while dextropimaric and cryptopimaric acid are epimeric at C_7. These results agree with the conclusions drawn by Bruun, Ryhage, and Stenhagen [1] from mass spectrometric data, and with the chemical evidence recently reported [10,11].

Experimental. The apparatus employed is described in Ref.[3], and the physical and chemical properties of the acids studied are given in Ref.[1]. The wavelength measurements are accurate to $\pm$ 0.015 μ.

Acknowledgements. We are grateful to Mrs. K. Nilsson for the spectrophotometric measurements, and to Mr. L. Ahlquist for assistance in the preparation of material. The visit of one of us (H.B.) to Sweden has made been possible by grants from the *Finnish State Commission for Science.*

1. Bruun, H. H., Stenhagen, E. and Ryhage, R. *Acta Chem. Scand.* **12** (1958) 789.
2. Bruun, H. H. *Acta Acad. Aboensis. Math. et Phys.* **19** (1954) (3), 7.
3. Bruun, H. H. *Paper and Timber Finland* **38** (1956) 577; **39** (1957) 221.
4. Cole, A. R. H., Jones, R. N. and Dobriner, K. *J. Am. Chem. Soc.* **74** (1952) 5571.
5. Jones, R. N., Ramsay, D. A., Herling, F. and Dobriner, K. *J. Am. Chem. Soc.* **74** (1952) 2828.
6. Jones, R. N., Humphries, P., Herling, F. and Dobriner, K. *J. Am. Chem. Soc.* **73** (1951) 3215.
7. Fürst, A., Kuhn, H. H., Scotoni, R. Jr. and Günthard, Hs. H. *Helv. Chim. Acta* **35** (1952) 951.
8. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules*, Methuen & Co., London 1954, p. 25.
9. McMurry, H. L. and Thornton, V. *Anal. Chem.* **24** (1952) 318.
10. Edwards, O. E. and Howe, R. *Chem. and Ind. London* **1958** 629.
11. Green, B., Harris, A. and Whalley, W. B. *Chem. and Ind. London* **1958** 1084; *J. Chem. Soc.* **1958** 4715.

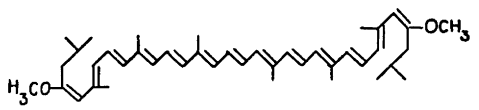
Received January 15, 1959.

A Note on the Constitutions of Spirilloxanthin and P-481

SYNNÖVE LIAAEN JENSEN

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

The molecular formula of the carotenoid spirilloxanthin, shown to be identical with that of rhodoviolascin [1], was established by Karrer *et al.*[2,3] and van Niel *et al.*[4,1]. The following constitution (I) was suggested by Karrer *et al.*[5], who isolated from the potassium permanganate oxidation products,



(I)

bixindialdehyde and a methoxyl-free dialdehyde with eleven conjugated double bonds. Karrer *et al.* preferred a symmetrical molecule, and by analogy with the generally occurring 3-substituted carotenoids, they placed the methoxyl groups in the 3,3′-positions. This formula (I) does not contain any *iso*propylidene end groups. Karrer *et al.*[6], however, obtained 0.60–0.73 moles of acetone by ozonolysis. Carotenoids of the lycopene type (two *iso*propylidene end groups) have given 1.24–1.68 moles of acetone by ozonolysis [7,8]; the corresponding values for the γ-carotene type (one *iso*propylidene end group) ranging from 0.6–0.94 [9,10]. The values reported by Karrer *et al.* are unexpectedly high for the structure given (I). By ozonolysis (I) would give two moles of methyl *iso*valerate, from which no measurable quantity of acetone is to be expected. The failure of formula (I) to account for the acetone formed by ozonolysis has already been pointed out by van Niel [11].

Micro determination of *iso*propylidene has been repeated, using the method of Kuhn and Roth [12], on crystalline, chromatographically purified spirilloxantin isolated from *Rhodospirillum rubrum*. The result is presented in Table 1 together with values simultaneously obtained for the carotenoid P-481 [13] from the same source and for chromatographically pure lycopene from tomatoes.

Table 1.

Carotenoid	Found: Moles acetone/mole	Number of *iso*propylidene groups
Spirilloxanthin	0.42	0
P-481	0.82	1
Lycopene	1.70	2

Although our value for spirilloxanthin is appreciably lower than the values found by Karrer *et al.*, it still exceeds the amount found for carotenoids of the β-carotene type (no *iso*propylidene groups). The reported value for *iso*propylidene determination of β-carotene is 0.2 [9]. We therefore assume that the methoxyl groups of spirilloxanthin belong to a structural element from which some acetone is formed upon ozonolysis.

Assuming a symmetrical formula, Karrer's potassium permanganate oxidation data restrict the location of the methoxyl groups to positions 1, 2 or 3 and 1′, 2′ or 3′. As already mentioned the *iso*propylidene determination does not favour a 3-methoxy-structure. In addition, if the methoxyl groups were placed in the 3,3′-positions the stability of the so-called OH-spirilloxanthin [14] would be difficult to explain. This compound is a bacterial carotenoid with a visible spectrum similar to that of spirilloxanthin, but which according to partition tests and chromatographic behaviour contains one hydroxyl group [15,14]. It has been regarded by Goodwin [15] as a mono-demethylated spirilloxanthin. This assumption seems to agree with the results from kinetic studies of carotenoid synthesis in *Rhodospirillum rubrum* [14]. Mono-demethylated spirilloxanthin, according to formula (I) would be an enol, rearranging in neutral or acidic media to a carotenoid with twelve conjugated double bonds and an isolated keto group. OH-Spirilloxanthin should thus differ remarkably from spirilloxanthin in its visible spectrum. On addition of alkali a pronounced bathochromic shift in the visible spectrum should occur; this is not the case. It may be mentioned that 4,4′-dihydro-rhodoxanthin has a pure β-carotene spectrum [16] with no tendency to prolongation of the chromophore by enolization in neutral solvents.

Carotenoids with allylic methoxyl groups are known to yield products with extended conjugated chains by treatment with

chloroform-hydrochloric acid [17,7]. A series of experiments with chloroform-hydrochloric acid treatment [18] of spirilloxanthin, resulted only in a certain *trans-cis* isomerization of spirilloxanthin and gave no product with an extended conjugated chain. Methoxyl groups are therefore not likely to be present in the 2,2′-positions.

The arguments presented above indicate, by elimination, positions 1,1′ as the most likely sites for the methoxyl groups of spirilloxanthin. Further support for this hypothesis may be obtained from the IR-absorption bands of the methoxyl groups of a series of related compounds (Table 2), although very little data are available.

Table 2.

Compound	C—O Stretching Vibration Frequency cm^{-1}
Spirilloxanthin (KBr)	1 080
4,4′-dimethoxy-β-carotene (KBr) [18] *	1 082—1 097
Pigment Y [7]	1 064
Spheroidenone [7]	1 066
=C—O—C [19]	near 1 250

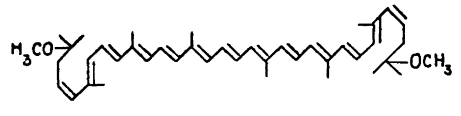
* Private communication from Dr. R. Entschel.

The frequency of the C—O—C stretching vibration of spirilloxanthin is different from that of an enolic methoxyl group.

A relatively intense band around 1 660 cm^{-1} has been shown by Rosenkrantz and Gut [20] to be characteristic for compounds containing the —CH=C—O— grouping. Spirilloxanthin has no absorption band in this region. IR-data are thus not in accordance with formula (I).

As a consequence of the above discussion the constitution (II) is suggested for spirilloxanthin.

As long as the elementary composition of P-481 is unknown, it is premature to indicate a structure for this carotenoid. As seen from Table 1 this carotenoid seems to contain one *iso*propylidene group. A chromophore of twelve conjugated double bonds [14] and the presence of one methoxyl group [21] have already been reported. The IR-absorption band of the methoxyl group [21] at 1 078 cm^{-1} has the same position as that of spirilloxanthin.



(II)

The biological role of P-481 as an intermediate between lycopene and spirilloxanthin in the biosynthesis of carotenoids of *Rhodospirillum rubrum* has been established by Stanier *et al.*[14].

Combining chemical and biological evidence, it is tentatively suggested that the P-481-molecule consists of half a lycopene molecule and half a spirilloxanthin molecule.

The author is gratefully indebted to Prof. N. A. Sörensen for helpful advice and valuable discussions throughout this work.

This work was supported by a grant from *Norges Tekniske Högskole*.

1. Polgàr, A., van Niel, C. B. and Zechmeister, L. *Arch. Biochem.* **5** (1944) 243.
2. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **18** (1935) 1306.
3. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 3.
4. van Niel, C. B. and Smith, J. H. C. *Arch. Mikrobiol.* **6** (1935) 219.
5. Karrer, P. and Koenig, H. *Helv. Chim. Acta* **23** (1940) 460.
6. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 1019.
7. Goodwin, T. W., Land, D. G. and Sissins, M. E. *Biochem. J.* **64** (1956) 486.
8. Rabourn, W. J. and Quackenbush, F. J. *Arch. Biochem.* **61** (1956) 111.
9. Haagen-Smit, A. J., Pinchard, J. H. and Zechmeister, L. *Arch. Biochem.* **26** (1950) 358.
10. Kuhn, R. and Grundmann, Chr. *Ber.* **67** (1934) 339.
11. van Niel, C. B. *Bacteriol. Rev.* **8** (1944) 1.
12. Kuhn, R. and Roth, H. *Ber.* **65** (1932) 1285.
13. Goodwin, T. W. and Land, D. E. *Arch. Mikrobiol.* **24** (1956) 305.
14. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochim. et Biophys. Acta* **29** (1958) 477.
15. Goodwin, T. W. and Osman, H. G. *Biochem. J.* **56** (1954) 222.
16. Kuhn, R. and Brockmann, H. *Ber.* **66** (1933) 828.
17. Wallcave, L. and Zechmeister, L. *J. Am. Chem. Soc.* **75** (1953) 4495.

18. Entschel, R. and Karrer, P. *Helv. Chim. Acta* **41** (1958) 402.
19. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules.* 2nd Ed. Methuen and Co. Ltd., London 1958, p. 114.
20. Rosenkrantz, H. and Gut, M. *Helv. Chim. Acta* **36** (1953) 1000.
21. Liaaen Jensen, S. *Acta Chem. Scand.* **12** (1958) 1698.

Received January 17, 1959.

Synthesis of Certain S-Substituted L-Cysteines

DIMITRIOS THEODOROPOULOS*

Department of Medical Biochemistry, University of Gothenburg, Sweden

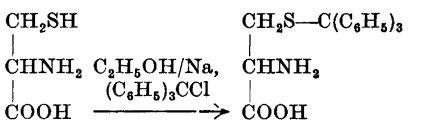
Twenty years ago du Vigneaud [1] introduced the benzylation of the thiol group of cysteine by adding benzyl chloride to the liquid ammonia solution of the sodium salt of cysteine. The S-benzyl group was reversibly split off by reduction with sodium in liquid ammonia. This method has played a fundamental role in the synthesis of various cysteinyl peptides and more recently in the dramatic synthesis of oxytocin [2].

Since the discovery by Weisberger and Levine [3] that leukaemic leukocytes have a high initial cystine-cysteine requirement, several S-cysteine analogues have been synthesized and tested as possible anticancer agents [4]. In most cases du Vigneaud's method has been employed with excellent results. Thus, S-dichlorovinyl-L-cysteine was prepared by McKinney [5] *et al.* in 60—70 % yield.

An alternative procedure for the synthesis of S-alkyl cysteines has been introduced by Zahn and Traumann [6]. Cysteine hydrochloride was treated with the appropriate alkyl bromide in a bicarbonate solution in the presence of nitrogen. Among the products thus produced, S-methyl-L-cysteine and S-ethyl-L-cysteine were obtained in 20 and 10 % yield, respectively. In a similar manner, but in alkaline solution with sodium hydroxide instead of bicarbonate, aminoethylcysteine[7] was synthesized.

In this respect it was found that treatment of cysteine in absolute ethanol with sodium, followed by addition of the appropriate alkyl or phenyl halide, results in the production of the desired S-derivative in excellent yield. Thus, S-benzyl-L-cysteine was synthesized in 70 % yield and its optical value shown to be identical to that reported. Excellent yields were also obtained in the cases of S-butyl-L-cysteine, S-ethyl-L-cysteine and S-methyl-L-cysteine. The thus obtained products were found to be homogeneous according to paper chromatography, as far as the method can detect. This finding suggests that the oxidation of cysteine to cystine is extremely limited or avoided under the experimental conditions described here. On the contrary, the diazonium salt procedure [8,9] for the preparation of S-aryl-L-cysteines gives products always contaminated with cystine, which is difficult to remove.

Treatment of cysteine hydrochloride with trityl chloride in a similar manner, produces S-trityl-L-cysteine in about 20 % yield.



A similar product has been synthesized by selective detritylation of S,N-ditrityl-L-cysteine [10], prepared by direct tritylation [11] of cysteine in the presence of diethylamine, but its reported optical value is entirely different to that described in this parer (see Experimental).

Experimental. S-Benzyl-L-cysteine. To a suspension of 1.75 g (0.01 mole) of L-cysteine hydrochloride monohydrate in 30 ml of absolute ethanol, 0.92 g (0.04 mole) of sodium was added within 10 min at room temperature. When the last piece of sodium was about to disappear, 1.26 g (0.01 mole) of benzyl chloride was added with stirring. After 2 min the reaction mixture was poured into 50 ml of water * and the solution acidified with acetic acid to pH 5—5.5. The desired product precipitated immediately. It was cooled in the refrigerator, filtered and washed successively with water and ethylether. Yield 1.5 g (71 %) m. p. 216—218° (decomp.), reported [1] 216—218° (decomp.),

* Swedish Medical Research Council Postdoctoral Fellow.

* Provided that all the sodium had disappeared.

18. Entschel, R. and Karrer, P. *Helv. Chim. Acta* **41** (1958) 402.
19. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules.* 2nd Ed. Methuen and Co. Ltd., London 1958, p. 114.
20. Rosenkrantz, H. and Gut, M. *Helv. Chim. Acta* **36** (1953) 1000.
21. Liaaen Jensen, S. *Acta Chem. Scand.* **12** (1958) 1698.

Received January 17, 1959.

Synthesis of Certain S-Substituted L-Cysteines

DIMITRIOS THEODOROPOULOS*

Department of Medical Biochemistry, University of Gothenburg, Sweden

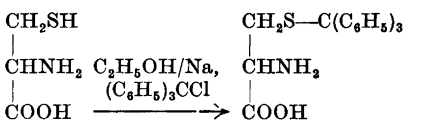
Twenty years ago du Vigneaud [1] introduced the benzylation of the thiol group of cysteine by adding benzyl chloride to the liquid ammonia solution of the sodium salt of cysteine. The S-benzyl group was reversibly split off by reduction with sodium in liquid ammonia. This method has played a fundamental role in the synthesis of various cysteinyl peptides and more recently in the dramatic synthesis of oxytocin [2].

Since the discovery by Weisberger and Levine [3] that leukaemic leukocytes have a high initial cystine-cysteine requirement, several S-cysteine analogues have been synthesized and tested as possible anticancer agents [4]. In most cases du Vigneaud's method has been employed with excellent results. Thus, S-dichlorovinyl-L-cysteine was prepared by McKinney [5] *et al.* in 60—70 % yield.

An alternative procedure for the synthesis of S-alkyl cysteines has been introduced by Zahn and Traumann [6]. Cysteine hydrochloride was treated with the appropriate alkyl bromide in a bicarbonate solution in the presence of nitrogen. Among the products thus produced, S-methyl-L-cysteine and S-ethyl-L-cysteine were obtained in 20 and 10 % yield, respectively. In a similar manner, but in alkaline solution with sodium hydroxide instead of bicarbonate, aminoethylcysteine[7] was synthesized.

In this respect it was found that treatment of cysteine in absolute ethanol with sodium, followed by addition of the appropriate alkyl or phenyl halide, results in the production of the desired S-derivative in excellent yield. Thus, S-benzyl-L-cysteine was synthesized in 70 % yield and its optical value shown to be identical to that reported. Excellent yields were also obtained in the cases of S-butyl-L-cysteine, S-ethyl-L-cysteine and S-methyl-L-cysteine. The thus obtained products were found to be homogeneous according to paper chromatography, as far as the method can detect. This finding suggests that the oxidation of cysteine to cystine is extremely limited or avoided under the experimental conditions described here. On the contrary, the diazonium salt procedure [8,9] for the preparation of S-aryl-L-cysteines gives products always contaminated with cystine, which is difficult to remove.

Treatment of cysteine hydrochloride with trityl chloride in a similar manner, produces S-trityl-L-cysteine in about 20 % yield.



A similar product has been synthesized by selective detritylation of S,N-ditrityl-L-cysteine [10], prepared by direct tritylation [11] of cysteine in the presence of diethylamine, but its reported optical value is entirely different to that described in this parer (see Experimental).

Experimental. S-Benzyl-L-cysteine. To a suspension of 1.75 g (0.01 mole) of L-cysteine hydrochloride monohydrate in 30 ml of absolute ethanol, 0.92 g (0.04 mole) of sodium was added within 10 min at room temperature. When the last piece of sodium was about to disappear, 1.26 g (0.01 mole) of benzyl chloride was added with stirring. After 2 min the reaction mixture was poured into 50 ml of water * and the solution acidified with acetic acid to pH 5—5.5. The desired product precipitated immediately. It was cooled in the refrigerator, filtered and washed successively with water and ethylether. Yield 1.5 g (71 %) m. p. 216—218° (decomp.), reported [1] 216—218° (decomp.),

* Swedish Medical Research Council Postdoctoral Fellow.

* Provided that all the sodium had disappeared.

$[\alpha]_D^{20}$ +21.9° (*c*, 2 in N NaOH), reported [1] $[\alpha]_D^{26.5}$ +23.5° (*c*, 1 in N NaOH).

S-Butyl-L-cysteine. It was prepared from cysteine hydrochloride and butyl bromide in a similar manner as described for the S-benzyl-cysteine derivative. Yield 1.6 g (94 %), m. p. 218—220° (decomp.), $[\alpha]_D^{20}$ —11.2° (*c*, 1 in 2 N HCl). (Found: N 7.50; S 17.82. Calc. for $C_7H_{15}O_2NS$: N 7.89; S 18.08.)

S-Ethyl-L-cysteine. This product was synthesized from L-cysteine hydrochloride and ethyl iodide as previously described, but the reaction mixture was diluted with no more than the necessary amount of water to form a clear solution. The desired product then precipitated upon acidification with acetic acid and cooling. Yield 1 g (82 %), m. p. 226—229° (decomp.), reported [6] m. p. 229°, $[\alpha]_D^{20}$ —27.9° (*c*, 2 in water), reported [6] $[\alpha]_D^{20}$ —25.8° (*c*, 5 in water). (Found: N 9.60; S 21.2. Calc. for $C_5H_{11}O_2NS$: N 9.39; S 21.40.)

S-Methyl-L-cysteine. Methyl iodide was used as the alkylating agent. The product being soluble in water was isolated in a similar manner as described for the S-ethylcysteine derivative. Yield 60 %, m. p. 245—250° (decomp.), reported [6] 251°, $[\alpha]_D^{20}$ —32.3° (*c*, 1 in water), reported [6] $[\alpha]_D^{20}$ —34.5° (*c*, 5 in water). (Found: N 10.15; S 23.20. Calc. for $C_4H_9O_2NS$: N 10.35; S 23.74.)

S-Trityl-L-cysteine. L-Cysteine hydrochloride monohydrate (1.75 g, 0.01 mole) was suspended in absolute ethanol and treated with sodium as described before. Trityl chloride (2.88 g, 0.01 mole) diluted in 6 ml of dry and freshly distilled tetrahydrofuran was added in 4—5 portions during 10 min with stirring. The reaction mixture was then acidified with acetic acid and diluted by addition of 100 ml of dry ether. Upon cooling for 1 h a white precipitate was deposited, which was filtered, washed well with water and finally with ethyl ether. Yield 0.8 g (20 %), m. p. 198—202° (decomp.), reported [10] 202—205° $[\alpha]_D^{20}$ —11.3° (*c*, 1 in 0.1 N NaOH), reported [10] $[\alpha]_D^{20}$ +19°± 1 (*c*, 2 in 0.1 N NaOH). (Found: N 3.5; S 8.6. Calc. for $C_{22}H_{21}I_2NS$: N 3.8; S 8.8.)

Foot note added in proof. Replacement of tetrahydrofuran by ethylether increased the yield in S-trityl-L-cysteine up to 60—70 %. It has also been found that the S-trityl group splits off by reduction with sodium in liquid ammonia at its boiling point. Thus, S-trityl-L-cysteine was converted to S-benzyl-L-cysteine and the optical rotation of the latter was in agreement with that reported above. However, the S-trityl group survives almost completely under the conditions used for the acidic hydrolysis of N-tritylamino acids and peptides [11].

This work was supported by the *Swedish Medical Research Council.*

1. Wood, L. and du Vigneaud, V. *J. Biol. Chem.* **130** (1939) 109.
2. du Vigneaud, V., Ressler, C., Swan, J., Roberts, W., Katsoyannis, P. and Gordon, S. *J. Am. Chem. Soc.* **75** (1953) 4879.
3. Weisberger, A. and Levine, B. *J. Hematol.* **79** (1954) 1082.
4. Goodman, L., Ross, L. and Baker, B. *J. Org. Chem.* **23** (1958) 1251.
5. McKinney, L., Weakley, F., Eldridge, A., Campbell, R., Cowan, J., Picken, J. and Biester, H. *J. Am. Chem. Soc.* **79** (1957) 3932.
6. Zahn, H. and Traumann, K. *Ann.* **581** (1953) 168.
7. Cavallini, D., DeMarco, C., Mondovi, B. and Azzone, G. *Experientia* **11** (1955) 61.
8. du Vigneaud, V., Wood, J. and Binkley, F. *J. Biol. Chem.* **138** (1941) 369.
9. West, H. and Mathura, G. *J. Biol. Chem.* **208** (1954) 315.
10. Amiard, G., Heymes, R. and Velluz, L. *Bull. soc. chim. France* **1956** 698.
11. Zervas, L. and Theodoropoulos, D. *J. Am. Chem. Soc.* **78** (1956) 1359.

Received January 20, 1959.

Enthalpy Titrations with Temperature Adjustment

KURT SCHLYTER and
LARS GUNNAR SILLÉN

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

With the revival of interest in complicated solution equilibria in the last two decades, a considerable amount of data has been collected about systems with several species formed (step-wise complex formation, polynuclear complexes) [1]. In many systems, there is very good evidence for the formulas of the species formed; the equilibrium constants of their formation, and thus the ΔG's of the reactions, are moreover known with a fair accuracy. In order to get a better understanding of such reactions, it would be desirable to break up the ΔG's into their ΔH and ΔS parts; many attempts have been made to calculate these from the variation of equilibrium constants with temperature. However, with the accuracy at present obtainable in systems with several species, and the limited temperature range available, the data of different careful workers very often disagree, even as to the sign of the quantities [2].

One might hope to get much better values for ΔH with a calorimeter, even of moderate accuracy. The traditional way of mixing two solutions, T and S, containing the reagents, would be keeping solution S and solution T in a sealed tube, in a calorimeter, until they have the same temperature, then mixing the solutions by breaking the tube with T and measuring the change of temperature. If only one reaction occurs, its ΔH may be obtained from the average of a relatively small number of such measurements.

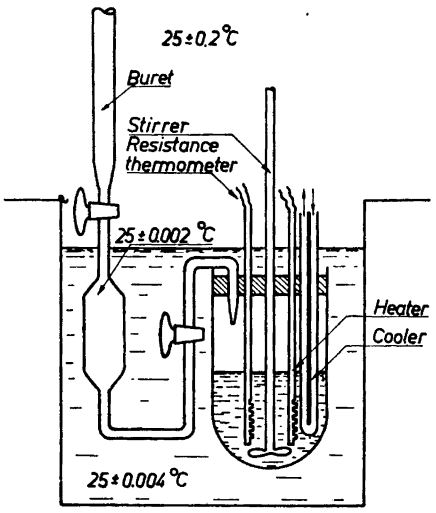


Fig. 1. Principle of calorimeter.

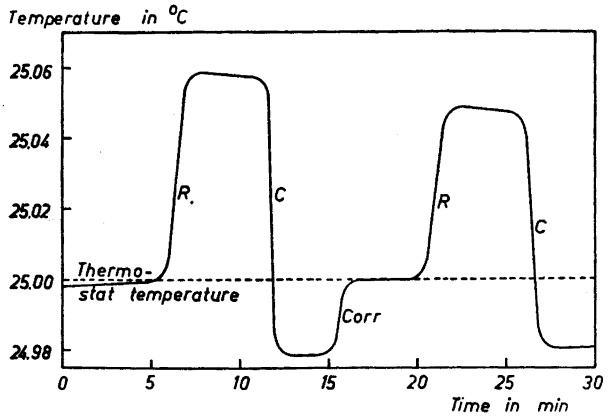


Fig. 2. Temperature history of part of a titration: R = reaction heat evolved; C=cooling applied; corr = temperature corrected by means of heater *etc.*

If, on the other hand, several reactions must be considered simultaneously, a much larger number of experiments of this type, varying the concentrations of both the reactants, would be needed and the method would become rather laborious and time-consuming.

We would suggest, for ΔH work, a titration technique which might give similar advantages over "point measurements" as does the titration technique introduced by Leden [3] for determining equilibrium constants: a much larger number of data for a given amount of time and labor, and a better check for small analytical errors.

It would, however, not be perfectly satisfactory to keep S in a calorimeter, and T in a buret (albeit thermostated), to add T portion-wise to S and measure the temperature in S after each addition. The temperature in S would naturally change during the reactions, so that one would have to make an increasing correction for the temperature difference between T and S. Moreover, the temperature in S might finally drift several degrees from the original one, say 25.0°C. Even if one knows the equilibrium constants for the various reactions at 25.0°C, the constants would now be different, which one would have to consider in the calculations.

These considerations have led us to try another simple device which we think has not been described before. The main parts are shown in Fig. 1, and a typical temperature history during the titration in Fig. 2.

The apparatus is kept in a thermostated room of approximately the standard temperature desired, 25.0°C. The upper part of the buret with solution T is thus air-thermostated. From the buret, T passes through a bulb, which is immersed in a water thermostat which keeps the temperature more accurately, and which also contains the calorimeter with S. The calorimeter is, as usual, provided with a thermometer and an electric heater, used for calibration. The unusual feature is a cooler of thin glass tubing, through which cool air may be blown; heat is then conducted away from the solution through the tube wall to the air. The temperature of S may be adjusted with any reasonable accuracy to equal the standard temperature of the thermostat bath and T; in practice, the final adjustment is best made with the heater, so the cooler is applied to produce a temperature somewhat below the standard one, after which the heat is applied.

The temperature history of the solution S will be as is seen in Fig. 2. Before each addition, the temperature in S is made equal to that in T; the addition is made, the temperature rise is recorded, and then the temperature of S is again brought back to the standard.

In this way, there will be practically no correction for temperature difference between S and T, and the temperature in S, even after an addition, may be kept within, say, 0.1° from the standard so that the equilibrium constants for the standard temperature may be used in the calculations.

An apparatus has been constructed according to these principles by one of us (K.S.) and is now being used to study a number of equilibrium systems. The main advantage is the increased speed of working, and the accuracy is quite satisfactory. A detailed description of the apparatus will appear elsewhere [4].

This work has been supported by *Statens Tekniska Forskningsråd (Swedish Technical Research Council)* and *Statens Naturvetenskapliga Forskningsråd (Swedish Natural Science Research Council)*.

1. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Tables of stability constants, Chem. Soc. Spec. Publ.* **6** (1957) and **7** (1958).
2. Sillén, L. G. *J. Inorg. & Nuclear Chem. 8* (1958) 176.
3. Leden, I. *Z. physik. Chem.* **188 A** (1941) 160.
4. Schlyter, K. *Trans. Roy. Inst. Technol. Stockholm* (1959) no. 132 (=Chem. 2).

Received January 21, 1959.

The Occurrence of Endocrocin in *Penicillium islandicum*

STEN GATENBECK

Institute of Biochemistry, University of Lund, Lund, Sweden

Earlier investigations of the biogenesis of polyhydroxyanthraquinones have shown that the anthraquinone molecule originates from acetate through head to tail couplings of acetate units. As discussed in another paper the experiments with labelled acetate as substrate do not explain the formation of the ring system since the intramolecular

If, on the other hand, several reactions must be considered simultaneously, a much larger number of experiments of this type, varying the concentrations of both the reactants, would be needed and the method would become rather laborious and time-consuming.

We would suggest, for ΔH work, a titration technique which might give similar advantages over "point measurements" as does the titration technique introduced by Leden [3] for determining equilibrium constants: a much larger number of data for a given amount of time and labor, and a better check for small analytical errors.

It would, however, not be perfectly satisfactory to keep S in a calorimeter, and T in a buret (albeit thermostated), to add T portion-wise to S and measure the temperature in S after each addition. The temperature in S would naturally change during the reactions, so that one would have to make an increasing correction for the temperature difference between T and S. Moreover, the temperature in S might finally drift several degrees from the original one, say 25.0°C. Even if one knows the equilibrium constants for the various reactions at 25.0°C, the constants would now be different, which one would have to consider in the calculations.

These considerations have led us to try another simple device which we think has not been described before. The main parts are shown in Fig. 1, and a typical temperature history during the titration in Fig. 2.

The apparatus is kept in a thermostated room of approximately the standard temperature desired, 25.0°C. The upper part of the buret with solution T is thus air-thermostated. From the buret, T passes through a bulb, which is immersed in a water thermostat which keeps the temperature more accurately, and which also contains the calorimeter with S. The calorimeter is, as usual, provided with a thermometer and an electric heater, used for calibration. The unusual feature is a cooler of thin glass tubing, through which cool air may be blown; heat is then conducted away from the solution through the tube wall to the air. The temperature of S may be adjusted with any reasonable accuracy to equal the standard temperature of the thermostat bath and T; in practice, the final adjustment is best made with the heater, so the cooler is applied to produce a temperature somewhat below the standard one, after which the heat is applied.

The temperature history of the solution S will be as is seen in Fig. 2. Before each addition, the temperature in S is made equal to that in T; the addition is made, the temperature rise is recorded, and then the temperature of S is again brought back to the standard.

In this way, there will be practically no correction for temperature difference between S and T, and the temperature in S, even after an addition, may be kept within, say, 0.1° from the standard so that the equilibrium constants for the standard temperature may be used in the calculations.

An apparatus has been constructed according to these principles by one of us (K.S.) and is now being used to study a number of equilibrium systems. The main advantage is the increased speed of working, and the accuracy is quite satisfactory. A detailed description of the apparatus will appear elsewhere [4].

This work has been supported by *Statens Tekniska Forskningsråd (Swedish Technical Research Council)* and *Statens Naturvetenskapliga Forskningsråd (Swedish Natural Science Research Council)*.

1. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Tables of stability constants, Chem. Soc. Spec. Publ.* **6** (1957) and **7** (1958).
2. Sillén, L. G. *J. Inorg. & Nuclear Chem. 8* (1958) 176.
3. Leden, I. *Z. physik. Chem.* **188 A** (1941) 160.
4. Schlyter, K. *Trans. Roy. Inst. Technol. Stockholm* (1959) no. 132 (=Chem. 2).

Received January 21, 1959.

The Occurrence of Endocrocin in *Penicillium islandicum*

STEN GATENBECK

Institute of Biochemistry, University of Lund, Lund, Sweden

Earlier investigations of the biogenesis of polyhydroxyanthraquinones have shown that the anthraquinone molecule originates from acetate through head to tail couplings of acetate units. As discussed in another paper the experiments with labelled acetate as substrate do not explain the formation of the ring system since the intramolecular

condensation of a C—16 polyketoacid and the condensation of two C—8 benzene derivatives will give rise to the same isotopic pattern [1]. In both cases one has to presume a polyhydroxyanthraquinone-carboxylic acid as an intermediate step in the formation of the polyhydroxyanthraquinone. The biological formation of the anthraquinones *via* anthraquinone-carboxylic acids is indicated by the occurrence in nature of a polyhydroxyanthraquinone-carboxylic acid with the carboxyl group in the expected position in the molecule.

Asahina and Fuzikawa [2] have found 4,5,7 - trihydroxy - 2 - methylanthraquinone-3-carboxylic acid (endocrocin) in the lichen *Nephromopsis endocrocea*. The same lichen also produces physcion, the 7-methylether of the decarboxylated endocrocin. Shibata and Natori [3] have isolated endocrocin from the mold *Aspergillus amstelodami*, which also produces catenarin. The formation of catenarin from endocrocin is easily explained by hydroxylation at the 1-position and decarboxylation of endocrocin.

Endocrocin has now been found in a UV-mutant of *Penicillium islandicum*. This strain produces in addition both emodin [4], *i. e.* decarboxylated endocrocin, and catenarin as well as islandicin. This gives further support to anthraquinone-carboxylic acids being intermediates in the formation of the anthraquinones.

In spite of the slight solubility of endocrocin in water, it was mainly found (about 3 mg/l) in the culture medium (pH 4.5) of *Penicillium islandicum*.

The isolated endocrocin decomposed slowly over the range 290—320°C. Asahina and Fuzikawo describe the melting point of endocrocin as decomposition up against 318°C.

The isolated product was soluble in 0.5 N $NaHCO_3$ with a brown red color and gave all the color reactions reported by Asahina and Fuzikawa (ethanolic ferric chloride, a red brown coloration; concentrated H_2SO_4, a red coloration; methanolic magnesium acetate, an orange red coloration). It had an absorption curve that was very close to that of emodin in appearance. The acetylated product was soluble in $NaHCO_3$ and had m. p. 212°C (Asahina and Fuzikawa 212°C).

Thermal decomposition of the isolated endocrocin yielded emodin. The emodin was identified by its absorption spectrum and its m. p. (257°C) which was not depressed on admixture with authentic emodin.

Experimental. Culture conditions. Fourteen flasks, each containing 500 ml of Czapek-Dox medium were cultivated according to Howard and Raistrick [5] for thirty days. The mycelium was filtered off and washed with distilled water.

Isolation of endocrocin. The culture medium was acidified with concentrated HCl and extracted with ether (3 l). After concentrating to about 500 ml the ether solution was treated with 0.5 N $NaHCO_3$ until no more pigment could be removed. The pigments in the aqueous solution were taken up in ether after acidification of the solution with HCl. The ether phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by developing on a thick paper chromatogram (Whatman 3 MM) in chloroform-methanol-formic acid (4 %) (10:1:1). The region containing endocrocin (R_F 0.75) was extracted with acetone. The endocrocin was with difficulty recrystallized from glacial acetic acid, m.p. 290—320°C (decomp.).

Decarboxylation of endocrocin. A small portion of the endocrocin was heated at 320°C for half an hour in a test tube. The emodin which sublimed on the walls of the test tube was rinsed out with acetone and resublimed in vacuum at 200—220°C. The emodin had m.p. 257°C.

Acetylation of endocrocin. About 20 mg of endocrocin were boiled for 2 min with 2 ml of acetic anhydride and a few drops of concentrated H_2SO_4. The reaction solution was poured onto crushed ice after cooling to room temperature. The precipitate formed was filtered off and recrystallized twice from methanol yielding small pale yellow needles with constant melting point (212°C).

1. Gatenbeck, S. *Acta Chem. Scand.* **12** (1958) 1211.
2. Asahina, Y. and Fuzikawa, F. *Ber.* **68** (1935) 1558.
3. Shibata, S. and Natori, S. *Pharm. Bull. Japan* **1** (1953) 160.
4. Gatenbeck, S. *Acta Chem. Scand.* **12** (1958) 1985.
5. Howard, B. H. and Raistrick, H. *Biochem. J.* **44** (1949) 227.

Received February 6, 1959.

Über die equatoriale oder axiale Anknüpfung der Hydroxylgruppe bzw. der Alkoxylgruppe am glycosidischen Kohlenstoffatom der α- und β-D-Glucopyranosen und der α- und β-D-Galactopyranosen

JAKOB BLOM

Laboratorium der Brauerei Tuborg, Kopenhagen, Dänemark

In sechsgliedrigen Ringsystemen, ob carbocyclisch oder heterocyclisch, sind die stabileren Strukturen als Stuhl-Konformationen aufgebaut. In einer Stuhl-Konformation können die Substituenten entweder eine axiale a- oder eine equatoriale e-Anknüpfung am Ring haben [1]. Die stabilere Stuhl-Konformation ist diejenige, welche die grösste Anzahl von e-Substituenten trägt. In einer Stuhl-Konformation des *Cyclo*hexans oder des Tetrahydropyrans stehen 1—4, 2—5, 3—6, also *para* Substituenten paarweise in *cis* Stellung, wenn die Anknüpfung e, a oder a, e ist, in *trans* Stellung, wenn die Anknüpfung e, e oder a, a ist [2].

I. Physikalisch - chemische Methoden

Schmelzpunkte (Tab. 1). Bekanntlich hat eine *trans*-Äthylenverbindung einen höheren Schmelzpunkt als die isomere *cis*-Verbindung. Erweitert man die Doppelbindung zu einem *Cyclo*hexanring hat man dasselbe Bild. Selbst bei hexa- und pentasubstituierten *Cyclo*hexanen gilt diese Regel: Eine Verbindung mit einer höheren Anzahl von *para-trans*-Substituenten hat einen höheren Schmelzpunkt als die isomere Verbindung mit einer geringeren Anzahl. In Verbindungen mit einem Tetrahydropyranring wie Methyl- und Äthyl-Glycoside der α- und β-D-Glucose und der α- und β-D-Galactose gilt dieselbe Regelmässigkeit, wenn man der Alkoxylgruppe in den α-Derivaten eine equatoriale, in den β-Derivaten eine axiale Anknüpfung gibt. Alle α-Methyl-Glycoside der D-Aldopyranosen haben dieselbe Konfiguration bei C_1, alle β-Methyl-Glycoside dieselbe aber umgekehrte Konfiguration bei C_1 [3]. D-Glucose und D-Galactose unterscheiden sich nur durch die e- bzw. a-Anknüpfung der Hydroxylgruppe bei C_4. Diese beiden Facta bedingen, dass von den Glycosiden der D-Glucose das Glycosid mit equatorialer Alkoxylgruppe den höheren Schmelz-

Tabelle 1. Schmelzpunkte.

Äthylen							Anzahl *trans*			COOH
Fumarsäure			C=C				1			287
Maleinsäure			C=C				0			130
*Cyclo*hexan	C_1	C_2	C_3	C_4	C_5	C_6	Anzahl *para trans*	OH	Cl	COOH
Di-	e	C	C	e	C	C	1	143		309
Di-	a	C	C	e	C	C	0	112		171
Scyllo-Inositol	e	e	e	e	e	e	3	352	300	
Neo-	a	e	e	a	e	e	3	315	217	
Myo-	a	e	e	e	e	e	2	227	137	
Deoxy-*scyllo*-I	e	e	e	e	e	C	2	235		
Deoxy-*myo*-I	a	e	e	e	e	C	1	181		
Tetrahydropyran	C_1	C_2	C_3	C_4	C_5	O		$-CH_3$	$-C_2H_5$	
D-Glucose-α	e	e	e	e	e	O	2	166	114	
D-Glucose-β	a	e	e	e	e	O	1	105	90	
D-Galactose-β	a	e	e	a	e	O	2	178	161	
D-Galactose-α	e	e	e	a	e	O	1	116	141	

Tabelle 2. $[M]_D$.

*Cyclo*hexan		C_1	C_2	C_3	C_4	C_5	C_6	$[M]_D$
Myo-Inositol		a	e	e	e	e	e	0
Myo-2-deoxy-Inositol		a	C	e	e	e	e	— 81
l-Inositol		a	a	e	e	e	e	—117
Tetrahydropyran		**C_1**	**C_2**	**C_3**	**C_4**	**C_5**	**O**	
D-Glucose	α-CH_3	α	e	e	e	e	O	+309
D-Glucose-2-deoxy	α-CH_3	α	C	e	e	e	O	+246
D-Mannose	α-CH_3	α	a	e	e	e	O	+154
D-Glucose	β-CH_3	β	e	e	e	e	O	— 66
D-Glucose-2-deoxy	β-CH_3	β	C	e	e	e	O	— 86
D-Mannose	β-CH_3	β	a	e	e	e	O	—136
D-Galactose	α-CH_3	α	e	e	a	e	O	+381
D-Galactose-2-deoxy	α-CH_3	α	C	e	a	e	O	+303
D-Galactose	β-CH_3	β	e	e	a	e	O	0
D-Galactose-2-deoxy	β-CH_3	β	C	e	a	e	O	0
D-Glucose	α	e	e	e	e	e	O	+202
D-Glucose-1-deoxy		C	e	e	e	e	O	+ 70
D-Glucose	β	a	e	e	e	e	O	+ 34
D-Galactose	α	e	e	e	a	e	O	+272
D-Galactose-1-deoxy		C	e	e	a	e	O	+126
D-Galactose	β	a	e	e	a	e	O	+ 95

punkt haben muss, während von den Glycosiden der D-Galactose das Glycosid mit axialer Alkoxylgruppe diese Position einnimmt.

Molekulare Drehung, $[M]_D$. (Tab. 2). Ersetzt man in der Ring-Methylen-Gruppe, von Monodeoxy-*Myo*inositol oder von 2-Deoxy-Glycosiden [4] von D-Glucose und D-Galactose ein Wasserstoffatom mit einer Hydroxylgruppe so steigt $[M]_D$ bei Einführung einer e-Hydroxylgruppe und fällt bei Einführung einer a-Hydroxylgruppe. Dieser Schluss lässt sich ziehen, da die e- bzw. die a-Anknüpfung der Hydroxylgruppe bei C_2 in den gebildeten Inositolen und Zuckerderivaten bekannt ist. Um ein entsprechendes Steigen oder Fallen von $[M]_D$ bei Einführung einer Hydroxylgruppe in der Ring-Methylengruppe von 1-Deoxy-D-Glucose und 1-Deoxy-D-Galactose zu erreichen, muss in der α-D-Glucose und in der α-D-Galactose die Hydroxylgruppe bei C_1 equatorial, in der β-D-Glucose und in der β-D-Galactose axial gebunden sein.

II. Biochemische und chemische Methoden

Oxydation. Bei der chemischen Oxydation einer sekundären Alkoholgruppe in einem *Cyclo*hexan-Ringsystem werden Verbindungen, welche eine a-Hydroxylgruppe tragen, schneller oxydiert als ihre Epimere, welche eine e-Hydroxylgruppe tragen [5]. Man ist der Ansicht, dass dieses Faktum darauf beruht, dass ein e-Wasserstoffatom sterisch leichter zugänglich ist als ein a-Wasserstoffatom. Bei der Oxydation von Inositolen und Monodeoxy-Inositolen durch *Acetobacter suboxydans* hat man gewisse Relationen zwischen Oxydabilität und Konstitution ableiten können [6]. In diesem Zusammenhang ist der wichtigste Punkt, dass in der vorgezogenen Konformation nur sekundäre Alkoholgruppen mit einer a-Hydroxylgruppe oxydiert werden. Durch Sauerstoff und Platinoxyd wird in *Myo*inositol dieselbe sekundäre Alkoholgruppe oxydiert als durch *Acetobacter suboxydans*.

Glucoseoxydase [7] ist ein specifisch wirkendes Enzym, es oxydiert α-D-Glucose zu D-Glucono-δ-Lacton, während es α-D-Glucose kaum angreift [8]. Die Oxydation von D-Aldohexosen durch Bromwasser zu δ-Lactonen, verläuft bei den β-Anomeren weit schneller als bei den α-Anomeren [9]. β-Methylglycoside werden bei C_1 durch Chlorwasser oxydiert, α-Methylglycoside langsam oder nicht [10].

Wenn es die leichtere Zugänglichkeit eines e-Wasserstoffatoms ist, welche die Oxydationsgeschwindigkeit von Derivaten von sowohl *Cyclo*hexan als von Tetrahydropyran bestimmt, müssen β-Verbindungen der Zucker am glycosidischen Kohlenstoffatom ein e-Wasserstoffatom tragen, da β-Verbindungen ja weit schneller oxydiert werden als die anomeren α-Verbindungen. β-D-Glucose und β-Methylglucosid haben demnach eine axiale Hydroxylgruppe oder Methoxylgruppe bei C_1.

Hydrolyse von 1,2-Anhydro-D-Glucose. 1,2-Anhydro-D-Glucose-3,4,6-triacetat wird Brigl-Anhydrid [11] genannt. Es enthält einen Äthylenoxyd- oder Epoxyd-Ring. Sowohl in *Cyclo*hexan- als in Tetrahydropyransystemen haben Epoxydringe einen a- und einen e-Brückenkopf. 1,2-Anhydro-D-Glucose hat bei C_2 einen e-Brückenkopf und muss daher bei C_1 einen a-Brückenkopf haben. Bei lang andauerndem Erhitzen (16—104 St.) auf 100° oder 120° addiert das Brigl-Anhydrid verschiedene Verbindungen unter Bildung von α-Glucosiden (Maltose, α,α-Trehalose, Saccharose). Die Ausbeute ist meistens nur gering. Das Brigl-Anhydrid addiert Alkohole mit ausserordentlicher Leichtigkeit. Die Reaktionsprodukte sind sterisch einheitliche β-D-Glucoside. Aus dem Brigl-Anhydrid werden also einerseits α-Glucoside andererseits β-Glucoside gebildet. Eine der Reaktionen muss unter Umlagerung verlaufen: die Frage ist: Welche? Die Umsetzung mit Carbinol verläuft schon bei Zimmertemperatur in wenigen Stunden quantitativ [12]. Unter diesen milden Bedingungen erscheint eine Umlagerung unwahrscheinlich. Es gibt nur eine plausible Erklärung: β-Methyl-D-Glucosid wird ohne Umlagerung gebildet; es hat eine axiale Methoxylgruppe.

Das *Resultat* dieser Untersuchungen steht im Widerspruch mit der heutigen Anschauung. Auf Grund der Übereinstimmung der Ergebnisse mehrerer von einander unabhängigen Methoden möchte ich schon jetzt die Resultate meiner Untersuchungen als "kurze Mitteilung" veröffentlichen. Ein ausführlicher Bericht wird in *Acta Chem. Scand.* erscheinen.

1. Hassel, O. *Tidsskr. Kjemi, Bergvesen, Met.* **3** (1943) 32.
2. Hassel, O. und Ottar, B. *Acta Chem. Scand.* **1** (1947) 929.
3. Jackson, E. L. und Hudson, C. S. *J. Am. Chem. Soc.* **59** (1937) 994.
4. Overend, W. G. und Stacey, M. *Advances in Carbohydrate Chem.* **8** (1953) 94.
5. Barton, D. H. R. *J. Chem. Soc.* **1953** 1035.
6. Magasanik, B., Franzl, R. E. und Chargaff, E. *J. Am. Chem. Soc.* **74** (1952) 2618.
7. Müller, D. *Biochem. Z.* **199** (1928) 136.
8. Keilin, D. und Hartree, E. F. *Biochem. J.* **50** (1952) 331.
9. Isbell, H. S. und Pigman, W. W. *J. Research Natl. Bur. Standards* **18** (1937) 141.
10. Dyfverman, A., Lindberg, B. und Wood, D. *Acta Chem. Scand.* **5** (1951) 253; Lindberg, B. und Wood, D. *Ibid.* **6** (1952) 791.
11. Brigl, P. *Z. physiol. Chem. Hoppe-Seyler* **122** (1922) 245.
12. Hardegger, E. und de Pascual, J. *Helv. Chim. Acta* **47** (1948) 281.

Eingegangen am 21. Januar 1959.

Column Electrophoresis of Nucleotides from Ribonucleic Acid of Brain Microsomes

NORA FRONTALI*

Institute of Biochemistry, University of Uppsala, Sweden

Analysis of the nucleotide composition of ribonucleic acids (RNA) is usually accomplished by ion exchange chromatography or by paper electrophoresis. This latter technique is extremely simple and quick, but it allows the handling of only limited amounts of material. In the present work column electrophoresis has been used, and has proved a good tool for this purpose. It has been employed for analysing the nucleotide composition of RNA from brain microsomes, which are similar to liver and pancreas microsomes in showing a high content of RNA [1].

The separations were performed on a cellulose powder column according to Porath [2], and with elution beginning before the end of the electrophoretic run for better resolution [3]. Samples of hydrolysates from about 6 mg RNA were handled in the present experiments, but at least four times higher amounts can be as well separated on the same column (2 cm diameter, 70 cm length). Microsomes were prepared by

* Permanent adress: Istituto Superiore di Sanità, Rome.

Wenn es die leichtere Zugänglichkeit eines e-Wasserstoffatoms ist, welche die Oxydationsgeschwindigkeit von Derivaten von sowohl *Cyclo*hexan als von Tetrahydropyran bestimmt, müssen β-Verbindungen der Zucker am glycosidischen Kohlenstoffatom ein e-Wasserstoffatom tragen, da β-Verbindungen ja weit schneller oxydiert werden als die anomeren α-Verbindungen. β-D-Glucose und β-Methylglucosid haben demnach eine axiale Hydroxylgruppe oder Methoxylgruppe bei C_1.

Hydrolyse von 1,2-Anhydro-D-Glucose. 1,2-Anhydro-D-Glucose-3,4,6-triacetat wird Brigl-Anhydrid [11] genannt. Es enthält einen Äthylenoxyd- oder Epoxyd-Ring. Sowohl in *Cyclo*hexan- als in Tetrahydropyransystemen haben Epoxydringe einen a- und einen e-Brückenkopf. 1,2-Anhydro-D-Glucose hat bei C_2 einen e-Brückenkopf und muss daher bei C_1 einen a-Brückenkopf haben. Bei lang andauerndem Erhitzen (16—104 St.) auf 100° oder 120° addiert das Brigl-Anhydrid verschiedene Verbindungen unter Bildung von α-Glucosiden (Maltose, α,α-Trehalose, Saccharose). Die Ausbeute ist meistens nur gering. Das Brigl-Anhydrid addiert Alkohole mit ausserordentlicher Leichtigkeit. Die Reaktionsprodukte sind sterisch einheitliche β-D-Glucoside. Aus dem Brigl-Anhydrid werden also einerseits α-Glucoside andererseits β-Glucoside gebildet. Eine der Reaktionen muss unter Umlagerung verlaufen: die Frage ist: Welche? Die Umsetzung mit Carbinol verläuft schon bei Zimmertemperatur in wenigen Stunden quantitativ [12]. Unter diesen milden Bedingungen erscheint eine Umlagerung unwahrscheinlich. Es gibt nur eine plausible Erklärung: β-Methyl-D-Glucosid wird ohne Umlagerung gebildet; es hat eine axiale Methoxylgruppe.

Das *Resultat* dieser Untersuchungen steht im Widerspruch mit der heutigen Anschauung. Auf Grund der Übereinstimmung der Ergebnisse mehrerer von einander unabhängigen Methoden möchte ich schon jetzt die Resultate meiner Untersuchungen als "kurze Mitteilung" veröffentlichen. Ein ausführlicher Bericht wird in *Acta Chem. Scand.* erscheinen.

1. Hassel, O. *Tidsskr. Kjemi, Bergvesen, Met.* **3** (1943) 32.
2. Hassel, O. und Ottar, B. *Acta Chem. Scand.* **1** (1947) 929.
3. Jackson, E. L. und Hudson, C. S. *J. Am. Chem. Soc.* **59** (1937) 994.
4. Overend, W. G. und Stacey, M. *Advances in Carbohydrate Chem.* **8** (1953) 94.
5. Barton, D. H. R. *J. Chem. Soc.* **1953** 1035.
6. Magasanik, B., Franzl, R. E. und Chargaff, E. *J. Am. Chem. Soc.* **74** (1952) 2618.
7. Müller, D. *Biochem. Z.* **199** (1928) 136.
8. Keilin, D. und Hartree, E. F. *Biochem. J.* **50** (1952) 331.
9. Isbell, H. S. und Pigman, W. W. *J. Research Natl. Bur. Standards* **18** (1937) 141.
10. Dyfverman, A., Lindberg, B. und Wood, D. *Acta Chem. Scand.* **5** (1951) 253; Lindberg, B. und Wood, D. *Ibid.* **6** (1952) 791.
11. Brigl, P. *Z. physiol. Chem. Hoppe-Seyler* **122** (1922) 245.
12. Hardegger, E. und de Pascual, J. *Helv. Chim. Acta* **47** (1948) 281.

Eingegangen am 21. Januar 1959.

Column Electrophoresis of Nucleotides from Ribonucleic Acid of Brain Microsomes

NORA FRONTALI*

Institute of Biochemistry, University of Uppsala, Sweden

Analysis of the nucleotide composition of ribonucleic acids (RNA) is usually accomplished by ion exchange chromatography or by paper electrophoresis. This latter technique is extremely simple and quick, but it allows the handling of only limited amounts of material. In the present work column electrophoresis has been used, and has proved a good tool for this purpose. It has been employed for analysing the nucleotide composition of RNA from brain microsomes, which are similar to liver and pancreas microsomes in showing a high content of RNA [1].

The separations were performed on a cellulose powder column according to Porath [2], and with elution beginning before the end of the electrophoretic run for better resolution [3]. Samples of hydrolysates from about 6 mg RNA were handled in the present experiments, but at least four times higher amounts can be as well separated on the same column (2 cm diameter, 70 cm length). Microsomes were prepared by

* Permanent adress: Istituto Superiore di Sanità, Rome.

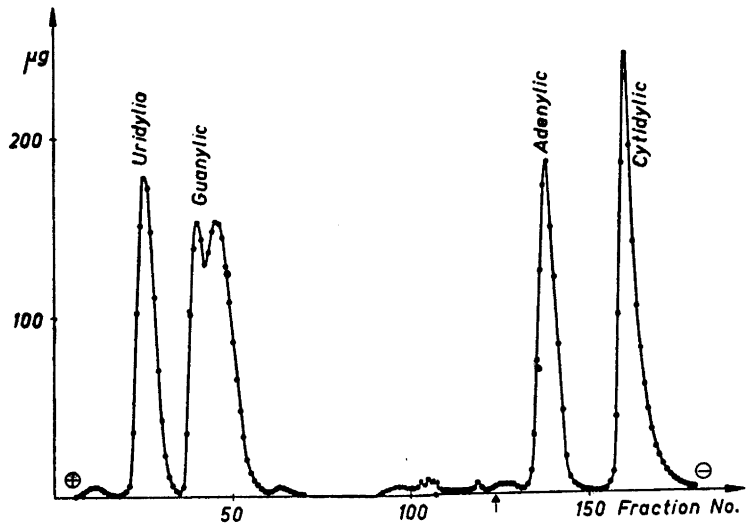


Fig. 1. Electrophoresis of an alkaline hydrolysate of RNA from brain microsomes on a 2 × 70 cm cellulose column with continuous elution device; 0.05 ammonium formate buffer, pH 3.5; 750 V, 22 mA. (Rubber or plastic joints or tubing were avoided as far as possible, as they were found to release UV absorbing material in the formate buffer.) Elution was started after 22 h, collecting fractions of 5 ml every 20 min for 19 h, fractions of 3 ml every 30 min for the next 20 h, and then fractions of 4 ml every 60 min till the end of the electrophoretic run, which lasted 88 h. From that point, marked by an arrow, elution in the usual way was started. The pattern is expressed as μg present in each fraction.

differential centrifugation from a homogenate of rat brain in 0.25 M sucrose. The fraction sedimenting between 18 000 × *g* (15 min) and 105 000 × *g* (60 min) was immediately precipitated with trichloroacetic acid (10 % final concentration). The precipitate was washed with trichloroacetic acid and extracted thoroughly with organic solvents. All these operations were performed keeping the temperature as close as possible to 0°, in order to minimize any possible enzymic RNA hydrolysis. The dry precipitate was hydrolysed with 0.3 M KOH at 37° for 18 h. The hydrolysate was neutralized with perchloric acid, and deproteinized by adding more perchloric acid (0.4 M final concentration). The supernatant after centrifugation was neutralized with KOH, and the perchlorate which precipitated overnight at −5° was discarded. This supernatant was used for electrophoresis, either as such or after freeze-drying.

The results of one electrophoretic separation with 0.05 M ammonium formate buffer pH 3.5 are shown in Fig. 1. The peaks of the four nucleotides, which have been identified from their relative mobilities in comparison with pure compounds, and from their spectra, are completely separated under these conditions. There is in addition an incomplete separation of the two isomers of guanylic acid, as has already been observed by paper electrophoresis at the same pH [4]. The small peaks are probably formed by a small aliquot of oligonucleotides.

The spectrophotometric measurement of the optical densities at 260 mμ of the fractions collected permitted the calculation of the relative amounts of the four nucleotides, as reported in Table 1. The extinction coefficients given by Beaven *et al.*[5] were used. The total sum accounts for 90 % of the total nucleotide content of the original hydrolysate, calculated according to Scott *et al.*[6] If the minor peaks are also taken into account, and their amount approximately calculated with the same factor, then the recovery is 94 %. The table reports for

Table 1. Nucleotide composition (in moles per 100 moles) of RNA of brain microsomes, compared with data on other tissues.

	rat brain microsomes	cat brain Ref.[7]	calf thymus microsomes *Ref.[8]*	rat liver Ref.[9]
Uridylic acid	19.7	20.6	19.3	20
Guanylic acid	32.5	31.8	33.6	32
Adenylic acid	18.1	21.6	19.1	21
Cytidylic acid	29.7	26.0	28.0	28

* These data have been recalculated, in order to render them comparable.

comparison the only available data on brain RNA [7]. They concern a purified preparation of RNA, obtained from slices of tissue after prolonged incubation. The way of preparation, besides the difference in species, can be the reason for the difference in nucleotide composition. The table reports also some recent data obtained by other authors who have performed, as we have done, direct hydrolysis of the RNA-protein. Although they concern different tissues, they show a close similarity with the present data on brain microsomes.

The fractions corresponding to each peak were pooled and lyophilized twice, in order to remove the ammonium formate. The dry powders, redissolved in a small volume, were used for further analysis. The four recovered nucleotides were submitted to chromatography with various solvents, in order to test their homogeneity. With the solvent *iso*butyric acid-ammonia [10] the uridylic acid gave rise to an additional spot. Its spectrum and chromatographic behaviour correspond to those of the "fifth nucleotide" described by Davis and Allen [11].

The same nucleotides were submitted to electrophoresis on paper at pH 2.5. One new spot separated ahead of guanylic acid. When a sample of guanylic acid was submitted to electrophoresis at similar conditions on a small cellulose column, a small faster migrating peak separated, as can be seen in Fig. 2. Its amount, calculated with the same extinction coefficient as guanylic acid, is 2.3 % of the total guanylic acid, and its spectrum is similar to that of the unknown component 1 B obtained by Kemp and Allen [12] by paper electrophoresis at pH 2.5 of guanylic acid from pancreas RNA.

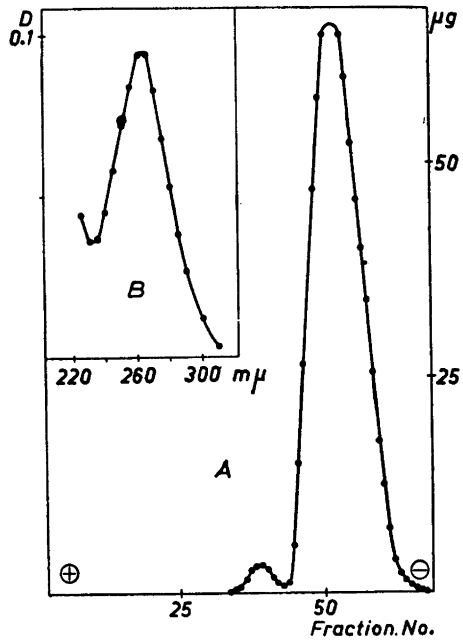


Fig. 2. a) Electrophoresis of brain RNA guanylic acid on a 2 × 18 cm cellulose column, with 0.2 M ammonium formate buffer pH 2.5; 600 V, 70 mA. The elution (5 ml every 5 min) was completed during the electrophoretic run, which lasted 6 h. b) Spectrum of the small, faster migrating peak obtained in a).

Paper electrophoresis of the four nucleotides was performed in borate buffer pH 9.2. One slower migrating compound separated from uridylic acid. It was present also in uridylic acid already deprived from the "fifth nucleotide" by chromatography.

The author wishes to express her gratitude to Dr. J. Porath for constant interest and advice.

1. Toschi, G. *Exptl. Cell Research. In press.*
2. Porath, J. *Biochim. et Biophys. Acta* **22** (1956) 151.
3. Porath, J., Lindner, E. B. and Jerstedt, S. *Nature* **182** (1958) 744.
4. Davidson, J. N. and Smellie, R. M. S. *Biochem. J.* **52** (1952) 599.

5. Beaven, G. H., Holiday, E. R. and Johnson, E. A. *The nucleic acids* edited by Chargaff, E. and Davidson, J. N., Academic press 1955, p. 493.
6. Scott, J. F., Fraccastoro, A. P. and Taft, E.B. *J. Histochem. and Cytochem.* **4** (1956) 1.
7. De Luca, H. A., Rossiter, R. J. and Strickland, K. P. *Biochem. J.* **55** (1953) 193.
8. Osawa, S., Takata, K. and Hotta, Y. *Biochim. et Biophys. Acta* **28** (1958) 271.
9. Ledig, M., Weill, J. D. and Mandel, P. *Bull. soc. chim. biol.* **40** (1958) 599.
10. Magasanik, B., Vischer, E., Donoger, R., Elson, D. and Chargaff, E. *J. Biol. Chem.* **186** (1950) 37.
11. Davis, F. F. and Allen, F. W. *J. Biol. Chem.* **227** (1957) 907.
12. Kemp, J. W. and Allen, F. W. *Biochim. et Biophys. Acta* **28** (1958) 51.

Received January 24, 1959.

Adenosine Triphosphatase in Chloroplasts

HERRICK BALTSCHEFFSKY

Wenner-Gren Institute, University of Stockholm, Sweden

The following two hypotheses are strongly supported by experimental evidence. 1. Light-induced phosphorylation (LIP) is an electron transport phosphorylation [1,2]. 2. The ATP-ase * reactions in mitochondria mirror oxidative phosphorylation (OP) reactions, in the reverse direction, going from ATP towards the electron transport chain [3].

Assuming that hypothesis 1 is correct, the question arises as to what extent similar or identical mechanisms are operating in the generation of ATP in OP and in LIP. Assuming that hypothesis 2 is correct, an investigation of the ATP-ase reactions in LIP systems should give information about the possible similarity between the two phosphorylation mechanisms.

Of the two known LIP systems, plant chloroplasts and bacterial chromatophores, the former have been obtained free from respiratory activity. In spinach, the most investigated plant, this activity is found in the mitochondria, which can be removed. In the photosynthetic bacteria, those fractions which show LIP have not yet been obtained free from the respiratory system. Thus the ATP-ase activity found in these bacteria [4-6] may reflect, at least partly, the OP rather than the LIP system.

A study was made of the ATP-ase activity in isolated spinach chloroplasts. They were prepared ** by the method of Allen, Whatley and Arnon [7]. Chloroplasts, freed of mitochondria, do not respire, according to these authors. In agreement with this, the oxygen uptake was zero with succinate as substrate and very low with DPNH. Arnon [8] has reported that the chloroplasts seem to be rather free of ATP-hydrolyzing enzymes. We obtain, however, a considerable ATP-ase activity in the presence of added Mg++.

The Mg++-dependent ATP-ase activity of whole chloroplasts and chloroplast fragments, prepared as in Ref.[8], and the strong inhibition caused by atebrin are shown in Table 1. Löw [9-11] has shown that atebrin and chloropromazine inhibit mitochondrial Mg++-activated ATP-ase and that some other ATP-hydrolyzing enzymes, which are not connected with electron transport systems, are unaffected. 1 mM chloropromazine gave about 85 % inhibition with chloroplast fragments.

Table 1. Mg++-dependent ATP-ase in spinach chloroplasts. Medium: 0.3 ml 0.1 M tris-(hydroxymethyl)aminomethane pH 7.5, 10 μmoles ATP, 8 μmoles $MgCl_2$. Where added: whole chloroplasts or chloroplast fragments equivalent to 0.5 mg chlorophyll, 14 μmoles atebrin. Final volume 2 ml, 20 min incubation, 30°C. Phosphate was measured according to Lindberg and Ernster [12].

	μmoles P liberated/h/mg chlorophyll
Whole chloroplasts	9
» » + atebrin	0.7
Chloroplast fragments	9
» » + atebrin	0.4

* Abbreviations: P, orthophosphate; ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; DPNH, reduced diphosphopyridine nucleotide; M, moles per liter.

** Many thanks are due to Dr. M. B. Allen for showing the author how to isolate spinach chloroplasts.

5. Beaven, G. H., Holiday, E. R. and Johnson, E. A. *The nucleic acids* edited by Chargaff, E. and Davidson, J. N., Academic press 1955, p. 493.
6. Scott, J. F., Fraccastoro, A. P. and Taft, E.B. *J. Histochem. and Cytochem.* **4** (1956) 1.
7. De Luca, H. A., Rossiter, R. J. and Strickland, K. P. *Biochem. J.* **55** (1953) 193.
8. Osawa, S., Takata, K. and Hotta, Y. *Biochim. et Biophys. Acta* **28** (1958) 271.
9. Ledig, M., Weill, J. D. and Mandel, P. *Bull. soc. chim. biol.* **40** (1958) 599.
10. Magasanik, B., Vischer, E., Donoger, R., Elson, D. and Chargaff, E. *J. Biol. Chem.* **186** (1950) 37.
11. Davis, F. F. and Allen, F. W. *J. Biol. Chem.* **227** (1957) 907.
12. Kemp, J. W. and Allen, F. W. *Biochim. et Biophys. Acta* **28** (1958) 51.

Received January 24, 1959.

Adenosine Triphosphatase in Chloroplasts

HERRICK BALTSCHEFFSKY

Wenner-Gren Institute, University of Stockholm, Sweden

The following two hypotheses are strongly supported by experimental evidence. 1. Light-induced phosphorylation (LIP) is an electron transport phosphorylation [1,2]. 2. The ATP-ase * reactions in mitochondria mirror oxidative phosphorylation (OP) reactions, in the reverse direction, going from ATP towards the electron transport chain [3].

Assuming that hypothesis 1 is correct, the question arises as to what extent similar or identical mechanisms are operating in the generation of ATP in OP and in LIP. Assuming that hypothesis 2 is correct, an investigation of the ATP-ase reactions in LIP systems should give information about the possible similarity between the two phosphorylation mechanisms.

Of the two known LIP systems, plant chloroplasts and bacterial chromatophores, the former have been obtained free from respiratory activity. In spinach, the most investigated plant, this activity is found in the mitochondria, which can be removed. In the photosynthetic bacteria, those fractions which show LIP have not yet been obtained free from the respiratory system. Thus the ATP-ase activity found in these bacteria [4-6] may reflect, at least partly, the OP rather than the LIP system.

A study was made of the ATP-ase activity in isolated spinach chloroplasts. They were prepared ** by the method of Allen, Whatley and Arnon [7]. Chloroplasts, freed of mitochondria, do not respire, according to these authors. In agreement with this, the oxygen uptake was zero with succinate as substrate and very low with DPNH. Arnon [8] has reported that the chloroplasts seem to be rather free of ATP-hydrolyzing enzymes. We obtain, however, a considerable ATP-ase activity in the presence of added Mg++.

The Mg++-dependent ATP-ase activity of whole chloroplasts and chloroplast fragments, prepared as in Ref.[8], and the strong inhibition caused by atebrin are shown in Table 1. Löw [9-11] has shown that atebrin and chloropromazine inhibit mitochondrial Mg++-activated ATP-ase and that some other ATP-hydrolyzing enzymes, which are not connected with electron transport systems, are unaffected. 1 mM chloropromazine gave about 85 % inhibition with chloroplast fragments.

Table 1. Mg++-dependent ATP-ase in spinach chloroplasts. Medium: 0.3 ml 0.1 M tris-(hydroxymethyl)aminomethane pH 7.5, 10 μmoles ATP, 8 μmoles $MgCl_2$. Where added: whole chloroplasts or chloroplast fragments equivalent to 0.5 mg chlorophyll, 14 μmoles atebrin. Final volume 2 ml, 20 min incubation, 30°C. Phosphate was measured according to Lindberg and Ernster [12].

	μmoles P liberated/h/mg chlorophyll
Whole chloroplasts	9
» » + atebrin	0.7
Chloroplast fragments	9
» » + atebrin	0.4

* Abbreviations: P, orthophosphate; ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; DPNH, reduced diphosphopyridine nucleotide; M, moles per liter.

** Many thanks are due to Dr. M. B. Allen for showing the author how to isolate spinach chloroplasts.

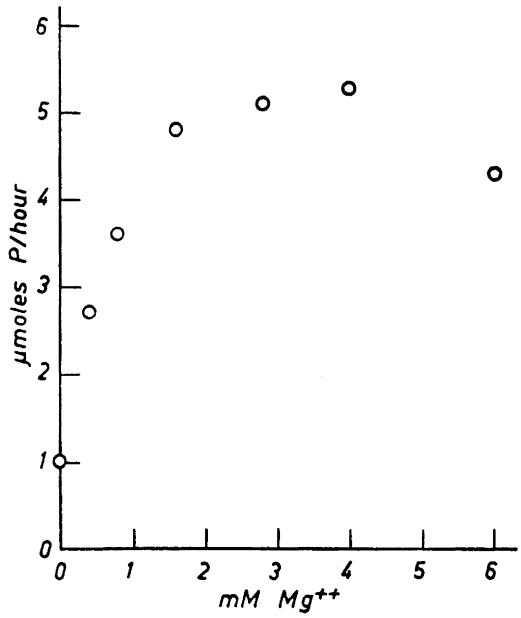


Fig. 1. Conditions as for Table 1, except that $MgCl_2$ was added as is shown in the figure, the amount of chloroplast fragments in each sample was equivalent to 0.68 mg chlorophyll and the incubation time was 30 min.

The requirement of added Mg++ is shown in Fig. 1. The chloroplast fragments used had been given two extra washings before the fragmentation in order to remove even more efficiently any mitochondrial contamination. No oxygen uptake was obtained with these fragments. The ATP-ase activity was optimal with 4 mM Mg++ and it was of roughly the same magnitude as after only one washing.

2,4-Dinitrophenol had no effect on the ATP-ase, neither in the presence nor in the absence of added Mg++.

The results reported show that isolated spinach chloroplasts exhibit a Mg++-dependent ATP-ase activity. As revealed by its sensitivity to atebrin and chloropromazine this reaction is similar to the Mg++-dependent ATP-ase of animal mitochondria. It is tempting to think that the ATP-ase found in chloroplasts is connected with LIP and that the results obtained support both hypotheses 1 and 2, showing a similarity between the phosphorylation mechanisms in OP and LIP.

1. Arnon, D. I., Whatley, F. R. and Allen M. B. *Biochim. et Biophys. Acta* **16** (1955) 607.
2. Frenkel, A. W. *J. Biol. Chem.* **222** (1956) 823.
3. Siekewitz, P., Löw, H., Ernster, L. and Lindberg, O. *Biochim. et Biophys. Acta* **29** (1958) 378.
4. Newton, J. W. and Kamen, M. D. *Biochim. et Biophys. Acta* **25** (1957) 462.
5. Baltscheffsky, H. and Baltscheffsky, M. *Acta Chem. Scand.* **12** (1958) 1333.
6. Cooper, C. *Biochim. et Biophys. Acta* **30** (1958) 529.
7. Allen, M. B., Whatley, F. R. and Arnon, D.I. *Biochim. et Biophys. Acta* **27** (1958) 16.
8. Arnon, D. I. *Ann. Rev. Plant Physiol.* **7** (1956) 325.
9. Löw, H. *Biochim. et Biophys. Acta* **32** (1959) 1.
10. Löw, H. *Ibid* **32** (1959) 11.
11. Löw, H. *Exptl. Cell Research* **16** (1959) 456.
12. Lindberg, O. and Ernster, L. in Glick, D. *Methods of Biochemical Analysis*, Interscience Publishers, New York 1956, Vol. 3 p. 1.

Received January 27, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 395—408

Acid-Soluble Nucleotides in the Unfertilized Eggs of the Sea-Urchin *Paracentrotus lividus*

ROY NILSSON

Department of Zoophysiology, University of Lund, Sweden

The acid-soluble nucleotides of the unfertilized eggs of the sea-urchin *Paracentrotus lividus* have been analysed by ion-exchange and paper chromatography. The following compounds have been identified: AMP*, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CTP and inosine. An UDP-derivative and two compounds containing CMP have not been fully identified.

In their recent work on the RNA and DNA content of the sea-urchin embryo during early embryonic life Agrell and Persson [1] have also studied the variations in the acid-soluble nucleotide fraction. Only optical densities in the ultraviolet were measured and no attempt was made to identify the components of this fraction. Other authors, however, have identified some of the nucleotide compounds in the unfertilized echinoderm egg. Chambers and Mende [2] have shown by the use of barium-precipitation and differential spectrophotometrical methods that the eggs of *Asterias forbesii* and *Strongylocentrotus dröbachiensis* contain ATP, ADP, and AMP. Whithley [3] using the same methods found that *S. purpuratus* contains ATP and White and Chambers [4] have shown that purified actomyosin dephosphorylates the calcium salt derived from the trichloroacetic acid extract of *Arbacia punctulata*. The methods hitherto applied do not, however, separate similar components, especially those occuring in small amounts; these disadvantages can be overcame by the use of chromatographic methods. In a recent analysis by ion-exchange chromatography of embryos of *Psamechinus miliaris* the presence of uridine- and guanosine derivatives as well as adenosine compounds was

* AMP = 5′-adenosinemonophospate
ADP = 5′-adenosinediphosphate
ATP = 5′-adenosinetriphosphate
GMP = 5′-guanosinemonophospate
GDP = 5′-guanosinediphosphate
GTP = 5′-guanosinetriphosphate
UMP = 5′-uridinemonophosphate
UDP = 5′-uridinediphosphate
UTP = 5′-uridinetriphosphate
CMP = 5′-cytidinemonophosphate
CTP = 5′-cytidinetriphosphate
TMP = 5′-thymidinemonophosphate
RNA = ribonucleic acid
DNA = deoxyribonucleic acid

demonstrated [5]. The present paper is concerned with an analysis of the acid-soluble nucleotides from sea-urchin eggs by ion-exchange and paper chromatography.

MATERIAL AND METHODS

The sea-urchin species used was *Paracentrotus lividus* collected at *Station Biologique Roscoff*. Most of the animals were of a shallow water form taken at low water; some were a deep water form taken from outside the harbour of Roscoff. The ovaries were placed in beakers containing sea water where the eggs were shed. The egg suspension was filtered through gauze to remove the rests of the ovaries. The eggs were then washed several times (4—5) with sea-water and then twice with a solution of 3.5 % sodium chloride. Before the last washing samples were taken for the counting of the eggs. The last washing was ended by gentle centrifugation (200 g for 2 min) to concentrate the eggs.

Preparation of the acid extract. The washed egg suspension was homogenized in a glass homogenizer or a Waring blendor with an equal volume of 10 % cold trichloroacetic acid and filtered through Hyflo Supercel. The extraction was repeated three times with 5 % trichloroacetic acid. The filtrates were pooled and extracted 6 times with ether to remove the bulk of the trichloroacetic acid, neutralized with a few drops of ammonia and freed from ether by aeration. All operations were performed in the cold (1—2°C).

Ion-exchange chromatography. Dowex-1 (200—400 mesh) in the formate form was used. The pH of the solution to be chromatographed was brought to 9—11 by addition of ammonia. After adsorption the column was washed with water to neutrality and elution was performed by the formic acid — sodium formate system previously used [6]. The effluent was fractionated by a time-operated fraction collector. The optical density of every fraction was read at 250, 260 and 270 mμ. Appropriate fractions were pooled and the nucleotides were concentrated by adsorption on norite from acid solution followed by elution with 50 % ethanol containing 1 % ammonia.

For re-chromatography of some of the most complex fractions obtained in the formate system, a chloride system was used [7], it having been found that nucleotides with identical or very similar elution positions in the formate system were generally well separated in the chloride system.

Paper chromatography. For determination of the purine or pyrimidine components in the different nucleotide fractions one dimensional chromatography in *iso*propanol-hydrochloric acid-water (98:25:27) was used after hydrolysis of the fraction in N hydrochloric acid at 100°C for one hour. For identification of the unhydrolysed fractions they were chromatographed in at least three different solvent systems, in general the following: propanol-ammonia (*d* 0.880)-water (60:30:10), *iso*butyric acid-ammonia (*d* 0.880)-water (66:1:33) and ethanol- M ammonium acetate pH 7.5 (75:30). Descending or ascending chromatography on Whatman No 1 paper was used with developing times of 20—70 h. In some cases the chromatogram was developed twice to secure better separation. The spots were located by photography in ultraviolet light. For quantitative estimations, the spots were cut out and eluted with 0.1 N hydrochloric acid. Phosphorus was located by spraying with the molybdate reagent of Hanes and Isherwood [8]. The chromatograms were further sprayed with ninhydrine to reveal amino acids, and with anilinium hydrogen phthalate to demonstrate the sugar components.

Analytical procedures. Phosphorus was determined by the method of Allen [9]; acid-labile phosphorus was determined after hydrolysis for 10 min at 100°C in N hydrochloric acid.

Purine-bound pentose was estimated by the orcinol method according to Albaum and Umbreit [10] and pyrimidine-bound pentose by the same method after treatment of the nucleotide with bromine according to Massart and Hoste [11]. For the determination of phosphorus and pentose a Klett-Summerson photoelectric colorimeter was used with a red filter.

Optical densities were measured in a Beckman Universal Spectrophotometer, model DU or in a Hilger Uvispek Spectrophotometer. The following extinction coefficients were used: 14 500 for adenosinephosphates at 258 mμ, 10 500 for guanosine phosphates at 256 mμ, 10 080 for uridine phosphates at 262 mμ and 13 000 for cytidine phosphates at 280 mμ. The measurements were generally made in 0.1 M hydrochloric acid.

Oxidation with periodate was carried out according to Dixon and Lipkin [12].

Dephosphorylation with 5'-nucleotidase. For these experiments dehydrated venom of *Crotalus adamenteus* (Ross Allen Reptile Institute, Silver Spring, Florida) was used, following the procedure of Johnson *et al.*[13] The reaction products were identified by paper chromatography and phosphorus analysis.

Determination of the egg count. During constant stirring of the egg suspension a sample was taken with a spoon and quantitatively transferred to a graduated cylinder, where its volume was estimated. The sample was then diluted to appropriate volume from which a 2-ml sample was taken and counted under a microscope with low magnification. The values obtained from triplicate samples agreed within 5 %.

RESULTS

Most of the phosphorus in the trichloroacetic acid extract from unfertilized *P. lividus* eggs was determined as inorganic phosphate. This fraction amounts to about 60 % of the total phosphorus, with the remaining 40 % equally distributed between acid-labile and acid-stable phosphates. The analytical data are shown in Table 1.

Table 1. Determination of the phosphorus content of the trichloroacetic acid extract from unfertilized *Paracentrotus lividus* eggs.

$\mu g/10^6$ eggs				
P_0	P_{10}	P_T	$P_{10}-P_0$	P_T-P_{10}
515	735	915	220	180
537	671	791	134	120
529	707	869	178	162
612	769	982	157	213
765	1 050	1 325	285	275

The phosphorus contents of different batches of eggs are in reasonable agreement with exception of the last one, which shows a considerably higher concentration of phosphorus (Table 1). But the eggs giving this value came from sea-urchins taken from deep water while the other samples originated from the shallow water form.

The ultraviolet absorption spectrum of the neutralized trichloroacetic acid extract had a maximum around 265 mμ and a minimum around 240 mμ (Fig. 1) indicating that the extract might contain ultraviolet absorbing components other than adenosine derivatives. Paper chromatography of the hydrolysed extract in *iso*propanol-hydrochloric acid-water showed that the extract contained derivatives of adenine, guanine, 5'-uridylic acid and 5'-cytidylic acid. Spraying with ninhydrine revealed several amino acids, which impaired the quantitative evaluation of the ultraviolet absorbing spots. It was possible, however, to obtain a rough estimation of the components by two dimensional paper chromatography (Fig. 2). This figure shows strong spots corresponding to AMP, ADP, and ATP and weaker spots with R_F-values which indicate guanosine-, cytidine-, and uridine derivatives.

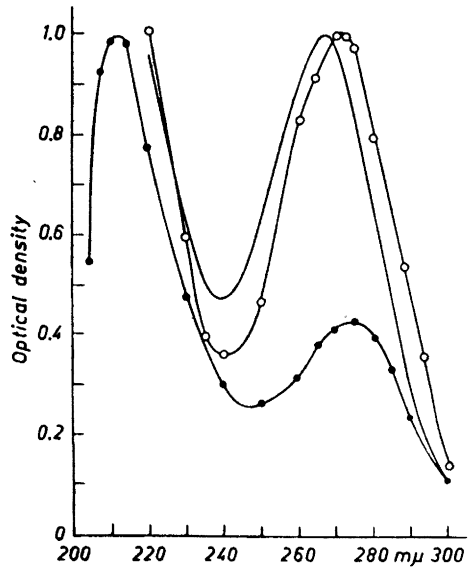


Fig. 1. Ultraviolet absorption curves 1) for the trichloroacetic acid extract from *P. lividus* eggs (—), 2) for the filtrate from the ion-exchange chromatogram of the extract (O—O) and 3) for the norite-eluate derived from the ion-exchange filtrate (●—●).

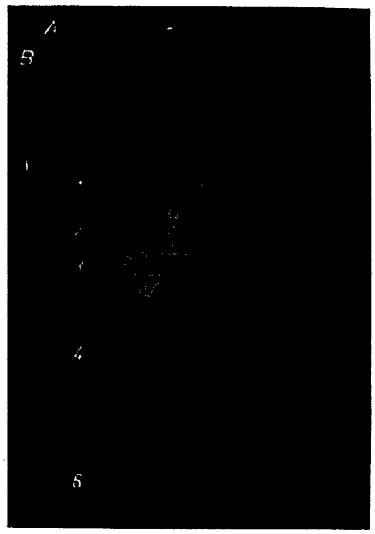


Fig. 2. Two-dimensional paper chromatogram of the extract from *P. lividus* eggs. Solvent: (A) *n*-propanol-ammonia-water (60:30:10) 72 h, (B) saturated ammonium sulphate-*iso*propanol-water (79:2:18) 12 h, Whatman No. 1 paper. (1) AMP, (2) ADP, (3) ATP, (4) guanosine derivatives, (5) uridine derivatives, and (6) cytidine derivatives.

The separation of the different components in the extract can be accomplished by ion-exchange chromatography on Dowex-1; Fig. 3 shows such a separation. While all the nucleotide material was adsorbed on the resin, most of the ultraviolet absorbing non-nucleotide components were removed in the filtrate. The present results indicate that about half of the ultraviolet absorption at 260 mμ in the acid extract is ascribable to non-nucleotide material; of an optical density at 260 mμ of 16 800 put on the column 8 600 were recovered in the filtrate and wash-water giving an ultraviolet absorption curve with a maximum around 273 mμ (Fig. 1). This fraction might contain purine and pyrimidine bases and ribosides which should be adsorbed on norite and then eluated with ammoniacal ethanol or with pyridine. Only about 40 % of the ultraviolet-absorbing material at 260 mμ from the filtrate and wash-water from the column was, however, adsorbed on norite in acid solution, and of the adsorbed material less than 10 % was eluted with 50 % ethanol (2 % ammonia) or with 25 % pyridine indicating the absence of any appreciable amounts of bases or nucleosides. Furthermore the eluates showed a strong ninhydrine reaction and an ultraviolet spectrum in 0.1 N hydrochloric

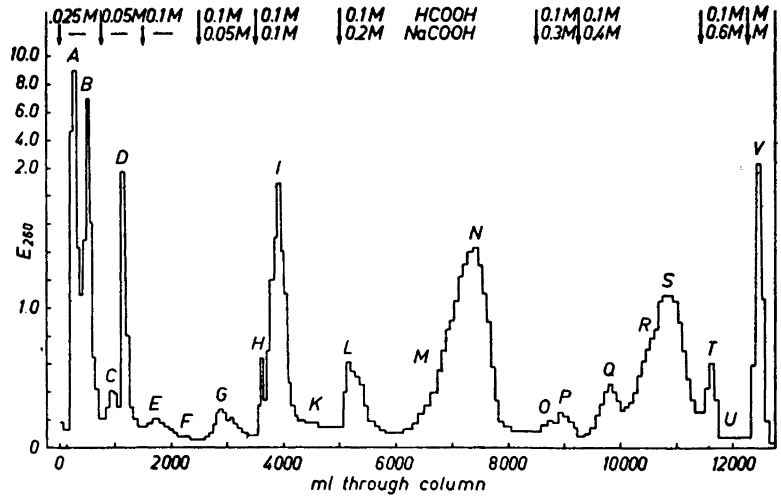


Fig. 3. Ion-exchange chromatogram of a trichloroacetic acid extract from *P. lividus* eggs on a Dowex-1 column (200—400 mesh, 2 cm² × 40 cm) in the formate system. Adsorbed material: extract with optical density E_{260} = 16 800. Recovery 103 %.

acid with maxima at 212 mμ and 275 mμ and a minimum at 250 mμ corresponding to the spectrum of tyrosine (Fig. 1).

Fractional elution of the nucleotides adsorbed on the column gave the separation shown in Fig. 3, resulting in the fractions indicated by the letters A—V. The fractions were concentrated by treatment with norite as described above. The concentrated eluates were then submitted to further analysis.

The different purine and pyrimidine components in the ion-exchange fractions were determined by paper chromatography of the hydrolysed fractions. R_F-values and ultraviolet absorption curves of the eluted spots were used for identification (Table 2).

It is clear from the table, that some of the fractions contained more than one component showing that the ion-exchange separation of the complex mixture of nucleotides in the acid extract of sea-urchin eggs had not been complete. In the first half of the chromatogram the separation was good but some overlapping had occurred between consecutive fractions, *e.g.* between B and C, C and D *etc.* In these cases complete separation could be achieved by preparative paper chromatography in the propanol-ammonia-water system. The mixture was applied to the paper as a line and after development, the formed zones were eluted with 0.01 N hydrochloric acid and concentrated by the norite-method. In those cases where it was obvious that the same components were present in more than one fraction, the eluates were pooled; in other cases the components were analysed separately. In the later part of the ion-exchange chromatogram the separation was not good, and some of the fractions were composed of a mixture of two compounds (fractions R and V)

Table 2. Paper chromatography of the hydrolysed fractions from the ion-exchange chromatogram shown in Fig. 3. R_F-values and ultraviolet-maxima of the eluted spots, together with those of authentic substances. Solvent: *iso*propanol-hydrochloric acid-water (98:25:27). Time: 24 h.

Fraction	R_F-value	UV max mμ	Fraction	R_F-value	UV max mμ
A	0.07	<220	M	0.40	262
	0.35	250		0.67	262
	0.50	280	N	0.40	262
B	0.38	262	O	0.39	262
	0.67	262		0.50	280
C	0.40	262	P	0.39	262
	0.67	262		0.50	280
D	0.50	280	Q	0.22	250
	0.67	263		0.68	262
E	0.38	—	R	0.38	262
F	0.38	—		0.68	262
G	0.22	249	S	0.38	262
H	0.22	249	T	0.38	262
	0.67	262	U	0.38	262
I	0.67	262	V	0.21	250
K	0.67	262		0.38	262
L	0.40	262	Guanine	0.22	249
Adenine	0.38	262	2′-CMP	0.55	280
2′-UMP			3′-CMP		
3′-UMP	0.75	262	5′-CMP	0.50	280
5′-UMP	0.67	262			

which could not be separated by paper chromatography in the propanol-ammonia system. In these cases the separation was achieved by re-chromatography of the fraction on Dowex-1 in the chloride system previously used (Fig. 4).

By these further partitions most of the fractions obtained in the original ion-exchange chromatogram could be divided into subfractions which were homogeneous in the following respects: On paper chromatography in propanol-ammonia-water they gave only one spot, their ultraviolet absorption spectra corresponded to one of the four ribosides adenosine, guanosine, uridine, and cytidine and after hydrolysis they gave only one ultraviolet absorbing spot on paper chromatography in *iso*propanol-hydrochloric acid-water (Table 3). Most of the original ion-exchange fractions were thus divided into components which were well separated and characterized at least with regard to the purine and pyrimidine part of the nucleotides. In some of the smaller fractions, however, the amounts available were not large enough to permit a final identification of the base-component. After hydrolysis fraction F gave a spot with an R_F-value identical with that of adenine; the spot, however, was too weak for determination of the ultraviolet absorption curve. The unpurified fraction did not give any definite nucleotide absorption spectrum; no maximum was obtained, only a shoulder between 250 and 265 mμ indicating the presence of a nucleotide heavily contaminated with non-nucleotide impurities. On

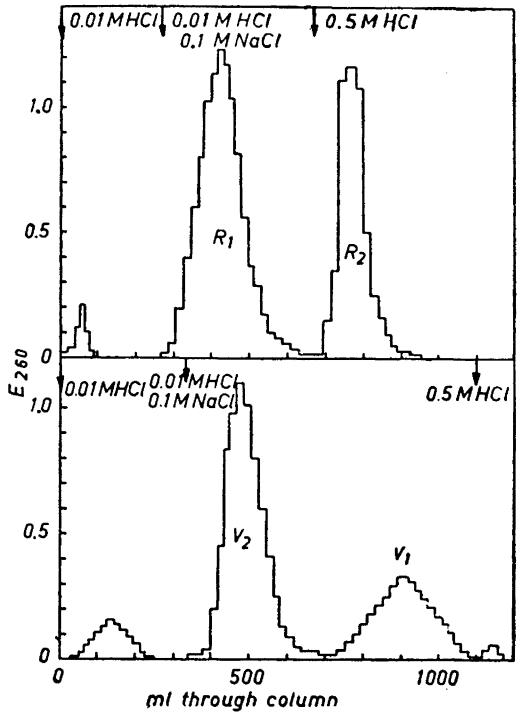


Fig. 4. Chromatogram on a Dowex-1 column (200—400 mesh, 0.3 cm² × 10 cm) in the chloride system of two complex fractions from the ion-exchange chromatogram shown in Fig. 3. Lower diagram fraction V. Upper diagram fraction R.

Table 3. Nucleotide fractions from the ion-exchange chromatogram shown in Fig. 3 after further purification by chromatography on paper or on Dowex-1 chloride. The products of hydrolysis were determined by paper chromatography in *iso*propanol-hydrochloric acid after hydrolysis for one hour at 100°C in N hydrochloric acid.

Fraction	UV max. mμ	Product of hydrolysis	Fraction	UV max. mμ	Product of hydrolysis
B	257	adenine	M_2	258	adenine
C	263	5′-UMP	N	258	adenine
D	265	5′-UMP	O	258	adenine
E	—	adenine	P	280	5′-CMP
F	—	—	Q	256	guanine
G	256	guanine	R_1	258	adenine
H_1	256	guanine	R_2	263	5′-UMP
H_2	263	5′-UMP	S	258	adenine
I	263	5′-UMP	T	258	adenine
K	263	5′-UMP	U	258	adenine
L	258	adenine	V_1	256	guanine
M_1	263	5′-UMP	V_2	258	adenine

hydrolysis fraction C gave not only 5'-UMP but also a faint spot corresponding to TMP, and the same spot was also found when the unhydrolysed fraction was chromatographed in *iso*propanol-hydrochloric acid-water. The ultraviolet absorption curve of the eluted spot had the same shape as that of thymidine with a maximum between 265 and 270 mμ. The amount obtained was, however, too small for further analysis. Fraction A was not included in the table, as it consisted of a mixture of at least four components which could not be resolved by the propanol-ammonia-water solvent. This fraction will be dealt with separately below.

Further identification of the purified fractions was then carried out by determination of acid-labile and total phosphorus and of pentose. The results of the analyses are shown in Table 4.

Some of the fractions from Table 3 are not mentioned in Table 4. Fractions E and F could not be analysed on account of the small amounts available. Furthermore, as mentioned above, some of the consecutive ion-exchange fractions contained the same component; on paper chromatography before and after hydrolysis they thus gave one and the same ultraviolet absorbing spot. Such cases are H_1 which is identical with G, H_2, identical with I, M_2 and O identical with N and fraction R_1, identical with S. T is probably the same substance as S; it appeared as a well defined fraction as the elution solution was changed before the base absorption in fraction S was low enough. Fraction V_2 gave the same analysis and R_F-value as fraction S.

The analysis of the purified ion-exchange fractions together with the establishment of the products of hydrolysis resulted in the identification of the different nucleotide compounds with reference to the purine or pyrimidine

Table 4. Analysis of the purified fractions from the ion-exchange chromatogram shown in Fig. 3.

Fraction	labile P μmole	total P μmole	base μmole	pentose μmole	base:pentose labile P: total P
B	0	0.117	0.118	0.117	1:0.00:—:0.99
C	0	0.122	0.122	—	1:—:—:1.00
D	0	0.170	0.170	0.156	1:0.92:—:1.00
G	0	0.155	0.154	0.151	1:0.98:—:1.01
I	0.109	0.216	0.110	—	1:—:0.99:1.96
L	0.076	0.227	0.181	0.174	1:0.96:0.41:1.25
M_1	0.066	0.134	0.066	0.070	1:1.06:1.00:2.03
N	0.128	0.252	0.126	0.126	1:1.00:1.02:2.00
O	0.094	0.198	0.093	—	1:—:1.01:2.12
P	0.180	0.270	0.091	0.086	1:0.94:1.98:2.96
Q	0.114	0.228	0.114	0.123	1:1.08:1.00:2.00
R_1	0.181	0.272	0.095	0.095	1:1.00:1.91:2.86
R_2	0.306	0.460	0.151	0.141	1:0.93:2.04:3.06
S	0.206	0.312	0.103	0.102	1:0.98:2.00:3.02
T	0.160	0.248	0.082	0.081	1:0.99:1.95:3.02
U	0.140	0.216	0.071	0.072	1:0.99:1.95:3.00
V_1	0.185	0.551	0.185	0.184	1:1.00:1.00:3.00
V_2	0.211	0.309	0.104	0.112	1:1.07:2.03:2.97

Table 5. R_F-values of the nucleotides obtained by ion-exchange chromatography of the trichloroacetic acid extract from unfertilized *P. lividus* eggs. Solvents: (1) *n*-propanol-ammonia (*d* 0.880)-water (60:30:10) 70 h, (2) *iso*butyric acid-ammonia (*d* 0.880)-water (66:1:33) 60 h, (3) ethanol-M ammonium acetate pH 7.5 (75:30) 48 h. R_F-values of authentic specimens are included.

Fraction	R_F-value in system			
	1	2	3	Identified as
B	0.30	0.62	0.25	AMP
C	0.21	0.44	0.32	UMP
D	0.21	0.44	0.31	UMP
E	0.30	0.62	0.25	?
F	0.30	0.62	0.25	?
G	0.15	0.41	0.16	GMP
H_1	0.15	0.40	0.16	GMP
H_2	0.35	0.36	0.50	UDPX
I	0.35	0.36	0.50	UDPX
K	0.35	0.37	0.50	UDPX
L	0.29	0.62	0.25	?
M_1	0.18	0.30	0.17	UDP
M_2	0.22	0.50	0.12	ADP
N	0.21	0.50	0.12	ADP
O	0.21	—	—	ADP ?
P	0.12	0.32	—	CTP
Q	0.12	0.30	0.06	GDP
R_1	0.17	0.45	0.08	ATP
R_2	0.13	0.24	0.10	UTP
S	0.17	0.45	0.08	ATP
T	0.17	0.42	0.09	ATP
U	0.17	0.43	0.08	ATP
V_1	0.11	0.23	0.06	GTP
V_2	0.17	0.44	0.08	ATP
AMP	0.30	0.62	0.25	
ADP	0.21	0.50	0.12	
ATP	0.17	0.42	0.08	
GMP	0.15	0.40	0.16	
GDP	0.13	0.30	0.10	
GTP	0.11	0.23	0.05	
UMP	0.22	0.44	0.32	
UDP	0.17	0.30	0.19	
UTP	0.13	0.25	0.10	
CMP	0.23	0.50	—	
CDP	0.17	0.44	—	
CTP	0.12	0.33	—	

component and to the degree of phosphorylation. The identification was finally checked by paper chromatography in three different solvent systems together with authentic compounds. As developing systems an alkaline, an acid and a neutral solvent were used. The R_F-values in the different systems are shown in Table 5, which also gives the identification of the fractions.

Most of the fractions have been definitely identified as the nucleotides indicated in Table 5 by the analyses shown in Table 4 and by chromatography. Some of the fractions may, however, need some further comments.

Fraction C was eluted from the ion-exchange column by the same eluent as D, which it preceded as a small peak. The main component of C showed the same analytical results and the same R_p-values in four solvent systems as fraction D, a fraction in all respects identical with authentic 5′-UMP. Fraction C also contained AMP but the occurrence of thymidylic acid or some other tymidine derivative as indicated by paper chromatography after hydrolysis in *iso*propanol-hydrochloric acid-water, mentioned above, could not be confirmed by chromatography before hydrolysis in the systems used in Table 5.

On paper chromatography fractions E and F behaved like 5′-AMP but their elution positions on ion-exchange were different from that of 5′-AMP. They may possibly be labile derivatives of 5′-AMP, transformed to 5′-AMP during the working up of the original ion-exchange fractions. They cannot be 2′- or 3′- derivatives as by treatment with 5′-nucleotidase they were completely dephosphorylated to adenosine.

The peak represented by fraction H was possibly a consequence of a premature change of eluting solution resulting in a nearly equimolar mixture of H_1 identical with GMP (G) and H_2 identical with I. Fraction I appeared as a well defined peak entirely free from interfering ultraviolet absorbing compounds. It is a derivative of UDP containing ninhydrine positive components; it has not yet been fully identified. L is also a complex fraction with an analysis suggesting a mixture of several components. The fraction gave, however, only one spot on paper chromatography in the three systems mentioned above. The R_p-values corresponded to those of 5′-AMP, indicating in fraction L the presence of a compound containing the 5′-AMP moiety which probably was released by the norite treatment. ATP was found to occur in several fractions, R_1, S, T, U, and V_2. The ATP samples recovered from all these fractions were, however, in all tested respects identical with ATP isolated from muscle. It was probably the unsatisfactory resolution in the formate system of a mixture of higher phosphorylated nucleotides which was the cause of the broad elution of ATP. The separation was further impaired by the high background absorption given by non-nucleotide components present in the crude extract. If, however, an extract purified by norite treatment was used an ion-exchange chromatogram was obtained in which ATP was eluted only in fractions R and S, T and U were absent and V consisted entirely of GTP.

To complement the identification described above, the fractions were also tested to verify the position of the phosphate groups in the 5′-position. Two analytical methods were used; periodate oxidation and the action of a specific 5′-nucleotidase from snake venom. The results of the analysis are shown in Table 6.

All fractions analysed by periodate oxidation and with 5′-nucleotidase were established as 5′-phosphates; no evidence was obtained for the occurrence of 2′- or 3′-derivatives. Dephosphorylation by snake venom 5′-nucleotidase resulted — under the conditions used — in the liberation of one mole of orthophosphate per mole of mono- or triphosphate, while two moles were liberated from one mole of nucleoside diphosphate. These findings are in agreement with the results of Johnson *et al.*[13]

Table 6. Action of periodate and 5′-nucleotidase on the purified nucleotides from unfertilized *P. lividus* eggs. Oxidation with periodate: the reaction mixture contained in 0.4 ml 0.050 μmole of nucleotide and 0.100 μmole of periodate. The change in extinction at 227 mμ was read against a periodate blank and a water blank. Reaction time 45 min. Action of 5′-nucleotidase: 0.5—1.0 μmole of nucleotide, 0.5 mg of snake venom, 10 μl of 0.5 M $MgCl_2$ and 0.05 M Tris-hydroxymethylaminomethane buffer (pH 8.5) to 2 ml. After 2 h at 38—39°C the reaction mixture was cooled to 0°C and deproteinized by the addition of 0.2 ml of 50 % trichloroacetic acid. After centrifugation the supernatant was analysed for total and inorganic phosphorus. The reaction products were determined by paper chromatography in butanol-formic acid-water (77:10:13).

Fraction	Oxidation with periodate μmole		Action of 5′-nucleotidase μmole		
	nucleotide used	periodate consumed	nucleotide used	P_0 formed	reaction product
B	0.050	0.047	0.52	0.52	adenosine
D			1.00	0.98	uridine
E + F			0.36	0.31	adenosine
G	0.050	0.051	0.55	0.50	guanosine
I	0.050	0.047	0.63	1.39	uridine
L			0.66	1.32	adenosine
M_1	0.050	0.053	0.61	1.18	uridine
M_2	0.050	0.046	0.61	1.18	adenosine
N	0.050	0.047	0.97	1.94	adenosine
P			0.50	0.50	cytidine
Q	0.050	0.048	1.07	2.05	guanosine
R_2	0.050	0.047	0.88	0.90	uridine
S	0.050	0.047	0.60	0.63	adenosine
T	0.050	0.047	1.03	1.07	adenosine
U	0.050	0.055	0.72	0.72	adenosine
V_1	0.050	0.048	0.86	0.89	guanosine
V_2	0.050	0.053	0.88	1.04	adenosine
5′-AMP	0.100	0.102	0.80	0.78	adenosine
2′-,3′-AMP	0.100	0.009	0.74	0.02	2′-,3′-AMP
ADP			0.64	1.26	adenosine
ATP			0.72	0.74	adenosine

Fraction A has hitherto only been mentioned in Table 2 where it was shown that on paper chromatography in *iso*propanol-hydrochloric acid-water the hydrolysed fraction gave three ultraviolet absorbing spots. The spot with the lowest R_F-value was not of purine or pyrimidine type since it did not show any maximum in the region between 220 and 300 mμ. The second spot corresponded to hypoxanthine possibly contaminated with small amounts of adenine, according to the R_F-values and ultraviolet spectra in acid and alkali. The third spot was 5′-CMP according to R_F-values and ultraviolet maxima at 280 mμ and 270 mμ at pH 1 respectively 11.

The components of the unhydrolysed fraction A were then separated by paper chromatography in butanol-formic acid-water (77:10:13). Four spots were obtained; spot I with $R_F = 0.03$, containing cytidylic acid and ninhydrine positive compounds, spot II ($R_F = 0.15$) with hypoxanthine spectrum, spot III ($R_F = 0.28$), a faint spot with an absorption maximum around 260 mμ

and spot IV ($R_F = 0.35$), also a faint spot which on account of the spectrum — maxima at 275 mμ and 213 mμ — and its positive reaction with ninhydrine was identified as tyrosine.

From spot I 5′-CMP was isolated free from ninhydrine reactive components by paper chromatography in *iso*propanol-hydrochloric acid-water and was further characterized by its R_F-values in propanol-ammonia-water (0.22) and *iso*butyric acid-ammonia-water (0.50). Analysis of the pure fraction gave the ration of 1:1.05:0:1.07 for cytosine:pentose:labile phosphate:total phosphate. Furthermore, a consumption of 0.98 moles of periodate per mole base was obtained and on treatment with 5′-nucleotidase 1.00 mole orthophosphate was released with cytidine as the other reaction product. As mentioned above, small amounts of cytidine compounds were also found in fractions B, C, and D and in two other ion-exchange chromatograms larger amounts of three cytidine derivatives were eluted before AMP. One of these was 5′-CMP; on hydrolysis the other two gave 5′-CMP but before hydrolysis they moved differently from 5′-CMP on paper chromatography in ethanol-ammonium acetate (pH 7.5). Both these substances contained phosphorus and pentose and are possibly cytidine derivatives of the type reported by Baddiley *et al.*[14] and by Kennedy and Weiss.[15] Further identification of these compounds had to be postponed.

Spot II obtained from fraction A was identified as inosine. The R_F-values obtained by paper chromatography in four different solvent systems, — propanol-ammonia-water $R_F = 0.35$ (0.36), butanol-formic acid-water (77:10:13) $R_F = 0.15$ (0.15), butanol saturated with 10 % urea $R_F = 0.18$ (0.19) and ethanol-M ammonium-acetate pH 7.5 $R_F = 0.31$ (0.30) — were the same as those of authentic inosine, shown within the brackets. The ultraviolet spectrum of the eluted spot in acid and alkali was identical with that of inosine. The ratio 1:0.95 was obtained for hypoxanthine and pentose; no phosphorus was found. In the same way, spot III was identified as adenosine.

Extracts from four different batches of unfertilized sea urchin eggs were analysed by ion-exchange either as the crude extract (3) or after treatment

Table 7. The nucleotide content of unfertilized *P. lividus* eggs by ion-exchange chromatography of trichloroacetic acid extracts.

Fraction	μmole/10^7 eggs	Fraction	μmole/10^7 eggs
AMP	3.0	UMP	1.0
ADP	3.1	UDP	1.1
ATP	6.0	UDPX	3.3
Unidentified		UTP	1.0
Ad-compounds	1.3	ΣUr-compound	6.4
ΣAd-compounds	13.4	CMP	2.1
GMP	0.3	CTP	0.3
GDP	1.6	Unidentified	2.8
GTP	1.1	Cy-compounds	
ΣGu-compounds	3.0	ΣCy-compounds	5.2
Inosine	0.7		

with norite and with essentially the same results. The amounts of the nucleotide derivatives obtained are shown in Table 7.

The nucleotide content had the following general pattern: nearly one half of the nucleotides consists of adenosine derivatives (49 %) and the other half of equal amounts of uracil- and cytosine compounds (22 % respectively 17 %) and a smaller quantity of guanine nucleotides (10 %).

Even if adenine nucleotides dominate, the distribution of the nucleotides from the different purine- and pyrimidine base is fairly even. The pattern of acid-soluble nucleotides is quite different from that of *e.g.*, skeletal muscle [16] and the body wall musculature of *Annelida* [6] where the nucleotide fraction consists of adenine compounds to 90 %, or from that of the oviduct of the hen which has a high content of uridine nucleotides [17]. Muscle and the oviduct may be taken as examples of organs with a higly specialized function, a function which is reflected in the nucleotide composition; in muscle, which for its work needs easily accessible energy, adenine nucleotides, especially ATP, predominate, while the oviduct — whose main function is thought to be the synthesis of glycoproteins containing hexosaminederivatives — has a high content of uridine compounds, particularly in the form of different UDP-derivatives, which are thought to participate in the biosynthesis of mucopolysacharides.

As an example of reversed conditiones, *i.e.* an organ with a variety of functions, the liver can be taken. The more even composition of the nucleotide fraction of the liver [16] with regard to the different purines and pyrimidines, compared with that of the muscle and the oviduct, may be taken as a consequence of the multiplicity of functions.

Even the unfertilized egg which shall develop into a complex organism, has to have a manifold equipment of different nucleotides as a consequence of the multiplicity of processes essential for the differentiation and development of the embryo. Interesting is the occurrence of rather large amounts of cytosine derivatives, a type of nucleotides until recently not found to be present in biological material in higher concentrations. Kennedy and Weiss isolated different cytidine nucleotides from the liver of the hen and the rat, and have also shown that these compounds take part in the biosynthesis of phospholipids [15]. Furthermore, in a preliminary note Sugino *et al.*[18,19] have reported the occurrence of deoxycytidine derivatives in several organisms, among others in sea-urchin eggs. The unidentified cytosine compounds in the present investigation may consist of similar substances.

The only nucleosides found in the ion-exchange fractions were inosine and traces of adenosine. Part of the ultraviolet absorbing compounds from the extract was, however, not retained by the ion-exchange column; bases and nucleosides could be included in this fraction. But, as mentioned above, the major part of the ultraviolet absorption in the filtrate and wash water from the column consisted probably of amino acids and the only purine or pyrimidine compounds found were small amounts of substances — detected by paper chromatography — with spectra resembling those of uracil and cytosine. Therefore it seems unlikely that the unfertilized sea urchin egg contained larger amounts of free bases and nucleosides.

The further identification of the unknown fractions and the variations in nucleotide content during the mitotic cycle and the early development of the sea-urchin embryo is receiving attention.

I wish to thank the staff of the *Station Biologique, Roscoff*, for their kind help in supplying the sea-urchin material. I also wish to thank Laborator A. Deutsch, Statens Mejeriförsök, Alnarp, for valuable discussions.

This work was financially supported by the *Swedish Natural Science Research Council.*

REFERENCES

1. Agrell, I. and Persson, H. *Nature* **178** (1956) 1396.
2. Chambers, E. L. and Mende, T. J. *Arch. Biochem. Biophys.* **44** (1953) 46.
3. Whiteley, A. H. *Am. Naturalist* **83** (1949) 249.
4. White, W. E. and Chambers, E. L. *Rev. pathol. comparée et hyg. gen*, **613** (1949) 647.
5. Hultin, T. *Exptl. Cell. Research* **12** (1957) 413.
6. Nilsson, R. *Acta Chem. Scand.* **11** (1957) 1003.
7. Deutsch, A. and Nilsson, R. *Acta Chem. Scand.* **7** (1953) 1288.
8. Hanes, C. S. and Isherwood, F. A. *Nature* **164** (1949) 1107.
9. Allen, R. J. L. *Biochem. J.* **34** (1940) 858.
10. Albaum, H. G. and Umbreit, W. W. *J. Biol. Chem.* **107** (1947) 369.
11. Massart, L. and Hoste, J. *Biochim. et Biophys. Acta* **1** (1947) 83.
12. Dixon, J. S. and Lipkin, P. *Anal. Chem.* **26** (1954) 1092.
13. Johnson, M., Kaye, M. A. G., Hems, R. and Krebs, H. A. *Biochem. J.* **54** (1953) 625.
14. Baddiley, J., Buchanan, J. G., Carss, B. and Mathias, A. P. *Biochim. et Biophys. Acta* **21** (1956) 191.
15. Kennedy, E. P. and Weiss, S. B. *J. Biol. Chem.* **222** (1956) 193.
16. Schmitz, H., Potter, V. R., Hurlbert, R. B. and White, D. M. *Cancer Research* **14** (1954) 66.
17. Strominger, J. L. *Biochim. et Biophys. Acta* **17** (1955) 283.
18. Sugino, Y. *J. Am. Chem. Soc.* **79** (1957) 5074.
19. Sugino, Y., Sugino, N., Okazana, R. and Okazaki, T. *Biochim. et Biophys. Acta* **26** (1957) 453.

Received November 24, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 409—414

The Degradation Product from Male Sperm Antagglutin with Molten Potassium Bisulphate

SVANTE ROSS

Institute of Zoophysiology, University of Uppsala, Sweden

The degradation product from male sperm antagglutin with molten potassium bisulphate has been compared with the corresponding products from some model compounds, especially tocopherols and hydroquinone. Some reactions taking place in the molten potassium bisulphate have been studied. The chemical structure of the antagglutin product has been discussed.

Male sperm antagglutin, a substance which in mammalian spermatozoa [1,2], prevents head-to-head agglutination, is a proteid containing sugar [2], sulphuric acid residues [3], and a further component which, probably in a somewhat modified form has been sporadically extracted with ethyl ether from antagglutin preparations. Judging from its ultra-violet spectrum this extractable artefact seems to be an aromatic compound of diphenolic character. It is easily oxidized to a quinone-like substance [4,5].

Heating of male sperm antagglutin in molten potassium bisulphate at 220°C gives a product which is soluble in ethanol and water, and which after oxidation shows a rather high optical density in ultra-violet light with a maximum at 256 mμ. The transformation of the antagglutin into this oxidized compound has been used as a method for quantitative determination of male sperm antagglutin [6]. This oxidized product is very similar to the oxidized form of the above mentioned aromatic substance which was extracted from the antagglutin solutions. As this degraded substance is rather easily formed under controlled conditions, experiments have been performed in order to obtain information about its properties and its precursor in the antagglutin molecule. Unfortunately the formed product is very easily further oxidized and therefore difficult to isolate. For this reason some model substances have been treated in the same way as the antagglutin, and the properties of the obtained products compared with those of the product from antagglutin. Some information about the chemical reactions that may occur in the molten potassium bisulphate have also been obtained from this model experiments.

EXPERIMENTAL AND RESULTS

The treatment of the male sperm antagglutin and the model substances with molten potassium bisulphate has been performed according to the method of quantitative determination of male sperm antagglutin [6], but with some modifications: about 1—3 μg male sperm antagglutin or 0.1—1 mg of the model substance is mixed with a large quantity (1—2 g) of dried, ether-extracted potassium bisulphate. This is done in a test tube with a diameter of one cm and a length of six cm. This reaction tube is placed in a bigger tube in which it is held upright by a layer of glass beads at the bottom. The bigger tube is immersed for one hour into an oil bath of 220°C. After cooling the reaction tube is cleaned externally with ethyl ether, and then reduced to powder in a mortar together with its contents. The powder is extracted with 3 ml of absolute ethanol in a centrifuge tube with a glass stopper. The centrifugated ethanol extract is evaporated under vacuum at 50°C, and the residue extracted several times with 3 ml of freshly distilled, dried ethyl ether. The residue in the distillation flask is taken up in 3 ml of absolute ethanol or distilled water, and its absorption spectrum in ultra-violet light read, especially between 250 and 260 mμ, where the degradation product from male sperm antagglutin has a maximum at 256 mμ. In some cases manganese dioxide is added to the powder during the extraction in order to act as an oxidizing agent, but in other cases the oxidation is performed through aeration in presence of platinum black. A Beckmann DU spectrophotometer was used for the absorption readings.

Hydroquinone disulphonic acid has been synthetized according to v. Hesse [7]. The identity and purity of the obtained product was acertained spectrophotometrically. The other chemicals used are commercial preparations of *pro analysi* purity.

Besides the male sperm antagglutin only few of the tested compounds give products after treatment with molten potassium bisulphate which have absorption maxima at 256 mμ (Table 1), *viz.* α- and δ-tocopherol on the one side, and hydroquinone and some substances which in molten potassium bisulphate may be transformed into hydroquinone, on the other.

The absorption maximum at 256 mμ of the degradation product from male sperm antagglutin disappears, when the solution is made alkaline, but reappears again when the solution is acidified (Fig. 1). The absorption maximum also disappears after hydrogenation with hydrogen and a quantity of platinum black, but reappears after aeration of this solution. The compound with absorption maximum at 256 mμ is very easily oxidized, and the maximum is then

Table 1. Compounds which have been tested with reference to the absorption maximum at 256 mμ after treatment with molten potassium bisulphate.

Aromatic compounds.		Aliphatic compounds.	
Phenol	—	Quinic acid	+
Hydroquinone	+	Inositol	+
Pyrocatechol	—	1,4-*Cyclo*hexadion	—
Resorcinol	—	Glucose	—
Durohydroquinone	—	Fructose	—
Phloroglucinol	—	Arabinose	—
p-Benzoquinone	+		
2,5-Dihydroxybenzoic acid	+		
p-Dimethoxybenzene	—		
α-Tocopherol	+		
δ-Tocopherol	+		
Tyrosine	—		
Tryptophane	—		
Coumarine	—		

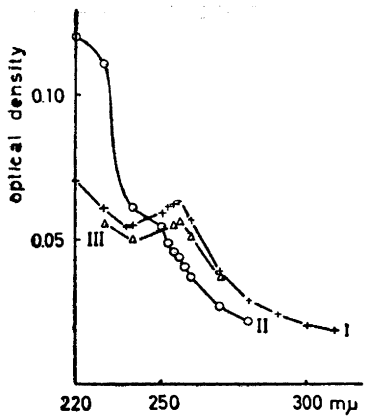


Fig. 1. The absorption in ultra-violet light of the degradation product of male sperm antagglutin with molten potassium bisulphate. I, pH about 4; II, pH about 12; III, pH restored to 4. Solvent distilled water.

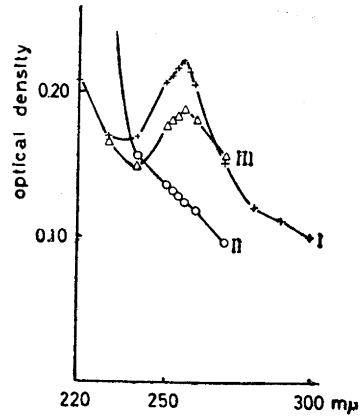


Fig. 2. The absorption in ultra-violet light of the product of α-tocopherylphosphate and molten potassium bisulphate. I, pH about 4; II, pH about 12; III, pH restored to 4. Solvent distilled water.

irreversibly lost. The degraded product from antagglutin is soluble in water and ethanol, but not in ethyl ether (Table 2).

The products from α-tocopherol and its acetate and phosphate-esters and δ-tocopherol after treatment with molten potassium bisulphate have the same properties as those of the degradation product from male sperm antagglutin (Fig. 2 and Table 2). The yields of the products are, however, very poor, only about one hundreth (w/w) of that of the degraded product from antagglutin. Trials to isolate the product from α-tocopherol have failed owing to its sensitivity to further oxidation.

From Table 1 it is obvious that hydroquinone as well as compounds that in molten potassium bisulphate may be transformed into hydroquinone, like quinic acid, inositol, and 2,5-dihydroxybenzoic acid, give a product with an absorption maximum at 256 mμ (Fig. 3:I). With these compounds also a maximum at 312 mμ is obtained which has never been observed with the products from antagglutin or the tocopherols. This absorption maximum at 312 mμ is identical with that of hydroquinone disulphonic acid (Fig. 3:II) which after oxidation with manganese dioxide in 80 % ethanol gives a compound with an absorption maximum at 256 mμ (Fig. 3:III).

This compound is *p*-benzoquinone disulphonic acid, the dipotassium salt of which in aqueous solution at pH 4 has a molar extinction at 256 mμ of about 14 500. Hydroquinone is hence sulphonated in the molten potassium bisulphate which explains the non-solubility of the reaction product in ethyl ether (Table 2).

The product obtained from hydroquinone, although not identical with the product obtained from antagglutin, shows qualities which are similar to those of the latter (Table 2). Thus the absorption maximum at 256 mμ disappears,

Table 2. Some properties of the compounds that give products with an absorption maximum at 256 mμ after treatment with molten potassium bisulphate.

Before treatment			After treatment					
Compound	Absorption maximum	Solubility	Absorption maximum	Absorption maximum after reduction	The pH-dependence of the absorption maximum	Solubility	Oxidability	The resulting product
[I]Male sperm antagglutin	None	Water	256 mμ	None	The maximum disappears in alkaline solution	Water, ethanol; insoluble in ether	Great	
[II]α-Tocopherol	292m μ	Ethanol, ether	»	»	»	»	»	
δ-Tocopherol	297m μ	Ethanol, ether	»	»	»	»	»	
[III]Hydroquinone	290m μ	Ethanol, ether	»	312 mμ	»	»	»	
								p-Benzoquinone disulphonic acid
Quinic acid	None	Water	»	312 mμ	»	»	»	»
Inositol	None	Water	»	312 mμ	»	»	»	»

when the solution is made alkaline, and reappears again, when the solution is acidified. The synthetic *p*-benzoquinone disulphonic acid likewise shows the same qualities (Fig. 4). These substances also agree in being very easily further oxidized, even at room temperature. By this reaction the absorption maxima are lost (Table 2).

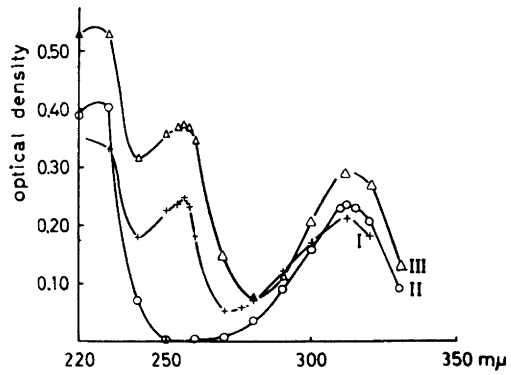


Fig. 3. The absorption in ultra-violet light of the product from hydroquinone and molten potassium bisulphate, I; synthetic hydroquinone disulphonic acid, II; partly oxidized synthetical hydroquinone disulphonic acid, III. Solvent distilled water.

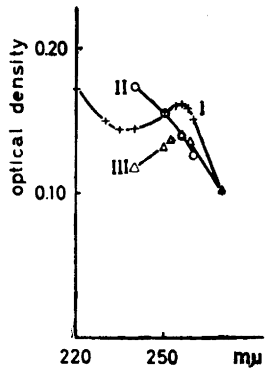


Fig. 4. The pH dependence of the ultra-violet spectrum of quinone disulphonic acid. I, pH about 4; II, pH about 12; III, pH restored to 4. Solvent distilled water.

DISCUSSION

The experiments with the hydroquinone group yield some information of the reactions that can take place in the molten potassium bisulphate. Sulphonation of hydroquinone in this milieu always gives the disulphonic acid. The wellknown dehydration reaction (compare the acrolein formation from glycerol in molten potassium bisulphate) occurs, when inositol and quinic acid are treated with the molten potassium bisulphate. Thus, by this treatment, an aliphatic carbocyclic structure with several hydroxylic groups can thus be transformed into an aromatic one. Sugars do not seem to react in this way (Table 1). The decarboxylation of 2,5-dihydroxybenzoic acid is certainly effected by the rather high temperature in the molten potassium bisulphate.

Of the tested compounds the products from α- and δ-tocopherol are most similar to the antagglutin degradation product. Yet it is surprising that both α- and δ-tocopherol give the same product, although there are three methyl groups in the benzene nucleus in the former, and only one in the latter substance. Durohydroquinone, that has the same benzene nucleus as α-tocopherol, does not give after corresponding treatment any product similar to the degradation product of antagglutin. It is therefore probable that the chromane structure of the α-tocopherol is at least partly retained in the product from α-tocopherol after heating in molten potassium bisulphate.

The aromatic compound of the male sperm antagglutin has been compared with tocopherols [4,5], and it has been shown that the derivative extracted from antagglutin is not identical with any of the known tocopherols [5]. It also appears most probable that this aromatic compound is an artefact originating from an aliphatic precursor constituent bound in the antagglutin molecule [5]. In fact, the intact antagglutin has no specific absorption in ultra-violet light like that of aromatic compounds.

The many resemblances between quinone disulphonic acid and the antagglutin degradation product point (Table 2) to a quinone-like structure, but no absorption maximum after reduction related to a hydroquinone has been detected. The situation of the absorption maximum of the antagglutin degradation product at 256 mμ in connection with the fact that an oxidation

is necessary in order to obtain this maximum makes it reasonable to propose that the chemical structure underlying the spectrum may be a carbonylic substance with two conjugated ethylenic bounds. The transformation in the molten potassium bisulphate of carbocyclic compounds like quinic acid and inositol into hydroquinone disulphonic acid suggests that the precursor constituent of the antagglutin degradation product and also of the extractable aromatic compound mentioned above is a carbocyclic residue with some hydroxylic groups.

Acknowledgment. The author is very much indebted to Professor P. E. Lindahl for advice and valuable discussions and for reading of the manuscript. This study was aided by grants from the *Population Council, New York*, and from the *Swedish Natural Scienes Research Council.*

REFERENCES

1. Lindahl P. E. and Kihlström J. E. *Report of the II International Congress of Physiology and Pathology of Animal Reproduction and of Artificial Insemination*, Copenhagen 1952 p. 70.
2. Lindahl P. E. and Kihlström J. E. *Fertility and Sterility* **5** (1954) 241.
3. Kihlström J. E. *Arkiv Kemi* **7** (1954) 399.
4. Lindahl P. E. and Kihlström J. E. *Nature* **174** (1954) 600.
5. Lindahl P. E., Kihlström J. E. and Ross S. *Acta Chem. Scand.* **10** (1956) 1997.
6. Lindahl P. E., Kihlström J. E. and Ross S. *Acta Chem. Scand.* **11** (1957) 1.
7. von Hesse, O. *Ann.* **110** (1956) 194.

Received November 3, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 415—419

The Crystal Structure of the So-Called δ-Titanium Oxide and Its Structural Relation to the ω-Phases of Some Binary Alloy Systems of Titanium *

STEN ANDERSSON

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

The crystal structure of the so-called δ-titanium oxide has been determined using X-ray powder techniques. The structure may be described as a deficient interstitial solution of oxygen in a metal atom lattice of the type present in the ω-phases of binary alloy systems of titanium.

In 1953 it was reported by Bumps, Kessler and Hansen [1] that, when titanium oxide preparations of compositions ranging from $TiO_{0.66}$ to $TiO_{0.75}$ are annealed at temperatures below 900°C, a previously unknown phase (called δ-titanium oxide) is formed in addition to the solid solution of oxygen in α-titanium and titanium monoxide. Possible formulae suggested for the δ-oxide were Ti_3O_2 or Ti_4O_3. The powder pattern with the exception of one reflexion was said to be in conformity with a tetragonal unit cell with the dimensions $a = 5.333$ Å and $c = 6.645$ Å. Schofield and Bacon [2] confirmed the data given by Bumps *et al.*

Koncz and Koncz-Déri [3] observed the formation of δ-titanium oxide when oxidizing titanium metal at low oxygen pressures within the temperature region 570—820°C. They reported a different tetragonal unit cell ($a = 3.20$ Å and $c = 5.12$ Å) and suggested the composition Ti_3O_2.

EXPERIMENTAL

In the present investigation, samples of compositions ranging from $TiO_{0.5}$ to $TiO_{1.0}$ were kept in sealed, evacuated silica tubes at temperatures between 600° and 800°C for a month. (Details about the titanium oxide specimens employed in this investigation have been given in a previous report [4].) The identification of the samples was performed by means of the X-ray powder patterns obtained in a Guinier focusing camera of 80 mm

* Research sponsored by the Office, Chief of Research and Development, U. S. Department of Army, through its European Office, under contract number DA-91-508-EUC-245.

diameter using strictly monochromatized Cu$K\alpha_1$ radiation and potassium chloride added to the specimens as an internal standard.

The intensities of the powder reflexions were measured by means of an X-ray diffractometer registering the pattern up to $2\Theta = 160°$ with Cu$K\alpha$ radiation. The specimen was diluted with gum arabic in order to avoid orientation as far as possible. The positive effect obtained in this way was strikingly demonstrated when observing the intensities of the reflexions of the Ti_2O oxide which was always present in the sample *(v. infra)*. While the latter intensities were highly anomalous when using an undiluted specimen, the addition of gum arabic lead to values close to the ideal ones.

For studying the oxide at elevated temperatures, the diffractometer was provided with an electric vacuum furnace which allowed the specimen to be heated to 1 000°C.

DETERMINATION OF THE STRUCTURE

The results of the phase analysis of the composition region $TiO_{0.5}$-$TiO_{1.0}$ reported in a previous article [4] were confirmed by the additional experiments included in the present study. Thus the δ-oxide was never obtained pure but was mixed with considerable amounts of the Ti_2O phase in preparations $TiO_{\sim 0.55}$ and with Ti_2O and TiO (low-temperature form) in samples $TiO_{0.6-0.9}$. Evidently it has not been possible to obtain the δ-oxide under equilibrium conditions.

As it was not possible to prepare single crystals of the δ-oxide, the structure determination had to be based exclusively on powder data. The specimen found to be the most suitable for this purpose was $TiO_{0.55}$. After subtracting the Ti_2O lines from the pattern given by this sample, all the remaining reflexions, which should be those of the δ-oxide, could be indexed assuming a hexagonal unit cell with the dimensions

$$a = 4.991_5 \text{ Å}, \qquad c = 2.879_4 \text{ Å}, \qquad V = 62.13 \text{ Å}^3$$

The powder pattern is given in Table 1.

The positions of the lines of the δ-oxide were not perceptibly different in samples of other gross compositions which shows that the homogeneity range of this phase must be narrow.

Table 1. The Guinier powder pattern of δ-TiO_x. Dimensions of the hexagonal unit cell: $a = 4.991_5$ Å, $c = 2.879_4$ Å.

I	$\sin^2\Theta_{obs}$	hkl	$\sin^2\Theta_{calc}$
st	0.09526	110	0.09525
st	0.10332	101	0.10331
w	0.16678	111	0.16681
m	0.19856	201	0.19856
m	0.28590	300	0.28575
		002	0.28623
w	0.29385	211	0.29381
w	0.38136	220	0.38100
		112	0.38148

The following structure was derived from the powder diffractogram:
Unit cell content: 3 TiO_x
Space-group: $P6/mmm$ (No. 191)
1 Ti in 1(*a*): 0,0,0
2 Ti in 2(*d*): $\frac{1}{3},\frac{2}{3},\frac{1}{2}$; $\frac{2}{3},\frac{1}{3},\frac{1}{2}$
3*x*O in 3(*f*): $\frac{1}{2},0,0$; $0,\frac{1}{2},0$; $\frac{1}{2},\frac{1}{2},0$

An attempt was made to estimate the oxygen content (x) of the δ-oxide by calculating the intensities corresponding to various values of x and comparing these data with the observed ones (*cf.* Table 2). This comparison was performed by means of an index of agreement

$$R = \frac{\Sigma \left| \sqrt{I_{obs}} - \sqrt{I_{calc}} \right|}{\Sigma \sqrt{I_{calc}}}$$

In this way the following values for R were obtained

composition	Ti	$TiO_{0.4}$	$TiO_{0.5}$	$TiO_{0.6}$	TiO
R	0.115	0.075	0.070	0.075	0.095

Table 2. Observed intensities and also calculated ones for different values of x in the formula δ-TiO_x. A temperature factor of $B = 1.2$ Å^2 was used.

hkl	I_{obs}	I_{calc}(Ti)	I_{calc}($TiO_{0.5}$)	I_{calc}(TiO)
100	—	0	2	10
001	—	0	0	0
110	100	130	118	103
101	86	94	82	67
200	—	0	3	12
111	24	12	19	25
201	53	36	58	86
210	—	0	0	1
300, 002	40	30	31	32
211	31	38	33	29
102	—	0	0	0
301	—	3	1	1
220, 112	30	30	33	35
310	—	0	0	0
202	—	0	0	1
221	—	2	1	0
311	—	11	10	9
400	—	0	0	0
212	—	0	0	0
302, 401	11	12	13.5	14
320	—	0	0	0
003	—	0	0	0
410, 222	18	12	14	14
321, 103	8	8	7	6
312	—	0	0	0
411	—	1	1	1
113	—	0	0	1
203	—	2	2	3

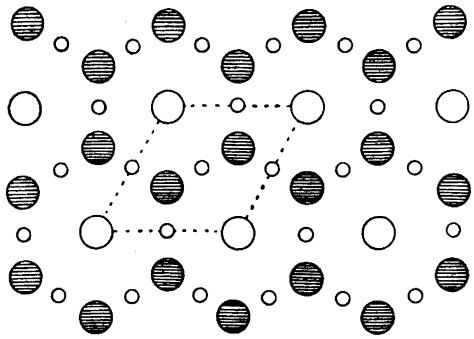


Fig. 1. The crystal structure of δ-TiO_x. Projection after c-axis. Large and small circles represent titanium and oxygen atoms, respectively. Open circles have $z = 0$ and shaded ones $z = \frac{1}{2}$. The oxygen positions are partly vacant in a random distribution.

The best fit should thus correspond to the composition $TiO_{0.5}$. The experimental evidence given above suggests a higher content of oxygen but it may be that the actual composition is not very different from $TiO_{0.5}$.

The thermal expansion of the δ-oxide, which was obtained from the diffractometer data at elevated temperatures (20°—800°C), was found to be 10×10^{-6}/°C in the *a* direction and 9×10^{-6}/°C in the *c* direction.

DISCUSSION OF THE STRUCTURE

The structure thus derived is illustrated in Fig. 1 and the interatomic distances are given in Table 3. The metal atoms of the two atomic positions have rather different environments. The Ti atom in 1(*a*) has two close Ti neighbours at a distance equal to the *c* axis (2.88 Å) and twelve additional Ti atoms at a considerably longer distance (3.22 Å) forming a hexagonal prism. Six oxygen atom positions in the equator plane of this prism occur at a distance of 2.50 Å from the central Ti atom. Each of the Ti atoms in the position 2(*d*) is situated at the common center of a trigonal Ti bipyramid, a trigonal Ti prism and a trigonal O prism (randomly defective). The distances from the central Ti atom to the various atoms of these coordination polyhedra are 2.88, 3.22 and 2.04 Å respectively. The O atoms are surrounded by six Ti atoms, four forming a square and the other two completing an elongated Ti octahedron.

A structure analogous to the one thus derived for δ-titanium oxide but with all the non-metal positions filled is represented by the ε-TaN studied by Brauer [7,8] and Schönberg [9]. The δ-oxide may evidently be considered as having a non-metal deficient ε-TaN structure.

The structure can also be described as an interstitial solution of oxygen in the metal atom structure. This metal atom structure is actually present in the ω-phases occurring in the binary systems of titanium with vanadium [5] and chromium [6]. Thus it seems natural to describe δ-titanium oxide as an interstitial solution of oxygen in a titanium structure of ω-type (ω-TiO_x).

Table 3. Interatomic distances in δ-TiO_x.

Atom	Surrounding atoms	Interatomic distances, Å
Ti in 1(*a*)	2 Ti in 1(*a*)	2.879_4
	12 Ti in 2(*d*)	3.221_4
	6 O in 3(*f*)	2.495_8
Ti in 2(*d*)	2 Ti in 2(*d*)	2.879_4
	3 Ti in 2(*d*)	2.881_8
	6 Ti in 1(*a*)	3.221_4
	6 O in 3(*f*)	2.037_6
O in 3(*f*)	4 Ti in 2(*d*)	2.037_6
	2 Ti in 1(*a*)	2.495_8

Relationships between the structure of the δ-oxide and those of other phases occurring in various titanium systems will be discussed elsewhere.

The shorter Ti-Ti distances of δ-titanium oxide (2.88 Å) are similar in length to those observed for the ordered Ti_2O structure of *anti*-$Cd(OH)_2$ type (2.85 Å) [10]. In the δ-oxide, these close metal-metal contacts are present both parallel and normal to the *c* axis. In Ti_2O the short Ti-Ti distances are inclined to the *c* axis. The similarities between the coefficients of thermal expansion observed for the δ-phase (*v. supra*) and for Ti_2O ($\alpha_c = 10.3 \times 10^{-6}$/°C and $\alpha_a = 9.2 \times 10^{-6}$/°C within the temperature range 20°—500°C) are probably connected with the occurrence of these close metal-metal contacts.

Acknowledgement. The author wishes to express his sincere gratitude to the principal investigator of this research program, Dr. Arne Magnéli, for having suggested this investigation, for his never-failing interest and for many valuable discussions.

REFERENCES

1. Bumps, E. S., Kessler, H. D. and Hansen, M. *Trans. Am. Soc. Metals* **45** (1953) 1008.
2. Schofield, T. H. and Bacon, A. E. *J. Inst. Metals* **84** (1955—56) 47.
3. Koncz, J. and Koncz-Déri, M. *Periodica Polytechnica, Chemical Engineering* **1** No. 1 (1957) 67.
4. Andersson, S., Collén, B., Kuylenstierna, U. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1641.
5. Silcock, J. M., Davies, M. H. and Hardy, H. K. *J. Inst. Metals: Symposium on Mechanism of Phase Transformations in Metals* 1955, p. 93.
6. Bagaryatskii, Y. A., Nosova, G. I. and Tagunova, T. V. *Doklady Akad. Nauk SSSR* **105** (1955) 1225.
7. Brauer, G. and Zapp, K. H. *Naturwiss.* **40** (1953) 604.
8. Brauer, G. and Zapp, K. H. *Z. anorg. Chem.* **277** (1954) 129.
9. Schönberg, N. *Acta Chem. Scand.* **8** (1954) 199.
10. Andersson, S., Åsbrink, S., Holmberg, B. and Magnéli, A. *Symposium on Crystal Physics, National Institute of Sciences (India), Calcutta, Aug. 1958.*

Received November 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 420—424

Addition Compounds of Antimony Chlorides with Sulfoxides and Sulfones

INGVAR LINDQVIST and PALL EINARSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The liquidus curves have been studied for the systems $SbCl_5$—$SO_2(CH_3)_2$, $SbCl_3$—$SO(CH_3)_2$ and $SbCl_3$—$SO_2(CH_3)_2$. The following new compounds have been prepared: $SbCl_5 \cdot SO(CH_3)_2$, $SbCl_5 \cdot SO(C_6H_5)_2$, $SbCl_5 \cdot SO_2(CH_3)_2$, $SbCl_5 \cdot SO_2(C_6H_5)_2$, $SbCl_3 \cdot 2SO(CH_3)_2$ and $SbCl_3 \cdot SO_2(CH_3)_2$. The melting point relations between these compounds have been discussed.

The suggestion by Groeneveld [1] that in the oxychlorides in many cases the oxygen atoms function as electron pair donors by the formation of solid addition compounds has recently been confirmed by X-ray work [2] on $SbCl_5.POCl_3$ and by infrared spectroscopy on different addition compounds [3]. These results have led to further studies of the occurrence of addition compounds which might be of the same type. It was proved by Groeneveld in his thesis that $SbCl_5$ forms 1:1 addition compounds with $SOCl_2$ and SO_2Cl_2. His results have been confirmed by us (congruent melting points of +6°C and —22°C were found). A stronger tendency to compound formation should be expected with sulfoxides and sulfones where chlorine is replaced by the less electronegative alkyl and aryl groups, resulting in a drift of electrons towards the oxygen atom and thus increasing its donor properties. We have therefore studied the system $SbCl_5$—$SO_2(CH_3)_2$ and have made additional experiments with $SO(CH_3)_2$, $SO(C_6H_5)_2$ and $SO_2(C_6H_5)_2$. As a complement the systems $SbCl_3$—$SO(CH_3)_2$ and $SbCl_3$—$SO_2(CH_3)_2$ have also been investigated, $SbCl_3$ in contrast to $SbCl_5$ being a dipole molecule.

EXPERIMENTAL

Chemicals used. $SbCl_5$ was purified by vacuum distillation and $SbCl_3$ by sublimation directly into a cool glass bulb placed 3 cm above the bulk of impure solid. $SO(CH_3)_2$ was repeatedly distilled until it had a final melting point of +19°C. The other sulfoxide and the sulfones were prepared and purified by Dr. B. Sjöberg whose help is gratefully acknowledged. They had the following melting points: $SO(C_6H_5)_2$ +70°C, $SO_2(CH_3)_2$ +108—109°C and $SO_2(C_6H_5)_2$ +125°C.

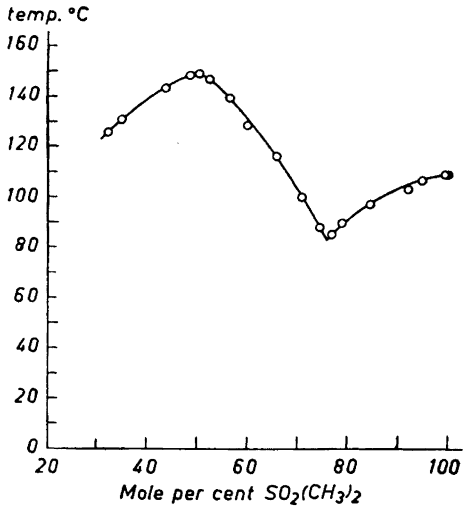


Fig. 1. The system $SbCl_5-SO_2(CH_3)_2$.

Phase diagrams. The liquidus curves were studied by the same methods as described earlier [4]. The reaction vessel was cooled down below 0°C before every addition of a reactant in order to prevent irreversible side reactions (elimination of organic halides, oxidation, *etc.*). If such precautions are not taken very vigorous reactions take place.

RESULTS

1. The liquidus curve of the system $SbCl_5$-$SO_2(CH_3)_2$ is given in Fig. 1. The addition compound $SbCl_5 \cdot SO_2(CH_3)_2$ has a congruent melting point of +149°C. This compound can also be prepared from solutions in carbon tetrachloride. The crystals are very hygroscopic.

2. Analogous compounds were also prepared with $SO(CH_3)_2$, $SO(C_6H_5)_2$ and $SO_2(C_6H_5)_2$ by melting the reactants together or by precipitation from carbon tetrachloride solutions. The melting points of the colorless addition compounds, $SbCl_5 \cdot SO(CH_3)_2$ $SbCl_5 \cdot SO(C_6H_5)_2$ and $SbCl_5 \cdot SO_2(C_6H_5)_2$ are +135 C°, +150—155°C and +141°C. The compound $SbCl_5 \cdot SO(CH_3)_2$ is partially decomposed by melting and its melting point is continuously decreasing by remelting.

3. These addition compounds can all be dissolved in ethylene chloride and in benzene. It was observed that they give colourless solutions with benzene while the corresponding addition compounds with $POCl_3$ and $SeOCl_2$ give the same kind of dark colour as $SbCl_5$ itself.

4. The liquidus curve of the system $SbCl_3$-$SO(CH_3)_2$ is shown in Fig. 2*a*. At mole fractions of $SO(CH_3)_2$ smaller than 0.60 strong supercooling prevents a reliable study of the phase diagram. The addition compound $SbCl_3 \cdot 2SO(CH_3)_2$ has a congruent melting point of +70°C. Attempts to prepare the compound by precipitation from chloroform solution ($SbCl_3$ does not dissolve in carbon tetrachloride) only led to the formation of a liquid phase dispersed as oil drops in the solution.

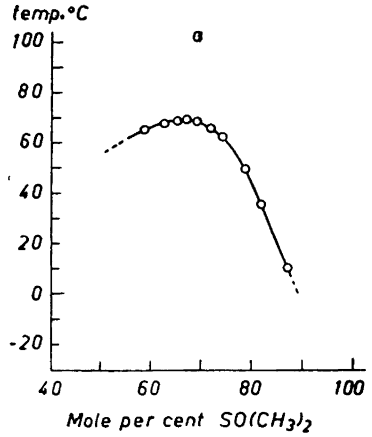


Fig. 2a. The system $SbCl_3$—$SO(CH_3)_2$.

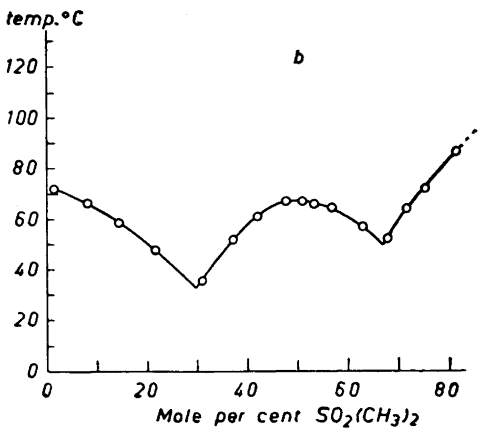


Fig. 2b. The system $SbCl_3$—$SO_2(CH_3)_2$.

5. The liquidus curve of the system $SbCl_3$—$SO_2(CH_3)_2$ is given in Fig. 2*b*. The addition compound $SbCl_3 \cdot SO_2(CH_3)_2$ has a congruent melting point of +67°C. The direct precipitation from chloroform solution offered the same difficulties as with the sulfoxide.

DISCUSSION

The melting points of compounds with appreciable dipole moments are mainly determined by two factors, the dipole moment and the mole volume. (The shape of the molecule is also of importance as it might permit or prevent particularly favourable packings of the dipole molecules. In the cases discussed here that effect will, however, probably parallel the effect of increasing volume if it is of any importance at all.) With low dipole moments the variations in the London-van der Waals attraction forces also play an important role. The effect of the two factors is clearly demonstrated in the sequence SO_2Cl_2, $SO_2(CH_3)_2$, $SO_2(C_2H_5)_2$ and $SO_2(C_6H_5)_2$ where the dipole moments are 1.80, 4.41, 4.41 and 5.04 while the melting points are —54°C, +109°C, +74°C and +125°C. The big difference in dipole moment between SO_2Cl_2 and the sulfones is reflected in a big difference in melting points. Within the sulfones with rather similar dipole moments the mole volumes are of larger importance and $SO_2(C_2H_5)_2$ has a appreciably lower melting point than $SO_2(CH_3)_2$. Of the sulfoxides only the dipole moment of $SO(C_6H_5)_2$ is known (~4), which is much larger than the value 1.44 for $SOCl_2$. The melting points are also very different: +70°C and —105°C.

If we now consider the addition compounds, the same effects should determine the melting points. (If the molecule is dissociated into one acceptor and one donor molecule by melting this is not true. In that case, with very weak acceptor-donor interaction, lower melting points might be obtained than would be expected from the total dipole moment and mole volume of the

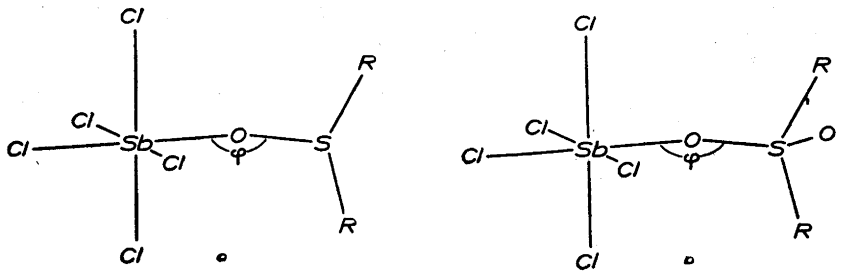


Fig. 3. *a* The probable structure of the addition compounds of $SbCl_5$ with $SOCl_2$ and the sulfoxides, *b* The probable structure of the addition compounds of $SbCl_5$ with SO_2Cl_2 and the sulfones.

addition compound molecule.) In the common case when the interaction between the complex molecules is the determining factor for the cohesion in the solid and liquid state we will first discuss the resultant dipole moment. Addition compounds with different donor molecules and the same acceptor molecule are easiest to compare. As a rule the donor molecules are not very much changed in structure by the complex formation while the acceptor molecules always are changed in shape because the acceptor atom has increased its coordination by new bonds to the donor atoms in the donor molecules. This is demonstrated in Fig. 3 for addition compounds with $SbCl_5$. The angle φ is generally not 180°. (For $SbCl_5 \cdot POCl_3$ the angle 146°5 is reported [2].) As a first approximation the dipole moment of the complex molecule is the vector sum of the dipole moments of the acceptor molecule (in the configuration which it has in the complex molecule) and of the donor molecule. The resultant dipole moment thus depends on the angle between the two vectors and it is obvious that the coupling probably will be less favourable for the sulfones than for the sulfoxides. The acceptor-donor bonds Sb-O will modify the results obtained by simple vector addition in such a way that the dipole moment is further increased by a strong acceptor-donor bond. Knowledge of the structures *and* the dipole moments might thus give information about the acceptor-donor bond. The resultant dipole moment is, however, mainly determined by the vector addition.

In the systems discussed here this would imply that the melting points of the addition compounds of $SbCl_5$ with $SOCl_2$ and sulfoxides would show a larger increase compared with the simple donor molecules than SO_2Cl_2 and the sulfones would do. The values are: +111°C for $SOCl_2$, +115°C for $SO(CH_3)_2$ and +80—85°C for $SO(C_6H_5)_2$, +32°C for SO_2Cl_2, +40°C for $SO_2(CH_3)_2$, +22°C for $SO_2(C_2H_5)_2$ and +16°C for $SO_2(C_6H_5)_2$. (The addition compound $SbCl_5 \cdot SO_2(C_2H_5)_2$ with a melting point of +96°C has been prepared by Klages [5].) The result is thus what could be expected from the simple considerations although a strong Sb—O bond might also have enhanced the effect.

With $SbCl_3$ one must also take into account the dipole moment of the free acceptor molecule (~4) and the possibility that the two dipole moments will be coupled in a very unfavourable way in the addition compound. The mole

volume increase might then be the dominating factor. The compounds $SbCl_3 \cdot 2SO(CH_3)_2$ and $SbCl_3 \cdot SO_2(CH_3)_2$ have thus both melting points below that of $SbCl_3$.

One should not try to elaborate too much on qualitative discussions of this type until the structures are known. The contribution of the London-van der Waals forces to the cohesion energy has also been considered to be equal for similar addition compounds. It is clear, however, that the strength of the acceptor-donor bond is not reflected in an obvious way in the melting point relations discussed here.

One indication about the strength of the bonds might be offered by the absence of colour in benzene solution. A dark colour is obtained with free $SbCl_5$ in benzene which readily leads to the conclusion that the acceptor-donor bond Sb-O in the addition compounds with sulfoxides and sulfones are stronger than with $POCl_3$ and $SeOCl_2$ which give a darker colour. Further studies are required, however.

An interesting feature is that $SbCl_3$ forms a 1:2 addition compound with $SO(CH_3)_2$. The same tendency for $SbCl_3$ to form 1:2 compounds has been observed in other connections. The compound $SbCl_3 \cdot 2POCl_3$ has thus been prepared [6] in contrast to only $AsCl_3 \cdot POCl_3$ described earlier [4].

This work has been sponsored by the *Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force*, through its European Office under contract No. AF 61(052)-43. This support is gratefully acknowledged.

Finally we wish to thank Professor G. Hägg for the facilities put at our disposal.

REFERENCES

1. Groeneveld, W. L. *Rec. trav. chim.* **75** (1956) 594.
2. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134.
3. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **80** (1958) *In press.*
4. Agerman, M., Andersson, L. H., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **12** (1958) 477.
5. Klages, F. *Private communication.*
6. Lindqvist, I. *Unpublished results.*

Received November 19, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 425—432

The Structures of Ni_6Si_2B, Fe_2P and Some Related Phases

STIG RUNDQVIST and FRANZ JELLINEK*

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

It has been found that the Fe_2P (*C* 22) structure previously proposed must be revised. The true symmetry is hexagonal, rather than trigonal. The phases Ni_6Si_2B, Mn_2P, and Ni_2P are isotypic with Fe_2P, but Co_2P possesses a different — orthorhombic — structure. Some structurally related phases are also discussed.

In the course of a study of the system Ni-B [1] it was observed that Ni-B mixtures containing more than approximately 35 at % of B, attack silica tubes at elevated temperatures. A ternary phase of composition Ni_6Si_2B is formed. The structure of the compound was determined and found to be very closely related to the Fe_2P structure (*C* 22-type) as proposed by Hendricks and Kosting [2], the only difference being that — while in Ni_6Si_2B the Si atoms are surrounded by 9 Ni — the corresponding P I atoms in the *C* 22-type are shifted 0.43 Å in the *c* direction in order to obtain a distorted octahedral environment. This shift lowers the symmetry of the structure from hexagonal (space group $P\bar{6}2m$) to trigonal (space group $P321$). As experimental evidence confirming this lowering of the symmetry was lacking, and the structural arguments which led Hendricks and Kosting [2] to their structure proposal are not longer very convincing, it was considered worth while to reinvestigate the structure of Fe_2P. Indeed it was found that the *C* 22-type must be revised: Fe_2P actually is isotypic with Ni_6Si_2B. The same applies to Mn_2P and Ni_2P, but for Co_2P a different structure — already determined by Nowotny [3] — was confirmed.

EXPERIMENTAL

The Ni_6Si_2B samples were prepared by heating mixtures of nickel powder (99.9 %), boron powder (99.7 %) and silicon powder (99.9 %) in evacuated and sealed silica tubes at 950°C. These mixtures did not attack the silica tubes appreciably, while mixtures of nickel and boron only, containing more than 35 at % boron attacked the tubes severely

* On leave from Anorganisch- en Fysisch-Chemisch Laboratorium der Rijksuniversiteit, Groningen, the Netherlands.

at temperatures above 900°C. The attack resulted in formation of Ni_6Si_2B, often in the form of needle-like crystals.

Fe_2P was prepared by dropping pellets of red phosphorus (99 %) into molten iron (97.5 %; main impurity oxygen). The iron was melted in an induction furnace under nitrogen atmosphere.

Weissenberg diagrams around the c axes of Ni_6Si_2B (layer lines 0—3) and Fe_2P (layer lines 0, 3, 4) were taken with MoK radiation; in the case of Fe_2P an equatorial diagram about the a axis was also made. Intensities were estimated visually. Some very weak reflexions were estimated only in a qualitative way; these were not used in the structure refinements. As the crystals used were quite small, no absorption correction was considered necessary.

Powder photographs were taken in Guinier-type cameras, using Cr$K\alpha$ (for Fe_2P, Mn_2P and Co_2P) and Cu$K\alpha$ (for Ni_6Si_2B and Ni_2P) radiations. The phosphides possess rather narrow homogeneity ranges, while in the case of Ni_6Si_2B a limited Si-B substitution was found. The unit-cell dimensions given in this paper are believed to correspond closely to the ideal compositions indicated. The accuracy of the lattice-parameter measurements is better than 0.05 %.

DETERMINATION OF THE Ni_6Si_2B STRUCTURE

The unit-cell dimensions of Ni_6Si_2B were found as $a = 6.105$ Å; $c = 2.895$ Å; $c/a = 0.4742$; $U = 93.4$ Å^3; with one formula unit in the cell. The density is calculated to be 7.45 g.cm^{-3}.

An inspection of the Weissenberg diagrams showed that — apart from normal decline — the intensities of ($hk2$) reflexions are the same as those of the corresponding ($hk0$) reflexions, while the same holds for ($hk3$) and ($hk1$). This indicates that all atoms (except possibly B) are arranged in layers with $z = 0$ and $z = 1/2$, respectively. Therefore, a Patterson section ($xy0$) was calculated. This section proved to be compatible with space group $P\bar{6}2m$, if 3 NiI are placed in 3(f), 3 NiII in 3(g) and 2 Si in 2(c). From structural arguments B was placed in 1(b) and, indeed, the Patterson synthesis gave support to this hypothesis.

Approximate Ni parameters were read from the Patterson map. Structure factors of the ($hk0$) reflexions were calculated; the scattering factors of Ni were interpolated from tables given by Thomas and Umeda [4], those of Si from Tomiie and Stam [5], and B scattering factors from Ibers [6]; the small correction for dispersion and anomalous scattering was neglected. Refinement of the Ni parameters by an algebraic method led to an R value of 5.5 % for the 23 measured ($hk0$) reflexions, when a temperature factor with $B = 0.52$ Å^2 was applied. Using the same temperature and scale factors, R was calculated to be 5.1 % for the 25 observed ($hk1$) reflexions. Therefore, no further refinement was considered worth while. Observed and calculated structure factors are compared in Table 1.

The final structure is the following:

Space group $P\bar{6}2m$ — (D^3_{3h});

3 NiI in 3 (f): $x00$; $0x0$; $\bar{x}\bar{x}0$ with $x = 0.247$

3 NiII in 3(g): $x0\frac{1}{2}$; $0x\frac{1}{2}$; $\bar{x}\bar{x}\frac{1}{2}$ with $x = 0.608$

2 Si in 2(c): $\frac{1}{3}\,\frac{2}{3}0$; $\frac{2}{3}\,\frac{1}{3}0$

1 B in 1(b): $00\frac{1}{2}$.

Table 1. Observed and calculated ($hk0$) and ($hk1$) structure factors for Ni_6Si_2B.

hk	$l = 0$			$l = 1$		
	F_o	F_c	α_c in°	F_o	F_c	α_c in°
00		201.0	0.0		16.9	0.0
10	w. *	4.3	0.0	21.6	21.4	0.0
20	w. *	2.3	0.0	61.5	56.0	180.0
30	66.4	67.6	0.0		3.0	180.0
40	31.1	26.6	0.0	43.3	44.0	0.0
50	44.1	45.9	0.0	26.1	27.1	180.0
60		1.0	180.0		0.5	0.0
70		11.1	0.0		6.3	180.0
80	35.7	35.1	0.0		0.7	0.0
90		7.8	0.0	17.1	17.0	0.0
100		7.4	0.0	16.2	19.7	180.0
11	29.2	29.1	192.1	84.6	84.5	74.3
21	63.9	68.4	114.6	31.3	28.8	152.0
31	31.7	31.3	250.8	38.0	37.6	51.4
41	21.3	20.8	340.2	31.0	33.7	8.3
51	31.7	31.3	125.7	19.4	18.9	126.4
61	23.5	26.5	186.4	35.9	39.8	93.7
71	16.3	15.7	34.7	15.3	15.1	331.8
22	29.3	28.5	104.7	23.5	21.4	283.3
32	32.9	29.5	277.8	45.4	47.1	209.3
42	37.9	39.0	142.6	23.1	25.9	262.6
52	23.9	23.1	60.1	20.2	20.8	126.7
62		12.3	132.0	15.9	15.3	198.7
72		14.1	183.8	23.7	24.9	259.2
82		11.8	36.2		13.1	211.4
92	19.2	22.3	121.0		1.8	191.0
33	37.2	36.6	275.2	14.3	14.0	244.0
43	14.0	15.6	268.5	14.0	14.9	63.3
53	21.8	23.4	5.3	14.5	13.3	178.6
63	30.7	27.7	254.8		6.8	0.0
73	16.9	15.3	265.9	18.3	20.1	210.9
44	30.5	29.0	27.8	41.4	42.8	342.3
55	24.4	21.9	31.3	19.2	20.4	148.2

* The reflexions designed by w. were discernible above the background, but it was not possible to estimate them with any accuracy.

Interatomic distances are listed in Table 2; the accuracy of the given values is better than 0.01 Å. Fig. 1 shows the environment of the B and Si atoms. Si is surrounded by 6 NiII forming a trigonal prism and by 3 NiI in a triangle, situated outside the centers of the rectangular prism faces; the 9 Ni atoms forming the described tetrakaidecahedron around Si are approximately equidistant from the central atom. The B environment is similar, but the 3 Ni in the triangle are much farther away than those forming the trigonal prism and cannot be regarded as true neighbours. Adopting r_{Ni} as 1.24 Å the plausible values of $r_{Si} = 1.12$ Å and $r_B = 0.84$ Å are derived.

Table 2. Interatomic distances in Ni_6Si_2B.

Si	—	3 NiI	2.34 Å	B	—	6 NiI	2.08 Å
	—	6 NiII	2.37		—	3 NiII	2.39
NiI	—	2 B	2.08	NiII	—	4 Si	2.37
	—	2 Si	2.34		—	1 B	2.39
	—	4 NiII	2.55		—	4 NiI	2.55
	—	2 NiI	2.60		—	2 NiI	2.64
	—	2 NiII	2.64		—	2 NiII	2.895
	—	2 NiI	2.895		—	4 NiII	3.26

REDETERMINATION OF THE Fe_2P STRUCTURE

For Fe_2P we found the unit-cell dimensions as $a = 5.865$ Å; $c = 3.456$ Å; $c/a = 0.5893$; $U = 103.0$ Å^3; in close agreement with the values given by Hägg [7] and Friauf [8], less so with the values by Nowotny and Henglein [9]. With three formula units in the cell the calculated density is 6.90 g.cm^{-3}, which compares favourably with the observed values of 6.83 [7], 6.89 [8] and 6.77 [10] g.cm^{-3}.

In the structure Hendricks and Kosting [2] proposed for Fe_2P, using Hägg's [7] experimental data, the Fe atoms occupy the same sites as the Ni atoms in Ni_6Si_2B, PII is situated in $00\frac{1}{2}$, but PI is shifted from the plane $z = 0$ to the position $\frac{1}{3}\,\frac{2}{3}\,\frac{1}{8}$, $\frac{2}{3}\,\frac{1}{3}\,\frac{7}{8}$. This shift lowers the symmetry from space group $P\bar{6}2m$ to $P321$.

A comparison of the intensities of ($hk0$) and ($hk4$) reflexions showed at once that Hendricks and Kosting's structure cannot be the correct one;

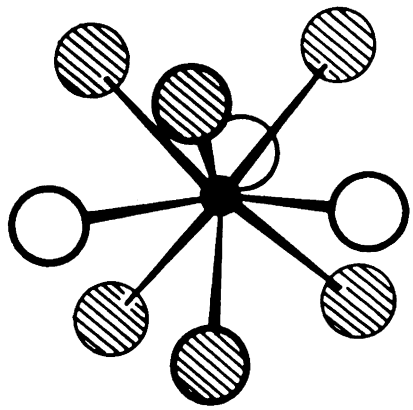


Fig. 1. The Si and B environments in Ni_6Si_2B. The nonmetal (black) is surrounded by six metal atoms (shaded) forming a trigonal prism, and by three metal atoms (white) in a triangle. The resulting coordination figure (a tetrakaidecahedron) is built up by fourteen triangles sharing all edges. The nine Ni atoms around Si are approximately equidistant from the central atom. The B environment is more distorted, as the three Ni in the triangle are much farther away from the central atom than the six Ni forming the trigonal prism.

adopting their proposed structure the intensity sequences in the zero and fourth layer lines about the c axis should be quite different, while actually they were identical. Furthermore, within experimental accuracy it was found that $|F(hkl)| = |F(\bar{h}kl)|$, indicating that the symmetry of Fe_2P is hexagonal and that PI lies in the plane $(xy0)$.

Nevertheless, we tried to interpret the small differences between $F(\bar{h}kl)$ and $F(hkl)$ as due to a minor deviation from hexagonal symmetry (and not to experimental inaccuracy), but no selfconsistent result was possible. Therefore, space group $P\bar{6}2m$ was adopted. Structure factors for $(h0l)$ reflexions were computed, using Fe scattering factors from Thomas and Umeda [4] and P scattering factors from Tomiie and Stam [5]. The Fe parameters derived by Hendricks and Kosting were provisionally adopted, and they proved to be surprisingly accurate. Refinement by an algebraic method indicated only minor shifts, the final structure being:

Space group $P\bar{6}2m$ — (D_{3h}^3);

3 FeI in 3(f): $x00$; $0x0$; $\bar{x}\bar{x}0$ with $x = 0.256$

3 FeII in 3(g): $x0\frac{1}{2}$; $0x\frac{1}{2}$; $\bar{x}\bar{x}\frac{1}{2}$ with $x = 0.594$

2 PI in 2(c): $\frac{1}{3}\frac{2}{3}0$; $\frac{2}{3}\frac{1}{3}0$

1 PII in 1(b): $00\frac{1}{2}$

A temperature factor with $B = 0.67$ Å^2 was employed, giving $R = 6.1$ % for the 34 measured $(h0l)$ reflexions. A comparison of observed and calculated structure factors is given in Table 3. In Table 4 interatomic distances are listed; the standard deviations are calculated to be less than 0.01 Å. The structure of Fe_2P is isotypic with that of Ni_6Si_2B and both PI and PII possess a distorted tetrakaidecahedral environment. However, the distortions are quite different in the two cases: while the three Fe atoms surrounding PI in a triangle are much closer to the central atom than the six Fe atoms forming the trigonal prism, just the opposite is true for the PII environment.

THE STRUCTURES OF SOME RELATED PHASES

Several binary phases have been described as isotypic with Fe_2P; as the isotypism generally was derived from a comparison of powder photographs, it is highly probable that the revised Fe_2P structure also applies to β-Co_2As [11], Pd_2Si [12,13], Pd_2Ge [12], and Pt_2Ge [12]. This was actually proved to be the case for Mn_2P and Ni_2P. The unit-cell dimensions were found to be:
for Mn_2P: $a = 6.074$ Å; $c = 3.454$ Å; $c/a = 0.5687$;

$U = 110.4$ Å^3; $Dx = 6.36$ g.cm^{-3};

in agreement with values by Fylking [14], Wiechmann [15], and Årstad and Nowotny [16];
for Ni_2P: $a = 5.864$ Å; $c = 3.385$ Å; $c/a = 0.5772$;

$U = 100.8$ Å^3; $Dx = 7.33$ g.cm^{-3};

Table 3. Observed and calculated (*h0l*) structure factors for Fe_2P.

h0l	F_o	F_c	*h0l*	F_o	F_c	*h0l*	F_o	F_c
000		+201.0	300	66.9	+65.9	800	22.6	+21.7
001	11.1	+ 11.6	301	v.w.	+ 5.1	801		+ 4.2
002	107.2	+119.6	302	49.9	+52.7	802	22.6	+19.4
003	w. *	+ 6.6	303		+ 3.8	12 00	14.3	+14.3
004	61.8	+ 67.2	304	33.7	+32.4	12 01		+ 1.8
005		+ 3.6	305		+ 2.1	12 02	12.2	+13.1
006	34.0	+ 35.0	306	18.8	+17.1	12 03		+ 1.5
007		+ 1.6	400	35.3	+32.7	12 04	11.7	+10.0
008	21.0	+ 17.1	401	26.7	+27.8			
100	6.4	+ 6.3	402	27.5	+26.8			
101	11.0	+ 10.9	403	20.8	+19.2			
102	v.w.	+ 4.0	404	19.0	+17.1			
103	v.w.	+ 5.3	405	w.	+11.8			
200	16.2	+ 14.8	406	v.w.	+ 9.7			
201	66.3	− 66.3	500	35.8	+37.4			
202	13.0	+ 10.7	501	35.4	−34.6			
203	40.7	− 41.9	502	30.0	+31.7			
204	v.w.	+ 6.2	503	27.3	−25.6			
205	22.8	− 23.4	504	22.0	+21.1			
206		+ 3.3	505	17.0	−15.4			
207	v.w.	− 11.7	700	25.2	+24.7			
208		+ 1.7	701		−11.3			
209	v.w.	− 5.6	702	24.3	+21.8			

* The reflexions designed by w. (weak) or v.w. (very weak) were observable on the diagrams, but it was not possible to estimate them with any accuracy.

which differs somewhat from Nowotny and Henglein's [17] values. There are reasons to believe that the phase "Pd_3B_2", which Buddery and Welch [13] prepared by heating Pd and B in silica tubes and to which a Fe_2P structure is ascribed, actually is the Pd analogue of Ni_6Si_2B. Indeed, in view of the structural non-equivalence of PI and PII in Fe_2P, it may be expected that several ternary phases containing two kinds of non-metals, crystallize in isotypic structures. FeI and FeII are also non-equivalent in Fe_2P, but — as far as we are aware of — no isostructural ternary phase containing two different transition metals, has yet been described. However, a superstructure of the Fe_2P type results, if in the structure proposed by Perlitz and Westgren [18] for $Al_8FeMg_3Si_6$ the Si positions and part of the (Al,Fe) positions are interchanged; such a change would be compatible with the published experimental data.

Table 4. Interatomic distances in Fe_2P.

PI	− 3 FeI	2.22 Å	PII	− 6 FeI	2.29 Å
	− 6 FeII	2.48		− 3 FeII	2.38
FeI	− 2 PI	2.22	FeII	− 1 PII	2.38
	− 2 PII	2.29		− 4 PI	2.48
	− 2 FeI	2.60		− 2 FeI	2.63
	− 2 FeII	2.63		− 4 FeI	2.71
	− 4 FeII	2.71		− 4 FeII	3.08

In agreement with Nowotny [3] we found for Co_2P an orthorhombic unit-cell, but the lattice dimensions observed by us are much smaller than those given by him. For the composition Co_2P we determined: $a = 6.608$ Å; $b = 5.644$ Å; $c = 3.512$ Å; $U = 131.0$ Å^3; $Dx = 7.55$ g.cm^{-3}. With increasing P content the unit-cell contracts in all directions, contrary to Nowotny's [3] observations.* Co_2P is isotypic with Co_2Si[19] — a limited mutual solubility exists — with δ-Ni_2Si [20], Rh_2Ge [21], Ru_2Si [23], Rh_2Si [23] (the alleged phase Rh_2B[22] probably actually was Rh_2Si too) and several Pd compounds [26]. The P environment is similar to that in Fe_2P, but the packing is different. This structure type is a distortion of the Ni_2In type, but — as was already pointed out by Nowotny [3] — the Co_2P structure can also be regarded as an anti-$PbCl_2$-type.

It is noteworthy that both the revised and the old — incorrect — Fe_2P structures also have their counterparts in inorganic-salt structures: β_1-K_2ThF_6 and β_1-K_2UF_6[24] are formally isotypic with Ni_6Si_2B and Fe_2P, while $SrCl_2.6H_2O$[25], β_2-Na_2ThF_6[24] and isotypes possess structures which are the anti-types to Hendricks and Kosting's [2] proposal for the *C* 22 structure.

Acknowledgements. The research described in this paper was sponsored by the *Statens Tekniska Forskningsråd* and by the *European Commission of Air Research and Development Command* under Contract No. AF61(052)—40.

F. J. wishes to thank Professor G. Hägg for the kind hospitality he enjoyed at his institute during the tenure of a stipendium by the *Netherlands Organization for Pure Research (Z.W.O.)* which is most gratefully acknowledged. S.R. wishes to express his sincere gratitude for a scholarship from the *Statens Tekniska Forskningsråd.*

REFERENCES

1. Rundqvist, S. *To be published.*
2. Hendricks, S. B. and Kosting, P. R. *Z. Krist.* **74** (1930) 511.
3. Nowotny, H. *Z. anorg. Chem.* **254** (1947) 31.
4. Thomas, L. H. and Umeda, K. *J. Chem. Phys.* **26** (1957) 293.
5. Tomiie, Y. and Stam, C. H. *Acta Cryst.* **11** (1958) 126.
6. Ibers, J. A. *Acta Cryst.* **10** (1957) 86.
7. Hägg, G. *Nova Acta Regiae Soc. Sci. Upsaliensis*, Ser. IV, **7** (1929) No. 1.
8. Friauf, J. B. *Trans. Am. Soc. Steel Treating* **17** (1930) 499.
9. Nowotny, H. and Henglein, E. *Monatsh.* **79** (1948) 385.
10. Franke, W., Meisel, K., Juza, R. and Biltz, W. *Z. anorg. Chem.* **218** (1934) 346.
11. Heyding, R. D. and Calvert, L. D. *Can. J. Chem.* **35** (1957) 449.
12. Anderko, K. and Schubert, K. *Z. Metallk.* **44** (1953) 307.
13. Buddery, J. H. and Welch, A. J. E. *Nature* **167** (1951) 362.
14. Fylking, K.-E. *Arkiv Kemi, Mineral. Geol.* **11 B** (1934) No. 48.
15. Wiechmann, F. *Z. anorg. Chem.* **234** (1937) 130.
16. Årstad, O. and Nowotny, H. *Z. physik. Chem.* **B38** (1937) 356.
17. Nowotny, H. and Henglein, E. *Z. physik. Chem.* **B40** (1938) 281.
18. Perlitz, H. and Westgren, A. *Arkiv Kemi, Mineral. Geol.* **15B** (1942) No. 16.
19. Geller, S. and Wolontis, V. M. *Acta Cryst.* **8** (1955) 83.
20. Toman, K. *Acta Cryst.* **5** (1952) 329.
21. Geller, S. *Acta Cryst.* **8** (1955) 15.

* *Note added in proof:* Professor H. Nowotny kindly informs us that a rechecking of his earlier investigations confirms our results.

22. Mooney, R. W. and Welch, A. J. E. *Acta Cryst.* **7** (1954) 49.
23. Åselius, J., *Private communication.*
24. Zachariasen, W. H. *Acta Cryst.* **1** (1948) 265.
25. Tovborg Jensen, A. *Kgl. Danske Videnskab. Selskab* **17** (1940) No. 9.
26. Schubert, K., Breimer, H., Burkhardt, W., Günzel, E., Haufler, R., Lukas, H. L., Vetter, H., Wegst, J. and Wilkens, M. *Naturwiss.* **44** (1957) 229.

Received November 7, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 433—441

X-Ray Investigations on Me-Si-B Systems (Me = Mn, Fe, Co)

I. Some Features of the Co-Si-B System at 1 000° C. Intermediate Phases in the Co-Si-B and Fe-Si-B Systems

BERTIL ARONSSON and (Miss) GUNVOR LUNDGREN

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

Some features of the Co-Si-B system at 1000°C have been determined with X-ray methods. It is found that the homogeneity ranges of the intermediate phases are small. The crystal structure of the ternary phase $Co_{4.7}Si_2B$ (isomorphous with $Fe_{\sim 4.7}Si_2B$) has been established from single crystal data and is closely related to the W_5Si_3($T1$) structure.

The Fe-Si-B system contains several ternary phases, the most remarkable of which is a cementite (Fe_3C) type phase, while the crystal structures of Fe_5SiB_2 and Mn_5SiB_2 are shown to be of the Cr_5B_3($T2$) type.

Since there are many similarities in the crystal chemistry of the borides and silicides of the transition metals, it seems very likely that a systematic study of the intermediate phases of Me-Si-B systems will give valuable information about both groups of compounds.

Investigations on Me-Si-B systems have been made by Nowotny and coworkers [1,2] who have been particularly interested in the Me_5X_3 phases (X = B, Si). At this Institute studies on Me-Si-B systems with Me = manganese, iron, and cobalt are in progress. This paper will deal with the Co-Si-B system and will also give some preliminary information about the intermediate phases of the Fe-Si-B and Mn-Si-B systems. In a second paper a more detailed account of the Fe-Si-B system will be given.

Our primary interest has been the crystal structures of the intermediate phases, and our investigations have been carried out chiefly by means of X-ray methods. However, no accurate determinations of the phase diagrams have been made. Before proceeding to the results of the present study of the Co-Si-B system, some reference to previous work on the Co-B and Co-Si systems will be given.

Table 1. Crystallographic data on intermediate phases in the Co-Si and Co-B systems.

	Lattice parameters (in Å)			Structure type	Ref.
	a	*b*	*c*		
Co_3B	4.408	5.225	6.629	*D* $O_{11}(Fe_3C)$	4
Co_2B	5.016		4.220	*C* 16 ($CuAl_2$)	3
CoB	5.253	3.043	3.956	*B* 27 (FeB)	3
Co_2Si	4.918	3.737	7.109	*C* 23 ($PbCl_2$)	5,6
CoSi	4.447			*B* 20 (FeSi)	7
$CoSi_2$	5.366			*C* 1 (CaF_2)	8,9

PREVIOUS WORK ON THE Co-B and Co-Si SYSTEMS

Reported crystallographic data on the intermediate phases of the binary Co-Si and Co-B systems are collected in Table 1. Bjurström [3] determined the structures of Co_2B and CoB to be of the *C* 16 ($CuAl_2$) and *B* 27 (FeB) types, respectively. Co_3B was recently discovered by Rundqvist [4,5]. Borén [6] determined the unit cell dimensions of Co_2Si and CoSi and found that the structure of the latter was of the *B* 20 (FeSi) type. Borén *et al.*[7] also gave a structure for Co_2Si, and this has recently been corrected by Geller [8]. The correct Co_2Si structure, which Geller [8] has shown to be isomorphous with δ-Ni_2Si [10], has been pointed out [9] to be indeed that of the *C* 23 ($PbCl_2$) type. Finally, $CoSi_2$ is of the *C* 1 type (Schubert and Pfisterer [11], Bertaut and Blum [12]). A hightemperature modification of Co_2Si has been reported [13,14], as well as a phase with the composition Co_3Si [13,14]. None of these phases is stable below 1 170°C and no crystallographic constants have been published for them. The solid solubility of silicon in cobalt has not been determined accurately but Hashimoto [14] reported a solubility of about 10 at % Si at 1 000°C.

No studies on ternary Co-Si-B alloys have been noted in the literature.

EXPERIMENTAL

The following chemicals were used:

Cobalt (99.2 %, containing 0.45 % Ni) kindly donated by Societé Générale Metallurgique de Hoboken.

Silicon (99.9 %) obtained from Pechiney & C:ie, Paris.

Boron (99 %) kindly donated by Borax Consolidated Ltd., London.

When a rough knowledge of the system had been obtained from arc melted and sintered alloys, 2—3 g samples of suitable compositions were prepared by sintering pressed pellets of the elements in evacuated silica tubes at 1 000°C. After four days, each sample was crushed, thoroughly mixed, then sintered for another two days and finally quenched in oil. After this treatment, all samples gave very sharp powder diffraction lines and the l.p. (lattice parameters) of the phases in a three phase region were found to be the same in different samples from this region. Therefore, it was concluded that equilibrium had been attained.

The powder photographs were taken in a Guiniertype powder camera using Cr*Ka* radiation and employing CaF_2 (a = 5.4630 Å at 21°C) as a calibration standard. The l.p. are estimated to be accurate to ± 0.03 %.

The oxygen content (weight percent) of two 2—3 g samples, determined by a vacuum fusion technique, was found to be 0.211 and 0.212, respectively. Since the composition of all samples was approximately Co_2Si_2B and the method of preparation was the same, the oxygen content of all samples has been assumed to be 0.21 % by weight. The analyses for cobalt and boron were carried out by Dr. L. Gustafsson at the Analytical Department of this Institute. For the cobalt analyses, the samples were dissolved in a mixture of nitric and hydrofluoric acid. The hydrofluoric acid was then removed by evaporating with sulfuric acid and the cobalt content (including traces of other heavy metals) was subsequently determined by titration with EDTA. For the boron determinations, the samples were first dissolved in dilute hydrochloric acid plus chlorine (0.3 g samples were

completely dissolved within 7—10 h). Cobalt was removed from the acid solution by passing the solution through a cationic exchanger (Dowex 50) and the cobalt-free solution was then neutralized to pH 6.80. After addition of mannitol, the solution was again titrated to pH 6.80. The results of the chemical analyses are given below (weight %).

Sample	Co *	B	Si as difference	Σ (see text)
1	79.75 ± 0.17	2.99 ± 0.20	16.7	99.4
2	77.51	4.61	17.3	99.4
4	83.30	5.33	10.8	99.4
6	82.45	3.34	13.6	99.4
9	79.58	2.13	17.7	99.4

* Traces of nickel and iron computed as cobalt are included in the values of this column.

The accuracy of the cobalt analyses is estimated to be ± 0.2 %, and that of the boron analyses ± 0.4 %. The impurities of the original chemicals (except oxygen and nickel) amounted to about 0.4 % by weight. By adding 0.2 % oxygen, a total of 0.6 % impurities is obtained. The silicon content has been obtained as the difference between 99.4 % and the sum of the cobalt and boron contents. This procedure was unsatisfactory, but any direct analysis for silicon would not give more reliable results unless much larger samples were available. However, very accurate analytical figures are of questionable value since the form in which oxygen is present in the sample is unknown. Oxygen may be bound to boron and silicon, and this would slightly reduce the amounts of the latter elements which actually participate in the Co-Si-B equilibria. Therefore, an alloy for which the analytical composition has been found to be $Co_{4.60}Si_2B$ plus traces of oxygen should rather be written $Co_{4.65}Si_2B$ plus traces of B_2O_3 and SiO_2. In addition, a spectrographic analysis of one sample revealed the presence of traces of nickel and iron. Small amounts of these metals can probably substitute for cobalt in the different alloys and therefore should be added to the cobalt content.

The densities of two samples which contained 80—90 % of the ternary phase $Co_{4.7}Si_2B$ were determined by weighing in air and benzene. The amounts of other phases present in the samples were estimated from the powder photographs, while the density of the ternary phase could be calculated. The values were reproducible to within 0.5 % and a test of the method on some known substances proved satisfactory. The results of the determination of the densities are given below:

	Density of sample	Calculated density of $Co_{4.7}Si_2B$
Sample 36 (arc melted, contained $Co_{4.7}Si_2B + CoB$)	7.27 g/cm³	7.26 g/cm³
Sample 10 (sintered, contained $Co_{4.7}Si_2B + Co_2B + Co_2Si$)	7.21 g/cm³	7.16 g/cm³
	Average	7.21 g/cm³

A small single crystal of the $Co_{4.7}Si_2B$ phase was picked out of an arc melted alloy that contained $Co_{4.7}Si_2B$ and small amounts of CoB. The l.p. of $Co_{4.7}Si_2B$ of this alloy were found to be $a = 8.615$ Å and $c = 4.250$ Å. These values are intermediate between those found in the neighbouring three-phase triangles. Weissenberg photographs were taken around the tetragonal axis by use of Mo$K\alpha$ radiation, and intensities of 92 nonequivalent reflexions from the layer lines 0—4 were visually estimated. An empirical correction for thermal movement and Θ-dependent absorption was obtained by plotting log F_o/F_c against $\sin^2\Theta$. The Fourier summations were made on the Hägg-Laurent [15] machine.

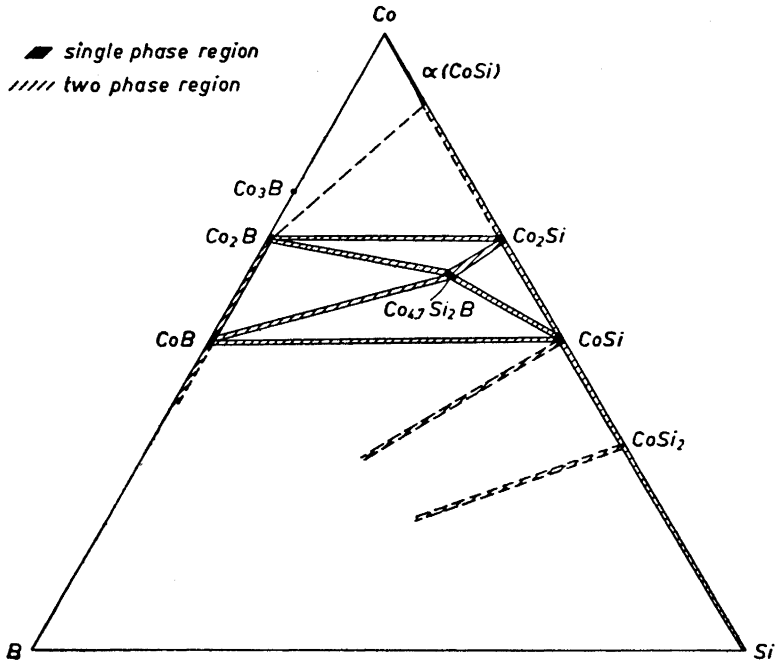


Fig. 1. Some features of the Co-Si-B system at 1 000°C. The one- and two-phase regions are greatly exaggerated for clarity.

RESULTS

The Co-Si-B system at 1 000°C. The results of the present investigation have been used for the construction of a partial isothermal section of the Co-Si-B system at 1 000°C (Fig. 1). The quasi-binary systems Co_2B-Co_2Si and CoB-CoSi divide the section into three parts. The main interest has been devoted to the Co_2X-CoX (X = Si,B) region, which was found to contain a ternary phase.

The l.p. (lattice parameters) reported for the binary phases Co_2B, CoB, Co_2Si and CoSi are given in Table 1. In ternary Co-Si-B alloys, the l.p. of these phases differ only slightly (not more than 0.2 %) from the values of Table 1, indicating that at 1 000°C the homogeneity ranges of Co_2B, CoB, Co_2Si and CoSi are small in the ternary Co-Si-B system.

In the metal-rich part of the system (> 66.7 at % Co) hexagonal cobalt with dissolved silicon was found. This phase will be called α(CoSi) in the following. (It has not been determined if the hexagonal α(CoSi) phase is stable up to 1 000°C or if it is formed during quenching.) The three-phase region Co_2B-Co_2Si-α(CoSi) and the two-phase region Co_2B-α(CoSi) were established. In Co_2B-Co_2Si-α(CoSi) alloys the l.p. of the hexagonal α(CoSi) phase were found to be $a = 2.502$ Å and $c = 4.062$ Å. These values are smaller

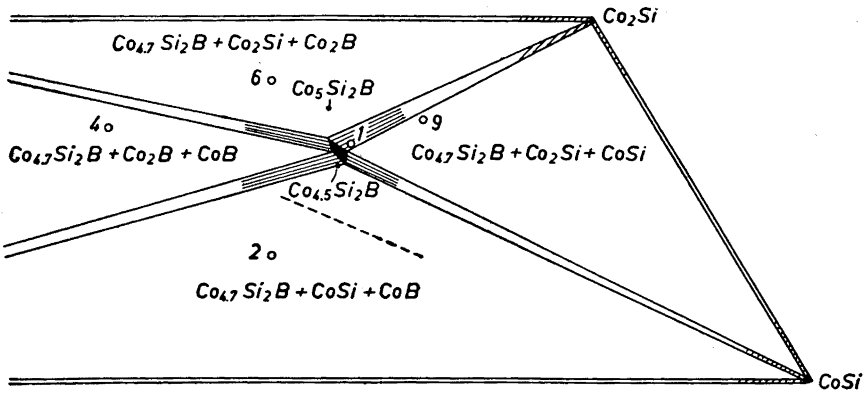


Fig. 2. The Co-Si-B system around Co_5Si_2B. The numbers refer to samples the analyses of which are given in the text.

than those reported for pure α-Co($a = 2.507$ Å, $c = 4.069$ Å)[16] and show that the α(CoSi) phase in equilibrium with Co_2B and Co_2Si contains considerable amounts of silicon. The existence of Co_3B was confirmed in binary Co-B alloys. No attempt was made to determine the equilibria in the Co-Si-B system in which this phase was involved. Evidently, the phase appears only in silicon-poor alloys.

The composition of the ternary phase could be determined quite well from analysed samples of approximate composition Co_5Si_2B (Fig. 2). The two-phase sample 1 (the l.p. of the $Co_{4.7}Si_2B$ phase in this sample were intermediate between those found in neighbouring three-phase regions) contained only small amounts of Co_2Si(~ 5 %). The limit between the two phase range $Co_{4.7}Si_2B$-Co_2Si and the three-phase region $Co_{4.7}Si_2B$-Co_2Si-CoSi was obtained from samples 1 and 9. These facts established the composition of the ternary phase to be $Co_{4.6}Si_2B$ (Fig. 2). This composition was further supported by the observation (from the intensities of the powder lines) that samples 6 and 9 were situated nearer to the ternary single-phase range than samples 2 and 4. As pointed out in the preceding paragraph, some boron and silicon are probably bound to oxygen and the amounts of these elements which actually participate in the Co-Si-B equilibria are slightly smaller than the analytically obtained values used in the construction of Fig. 2. Therefore, we have assigned the formula $Co_{4.7}Si_2B$ to the ternary phase. This composition gives a better agreement between observed and calculated densities than does $Co_{4.6}Si_2B$. The calculated density of $Co_{4.7}Si_2B$ is 7.24 g/cm^3 and that of $Co_{4.6}Si_2B$ is 7.09 g/cm^3 as compared with the observed value of 7.21 g/cm^3. The results of the single crystal investigation are also more consistent with the formula $Co_{4.7}Si_2B$ rather than with the formula $Co_{4.6}Si_2B$.

The l.p. of $Co_{4.7}Si_2B$ (Table 2) are nearly the same in the different three-phase regions indicating a small homogeneity range of this phase. It seems reasonable to assume that the homogeneity range depends on a variation in

Table 2. Lattice parameters of the $Co_{4.7}Si_2B$ phase. (Estimated accuracy ± 0.03 %.)

Three-phase region	Lattice parameters in Å			
	a	*c*	*c/a*	*V*
$Co_2B-Co_2Si-Co_{4.7}Si_2B$	8.624	4.248	0.4926	316.0 Å³
$Co_2Si-CoSi-Co_{4.7}Si_2B$	8.618	4.252	0.4934	315.8
$Co_2B-CoB-Co_{4.7}Si_2B$	8.621	4.247	0.4926	315.6
$CoSi-CoB-Co_{4.7}Si_2B$	8.605	4.249	0.4938	314.6

cobalt content (meaning a varying amount of vacancies in the cobalt skeleton) and on a B-Si substitution. Increasing the cobalt content should increase the volume of the unit cell while this should decrease with increasing B/Si ratio. As the unit cell volume is nearly constant for the first three of the three-phase regions shown in Table 2, we have sketched the single-phase range (Fig. 2) so that with increasing cobalt content, the B/Si ratio also increases. With a simultaneous increase of these two parameters, the volume of the unit cell will be very little affected.

Alloys of approximate composition Co_5Si_2B were annealed at temperatures between 950°C and 1 150°C, but no transformation of the ternary phase was observed, showing that $Co_{4.7}Si_2B$ is probably stable up to the melting point. Since a phase with the composition Me_5SiB_2 has been found both in the Fe-Si-B and in the Mn-Si-B systems, alloys of approximate composition Co_5SiB_2 were also annealed between 950°C and 1 150°C. However, only the three phases Co_2B, CoB and $Co_{4.7}Si_2B$ were found in the samples and it was concluded that no phase with the composition Co_5SiB_2 exists above 950°C.

Some alloys were prepared in the "cobalt-poor" part of the system (< 50 at % Co). CoSi and $CoSi_2$ were found to be in equilibrium with some very "boron-rich" phase, but no lines from this phase were observed on the powder photographs. The phase is amorphous and possibly is amorphous boron.

The crystal structure of $Co_{4.7}Si_2B$. The powder photograph of $Co_{4.7}Si_2B$ showed that the crystal structure of this phase must be very similar to that found for the tetragonal Me_5Si_3 silicides of the W_5Si_3($T1$) type [17–19]. It was considered important to study the $Co_{4.7}Si_2B$ structure more closely for two reasons. Firstly, it would be of interest to know whether the metal skeleton of $Co_{4.7}Si_2B$ is, in fact, entirely analogous to that of the W_5Si_3 type silicides

Table 3. Results of the single crystal investigation on $Co_{4.7}Si_2B$. (Space-group $D_{4h}^{18}-I4/mcm$.)

Atomic parameters		Relative scattering parameters from the $\varrho(xy)$ proj.	$\varrho(xy0)$ and $\varrho(xy1/4)$ sections	Corresponding parameters in W_5Si_3	Corresponding parameters in Cr_5Si_3
16 Co_I in 16(k)	$x = 0.0757$	27.4	27.0	$x = 0.074$	$x = 0.075$
	$y = 0.2036$			$y = 0.223$	$y = 0.224$
2.5–3 Co_{II} in 4(b)		18.0	19.6		
8 Si in 8(h)	$x = 0.162$	14.0		$x = 0.17$	$x = 0.165$
4 B in 4(a)		4.8			

as this situation would imply the existence of Co-Co contacts shorter than 2.13 Å (= $c/2$). Secondly, it would be interesting to know whether or not the boron and silicon atoms are statistically distributed in the structure. A detailed study of the crystal structure of $Co_{4.7}Si_2B$ might also give some explanation for the deviation from stoichiometry.

The result of the single crystal investigation is shown in Table 3. (For comparison some data on W_5Si_3[17] and Cr_5Si_3[18] are included.) The systematic extinctions were consistent with the space-group D_{4h}^{18}—$I\ 4/mcm$. The atomic parameters were refined from successive twodimensional $\varrho_o(xy)$ and $\varrho_c(xy)$ syntheses with the last backshift-corrections being 0.0003 for the cobalt parameters and 0.001 for the silicon parameter. This procedure gave the final parameters $x_{Co} = 0.0757$, $y_{Co} = 0.2035$ and $x_{Si} = 0.162$ in excellent agreement with the values $x_{Co} = 0.0758$, $y_{Co} = 0.2037$, and $x_{Si} = 0.162$ derived from the threedimensional ϱ_o ($xy0$) section. The scattering parameters were obtained from the $\varrho(xy)$ projection. By choosing the scattering parameters proportional to 27.0, 18.0, 14.0 and 5.0 the final (ϱ_o—ϱ_c) synthesis was very nearly zero for all values of x and y. The final R-value of the 26 observed nonequivalent $hk0$ reflexions was 6.1 %. A temperature factor with $B = 0.72$ Å^2 was employed. If the scattering parameters were assumed to be proportional to 27.0, 27.0, 14.0 and 5.0 respectively the R-value did not drop below 16 %.

Evidently, the 16(k) positions are occupied mainly by cobalt atoms; the 8(h) positions, mainly by silicon atoms and the 4(a) positions, mainly by boron atoms. The number of electrons found in the 4(b) positions, however, is much smaller than one would expect if this position was entirely occupied by cobalt atoms. (A reinspection of the ϱ_o (xy) projection which was evaluated during the determination [17] of the structure of W_5Si_3 showed that in W_5Si_3 there is no significant difference in the scattering parameters for atoms occupying 16(k) and 4(b), respectively.) The small number of electrons found in the 4(b) position can be explained either by assuming that this position is only partly (to 70 %) occupied by cobalt atoms or that it is occupied by a mixture of cobalt, silicon, and boron atoms. The composition of the ternary phase required for the second alternative is shown by the dotted line in Fig. 2. This line has been constructed with the assumption that the 4(b) positions are occupied

Table 4. Interatomic distances in $Co_{4.7}Si_2B$. (Estimated accuracy ± 0.03 Å.) (Distances have an asterisk when one of the atoms are in the partly occupied position 4(b).)

			Average (Å)
Co_I	— 9 Co	2.49(2); 2.69; 2.64(2); 2.65(2); 2.84(2) *	2.66
	— 4 Si	2.29; 2.35; 2.53(2)	2.42
	— 2 B	2.15 (2)	2.15
Co_{II}	— 10 Co	2.13(2) **; 2.84(8) *	2.70
	— 4 Si	2.24(4) *	2.24
Si	— 10 Co	2.24(2) *; 2.29(2); 2.35(2); 2.53(4)	2.38
	2 Si	3.02(2)	
B	— 8 Co	2.15(8)	2.15
	— 2 B	2.13(2)	2.13

by a mixture of cobalt, silicon and boron with a weighted mean atomic number of 20. Evidently this alternative is not the correct one. The first alternative corresponds to a composition of $Co_{4.67}Si_2B$, in excellent agreement with the formula $Co_{4.7}Si_2B$ arrived at in the preceding paragraph.

The atomic parameters in $Co_{4.7}Si_2B$ are different from those in W_5Si_3[17] and Cr_5Si_3[18], which means that the antiprismatic holes of the metal skeleton (where the boron atoms are situated in $Co_{4.7}Si_2B$) are relatively smaller in $Co_{4.7}Si_2B$ than in W_5Si_3 and Cr_5Si_3. The distance from the centre of the antiprismatic hole to the eight neighbouring metal atoms is only 2.15 Å in $Co_{4.7}Si_2B$ while the corresponding distance in Cr_5Si_3[18] is 2.46 Å. The different atomic parameters also imply that Si-Me distances much shorter than in Cr_5Si_3 and W_5Si_3 have been created in $Co_{4.7}Si_2B$. The distance between the silicon atoms and the cobalt atoms in the partly occupied position is only 2.24 Å while the shortest Me-Si contact in Cr_5Si_3 is 2.42 Å[18]. The *average* Co-B and Co-Si distances in $Co_{4.7}Si_2B$ (2.15 Å and 2.38 Å) are very similar to those found in Co_2B[3] (2.15 Å) and Co_2Si (2.42 Å)[8].

This detailed inspection of $Co_{4.7}Si_2B$ has revealed some significant differences between the crystal structure of this phase and that of the $W_5Si_3(T1)$ type silicides. The deviation from stoichiometry for $Co_{4.7}Si_2B$ is dependent upon the fact that the 4(*b*) positions are only partly (to 65—70 %) occupied by cobalt atoms, thus leaving vacancies in the structure. The difference in sizes of the two types of holes in the metal skeleton is larger in $Co_{4.7}Si_2B$ than in the binary Me_5Si_3 phases of $W_5Si_3(T1)$ type. The boron and silicon atoms are ordered in $Co_{4.7}Si_2B$ with the boron atoms occupying the smaller holes of the metal skeleton and the silicon atoms, the larger.

INTERMEDIATE PHASES OF THE Fe-Si-B AND Mn-Si-B SYSTEMS

The Fe-Si-B system is more complicated than the Co-Si-B system. In the metal-rich (> 50 at % Fe) part of the system, four ternary phases have been found in alloys sintered at 975°C (Table 5). One of these phases is isomorphous with $Co_{4.7}Si_2B$ and probably has a similar composition. Fe_5SiB_2 crystallizes in the $Cr_5B_3(T2)$[20,19] structure and thus is isomorphous with Mo_5SiB_2[21] and Fe_5PB_2[22]. The crystal structure of a phase with the approximate composition Fe_4BSi is apparently unknown. A cementite (Fe_3C) type phase with slightly varying l.p. has also been found. Since the volume of the unit cell has been found to be smaller for this phase than for $Fe_3C_{0.2}B_{0.8}$[23],

Table 5. Intermediate phases in the Fe-Si-B and Mn-Si-B systems at 975°C.

	Approximate compositions and lattice parameters (in Å)	Structure type
$Fe_{4.7}Si_2B$	$a = 8.82$; $c = 4.34$	$Co_{4.7}Si_2B$ ($T1$)
Fe_5SiB_2	$a = 5.54$; $c = 10.32$	Cr_5B_3 ($T2$)
$Fe_3(Si,B)$ or		
$(Fe,Si)_3B$ (boron-rich)	$a = 4.46$; $b = 5.36$; $c = 6.66$; $V = 159$ Å^3	Fe_3C (cementite)
(boron-poor)	$a = 4.47$; $b = 5.30$; $c = 6.67$; $V = 158$ Å^3	
Fe_4BSi		unknown
Mn_5SiB_2	$a = 5.61$; $c = 10.44$	Cr_5B_3 ($T2$)

it has been assumed that the formula should be written $(Fe, Si)_3B$. However, the small unit cell volume could also be explained by assuming the formula to be $Fe_3(Si, B)$ with vacancies in the iron skeleton.

In the Mn-Si-B system Mn_5SiB_2 (isomorphous with Fe_5SiB_2) has been found but no phase analogous with $Co_{4.7}Si_2B$ seems to exist in this system.

A more detailed account of the Fe-Si-B system will soon appear in this journal.

Acknowledgements. The research described in this paper was sponsored by the *European Commission of Air Research and Development Command* under Contract no. AF61(052)-40. The financial support from this authority is gratefully acknowledged.

We want to thank professor G. Hägg for many valuable suggestions and Dr. S. Rundqvist for stimulating discussions. We want to express our gratitude to Dr. Lilly Gustafsson who devoted much time to the chemical analyses. The oxygen analyses were performed at the Analytical Department of Avesta Jernverk and we are much indebted to Dr. G. Liljekvist and Mr. E. Gunnarsson of this factory. Thanks are also due to Mr. K. Nordgren who carried out the density determinations.

REFERENCES

1. Nowotny, H., Dimakopoulou, E. and Kudielka, H. *Monatsh.* **88** (1957) 180.
2. Nowotny, H., Kieffer, R. and Benesovsky, F. *Planseeber. Pulvermet.* **5** (1957) 86.
3. Bjurström, T. *Arkiv Kemi, Mineral. Geol.* **A11** (1933) No. 5.
4. Rundqvist, S. *Nature* **181** (1958) 259.
5. Rundqvist, S. *Acta Chem. Scand.* **12** (1958) 658.
6. Borén, B. *Arkiv Kemi, Mineral. Geol.* **A11** (1933) No. 10.
7. Borén, B., Stahl, S. and Westgren, A. *Z. Physik. Chem.* **B29** (1935) 231.
8. Geller, S. *Acta Cryst.* **8** (1955) 83.
9. Smithells, C. J. *Metal Reference Book*, London 1955, p. 221.
10. Toman, K. *Acta Cryst.* **5** (1952) 329.
11. Schubert, K. and Pfisterer, H. *Z. Metallkunde* **41** (1950) 433.
12. Bertaut, F. and Blum, P. *Compt. rend.* **231** (1950) 626.
13. Vogel, R. and Rosenthal, K. *Arch. Eisenhüttenw.* **7** (1934) 689.
14. Hashimoto, V. *Nippon Kinzoku Gakkaishi* **1** (1937) 135.
15. Hägg, G. and Laurent, T. *J. Sci. Instr.* **23** (1946) 155.
16. Anantharaman, T. R. and Christian, J. W. See Hume-Rothery, W. and Raynor, G. V. *The structure of Metals and Alloys*, London 1954.
17. Aronsson, B. *Acta Chem. Scand.* **9** (1955) 1107.
18. Dauben, C. H., Templeton, D. H. and Myers, C. E. *J. Phys. Chem.* **60** (1956) 443.
19. Parthé, E., Lax, B. and Nowotny, H. *Monatsh.* **86** (1955) 859.
20. Bertaut, F. and Blum, P. *Compt. rend.* **236** (1953) 1055.
21. Aronsson, B. *Acta Chem. Scand.* **12** (1958) 31.
22. Rundqvist, S. *Private communication.*
23. Nicholson, M. E. *J. Metals* **9** (1957) 1.

Received November 7, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 442—455

Absorption Spectra of Alkyl *iso*Thiocyanates and N-Alkyl Monothiocarbamates *

EMIL SVÁTEK, RUDOLF ZAHRADNÍK and ANDERS KJÆR

Research Institute for Pharmacy and Biochemistry, Prague, Czechoslovakia; Institute of Industrial Hygiene and Occupational Diseases, Prague, Czechoslovakia; Organic Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, Denmark

Ultra-violet absorption spectra of twenty-two *iso*thiocyanates and N-alkyl monothiocarbamates have been determined. Whereas the former class of substances invariably displays a maximum of moderate intensitity ($\varepsilon \sim 10^3$) in the 244—248 mμ region, the N-alkyl monothiocarbamates possess absorption bands in the range 226—232 mμ and an attempt has been made to correlate the wave-length position of the absorption maximum with the structure of the substituents.

In the infra-red range of the spectrum, alkyl *iso*thiocyanates exhibit characteristic bands in two regions. The asymmetric stretching mode of the cumulative double bond system of the —N=C=S-grouping produces a moderately intense band at 2 170—2 221 cm^{-1}, accompanied by a group of two to three strong bands at 2 050—2 150 cm^{-1}, as well as a very weak band in the 2 000 cm^{-1} region. The position and intensity of the bands depend on the polarity of the solvents employed. The second characteristic frequency of alkyl *iso*thiocyanates occurs at 1 318—1 347 cm^{-1} and is assigned to the bending vibrations of —CH- atoms adjacent to the -N=C=S-grouping. This very strong band has been observed in all compounds possessing the RCH_2-N=C=S grouping, whereas methyl, *tert.*-butyl, phenyl and α-naphthyl *iso*thiocyanates do not exhibit typical absorption in the vicinity of 1 330 cm^{-1}.

The electronic spectra of alkyl *iso*thiocyanates have attracted only little attention heretofore. Kremann and Riebl [1] determined the absorption curve of allyl *iso*thiocyanate, whereas Pestemer and Litschauer [2] in addition to this mustard oil studied the spectroscopical properties of the ethyl- and monomethoxy-derivatives. According to these authors, *iso*thiocyanates possess absorption maxima at approximately 250 mμ with log $\varepsilon \sim 3$. Although the absorption properties of N-alkyl monothiocarbamates in the ultra-violet region have been utilized analytically they do not seem to have been subjected to detailed studies previously.

* Part III of the series: Reaction Kinetics and Properties of Compounds Belonging to Homologous Series of Nonaromatic Compounds. Part II: *Collection Czechoslov. Chem. Communs. In press.*

There does not exist any comprehensive treatise of the infra-red spectra of alkyl *iso*thiocyanates. The first data on the frequencies characteristic for such compounds were presented by Coblenz [3] who studied methyl, ethyl, allyl and phenyl *iso*thiocyanate. In view of the imperfect technique used in 1905, however, these frequencies can hardly be considered as too reliable. Gordy and Williams [4] later reported methyl and ethyl *iso*thiocyanate to possess bands at (3 012 cm^{-1}, 2 127 cm^{-1}) and (3 039 cm^{-1}, 2 205 cm^{-1}), respectively. Williams [5] recorded frequencies of the phenyl *iso*thiocyanate bands in the 650—3 000 cm^{-1} range but payed particular attention to the vibration of the —N=C=S-grouping, which displayed a broad band at 2 100 cm^{-1}. This consists, according to geometrical analysis, of three bands at 2 130, 2 080 and 1 950 cm^{-1}. As an explanation of this phenomenon the author considered Fermi resonance of the —N=C=S-group vibration, accompanied by an overtone of the fundamental frequency at 685 cm^{-1}.

More recently, frequency values of some *iso*thiocyanates have been listed in the papers by Ettlinger *et al.* (3-butenyl, *trans*- and *cis*-crotyl [6], *m*-methoxybenzyl [7]), Kjær and colleagues (*trans*-crotyl [8], *p*-methoxy-benzyl [9] and *o*-methoxybenzyl [10]) and Luskin *et al.*[11] The last authors summarized previous data and quoted the range 2 040—2 180 cm^{-1} for the strong NCS-group absorption. On basis of own experiments they concluded that *iso*thiocyanates in carbon tetrachloride solution display a strong band at 2 095 $\pm$ 15 cm^{-1}, frequently accompanied by a weak band at 2 000 cm^{-1}.

Attention should be drawn also to the Raman spectra of *iso*thiocyanates. In these, two especially intensive lines were observed at 2 100 and 2 170 cm^{-1}, both attributable to the —N=C=S group, rather than the expected single line [12,13]. Bagder [14] explained this doubling of the line of the —N=C=S mode in terms of Fermi resonance of the —N=C=S vibration with an overtone of the fundamental line at 1 070 cm^{-1}. This explanation, however, was later criticized by Kohlrausch [13] because it only applies in some cases (methyl and ethyl *iso*thiocyanate), whereas it does not hold for other derivatives. On basis of the Raman spectra a strong contribution of the mesomeric structure $R—\overset{\oplus}{N} \equiv C—S^{\ominus}$ to the *iso*thiocyanate structure has been suggested.

The present paper records the results from investigations of the absorption spectra of a series of alkyl *iso*thiocyanates and N-alkyl monothiocarbamates in the ultra-violet region between 220 and 350 mμ. It further presents the infra-red spectra of twenty-two *iso*thiocyanates in the region of the —N=C=S vibration (2 100 cm^{-1}). Of these, twelve selected species were further studied in the 1 600—650 cm^{-1} region where the —N=C=S grouping has not formerly been characterized.

EXPERIMENTAL

Apparatus and methods. Absorption curves in the ultra-violet region were recorded with the Sowiet spectrophotometer SF 4 at 21 $\pm$ 2°. All solutions were measured in 1.00 cm cells. The concentrations employed were usually 10^{-3} M or 10^{-4} M, depending on whether *iso*thiocyanates or N-substituted monothiocarbamates were being examined. Extinction readings were taken with 1 mμ intervals in the vicinity of absorption maxima, otherwise in steps of 5 mμ. Wave-length values were controlled by a mercury lamp and

are regarded as accurate within ± 0.5 mμ in the 250 mμ region. Extinction values are considered accurate to ± 3 %. Most absorption curves of alkyl *iso*thiocyanates were recorded in dioxane solution, whereas samples of the N-alkyl monothiocarbamates were prepared *in situ* by allowing such an amount of alkyl *iso*thiocyanate to react with 0.1 N NaOH that the resulting solution became 10^{-4} M. Four hours later, the absorption curves of the N-alkyl monothiocarbamates, resulting from the reaction of alkyl *iso*thiocyanates with the OH-ions [15], were determined. At the end of this time, the reaction had proceeded virtually to completion in all cases except when the slower reacting *tert.*-butyl *iso*thiocyanate was employed.

Infra-red spectra were determined with a Perkin-Elmer Model 12 B single-beam spectrophotometer with rock salt optics, and with a Zeiss Jena UR 10 double-beam spectrophotometer equipped with a lithium fluoride prism (for the range 1 900—2 300 cm^{-1}). The accuracy of frequencies in well-developed bands is ± 1 cm^{-1}. The instrument was calibrated against ammonia, atmospheric water and carbon dioxide, as well as polystyrene, benzene, pyridine and chloroform. The spectra were registered at constant temperature (25°), maintained by a room thermostate.

Reagents. The alkyl *iso*thiocyanates examined were the same substances as those studied in a preceding paper [15]. Dioxane (Lachema, reagent grade) was refluxed prior to use with a tenth of a volume of 4 % hydrochloric acid in a stream of nitrogen. Water and hydrochloric acid were then removed by shaking with solid potassium hydroxide. Thereafter, the dioxane was boiled for 9 h with metallic sodium, the precipitate and excess sodium were removed and the product distilled over fresh sodium. The main run was used in the experiments. Every third day, the dioxane was redistilled over sodium and the solvent was stored in the solid state in a refrigerator.

Other solvents employed were purified by standard procedures. All materials used were of reagent grade purity (Lachema).

RESULTS

Ultra-violet spectra of alkyl iso*thiocyanates and N-alkyl monothiocarbamates.* In the region of electronic spectra alkyl *iso*thiocyanates are characterized by a single absorption band of medium intensity ($\varepsilon \sim 10^3$) possessing a maximum between 244 and 248 mμ. Absorption curves of all mustard oils examined display the same general pattern, exemplified by the absorption curve (No. 1)

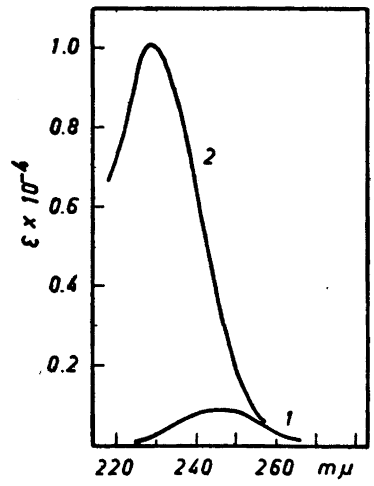


Fig. 1. The molecular extinction coefficient (ε) as a function of the wave-length. 1. *cyclo*Hexyl *iso*thiocyanate in dioxane; 2. N-*cyclo*hexyl monothiocarbamate in 0.1 M NaOH.

Table 1. Absorption data for ethyl *iso*thiocyanate (0.001 M solutions) in various solvents.

	Pentane 95 % Dioxane 5 %	Chloroform 90 % Dioxane 10 %	Dioxane	0.05 M HCl 50 % (aqueous) Ethanol 40 % Dioxane 10 %
λ_{max} (mμ)	250	245	245	241
log ε	2.94	2.86	2.86	2.82

Table 2. Position and intensity of ultra-violet absorption maxima of alkyl *iso*thiocyanates and N-alkyl monothiocarbamates.

No.	Alkyl R	Number of α-substituents	*iso*Thiocyanates: R—NCS[a]			Monothiocarbamates: R—NH—COS$^{\ominus}$[f]		
			$\Delta\lambda_{max}$, Et[b]	$\Delta\lambda_{max}$, Al[c]	log ε	$\Delta\lambda_{max}$, Et[b]	$\Delta\lambda_{max}$, Al[c]	log ε
I	Methyl	0	—1		2.8	—1		4.0
II	Ethyl	1	0[d]		2.9	0[g]		4.0
III	*iso*Propyl	2	1		2.90	2		3.9
IV	*tert.*-Butyl	3	3		3.05	6		—
V	*iso*Butyl	1	0		2.86	0		3.9
VI	*n*-Butyl	1	0		2.85	0.5		4.0
VII	*n*-Amyl	1	0		2.83	0.5		4.0
VIII	*n*-Hexyl	1	0		2.78	0.5		4.0
IX	*n*-Octyl	1	0		2.94	0.5		4.0
X	*cyclo*Hexyl	2	1.5		2.94	3		4.0
XI	Allyl	1	1	0[e]	2.94	1	0[h]	4.1
XII	DL-α-Methallyl	2	3	2	2.98	2	1	4.0
XIII	β-Methallyl	1	2	1	2.97	1	0	4.0
XIV	*trans*-Crotyl	1	1.5	0.5	3.04	1	0	4.1
XV	3-Butenyl	1	1	0	2.94	1	0	4.0
XVI	4-Pentenyl	1	0	—1	2.85	0	—1	4.0
XVII	Benzyl	1	3	2	3.08	3	2	4.1
XVIII	DL-α-Phenethyl	2	2.5	1.5	3.19	2	1	4.0
XIX	3-Methyl-thiopropyl	1	0.5		3.01	0		4.1
XX	4-Methyl-thiobutyl	1	0		2.98	0		4.0
XXI	5-Methyl-thiopentyl	1	0		2.97	0		4.0
XXII	Phenyl	1	25		3.98	32		4.2

[a] Measured in 10^{-3} M solutions in dioxane. [b] The relative absorption maxima $\Delta\lambda_{max, Et} = \lambda_{max, i} - \lambda_{max, Et}$, where $\lambda_{max, i}$ is the position of the absorption maximum of the ith derivative and $\lambda_{max, Et}$ is a similar symbol for the reference derivative (ethyl). [c] The same symbol as defined for [b], yet with the allyl derivative as the reference compound. [d] λ_{max} (ethyl) = 245 mμ; [e] λ_{max} (allyl) = 246 mμ; [f] Measured in 10^{-4} M solution in 0.1 N NaOH; [g] λ_{max} (ethyl) = 226 mμ. [h] λ_{max} (allyl) = 227 mμ.

of Fig. 1. The influence of solvent composition was investigated in the case of ethyl *iso*thiocyanate (Table 1). The position and intensity of the absorption maxima of all species studied are summarized in Table 2.

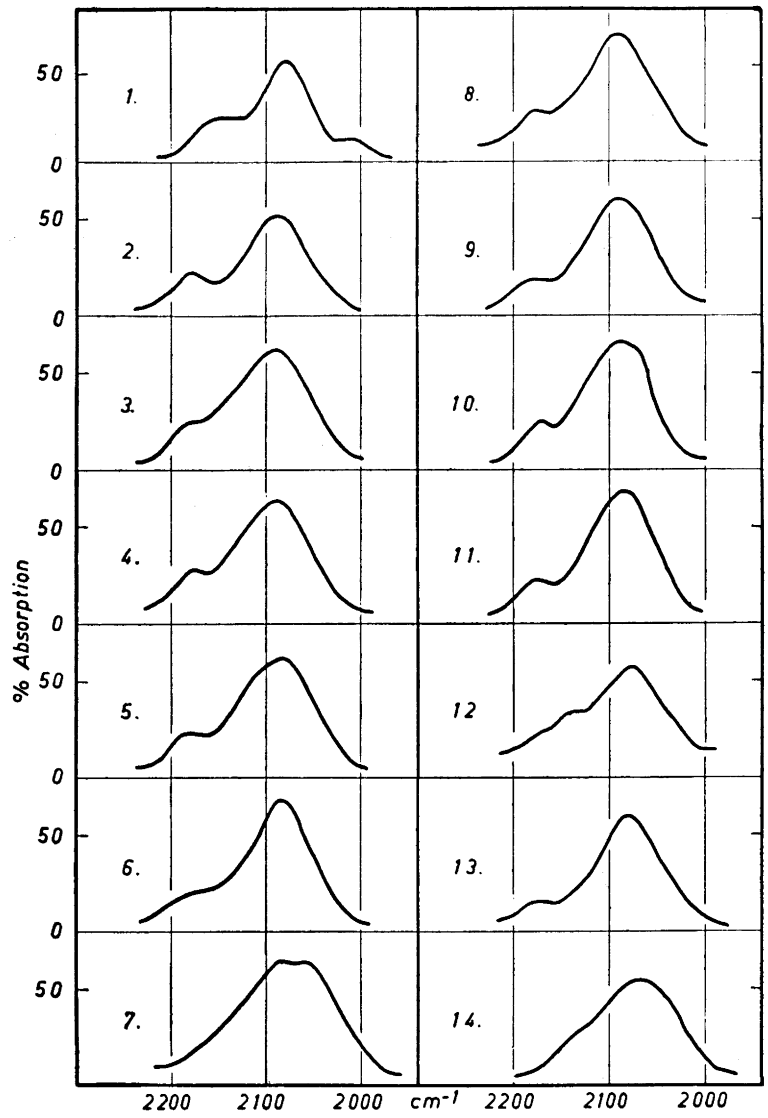


Fig. 2. Infra-red absorption bands of various *iso*thiocyanates in the 1 900—2 300 cm^{-1} region. (The thickness of the cell is 0.10 mm, concentration: 0.1 M in CCl_4, sodium chloride prism). 1. *iso*Propyl-, 2. *iso*butyl-, 3. *n*-hexyl-, 4. *n*-amyl-, 5. *iso*amyl-, 6. β-methallyl-, 7. DL-α-methallyl-, 8. *n*-octyl-, 9. 3-methylthiopropyl-, 10. 4-methylthiobutyl-, 11. 3-butenyl-, 12. *trans*-crotyl-, 13. benzyl-, 14. DL-α-phenethyl-*iso*thiocyanate.

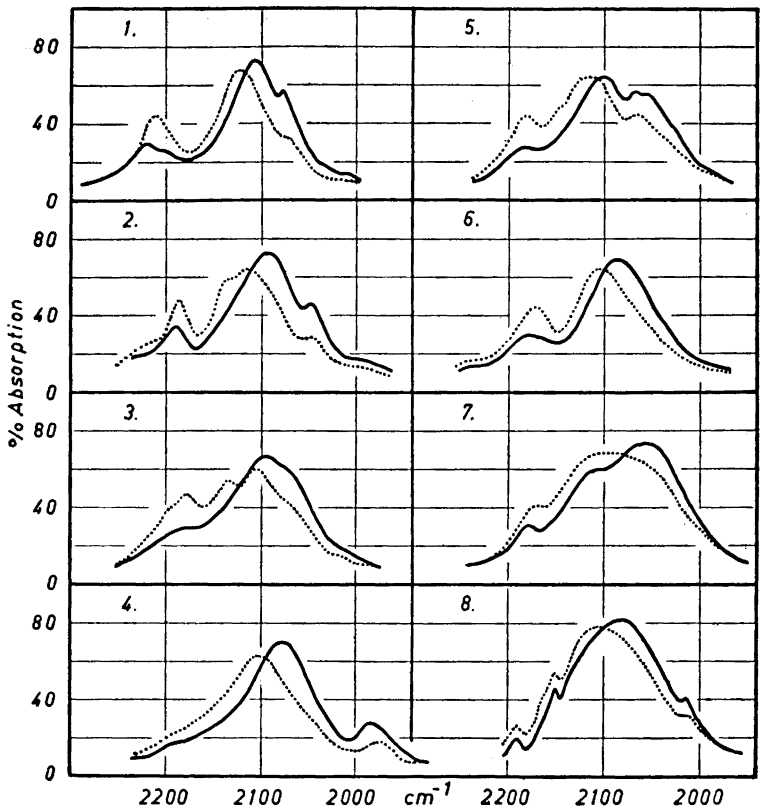


Fig. 3. Infra-red absorption bands of various *iso*thiocyanates in the 1 900—2 300 cm^{-1} region. (Concentration 0.1 M; in CCl_4-solution: full line; in $CHCl_3$: dotted line; absorption cell 0.10 mm, lithium fluoride prism). 1. Methyl-, 2. ethyl-, 3. *n*-butyl-, 4. *tert.*butyl-, 5. *cyclo*hexyl-, 6. allyl-, 7. phenyl-, 8. *α*-naphthyl- *iso*thiocyanate.

The general shape of the absorption curves of N-alkyl monothiocarbamates are similar to those of the mustard oils except for their higher intensity of the maximum absorption bands ($\varepsilon \sim 10^4$) (*cf.* Fig. 1, curve 2) which are located between 226 and 232 mμ (Table 2).

Infra-red spectra of alkyl *iso*thiocyanates

The 1 900—2 300 cm^{-1} region. In this region the spectra of twenty-two different *iso*thiocyanates were measured in carbon tetrachloride solution (0.1 M) in an 0.1 mm absorption cell (sodium chloride prism). Within this group, eight selected compounds were studied under comparable conditions at the higher resolving power of a lithium fluoride prism, and, in addition, in chloroform solution. Spectra of ethyl *iso*thiocyanate were recorded also

Table 3. Position (cm^{-1}) and intensity (% absorption) of bands of the —N=C=S-grouping in the 1 900—2 300 cm^{-1} region. (Concentration 0.1 M. (a): in CCl_4; (b): in $CHCl_3$. Thickness of the absorption cell 0.10 mm, lithium fluoride prism.)

Substituent	cm^{-1}	% A	cm^{-1}	% A	cm^{-1}	% A	cm^{-1}	% A	cm^{-1}	% A	Solvent
Methyl	2 221	30			2 106	74	2 077	56			a
	2 210	44			2 123	67	2 071	31			b
Ethyl	2 187	34			2 092	73	2 049	45			a
	2 184	47	2 135	58	2 113	63	2 046	28			b
n-Butyl	2 173	30 (i)*			2 097	66	2 068	56 (i)			a
	2 178	47	2 135	54	2 105	60	2 068	40 (i)			b
Allyl	2 172	31			2 087	68	2 072	63 (i)			a
	2 170	44			2 103	64	2 070	?			b
*cyclo*Hexyl	2 180	28			2 100	64	2 053	54			a
							2 067	56			a
	2 182	43	2 148	? (i)	2 113	63	2 064	44			b
tert.-Butyl					2 079	72			1 982	29	a
					2 104	64			1 976	19	b
Phenyl	2 177	30			2 106	60	2 054	74			a
	2 168	40			(2 100)	66	2 065	64			b
α-Naphthyl	2 190	20	2 150	45	2 080	82			2 015	41	a
	2 190	26	2 150	54	2 105	78			2 015	32	b

* (i): inflection.

in two extreme concentrations, *viz.* 0.01 M (1 mm cell) and 1.00 M (0.03 mm cell), as well as in the undiluted liquid and vaporous phase. The experimental curves are presented in Figs. 2 and 3, and the main frequencies and absorption data of eight selected compounds are reviewed in Table 3.

A typical feature of all the above compounds is the appearance of three to four bands which can be well differentiated: (i) a band of medium intensity in the 2 170—2 221 cm^{-1} region, (ii) a group of two to three strong bands in the range 2 050—2 150 cm^{-1}, and (iii) a weak band around 2 000 cm^{-1} which, in most cases, is poorly distinguishable and hence has not been studied in detail.

In chloroform solution a striking change of band intensities was observed on comparison with experiments where carbon tetrachloride served as the solvent. The band at 2 170—2 221 cm^{-1} rose at the expense of that located at 2 050—2 080 cm^{-1} whereas the strong band at 2 100 cm^{-1} was displaced towards higher wave-numbers ($\Delta\nu \sim 20$ cm^{-1}) and slightly decreased in intensity. The total band intensity, however, expressed as $\int A \, d\nu$ (the area of all bands from 1 950 cm^{-1} through 2 350 cm^{-1}), remained the same in carbon tetrachloride and chloroform.

In the above-mentioned region the spectra of the whole series of mustard oils are essentially identical, yet with three striking exceptions, *viz.* methyl, *tert.*-butyl and phenyl *iso*thiocyanate. Among all compounds examined, the methyl derivative displays bands at the highest frequencies. Tertiary butyl *iso*thiocyanate is devoid of any maximum in the 2 180 cm^{-1} region (in chloroform a shoulder only), but displays a maximum at 1 985 cm^{-1}. Finally, phenyl

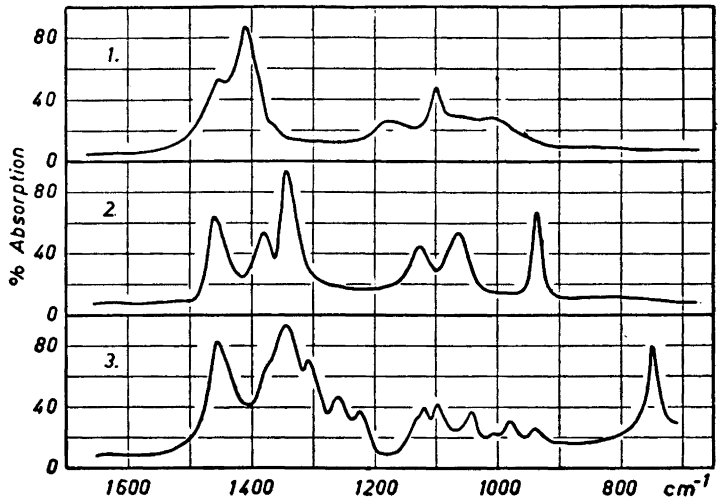


Fig. 4. Infra-red spectra of various *iso*thiocyanates. (5 % solutions in CCl_4 or CS_2). 1. Methyl *iso*thiocyanate. 2. Ethyl *iso*thiocyanate. 3. *n*-Butyl *iso*thiocyanate.

*iso*thiocyanate has its predominant bands at 2 054 cm^{-1} (in CCl_4) and also absorbs most intensely of all compounds listed in Table 3 in terms of $\int A\, d\nu$.

Ethyl *iso*thiocyanate, a typical representative of the present series, served as a model compound for the study of the dependence of the position and intensity of absorption bands on concentration, in carbon tetrachloride as

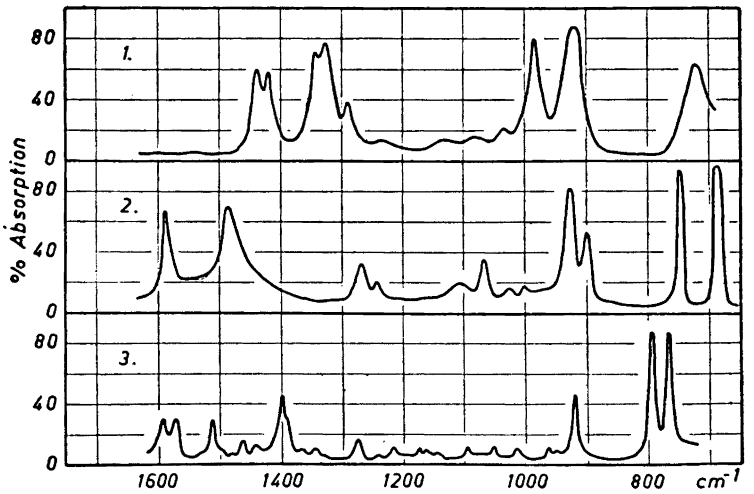


Fig. 5. Infra-red spectra of various *iso*thiocyanates (5 % solutions in CCl_4 or CS_2). 1. Allyl *iso*thiocyanate. 2. Phenyl *iso*thiocyanate. 3. α-Naphthyl *iso*thiocyanate.

well as chloroform. No conspicuous changes were noted, however. The spectra of the ethyl derivative as a film (liquid state) and in the vaporous phase did not deviate from those determined in solution.

The 1 800—1 500 cm^{-1} region. In this "double bond range" we have found no bands in the alkyl *iso*thiocyanates. Only derivatives of unsaturated alkyls and aromatic derivatives display absorption in agreement with literature assignments [18].

The 1 500—650 cm^{-1} region. Some typical regularities characteristic for the presence of the —N=C=S grouping were observed in this spectral range (Figs. 4 and 5). In the homologous series of normal substituents from ethyl to *n*-octyl, a strong absorption band at 1 346—1 348 cm^{-1} was apparent (ethyl 1 346 cm^{-1}, *n*-butyl 1 347 cm^{-1}, *n*-amyl 1 347 cm^{-1}, *n*-hexyl 1 348 cm^{-1}, *n*-octyl 1 347 cm^{-1}). Similarly, the allyl and benzyl derivatives displayed a band at 1 345 and 1 347 cm^{-1}, respectively. In *iso*propyl and *cyclo*hexyl *iso*thiocyanate the position of this band shifted to 1 329 and 1 318 cm^{-1}. Methyl, *tert.*-butyl, phenyl and α-naphthyl mustard oil again showed anomalous behaviour in this region compared with other compounds. None of them absorbed in the 1 350—1 300 cm^{-1} range, whereas methyl *iso*thiocyanate exhibited an intensive band at 1 412 cm^{-1} and the *tert.*-butyl derivative at 1 205 cm^{-1}. The position of the 1 347 cm^{-1}-band appeared to be independent of the polarity of the solvent and the concentration.

DISCUSSION

Ultra-violet spectra

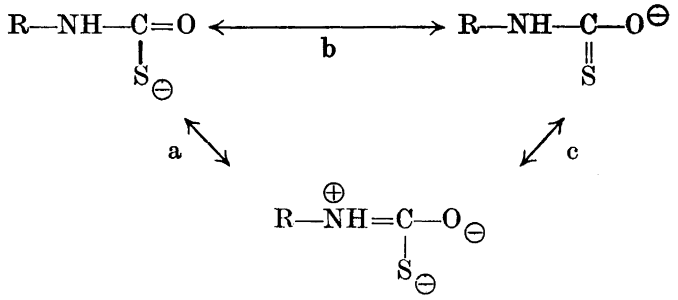
The molecular extinctions of the absorption maxima of alkyl *iso*thiocyanates (244—248 mμ) are approximately 10^3, a value intermediate between those characteristic for allowed and forbidden transitions. For the following reasons we believe that the absorption is assignable to the allowed N → V transition. An analysis of Raman as well as infra-red spectra suggests that the electron distribution in the NCS-grouping is approximately that of the dipolar form $R—\overset{\oplus}{N}\equiv C—\overset{\ominus}{S}$. The only likely N → V-transition in the present structure, however, is associated with the creation of cumulated double bonds ($R—\overset{\oplus}{N}\equiv C—\overset{\ominus}{S} \rightarrow R—N=C=S$) and such transitions are known to result in rather low extinction values [16]. The occurrence of an N → V-transition in the present case is supported also by the position of the absorption maximum. The range 245—260 mμ can be considered as the customary location for absorption bands attributable to transitions of the present type.

In this connexion attention should be drawn to the ultra-violet spectrum of the isomeric alkyl thiocyanates which exhibit [2] an absorption maximum of weak intensity ($\varepsilon = 30$) in the 240 mμ region, apparently belonging to a forbidden transition. This deviating pattern is not unexpected because the electron shift, characteristic for the N → V-transition, in this case would

imply not only the formation of cumulated double bonds but also, and more importantly, the participation of a polar ion containing trivalent sulphur.

$$R{-}S{-}C{\equiv}N \rightarrow R{-}\overset{\oplus}{S}{=}C{=}\overset{\ominus}{N}$$

The situation is more complicated in the N-alkyl monothiocarbamate series because three significant $N \rightarrow V$ transitions can be vizualized in molecules of this type, *viz.*:



It may be of interest to attempt an assignment of the absorption band (λ = 226—232 mμ) of high intensity ($\varepsilon \sim 10^4$), characteristic for alkyl monothiocarbamates, to one of these transitions (a, b, c). That represented by (a) is not active in the near ultra-violet region, as can be inferred, *inter alia*, from the insignificant absorption of N-alkyl carbamates in the 220—400 mμ region. Comparison of the position of the absorption maximum of N-alkyl monothiocarbamates with that of compounds containing N—C=S groupings (Fig. 6) suggests that the absorption maximum of the monothiocarbamates should be attributed to the transition (b) above. This interpretation, however, does not account for the fact that N-alkyl monothiocarbamates do not exhibit an additional maximum in the 245—260 mμ range, expectable from the transition (c) above. This is surprising, particularly because the formally similar dithiocarbamates and xanthogenates display maxima ascribable to the CSS- as well as to the N—C=S- or O—C=S-groupings.

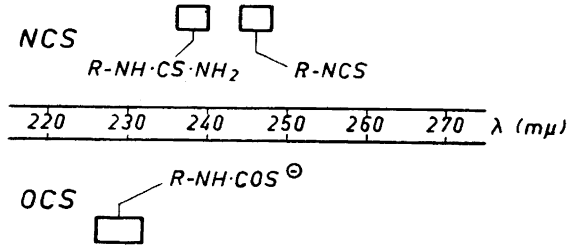


Fig. 6. Absorption maxima ranges of the —NCS and —OCS groupings in the ultra-violet region.

Influence of the structure of the R-group of iso*thiocyanates and monothiocarbamates on the position of the absorption maximum.* From Table 2 it can be seen that the introduction of any substituent in the methyl derivative in both series results in a bathochromic shift of the absorption maximum. This means that groups characterized by an $-I_S$ effect (*e.g.* allyl, benzyl, phenyl) as well as those displaying an $+I_S$ effect (*e.g. tert.*-butyl) displace the absorption maximum towards higher wave-lengths, *i.e.* the N → V-transition associated with the appearance of the absorption band requires lower energy. As both types of substituents ($+I_S$, $-I_S$) produce a qualitatively similar shift, we conclude that the extension of conjugation (due to hyperconjugation) is responsible, rather than the inductive effect. The data in Table 2 disclose the fact that replacing of the first hydrogen of the methyl grouping results in a shift of 1 mμ, whereas substitutions of the second and third hydrogens cause displacements of 2-4 mμ. Substitution at the β- and γ-carbon atom is not reflected by changes of absorption maxima, but in the case of "medium-size" *n*-alkyls (C_5, C_6, C_8) a shift of 1 mμ relative to the ethyl derivative was noticed in the monothiocarbamate series. In the *iso*thiocyanates these derivatives do not differ from the ethyl compound.

In the mustard oil series we consider hyperconjugation to be involved:

$$H_3\text{—}CH_2\text{—}\overset{\oplus}{N}\equiv C\text{—}\overset{\ominus}{S} \overset{h}{\longleftrightarrow} H\ \overset{\ominus}{\overline{C}}H_2\text{—}CH_2\text{—}\overset{\oplus}{N}\equiv C\text{—}\overset{\ominus}{S} \overset{(N\rightarrow V)}{\longleftrightarrow} H\ \overset{\oplus}{\overline{C}}H_2\text{—}CH_2\text{—}\overset{\ominus}{\overline{N}}=C=S$$

where h indicates a hyperconjugation displacement. Further substitution at the α-carbon atom of the ethyl derivative facilitates an extension of conjugation, whereas hyperconjugation in the methyl derivative operates less favourably, resulting in cumulation rather than conjugation of p electrons:

$$CH_3\text{—}\overset{\oplus}{N}\equiv C\text{—}\overset{\ominus}{S} \overset{h}{\longleftrightarrow} H\ \overset{\ominus}{\overline{C}}H_2\text{—}\overset{\oplus}{N}\equiv C\text{—}\overset{\ominus}{S} \overset{(N\rightarrow V)}{\longleftrightarrow} H\ \overset{\oplus}{\overline{C}}H_2\text{—}\overset{\ominus}{\overline{N}}=C=S$$

In both series, the ω-methylthioalkyl derivatives (XIX, XX and XXI) are closely comparable to the ethyl compound.

Apart from the number of substituents on the α-carbon atom (Table 2), the presence of π-electronic bonds in the substituting groups influences the magnitude of the bathochromic shift. Introduction of a double bond in β,γ-position (allyl derivatives) results in a shift equivalent to that produced by substitution of a hydrogen atom in the methyl derivative by a methyl grouping. Whereas in N-methallyl monothiocarbamate substitution at the α-carbon atom exclusively causes a bathochromic shift, the corresponding methallyl *iso*thiocyanate is changed by β-substitution as well. In the α-substituted mustard oil (XII) this displacement amounts to 3 mμ, which is more than the added influences of the allyl- (1 mμ) and the methyl-grouping (1 mμ) (estimated from the displacement on going from ethyl to *iso*propyl *iso*thiocyanate). However, considering the error attached to these measurements this difference does not warrant a more detailed discussion. It is interesting that the presence of a γ,δ-double bond, as in (XV), in both series produces an effect comparable to that observable in the allyl series. Previous examples of a corres-

ponding interaction between unconjugated chromophores across a dimethylene bridge have been recorded [17]. The presence of a δ,ε-double bond ((XVI) in Table 2) is without influence on the absorption pattern as estimated from the similarity between the spectra of (XVI) and the ethyl derivatives. In alkenyl *iso*thiocyanates the spectral displacement can again be ascribed to hyperconjugation, represented in the case of allyl *iso*thiocyanate by the following scheme:

$$CH_2{=}CH{-}CH_2{-}\overset{\oplus}{N}{\equiv}C{-}\overset{\ominus}{S} \xleftrightarrow{h} CH_2{=}CH{-}\overset{\ominus}{\underset{\oplus}{\underset{|}{CH}}}{-}\overset{\oplus}{N}{\equiv}C{-}\overset{\oplus}{S} \xleftrightarrow{(N \rightarrow V)}$$

$$\overset{\oplus}{H}\ \overset{\ominus}{\underset{|}{CH_2}}{-}CH{=}CH{-}\overset{\ominus}{N}{=}C{=}S$$

Besides *tert.*-butyl mustard oil, the benzyl and α-phenylethyl derivatives display the largest bathochromic displacement for reasons which we consider to be similar in nature to those suggested above for the alkenyl *iso*thiocyanates. A slight hypsochromic effect of the α-phenylethyl grouping, relative to benzyl mustard oil, rather than an expected displacement towards longer wave-lengths may be interpretable in terms of partial inhibition of the hyperconjugation effect or, rather, by steric interference of the conjugation between the aromatic ring and the NCS-grouping. The bathochromic shift associated with the introduction of a methyl-grouping in α-position of the allyl chain renders steric influence more likely as a deciding factor.

When the relative deviations in wave-lengths of maximum absorption in the *iso*thiocyanate and N-alkyl monothiocarbamate series are plotted (Fig. 7), a linear dependence appears. In case of unsaturated side chains the line acqui-

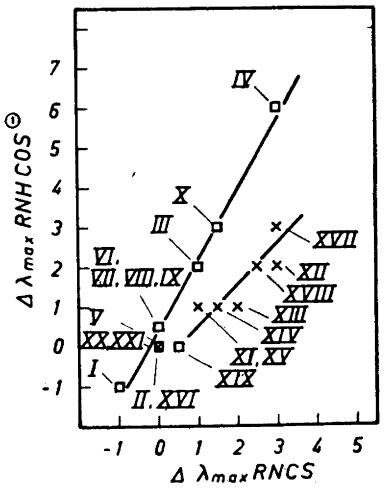


Fig. 7. Relationship between the relative absorption maxima of N-alkyl monothiocarbamates and alkyl *iso*thiocyanates (□ : compounds with saturated substituents; × : compounds with unsaturated substituents). The numbers of the compounds refer to Table 2.

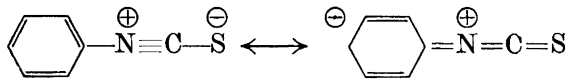
res a different slope, a deviation paralleling that observed in studies of the relative reaction velocities of alkyl *iso*thiocyanates.

Infra-red spectra

The 1 900—2 300 cm^{-1} region. In this wave-length region an increase of the absorption band at about 2 180 cm^{-1}, accompanied by an intensity decrease of the 2 090 cm^{-1} peak, is consistently observed on changing the solvent from CCl_4 to $CHCl_3$ (Fig. 3). The effect is presumably ascribable to an enhanced polarization of the NCS-grouping in the more polar solvent. The lacking band around 2 180 cm^{-1} in the spectrum of *tert.*-butyl *iso*thiocyanate (Fig. 3) is probably caused by steric influences.

The multiplet character of the band in this spectral region, attributed to the asymmetric stretching vibration of the —N=C=S grouping, is difficult to explain, for reasons similar to those outlined in the case of the Raman spectra [13]. Supplementary investigations of the Raman spectra are planned in order to clarify this problem. Association effects can hardly be responsible for the doubling of the bands, in view of the spectral independence of concentration or phase condition. Again, such phenomenons as tautomerism, hydrogen bonding or rotational isomerism seem very unlikely in case of the NCS-grouping.

The displacement of the absorption band from 2 100 cm^{-1} to 2 054 cm^{-1} in phenyl *iso*thiocyanate (Fig. 3) is explainable in terms of a conjugation effect diminishing the contribution from the triple-bonded hybride to the molecule which is assumed to be planar:



A qualitatively similar but less pronounced displacement is observable in the spectrum of α-naphthyl *iso*thiocyanate (Fig. 3), as would be expected.

The 1 500—650 cm^{-1} region. A strong band at 1 348—1 318 cm^{-1} can be assigned to the $-CH_2N=C=S$ or $>CH-N=C=S$ groupings. In the given region there has not been described, thus far, any intense, characteristic frequencies. Weak C—H-deformation modes (wagging and twisting) reaching into the 1 300 cm^{-1} region, such as those operating here, have received only little attention, particularly regarding the influence of substituents. The symmetric stretching vibration of the —N=C=S-grouping, which in Raman spectra gives rise to a strong line at 1 070 cm^{-1} (methyl and ethyl mustard oil) is, according to our findings, inactive or slightly active in the infra-red spectra examined. The strong band at 1 348—1 318 cm^{-1} can hardly be assigned to the symmetric stretching mode of the —N=C=S grouping, since methyl, *tert.*-butyl, phenyl and α-naphthyl *iso*thiocyanate do not absorb in this region. Furthermore, the Raman spectrum displays, in addition to the symmetric stretching vibration of the —N=C=S grouping at 1 070 cm^{-1}, a relatively strong line (in the ethyl derivative) of non-identified origin in the same region (1 340 cm^{-1}) where we find the band in the infra-red spectrum. It

is highly improbable that the stretching mode would be susceptible to that extent to the influence of substituents and be displaced only in methyl, *tert.*-butyl, phenyl and *α*-naphthyl *iso*thiocyanate from 1 340 cm^{-1} to another region. The most plausible interpretation of the latter bond is therefore that it represents a deformation vibration of the C—H grouping, the force constant of which is influenced by the presence of the polar $-N\overset{\oplus}{\equiv}C-\overset{\ominus}{S}$-grouping. A more exact characterization of the vibrational type would require a study of further derivatives. The introduction of deuterium to furnish the $-CD_2-N=C=S$-grouping would, of course, be advantageous in such investigations.

Methyl *iso*thiocyanate displays a strong band at 1 412 cm^{-1}, representing the symmetric deformation vibration of the methyl group and displaced by the influence of the —N=C=S-grouping from its normal position at 1 380 cm^{-1} in the linear aliphatic chain. Corresponding shifts of the methyl grouping, conditioned by the influence of polar substituents, have been reported and studied in detail [19–21]. The band at 1 453 cm^{-1} represents the asymmetric deformation vibration of the methyl group, practically unaffected by the —NCS-substitution (normal position: 1 450 cm^{-1}).

Tertiary butyl *iso*thiocyanate exhibits a broad, strikingly high band at 1 205 cm^{-1} within the region of the characteristic frequency of the *tert.* butyl-grouping. The intensity is probably influenced by the NCS-substitution.

REFERENCES

1. Kremann, R. and Riebl, R. *Z. physik. Chem.* **A 165** (1933) 347.
2. Pestemer, M. and Litschauer, B. *Monatsh.* **65** (1935) 233.
3. Coblenz, W. W. *Carnegie Institution of Washington, Publication* **35** (1905) 60.
4. Gordy, W. and Williams, D. *J. Chem. Phys.* **4** (1936) 85.
5. Williams, D. *J. Chem. Phys.* **8** (1940) 513.
6. Ettlinger, M. G. and Hodgkins, A. E. *J. Am. Chem. Soc.* **77** (1955) 1831.
7. Ettlinger, M. G. and Lundeen, A. J. *J. Am. Chem. Soc.* **78** (1956) 1952.
8. Kjær, A. and Rubinstein, K. *Acta Chem. Scand.* **8** (1954) 1335.
9. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 26.
10. Kjær, A. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 141.
11. Luskin, L. S., Gantert, G. E. and Craig, W. E. *J. Am. Chem. Soc.* **78** (1956) 4965.
12. Hibben, J. H. *The Raman Effect and its Chemical Applications*, Reinhold Publishing Corporation, New York 1939, p. 282.
13. Kohlrausch, K. W. F. *Ramanspektren. Hand und Jahrbuch der Chemischen Physik* **9**, Becker and Erler, Leipzig 1943, p. 294.
14. Bagder, R. M. *J. Chem. Phys.* **5** (1937) 178.
15. Zahradník, R. *Collection Czechoslov. Chem. Communs. In press.*
16. Braude, E. A. and Nachod, F. C. *Determination of Organic Structures by Physical Methods*, Academic Press Inc. New York 1955, p. 147.
17. Braude, E. A. and Nachod, F. C. *Determination of Organic Structures by Physical Methods*, Academic Press Inc. New York 1955, p. 156.
18. Jones, R. N. and Sandorfy, C. *Technique of Organic Chemistry IX*, Interscience Publ. Inc., New York 1956, p. 368 ff., p. 394 ff.
19. Sheppard, N. *Trans. Faraday Soc.* **51** (1955) 1465.
20. Bellamy, L. J. *J. Chem. Soc.* **1956** 2753.
21. Wilmshurst, J. K. *J. Chem. Phys.* **26** (1957) 426.

Received December 9, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 456—460

The Binding of Chromate to Red Corpuscles, Haemoglobin and Globin

BENGT ZACHARIAS

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

This investigation has shown that the presence of calcium in the incubation fluid promotes ^{51}Cr loss by chromate-labelled erythrocytes and blocks chromate uptake by red corpuscles. A site is suggested for the binding of chromate to the globin molecule.

The practice of labelling red corpuscles with radio chromate has become considerably wide-spread in recent years [1]. In contrast to other *in vitro* labelling methods employing radioactive substances — such as have been applied in the determination of the circulating red corpuscle mass [2–5] — the binding of ^{51}chromate to the red corpuscles is so stable that not only the corpuscle volume can be determined, making use of ^{51}chromate-labelled red corpuscles, but even information concerning the lifespan of the erythrocytes can be obtained [6,7].

The chromate loss from the circulating red corpuscles is, however, only a relative measure of the lifespan of the erythrocytes owing to a leakage of about 1 % per day from viable erythrocytes, apart from the 0.85 % loss per day due to red corpuscle physiological destruction [8].

In the healthy human organism, half of the chromate taken up by the red corpuscles is given off in the course of about one month, whereas in many pathological cases the chromate loss from the erythrocytes has been found to be far more rapid [7].

In vivo-labelling of red corpuscles by radiochromate shows an uptake of about 10 % only of the amount injected [9–11].

The uptake of radiochromate by red corpuscles *in vitro* increases with increasing temperature and increasing acidity [8,12,13]. It is also increased when an isotonic acid citrate solution (called ACD solution) is used instead of heparin as an anticoagulant [14,15].

It is reported that when ^{51}chromate-labelled red corpuscles are incubated in plasma, the ^{51}Cr loss is larger than when incubated in saline [16].

One of the aims of this investigation is to explain the above-mentioned difference in the rate of ^{51}Cr loss by the erythrocytes. The other is to determine the site of the binding of the chromate in the globin molecule, which is known to be responsible to a very large extent for the fixation of the chromate.

Gray and Sterling found already that, under optimal conditions *in vitro*, 80—90 % of the added radiochromate is taken up by the erythrocytes [6]. About 97 % of the radiochromate taken up by the erythrocytes is bound to the haemoglobin and only 2 % to the stroma.

EXPERIMENTAL

Materials. Blood was drawn by heart puncture from rats and rabbits of our laboratory strain. Horse blood was supplied by the Royal Veterinary Institute and human blood was supplied by the Karolinska Hospital. The radiochromate used had a specific activity of 1 mC per 23.76 μg chromium.

Determinations. Protein concentration of the globin solutions was determined according to the method of Warburg as modified by Kalckar [17]. The haemoglobin was determined as alkaline oxyhaemoglobin as described by Evelyn [18]. While the molecular weight [19] of haemoglobin is 66 700, that of globin is 66 000. Radioactivity was determined in a scintillation crystal counter, the samples having a volume of 10 ml.

Preparations. Haemoglobin was prepared by haemolysing the red corpuscles after repeated washings. Haemolysis was carried out by adding the same volume of distilled water and 0.2 volume of toluene. The haemolysate was centrifuged and the clear supernatant withdrawn. Rat and horse haemoglobin was crystallized from the clear haemolysate as described by Drabkin [20]. The haemolysates prepared from human and rabbit blood were filtered through Celite No. 545, as described by Boyd [21].

Native globin was prepared according to the method described by Rossi-Fanelli *et al.*[22], at a temperature of −15°C.

Activation. 5—12 ml whole blood were incubated for 1 h at room temperature with sodium chromate having an activity of 0.125—0.250 μC. After incubation, the percentage of uptake of sodium chromate by the erythrocytes was determined.

5—10 ml of haemoglobin and globin solutions were incubated in the same way as blood (above). The pH of the acid globin solutions was adjusted to 7.4 by 1 N NaOH.

After 1 h of incubation at room temperature the percentage of protein-bound chromium was determined, as described by Clegg [23], the sample being centrifuged for 1.5 h at 6 000—7 000 r.p.m.

RESULTS

Table 1 demonstrates that no difference can be detected in the uptake of chromate by the red corpuscles and by globin isolated from human, rabbit, horse or rat blood. It can be seen from Fig. 1 how the presence of 10 mg of

Table 1. Comparison of chromate binding of red corpuscles and the corresponding amount of globin.

Animal	% Chromate bound to red corpuscles	% Chromate bound to globin
Human	58.5	62.5
Rabbit	59.0	63.2
Horse	61.1	64.2
Rat	60.7	63.6

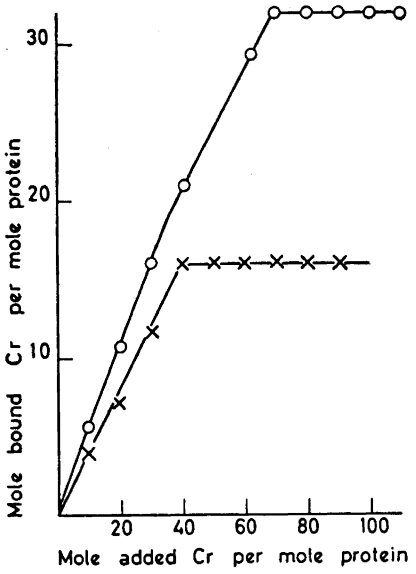


Fig. 1. The binding of ^{51}Cr by red corpuscles incubated in the presence of various amounts of chromate and in the presence and absence of calcium. Whole blood, haemoglobin and globin in the absence of calcium —O—O—; whole blood, haemoglobin and globin in the presence of calcium —×—×—.

calcium per 100 ml solution influences the uptake of chromate by human red corpuscles, haemoglobin and globin. The amount of chromate bound per molecule protein increases with increasing amounts of added chromate. In these experiments also, the presence of calcium ions reduces the amount of ^{51}chromium bound to about half of the amount fixed in the absence of cal-

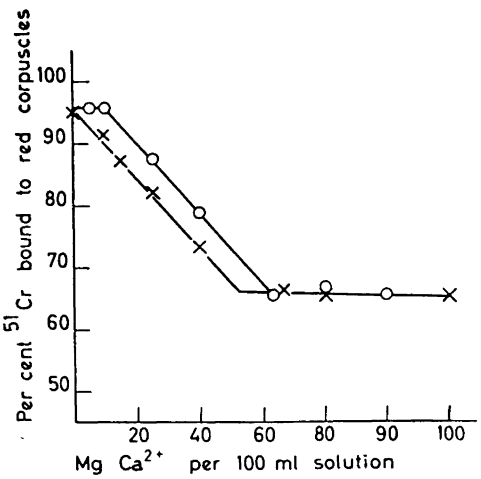


Fig. 2. The influence of varying amounts Ca^{2+} on the uptake of chromate by the red corpuscles. Saline —×—×—; ACD-plasma —O—O—.

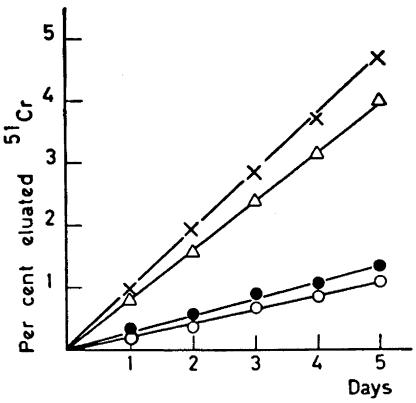


Fig. 3. Elution of ^{51}Cr from chromate labelled red corpuscles in the presence and absence of Ca^{2+} at 5°C. Saline —O—O—; Ca^{2+}-saline —×—×—; ACD-plasma —●—●—; Ca^{2+}-ACD-plasma —△—△

cium. In the presence of calcium ions, one molecule of protein can bind up to 16 molecules of chromate. In the absence of calcium, one molecule of protein can bind as much as 32 molecules of chromate.

The influence of varying amounts of calcium ions on the uptake of chromate by human red corpuscles in saline and ACD plasma is shown in Fig. 2. In calcium-free medium, the red corpuscles take up almost 100 % of the added chromate. Increasing amounts of calcium ions reduce the uptake of chromate until a plateau is reached at 60 %. In saline, this point is reached at a calcium concentration of 52 mg per 100 ml blood, and in ACD plasma at a calcium concentration of 64 mg per 100 ml. The fact that a part of the calcium is bound to the citrate of ACD-plasma explains the above difference. The amount of ionised calcium bound to ACD plasma was determined by conductometrical titration to be 12 mg per 100 ml.

Fig. 3 shows the elution of chromium from ^{51}Cr-labelled human red corpuscles incubated in different media at 5°C. Corrections were made for the small haemolysed fraction. The experiments, which took 5 days, were carried out at a low temperature as, when 37°C was sustained for 5 days, a marked haemolysis could not be avoided. The uptake of chromate by erythrocytes is known to depend very little on temperature. The elution rate in saline and ACD plasma was found to be 0.2 % per day. In saline containing 10 mg of calcium per 100 ml blood, the elution rate was 0.8 % per day, thus very much larger, and in ACD plasma containing 10 mg free calcium per 100 ml, the percentage was 0.75 per day.

DISCUSSION

We found that the presence of calcium in blood reduces the uptake of chromate by red corpuscles [15]. The uptake of chromate by haemoglobin and globin is also reduced to the same extent in the presence of calcium. This result suggests that globin and calcium compete for the chromate present and correspondingly the erythrocytes take up more chromate in the absence of calcium. This conclusion is supported by the observation that the elution

of chromate from the red corpuscles is increased when calcium is added to the blood. Cohn *et al.*[24] and Cannan [25] have determined the amino-acid groups present in haemoglobin and found among other amino-acid groups present a total of 33 histidine groups, all of which were dissociated at a physiological pH.

This result, and our finding that the number of chromate molecules bound by the globin molecule amounts to 32, suggests that the chromate is fixed to the histidine groups.

Acknowledgements. The author's thanks are due to Professor G. de Hevesy for the encouraging interest which he has taken in this work; to Dr. G. von Ehrenstein for stimulating discussions; to Dr. B. Gullbring for kindly supplying the human blood needed; and to the *Swedish Natural Science Research Council* for its generous support of this investigation.

REFERENCES

1. Crosby, W. H. *Proc. Intern. Conf. Peaceful Uses Atomic Energy*, Geneva 1955 Vol. **10**, p. 378.
2. Hevesy, G. and Zerahn, K. *Acta Physiol. Scand.* **4** (1942) 376.
3. Hevesy, G. and Nylin, G. *Acta Physiol. Scand.* **24** (1951) 285.
4. Hevesy, G. and Nylin, G. *Circulation Research* **1** (1953) 102.
5. Hevesy, G. *Arkiv Kemi* **4** (1951) 363.
6. Gray, S. J. and Sterling, K. *J. Clin. Invest.* **29** (1950) 1604.
7. Mollison, P. L. and Veall, N. *Brit. J. Haematol.* **1** (1955) 62.
8. Ebaugh, F. G., Emmerson, C. P. and Rodnan, G. P. *J. Clin. Invest.* **34** (1955) 629.
9. Jones, N. C. and Mollison, P. L. *Clin. Science* **15** (1956) 207.
10. Sutherland, D. A. and McCall, M. S. *Blood* **10** (1954) 646.
11. Rodnan, G. P., Ebaugh, F. G. and Spivey Fox, M. R. *Blood* **12** (1957) 355.
12. Donahue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Viranuvatti, V. and Finch, C. A. *Brit. J. Haematol.* **1** (1955) 249.
13. Read, R. C. *New England J. Med.* **250** (1954) 1021.
14. Neckeles, T. F., Weinstein, J. M. and LeRoy, G. V. *J. Lab. Clin. Med.* **42** (1953) 358.
15. v. Ehrenstein, G. and Zacharias, B. *Nature. In press.*
16. Stahl, P. R., and Dale, H. E. *Am. J. Physiol.* **193** (1958) 244.
17. Kalckar, H. M. *J. Biol. Chem.* **167** (1947) 461.
18. Evelyn, K. A. *J. Biol. Chem.* **115** (1936) 63.
19. Rhinesmith, H. S., Schroeder, W. A. and Pauling, L. *J. Am. Chem. Soc.* **79** (1957) 609.
20. Drabkin, D. L. *Arch. Biochem.* **21** (1949) 224.
21. Boyd, W. C. *J. Am. Chem. Soc.* **67** (1945) 1036.
22. Rossi-Fanelli, A., Antonini, E., Caputo, A. *Biochim. et Biophys. Acta* **28** (1958) 221.
23. Clegg, R. E. *Chemist Analyst* **38** (1949) 87.
24. Cohn, E. J., Green, A. A. and Blanchard, M. H. *J. Am. Chem. Soc.* **59** (1937) 509.
25. Cannan, R. K. *Chem. Revs.* **30** (1942) 395.

Received November 11, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 461—472

On the Isomerism of Hydroxyurea

XI. Absorption Spectra in the Infra-Red

HELMER KOFOD

The Royal Danish School of Pharmacy, Chemical Laboratory B, Copenhagen, Denmark

A brief survey of the Raman and infra-red spectroscopy of urea is presented. On this background recorded infra-red spectra of the hydroxyurea isomerides are discussed and assignments of the principal absorption frequencies have been attempted. Although no rigorous proof has been furnished the results are compatible with the proposed constitutions carbamhydroxamic acid (I) and O-carbaminoyl-hydroxylamine (II), respectively.

In a preliminary communication [1] the absorption in the frequency region 3 600—600 cm^{-1} of the two isomerides were briefly described. In the present paper a first attempt has been made at a more detailed interpretation of the spectra in terms of structure.

The compounds, as well as urea, which may be regarded as the mother substance of at least one of the isomerides, belong to the medium-size molecules, which are too complicated for a complete theoretical treatment by the spectroscopist, but which are nevertheless too small to leave them entirely to the purely empirical approach of the organic chemist.

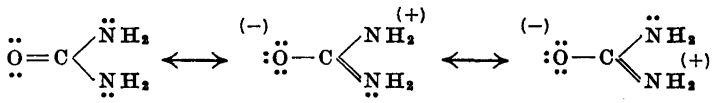
The structure of urea has been extensively studied by several authors. The Raman spectrum was recorded in 1934 by Kohlrausch and Pongratz [4] and also investigated in 1939 by Otvos and Edsall [5]. The latter work included a comparative investigation of deutero-urea. The absorption spectrum in the infra-red was recorded in 1941 by Kellner [2], who also subjected the molecule to a theoretical treatment [3]. Kellner found it possible to assign the observed frequencies to definite modes of vibration. The theoretical frequencies, calculated as usual from the evaluated force constants, were compared with the experimental infra-red and Raman frequencies, obtained by Kellner [2] and previous investigators [4,5]. Although quite satisfactory agreement was observed, Kellner pointed out that the proposed interpretation was by no means definite. The results of Kellner are compatible with the hypothesis advanced in 1935 by

Table 1. Infra-red and Raman frequencies of urea (cm^{-1}).

	IR (Kellner [3]) (solid)	Raman (Kohlrausch and Pongratz[4]) (in H_2O)	Raman (Otvos and Edsall [5] (in H_2O)	Assign-ments by Kellner[3]	IR (Davis & Hopkins [26]) (solid)	
			3 496 D (5b)	N—H a—st		
		3 462 (2sb)				
	3 434			N—H a—st	3 444 (s)	NH_2 a—st
A	3 410			N—H s—st		
	3 376	3 383 (3sb)	3 385 P (6bv)	N—H s—st	3 348 (s)	NH_2 s—st
	3 218	3 218 (1b)	3 235 P (5b)	first over-tone of 1680		
C		1 655 (0)	1 680 P (3b)	C=O st	1 610 (s)	C=O st
		1 593 (2b)	1 604 P? (4b)	b	1 620 (m)	NH_2 d
		1 458 (0)	1 478 D (2b)	a—st	1 476 (s)	NCN a—st
		1 350 (1/2)		b		
		1 157 (1b)	1 167 P (4)	b	1 155 (s)	NH_2 rock
D					1 055 (m)	NH_2 rock
		1 000 (8)	1 008 P (10)	s—st	1 004 (mw)	NCN s—st
					787 (m)	C=O b out of plane
					712 (m)	NH_2 torsio-nal
		685 (1b)				
			601 D (2)	b		
		525 (2b)	534 D (2)	b	574 (m)	C=O b in plane
					559 (w)	NCN d

a- asymmetric s- symmetric
st stretching b bending d deformation vibration
P polarized D depolarized
(s) strong, (m) medium (w) weak

Pauling [8] that the urea molecule is mesomeric, two ionic structures contributing approximately 20 % each to the actual electron distribution



This picture is also in agreement with X-ray measurements of the nuclear distances [9], $r(C{=}O) = 1.25$ Å and $r(C{-}N) = 1.37$ Å, and with the coplanarity of the carbon, oxygen and nitrogen atoms, as first deduced from crystallographic data [9], and later confirmed by absorption spectroscopy with polarized infra-red radiation by Keller [10] and by Waldron and Badger [11] from more

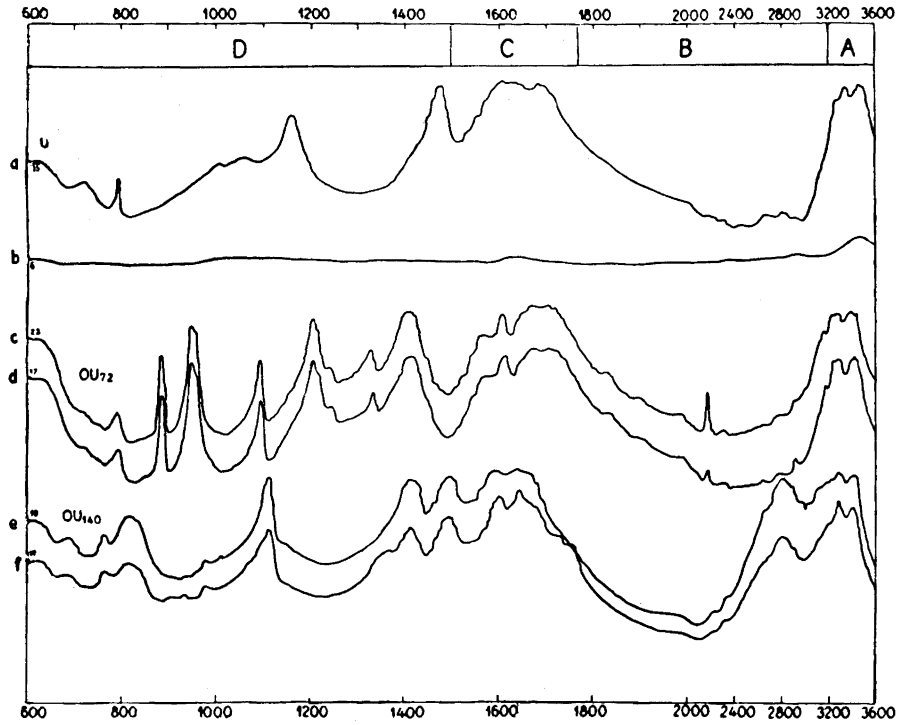


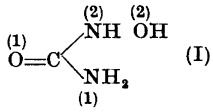
Fig. 1. Absorption spectra in the infra-red. Solid state, 1 mg substance/300 mg KBr. a, Urea; b, Potassium bromide (blank) against the atmosphere; c, »Hydroxyurea», m.p. 72°C, O-carbaminoyl-hydroxylamine, freshly prepared sample; d, As c, but sample stored at 0°C for 2 ½ years; e, Hydroxyurea, m.p. 140°C, carbamhydroxamic acid, prepared according to the present author [33]; f, As e, but sample prepared according to Runti and Deghenghi [33].

accurate measurements. Further corroboration of the latter work was presented in 1953 by Robinson and Price [6] who deduced the IR-absorption spectrum from the reflection spectrum using a single-crystal technique. Vaughan and Donohue [28] in 1952 obtained evidence that not only are the principle atoms coplanar but the hydrogen atoms lie in the plane of the rest of the molecule. Indications of this position had been observed in earlier work [10,11] and the coplanarity of the entire molecule was further supported by proton magnetic resonance studies [29] and by neutron diffraction work [27]. Davies and Hopkins [26] have recently reinvestigated the absorption of urea in the infra-red (solid and vapour state) and also recorded the spectrum of the uronium ion. Their frequency data, obtained with urea, are given in Table 1 together with the values and assignments by the previously quoted authors. Davies and Hopkins presented a detailed discussion of the bonding in urea in the solid state. The qualitative picture is in agreement with previous information, but the authors give revised values of the N—H bond lengths. They

state that only weak hydrogen bonding exists in the urea molecule, and their computation of the delocalization ("resonance"-) energy based upon the spectroscopic data yielded a value, which is considerably lower than the thermochemical value of 37 kcal/mole. The discrepancy is explained by the fact that the thermochemical value includes contributions from changes in the σ-bonds and from additional hydrogen bond energy made available by the planar configuration.

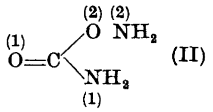
The absorption spectra of urea and of the isomeric "oxyureas", are reproduced in Fig. 1. A catalogue of frequencies of the principle absorption-bands is given in Table 2.

Among the proposed constitutions, those which seem to account for most chemical and physical properties of the oxyureas are



(I)

OU_{140}, Carbamhydroxamic acid



(II)

OU_{72}, O-Carbaminoylhydroxylamine

There is ample evidence that (I) represents the correct constitution of OU_{140}, *vide, e.g.*, parts III, IV, VI in the present series. Formula II was proposed by Exner [12] mainly from classical chemical arguments and on the basis of the results of the present author. It was, however, felt desirable to obtain further support by physical methods. At the time, when the preliminary communication [1] on the infra-red absorption appeared, only two O-acylated hydroxylamines were described in the literature, *viz.* O-anthranoyl-hydroxylamine [31] and the proposed structure II for OU_{72}. Since then the preparation of further two representatives of this type of compounds [30] has been reported, and this adds considerably to the probability of formula II. The need for a rigorous proof and the general interest [18] in the infra-red absorption of the biochemically important groupings CO—NH and NHOH is the author's justification for the following attempt at a detailed discussion of the spectra of the hydroxyureas.

The observed frequencies listed in Table 2 will be discussed with particular reference to the compatibility with the proposed constitutions I and II.

In the frequency region A all three compounds show an intense, partly resolved doublet with a slightly less intense shoulder on the low-frequency side. This is the region, where the N—H stretching vibration of amides are normally found, and the duplex nature of the peak is characteristic for the unsubstituted NH_2-group. The A-region of the urea spectrum has been carefully studied by many investigators [2-7,11], and there is general agreement that the frequencies are N—H stretching fundamentals *cf* Table 1. Mathis [13] reported a characteristic, intense band at 3 μ (3 333 cm^{-1}) in the spectra of hydroxamic acids in the solid state. He too regards it as being due to N—H stretching vibrations and takes the absorption bands as a proof of the constitution $R—C\langle^{O}_{NHOH}$. In 1958 Jencks [30] has recorded the absorption spectra

Table 2. Principle infra-red frequencies (cm^{-1}) of urea and the isomeric "oxyureas" in the solid state, *cf.* Fig. 1.

	U	(proposed) OU_{140}	(proposed [12]) OU_{72}	Proposed assignments
	Fig 1 a	Fig 1 e	Fig 1 d	
A	3 440 (s) 3 320 (s) 3 250 (s)	3 400 (s) 3 280 (s) 3 200 (s)	3 400 (s) 3 270 (s) 3 180 (s)	NH_2 a−st NH_2 s−st overtone?
B		2 800 (s)	 2 170 (w)	OH (hydrogen bonded) ?
C	 1 685 (s) 1 620 (s)	 1 650 (s) 1 600 (s)	1 715 (s) 1 675 (s) 1 610 (s) 1 565 (m)	C=O(1) (ester) C=O (amide I) C−O(2) (?) C=O (amide I) C−N st or NH_2 d (amide II ?) ?
D	 1 470 (s) 1 155 (m) 1 050 (m) 790 (w) 715 (w) 620 (m)	1 490 (s) 1 415 (s) 1 115 (s) 820 (m) 760 (m) 685 (m) 620 (m)	 1 415 (s) 1 335 (m) 1 250 (m) 1 210 (s) 1 095 (m) 950 (s) 885 (m) 790 (w) 620 (s)	C−N(1) st st C−N(2) st C−O(2) (ester) ? NH_2 rock N−O st? C−N st ? ? ? N−O st ? C−O st ? C−O st N−O st ? ? N−H rock ?

of various hydroxamic acids and a few O-acylhydroxylamines in chloroform solution. The data obtained with benzohydroxamic acid and O-benzoylhydroxylamine are representative and of interest in the present discussion, since the formulae of the compounds are analogous to I and II, respectively. The wave lengths, converted to wave numbers, are quoted in Table 3, and the assignments by Jencks are included. There is good agreement between the N—H stretching frequencies in Tables 2 and 3.

Table 3. Infra-red frequencies of benzoyl-hydroxylamines in chloroform-solution. Data and assignments by W. P. Jencks [30].

	$O=C(NHOH_2)(C_6H_5)$ Benzohydroxamic acid	$O=C(ONH_2)(C_6H_5)$ O-Benzoylhydroxyl-amine	
A	3 400 (m) N—H 3 180 (m) OH	3 290 (m) N—H 3 180 (w) N—H	
C	1 660 (s) C=O (amide)	1 730 (s) C=O (ester) 1 640 (w) (?) 1 533 (m) (?)	
D	1 385 (m) C—N st 895 (m) N—O (?)	1 454 (s) (?) 1 317 (m) (?) 1 180, 1 280 (s) C—O ester 925 (m) N—O (?)	

The assignment of the third (low-frequency) band is uncertain. In the urea molecule (*cf.* Table 1) it has been interpreted as the first overtone of the frequencies around 1 600 cm^{-1}. In benzohydroxamic acid on the other hand (*cf.* Table 3) a corresponding band has been assigned to a hydrogen bonded O—H group and in O-benzoyl hydroxylamine to a hydrogen bonded N—H group. It is emphasized that the latter assignments refer to solution spectra.

Since it is virtually impossible to distinguish between N—H and O—H bonds in this region the presence of a hydroxy-group in OU_{72} is not directly disproved; it is, however, unlikely for chemical reasons.

In the frequency-region B there is a broad band at 2 800 cm^{-1} in the spectrum of OU_{140}. It is one of the most intense bands in the spectrum and is absent or extremely weak in U and OU_{72}. It has therefore tentatively been assigned to the hydroxy-group [1]. Since this assignment would rule out all the possible formulations of OU_{72} containing free hydroxy-groups and would be in favour of the set of formulae I/II its validity has to be carefully considered. The normal stretching vibration of the unassociated O—H group is found at much higher frequencies in the A region. It is, however, well known [14] that the frequency is very considerably lowered by hydrogen bonding, of which there are ample possibilities in OU_{140}. For the O—H....O bond in the crystalline state the O—H frequency shift has been shown to be a monotonic and nearly linear function of the H....O distance [15,16]. Similar regularities have been demonstrated [17] for a number of other "straight" hydrogen bonds, such as OH....N, NH....O, NH....N, which may also be present in crystals of OU_{140}. The linear relationship is valid only up to a distance of *ca.* 2.8 Å. For short,

i. e. strong hydrogen bonds the OH frequency is reduced very considerably. When straight OH....O bonds are involved, stretching frequencies of the O—H bond as low as 2 000 cm^{-1} have in fact been recorded in the crystalline state. The observed frequency of 2 800 cm^{-1} is, therefore, by no means exceptionally low. Taking 3 700 cm^{-1} as the normal frequency of the non-hydrogen-bonded O—H group, 2 800 cm^{-1} would correspond [17] to a hydrogen bond distance OH....O of 2.67 Å.

The flattened shape of the maximum at 2 800 cm^{-1} in Fig. 1 is also in accordance with general experience concerning the hydrogen-bonded OH group in this frequency region [17, Fig. 2].

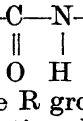
Mathis [13] has recorded the IR-spectra of a number of hydroxamic acids, which are, of course, closely related to the proposed formula (I) for OU_{140}. He reported "une bande d'absorption intense et très large" at 3.6 μ corresponding very closely to 2 800 cm^{-1}. This band is visible in the solid state only, *cf* Table 3. In carbon tetrachloride solution it disappeared and was replaced by a new, intense band at 3 450 cm^{-1} (2.9 μ). From these observations Mathis concluded that the 2 800 cm^{-1} band was *not* due to a hydrogen-bonded OH group and assigned it tentatively to a C—H stretching vibration, perturbed by the proximity of an oxygen atom, a proximity, which Mathis assumed to exist in the solid state only. The new band at 3 450 cm^{-1}, appearing in solution, was assigned to a hydrogen bonded hydroxy-group, intermolecular hydrogen bonds being postulated in solution only.

In the opinion of the present author the observations of Mathis might equally well be explained by assigning the band at 2 800 cm^{-1} to a hydrogen-bonded OH group in the crystalline state. Since the N—H group too may be involved in hydrogen bonds in the crystalline state, it would be reasonable to assume, that the new bond appearing at 3 450 cm^{-1} in solutions of hydroxamic acids represents the normal frequency of either the non-bonded OH group or of an unassociated N—H group.

Unfortunately the insolubility of OU_{140} in non-polar solvents prevents the recording of a solution spectrum, which might otherwise throw further light upon the origin of the 2 800 cm^{-1} band. Some further support for the assignment of the band at 2 800 cm^{-1} to a strongly hydrogen-bonded hydroxy-group is provided by observations by Nightingale and Wagner [34]. They reported a broad, intense band at 2 876 cm^{-1} in the infra-red spectrum of solid hydroxylamine at low temperature and attributed it to the O—H stretching vibration. The surprisingly low frequency was interpreted as indicating extremely strong hydrogen bonding in the crystal.

In the double-bond region C urea as well as the two oxyureas show an intense, broad absorption band, consisting of two incompletely resolved maxima.

This feature corresponds closely to what is known from amides of the general formula



in which the R groups must have a carbon adjacent to the amide group and cyclic connections by way of the R groups must be absent. These compounds all display two typical bands in the double-bond region. One is located at approximately 1 650 cm^{-1} ("Amide I") and is assigned to the carbonyl stretch-

ing vibration. For the other one at approximately 1 550 cm^{-1} ("Amide II") three different explanations have been proposed, the most probable one assigning it to the stretching vibrations of the C—N bond, which is supposed to be enhanced in bond strength by a mesomerism involving the adjacent C=O bond [25].

Hydroxamic acids similarly show an intense absorption in this region. In benzohydroxamic acid (*cf.* Table 3) the frequency of 1 666 cm^{-1}(6.0 μ) has been reported in the solid state [13] and 1 660 cm^{-1} in solution [30]. Both authors assigned it to carbonyl stretching vibration.

In urea a Raman frequency, which has been assigned to C=O, occurs at 1 680 cm^{-1}, other authors report the value of 1 665 cm^{-1} (*cf.* Table 1). Thompson *et al.*[18] have demonstrated that in the infra-red spectra of nearly all mono-, di- and tetra-substituted ureas the C=O absorption is located close to 1 660 cm^{-1}. The normal frequency in aldehydes, ketones and fatty acids is approximately 1 700 cm^{-1}. The reason for the value being considerably lower in amides, hydroxamic acids and ureas obviously is the weakening of the C=O bond by delocalisation.

We assume that the high frequency component of the intense, broad bands in OU_{72} and OU_{140} similarly represents a C=O stretching frequency. According to the above hypothesis the fact that in OU_{140} the high frequency component is 1 650 cm^{-1}, very close to the urea value, suggests that the delocalization and hence the bond length r(C=O) are nearly equal. In OU_{72}, on the other hand, the supposedly corresponding band is located at a considerably higher frequency than in urea, in fact very close to the normal value of 1 700 cm^{-1}, observed for simple ketones. Consequently the C=O bond should be very nearly a pure double bond. This trend might be expected from the proposed constitutions, since the symmetrical arrangements of two equivalent C—N bonds in urea and OU_{140} in all probability will cause more delocalization of the C=O bond than will the $C\langle^{O}_{N}$ arrangement in OU_{72}. On the assumption that formula II represents the correct constitution of OU_{72}, the carbonyl bond is in fact of the ester-type and the location of its frequency fit quite well with general experience. Furthermore the observation of a strong absorption at 1 730 cm^{-1} in O-benzoylhydroxylamine (*cf.* Table 3) assigned to the carbonyl group [30] may be taken as further corroboration of the assignment of the frequency.

The relation between bond length and stretching frequency of the carbonyl group has been studied by Rundle *et al.*[19]. For bond lengths ranging from 1.13 to 1.31 Å the points were found to fit a smooth curve, from which the C=O bond lengths in OU_{140} and OU_{72} may be read as 1.25 Å and 1.22 Å, respectively; that of urea is 1.26 Å.

Although the assignment of the high frequency absorption bands in region C to a carbonyl group is compatible with the formulae I and II it should be pointed out that no direct proof is delivered. This should be particularly emphasized with respect to formula II. The reason for this is that the C=N bond is known to produce a stretching frequency in the same region and of similar intensity, and it is virtually indistinguishable from that of C=O.

Molecular structures containing a C=N bond instead of a C=O bond, which have been proposed for both oxyureas, are therefore not entirely ruled out by the present results. Relatively few investigations have been carried out on the infra-red absorption of acyclic C=N compounds. Bonino and Manzoni-Ansidei [20] reported an absorption band at 1 650—1 660 cm^{-1} in acyclic oximes and assigned it to the C=N bond. Mathis [13] similarly observed a characteristic band at 5.95 —6 μ (1 680—1 666 cm^{-1}) in acyclic oximes and made the same assignment. Duyckaerts [21] reported the value 1 630 cm^{-1} in free oximes and assigned it to the C=N—OH group without specification; in salts and complexes of oximes he observed a band at 1 550 cm^{-1}, which he assigned to $\overset{+}{N}—\overset{-}{O}$. Borello and Henry [22] have investigated glyoximes and assigned a frequency at 1 625 cm^{-1} to the C=N bond. Some iminoethers and guanidines have also been investigated. It appears from the above and is generally agreed that the C=N absorption occurs at somewhat lower frequencies (1 625—1 650 cm^{-1}) than that of the C=O group, although values up to 1 683 cm^{-1}, have been found (acetoxime, Ref.[25], p. 182). It will be noticed that some of the values reported of the C=N frequency are very close to the low frequency component of the composite bands in region C in Table 2. This component at 1 620, 1 610 and 1 600 cm^{-1}, respectively, has therefore tentatively been assigned to the C—N bond, which is assumed to be enhanced in bond strenght by mesomerism involving the carbonyl group. It may possibly be identified with the "Amide II" band, for which a similar interpretation has been presented [25]. The fact that in OU_{72} the supposed "Amide II" band is distinctly separate from the C=O band, whereas in U and OU_{140} it is poorly resolved, may be correlated with the smaller degree of bond delocalization or "resonance" in the OU_{72} molecule as discussed above.

The alternative explanations [25] of the "Amide II" band may also be considered as a possibility, particularly the one assigning the band to NH_2 deformation, *cf.* Table 1.

As an aid in the assignment of the frequencies in region C the absorption of O-methyl-isourea might be of interest, since the contribution from structures containing C=N double bonding should be even more pronounced in this compound than in urea and the oxyureas. The Raman spectrum of an aqueous solution containing 50 % by weight of O-methylisouronium chloride has been determined by Edsall [23], but the results obviously refer to the cation, in which the delocalization of the C=N bonds is large, and no assignments have been made by the author except for some lines arising from the methyl-group. Investigations of the infra-red spectrum of the base are in progress in this laboratory.

Finally in the C region there is a shoulder at 1 565 cm^{-1}, also observed in O-benzoylhydroxylamine (*cf.* Table 3). In benzohydroxamic acid it is absent.

It is obvious from the discussion that at present a completely reliable assignment of the bands in the double-bond region C is hardly possible from the spectroscopic data alone, and the possibility is left open that the bands may

be entirely or partly due to C=N stretching vibrations and deformation of NH_2*.

In the frequency region D urea and OU_{140} display certain similarities, whereas the absorption curve of OU_{72} is much more complex. This is to be expected on the basis of the formulae I and II since it is the region, in which the C—N and C—O stretching frequencies normally appear, and with respect to these bonds U and OU_{140} are related and fundamentally different from OU_{72}.

At 1 470 cm^{-1} an intense band is noticed in urea (Table 2). It probably corresponds to the Raman lines observed at 1 458 and 1 478 cm^{-1}, respectively, by two different investigators (Table 1). These lines have been assigned to $C\langle^{N}_{N}$ asymmetric stretching. It is probably this band that reappears in OU_{140} at 1 490 cm^{-1}, but is absent or considerably displaced in OU_{72} in accordance with formula II, containing one C—N only.

At 1 415 cm^{-1} there is an intense band common to OU_{72} and OU_{140} but absent in U. If this band too be assigned to a C—N stretching vibration a reasonable explanation of the four bands appearing in the region 1 490—1 415 would be, that the two separate peaks in the "unsymmetric" OU_{140} are due to the two C—N bonds (formula I), and that these peaks in the symmetrical urea molecule coincide at the intermediate frequency 1 470. In OU_{72} in accordance with formula II there is one C—N band at 1 415 (in common with OU_{140}). The fact that all these frequencies are displaced from the normal C—N stretching frequency region (900—1 300 cm^{-1}) toward the double bond region may be understood as a result of the partial double bond character of the existing C—N bonds in all three compounds. In reasonable accordance with the results deduced from the C-region the relative displacement of the C—N frequencies indicate considerable bond delocalization in U and OU_{140} and much less in OU_{72}.

The low frequency part of the fingerprint-region D is still more difficult to interpret, and only vague suggestions may be attempted.

It would be reasonable to assign the bands located round 1 100 cm^{-1} in OU_{140} and OU_{72} to N—O stretching vibrations. Some support for this assignment is provided by investigations of Borello and Henry [22] who assigned a frequency at 1 150 cm^{-1} in a glyoxime to the N—O bond. Some evidence against this assignments is, however, found in the fact that absorption at 1 100 cm^{-1} is not completely absent in urea, the formula of which is devoid of N—O bonds. The frequency in question is of course in the normal position of C—N stretching vibrations (900—1 300) and this should be taken into account as an alternative assignment. A symmetrical stretching vibration of C—N has been reported at 1 000 cm^{-1} in urea (Table 1).

---

* *Added in proof.* A publication in Czech by O. Exner (*Chem. Listy* **52** (1958) 1613) has come to the author's knowledge after this paper had been delivered for publication. Exner has recorded the IR-absorption in the carbonyl-region of a number of hydroxylamine derivatives, *inter alia* the hydroxyureas. Exner reports a carbonyl frequency of 1 750 cm^{-1} in OU_{72} and a double band at 1 660 and 1 695 cm^{-1}, respectively, in OU_{140}. The numerical deviations from the values reported in Table 2 are probably due to the fact that Exner's measurements are carried out on dioxane solutions.

Formula II requires C—O single bond frequencies to exist in OU_{72}, and since this bond has no equivalent in the conventional formula of urea and probably not in that of OU_{140} either, any frequency assignable to C—O should be chosen amongst OU_{72}-bands, which are absent in U and OU_{140}. Three intense bands meeting these requirements, fall within the normal region of C—O stretching vibrations, namely those at 1 210 cm^{-1}, 950 cm^{-1} and at 885 cm^{-1}, respectively. It should be pointed out, however, that a band at 950 cm^{-1} observed in β-oximes in the solid state has been assigned to the N—O bond [24]. Since furthermore the band at 1 210 cm^{-1} has an equivalent in O-benzoylhydroxylamine (Table 3) we suggest that this band be assigned to the C—O stretching vibrations.

Finally the *E-region* is the one in which N—H rocking is normally observed and the band around 800 cm^{-1} observed in all three compounds may tentatively be assigned accordingly.

Summarizing the discussion of the absorption spectra it may be said that no details have been found, which are incompatible with the formulae I and II. Some observations are in favour of them, but no unambigous proof has been delivered as far as absorption spectroscopy in the infra-red is concerned.

EXPERIMENTAL

The preparation and characterization of the hydroxyureas (Fig. 1, curves *c*, *d*, and *f*) have been described in a previous communication [32]. The sample of the higher melting isomeride employed for the recording of curve *e* was prepared according to Runti and Deghenghi [33]. It is regarded as having a higher degree of purity than that used for curve *f*. The urea sample was a commercial analytical-grade product.

All results in Fig. 1 and Table 2 refer to the crystalline state. The pressed disc technique was used and the disc contained 1 mg substance dispersed in 300 mg of potassium bromide. The instrument was a Perkin Elmer double beam infra-red spectrophotometer model 21, equipped with sodium chloride optics. Resolution 927, response 1, gain 6.5, speed 5, suppression 2.

Acknowledgements. The author is grateful to professor, dr. Børge Bak for his kind interest and for valuable criticism. My thanks are also due to Mr. Henry E. Andersen for assistance in the recording of the spectra.

REFERENCES

1. Kofod, H. *Acta Chem. Scand.* **11** (1957) 1276.
2. Kellner, L. *Proc. Roy. Soc. London* **A 177** (1941) 447, table 6.
3. Kellner, L. *Proc. Roy. Soc. London* **A 177** (1941) 456.
4. Kohlrausch, K. W. F. and Pongratz, A. *Z.physik. Chem.* (*B*) **27** (1934) 176, p. 191.
5. Otvos, J. W. and Edsall, J. T. *J. Chem. Phys.* **7** (1939) 632.
6. Robinson, T. S. and Price, W. C. *Proc. Phys. Soc. London* **B 66** (1953) 969.
7. Waldron, R. D. and Badger, R. M. *J. Chem. Phys.* **18** (1950) 566.
8. Pauling, L. *Nature of the Chemical Bond*, New York 1945, p. 212.
9. Wyckoff, R. W. G. and Corey, R. B. *Z. Krist.* **89** (1934) 462.
10. Keller, W. E. *J. Chem. Phys.* **16** (1948) 1003.
11. Waldron, R. D. and Badger, R. M. *J. Chem. Phys.* **18** (1950) 566.
12. Exner, O. *Chem. listy* **51** (1957) 497.
13. Mathis, F. *Compt. rend.* **232** (1951) 505.
14. Badger, R. M. *J. Chem. Phys.* **11** (1940) 288.
15. Rundle, R. E. and Parasol, M. *J. Chem. Phys.* **20** (1952) 1487.

16. Lord, R. C. and Merrifield, R. E. *J. Chem. Phys.* **21** (1953) 166.
17. Nakamoto, K., Margoshes, M. and Rundle, R. E. *J. Am. Chem. Soc.* **77** (1955) 6480, Fig. 1.
18. Thompson, H., Nicolson, D. L. and Short, L. N. *Discussions Faroday Soc.* **9** (1950) 222.
19. Margoshes, M., Fillwalk, F., Fassel, V. A. and Rundle, R. E. *J. Chem. Phys.* **22** (1954) 381.
20. Bonino, G. B. and Manzoni-Ansidei, R. *Z. physik. Chem.* **B 22** (1933) 169.
21. Duyckaerts, G. *Bull. soc. roy. sci.* Liège **21** (1952) 196.
22. Borello, E. and Henry, L. *Compt. rend.* **241** (1955) 1280.
23. Edsall, J. T. *J. Am. Chem. Soc.* **65** (1943) 1814.
24. Palm, A. and Werbin, H. *Can. J. Chem.* **32** (1954) 858.
25. Randall, H. M., Fowler, R. G., Fuson, N. and Dangl. J. R. *Infra-red Determination of Organic Structures*, New York 1952, p. 11.
26. Davies, M. and Hopkins, L. *Trans. Faraday Soc.* **53** (1957) 1563.
27. Worham, J. E., Levy, H. A. and Peterson, S. W. *Acta Cryst.* **10** (1957) 319.
28. Vaughan, P. and Donohue, J. *Acta Cryst.* **5** (1952) 530.
29. Andrew, M. R. and Hyndman, D. *Proc. Phys. Soc. London* **A 66** (1953) 1187.
30. Jencks, W. P. *J. Am. Chem. Soc.* **80** (1958) 4582.
31. Scott, A. W. and Wood, B. L. *J. Org. Chem.* **1** (1942) 508.
32. Kofod, H. *Acta Chem. Scand.* **7** (1953) 938.
33. Runti, C. and Deghenghi, R. *Ann. triest. univ. Trieste, sez. 2a* **22—23** (1952—53) 9.
34. Nightingale, R. E. and Wagner, G. L. *J. Chem. Phys.* **22** (1954) 203.

Received December 8, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 473—479

Attempts to Prepare Heparin from Ox Liver Capsules with a Mild Method

ROLF OTTOSON* and OLLE SNELLMAN

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

Heparin has been prepared almost quantitatively by a very mild method from ox liver capsules. It has, however, not been freed from a phosphorus containing component of unknown nature which seems to be firmly bound to the heparin.

The aim for the present study has been to develop a mild method for the preparation of heparin in order to obtain it in an as undamaged state as possible. Previous attempts to fractionate commercial heparin with fractionated alcohol precipitation resulted in several fractions with decreasing anticlotting activities but with almost constant sulfur contents. It would be interesting to see whether these activity differences are a result of using too violent preparation methods or if they exist *in vivo*. Efforts have been made to avoid extreme pH values, high temperatures and enzymatic degradation resulting from the presence of carbohydrate attacking enzymes possibly admixed. Acetone denaturation and zone electrophoresis have been very effective in removing the proteins.

METHODS

The ox liver capsules were removed from the livers immediately after slaughter, put into Dewar bottles with ice and transported to the Institute. There they were cleaned from other tissue by scraping with a knife, chopped, weighed and frozen in fine-ground carbon dioxide ice. The mixture was ground to a fine powder in a plate mill and left at -10°C until the carbon dioxide had evaporated. The capsule powder was treated at $+2$°C for about 5 min in a porcelain mortar with a solution 0.10 M in potassium thiocyanate and 0.05 M in potassium hydrogen phosphate (K_2HPO_4). The result was a paste which was diluted with about five times its volume of buffer solution. The pH was adjusted to 7.7—7.9, usually with 0.10 M sodium hydroxide in

* Present adress: Research Institute of National Defence, Sundbyberg 4, Sweden.

the first extraction of a capsule batch and with acetic acid diluted about twenty times in the third and following extractions. The mixture was slowly stirred over night at +2°C. After centrifugation in an International Centrifuge, Model FS, size 3, for about one hour, the supernatant was spared and the residue was stirred with the same volume buffer solution two times more in the same way as before. In some cases the residue was treated with buffer solution several times more in order to control the efficiency of the extractions. The results for two preparations are shown in Table 1. The anticlotting activities are measured with the thrombin method described by Jaques and Charles [1]. In order to avoid delicate standardizations, commercial heparin (Vitrum) has been used as standard, and the activity of this heparin dried in vacuum over P_2O_5 has been set to 100. In Table 1 the activity measured directly on the extract neutralized to 7.2 has been recounted as mg heparin.

Table 1. Yields in the extracts from two preparations. Starting weight 83 g and 120 g capsules wet weight. Yield in mg heparin.

Extract number	Yield in mg heparin	
1	32	63
2	11	16
3	2	2
4	1	4
5	1	0
Mg per kg wet weight	567	708

The three first extracts were pooled, the pH adjusted to 7.0—7.2 with acetic acid diluted tenfold, and an equal volume acetone was added, and the precipitation mixture left at room temperature over night. The precipitate was centrifuged down and treated with about ten times its volume of distilled water with a Potter-Elvehjem homogenizing apparatus. The pH was kept at 7.7—7.9 during this procedure. The insoluble proteins were removed by centrifugation in a Spinco centrifuge, Model L, rotor 20, at 20 000 *g* for one hour. The residue was washed once with a small amount of water, centrifuged again and the two supernatants were combined. The pH was adjusted to 7.0—7.2, an equal volume acetone was added, the precipitate centrifuged down at about 2 000 *g* for one hour and then washed with acetone. The precipitate was treated with a homogenizer, Spinco centrifuged, washed with water and Spinco centrifuged once more. The supernatants were combined and dialysed in a cellophane bag (Visking Co) against several changes of distilled water for about 24 h altogether. During the dialysis the pH was controlled and kept at 7.5—7.9.

The residues after the extractions and the Spinco centrifugations were pepsin incubated at 38°C at pH 2 for about 3 days, with addition of chloroform to prevent bacterial growth. Part went into solution and all except some small grains dissolved when the pH was increased to 7.5. The total anticlotting activity in this fraction corresponded to at most 10 mg heparin Vitrum per kg capsule wet weight.

The supernatants after the acetone precipitations of the extracts were collected and concentrated in air stream at about 45°C, dialysed and investiga-

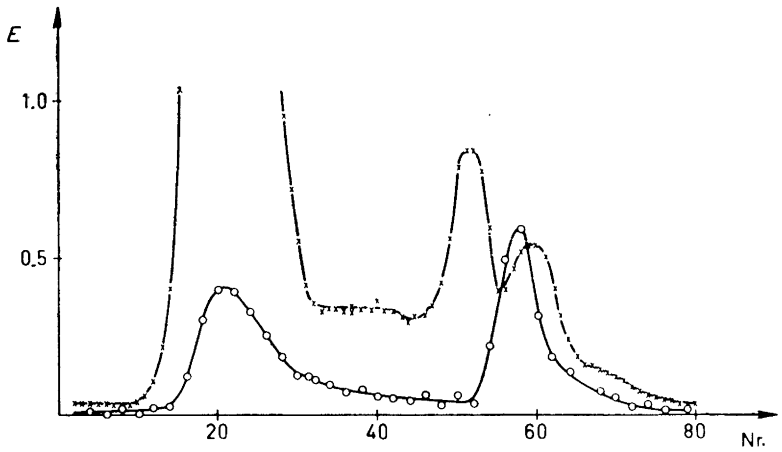


Fig. 1. Zone electrophoresis of two times acetone precipitated capsule extract: —o—o— = carbazole test curve; —x—x— = absorption at 280 mμ. Nr = number of eluated tubes, E = extinction.

ted for anticlotting activity. The activity corresponded to about 1 mg heparin Vitrum per kg capsule wet weight.

The dialysates were controlled for activity after concentration as described and redialysing in extra fine porous bags (Visking Co) but no heparin was found.

The activity not extracted with buffer and the activity lost in the precipitations correspond together to about 1 or 2 % of the total amount heparin activity in the capsules.

The dialysed crude heparin was subject to zone electrophoresis as described by Porath [2,3] with a cellulose powder, described by Flodin and Kupke [4], as supporting medium. The cellulose column was 70 cm long and 4.5 cm in diameter. The buffer used was 0.10 M potassium phosphate at pH 6.8. As DNP-leucine moves about half as fast as heparin at this pH and has a strong yellow colour, a little of this substance was added to make it possible to see how far the electrophoresis had proceeded. The sample was made only 0.03 M in phosphate buffer, which had a zone sharpening effect. This effect could be increased further by adding 5 ml distilled water to the sample. The starting zone contained about 50 ml solution. Each electrophoresis took about 80 h with a current of 0.1 A. After this time the zones were eluated with buffer in test tubes in a fraction collector changing tube every fifth minute. 15 ml was collected in each tube. One ml was taken from every second tube and tested for hexuronic acid with the Dische carbazole method [5]. All fractions were measured in a Beckman spectrophotometer, model DU, at 280 mμ.

A typical run is shown in Fig. 1. The first peak in the ultraviolet absorption curve is mainly due to nucleic acids, the second to DNP-leucine, and the third to the bulk of the proteins. In the carbazole reaction curve, the first peak (tubes 16—32) contained all the anticlotting activity. This fraction was

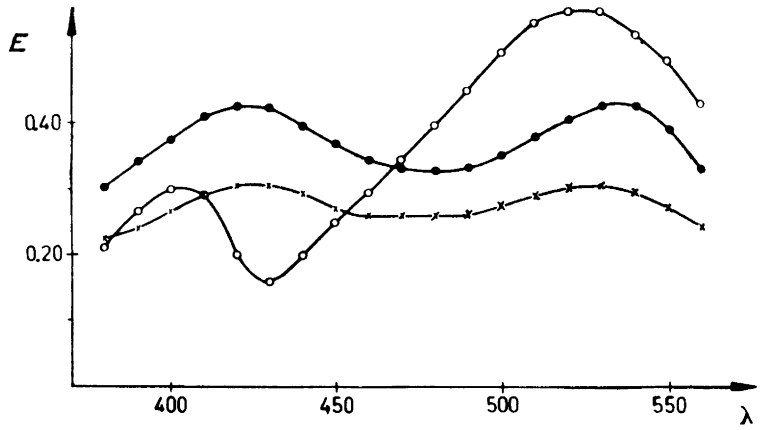


Fig. 2. Spectrophotometric absorption diagrams of carbazole tests on heparin = —o—o—; glycogen = —●—●—; the substance in peak 2 = —×—×—. E = extinction, λ = wavelength in mμ.

collected. Absolute alcohol was added until the alcohol concentration was 25 %. The precipitate, which formed after some hours, was discarded since a test for hexuronic acid was negative. Acetone was added to the supernatant to 50 %. The precipitate formed was centrifuged down, redissolved in a small volume of water and dialysed against distilled water in order to remove the precipitated phosphates.

The second zone (tube 53—64 in Fig. 1) was precipitated by adding 1 ml 4 M sodium chloride per 25 ml solution and two volumes alcohol. The precipitate formed was dried over P_2O_5 in vacuum and analyzed with the carbazole method. The absorption curve was compared with those of heparin and glycogen. The nitrogen content was 6.5 %. The data obtained indicated that this zone contained about 60 % glycogen and 40 % proteins.

The dialysate from zone one was run once more in zone electrophoresis. The result is shown in Fig. 3, which shows carbazole tests on the eluate. After this second electrophoresis the heparin containing zones from several preparations were pooled, dialysed against several changes of distilled water, and precipitated by adding 1 ml 4 M sodium chloride per 25 ml dialysate and two volumes alcohol. The precipitate was washed with alcohol and acetone and dried in vacuum over P_2O_5. The weight was 114 mg.

The final purification was made by dissolving the crude heparin in 26 ml distilled water and 20 ml of a buffer 0.05 M in sodium phosphate, pH 6.8, and 0.20 M in sodium chloride. Three fractions were obtained by adding absolute alcohol to 25, 54 and 66 %. The fraction before 25 % and the supernatant after 66 contained no hexuronic acid and were discarded. The fractions between 25 and 54 % and 54 and 66 % were washed with alcohol and acetone, dried and weighed. The fraction between 25 and 54 % weighed 104 mg. The fraction between 54 and 66 % weighed 3 mg, had an anticlotting activity of 8

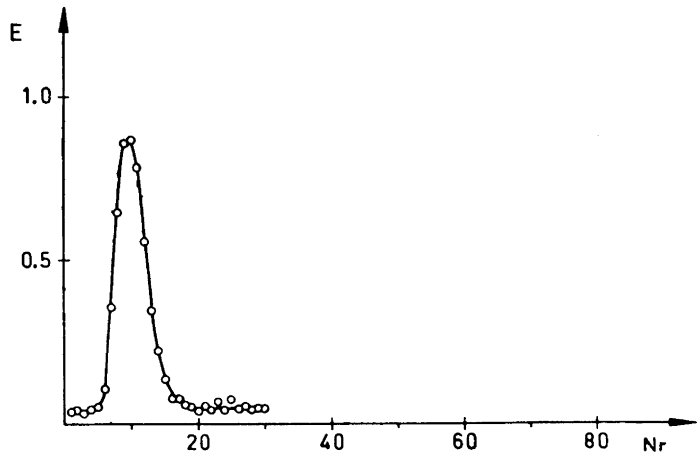


Fig. 3. Reelectrophoresis curve of the tubes No. 16—36 in the carbazole curve from the experiment shown in Fig. 1. Nr, *E* and —o—o— the same as in Fig. 1.

and a colour yield in the carbazole test of 36 % of that of commercial heparin (Vitrum).

The fraction between 25 and 54 % was subject to the following investigations, the results of which are tabulated in Table 2.

The ultraviolet absorption at 260 and 280 mμ was measured in a Beckman spectrophotometer, Model DU, in a 1 cm cuvette in order to get an estimation of the content of nucleic acid and proteins.

Ninhydrin tests were performed by taking some drops of a 3 % solution of the fraction, adding some drops of 0.2 % ninhydrin and heating in a water bath for 10 min.

The sulfur content was measured according to Ottoson and Snellman [6].

The anticlotting activity was measured.

The colour yield in per cent of the colour yield of the same weight commercial heparin (Vitrum) was measured with the carbazole method [5]. This measurement does not necessarily give even a relative value for the hexuronic acid content since the colour yield per mole hexuronic acid depends on how it is bound. Glucuronic acid in heparin gives a colour about 50 % more intense than that of free glucuronic acid.

Phosphorus was determined using a modification of a method described by Scheel [7]. In order to determine whether the phosphorus was organically bound or was a coprecipitation of inorganic phosphorus, tests were made without hydrolysis in sulfuric acid-perchloric acid mixture. The procedure exhibits a strong reaction for inorganic phosphorus in about one tenth of the phosphorus quantity present in the sample taken for analysis. The test was, however, negative.

One mg heparin was tested with the fuchsin-sulfurous acid test for aldehydes according to Feulgen, Boguth and Andresen [8]. The positive reaction for capsule heparin could, however, not be transferred by shaking into octyl alcohol.

To 1 mg heparin in 2 ml water 2.5 ml 0.1 % anthrone solution in concentrated sulfuric acid was added. The colour caused by hexoses is clearly visible for a few micrograms of glucose and strong for 50 μg micrograms.

Table 2. Data for capsule heparin and commercial heparin (Vitrum).

Determination	Capsule heparin	Heparin Vitrum
UV absorption at 260 mμ 1 mg per ml	0.304	0.360
UV absorption at 280 mμ 1 mg per ml	0.171	0.300
Ninhydrin test	negative	negative
Sulfur content, %	10.05	13.1 [a]
Anticlotting activity	112	100
Colour yield in the carbazole test	84	100
Phosphorus, %	1.10	—
Inorganic phosphorus	nil.	—
Fuchsin-sulfurous acid test	positive	negative
Anthrone test for hexoses	very weak	very weak

[a] Reprecipitated heparin Vitrum contains 12.2 % sulfur.

The results in Table 2 indicate that capsule heparin contains a component missing in the commercial heparin. Efforts to remove this component by refluxing a sample of heparin with ether-ethanol 5:1 or benzene-ethanol 5:1 on a water bath had no effect on the phosphorus content or the colour yield after 20 h refluxing. Shaking a heparin solution with phenol or reprecipitating it as barium salt has also without effect.

DISCUSSION

On comparing the commercial heparin and the capsule heparin here prepared, it seems probable that this heparin contains organically bound phophorus. The results indicate that this substance is not nucleic acid or a nucleotide, a protein or peptide or some hexose compound. The phosphorus content gives an equivalent weight of about 500.

The ratios of activity to sulfur content and to colour yield in the carbazole test seems to be almost the same as the corresponding ratios for one of the fractions of commercial heparin obtained by fractionated alcohol precipitations [9]. The heparin prepared has not yet sufficed for fractionation studies.

REFERENCES

1. Jagues, L. B. and Charles, A. F. *Quart. J. Pharm. and Pharmacol.* **14** (1941) 1.
2. Porath, J. *Acta Chem. Scand.* **8** (1954) 1813.
3. Porath, J. *Biochim. et Biophys. Acta* **22** (1956) 151.
4. Flodin, P. and Kupke, D. W. *Biochim. et Biophys. Acta* **21** (1956) 368.
5. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
6. Ottoson, R. and Snellman, O. *Acta Chem. Scand.* **11** (1957) 185.
7. Scheel, K. C. *Z. anal. Chem.* **105** (1936) 256.
8. Feulgen, R., Boguth, W. and Andresen, G. *Z. Physiol. Chem. Hoppe-Seyler* **287** (1951) 90.
9. Ottoson, R. and Snellman, O. *Unpublished.*

Received November 5, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 480—488

Analysis of Aqueous Mixtures by Gas Chromatography

BENGT SMITH

Institutionen för Organisk Kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

The utilization of some liquids as stationary phases in the analysis of various water-containing mixtures by gas liquid partition chromatography is described and examples given of the separations obtained. The application of the method to quantitative analysis is discussed.

Because of the rapidity with which water can be determined using gas-liquid partition chromatography (GLPC), this method has received considerable attention recently. In order to get good results, the liquid phase should preferably be able to dissolve some of the water. A too high solubility, however, is unsuitable because it results in long elution times. Examples of stationary phases previously used with success are polyethylene glycols [1,2] and a mixture of glycerol and tricresyl phosphate [3]. Data for the columns employed in this work and recommendations for their use are summarized in Table 1.

Polyethylene glycols. In the previous investigations [1,2] polyethylene glycols with an average molecular weight of 600 were used. Here, polymers with an average molecular weight of 200 and 400 were tried. There does not seem to be any special advantage in using the higher polymer since the thermal stability diminishes with increasing molecular weight and the vapour pressure of the lower ones is small enough to permit operation at 100°C for long periods. Fig. 1 shows the separation of an aqueous alcoholic mixture on column No. 1. As can be seen, the water band interferes with the *n*-butyl alcohol band. Water travels relatively more rapidly than the alcohols on polyethylene glycol 400 and this causes it to interfere, on column No. 2, with the *n*-propyl alcohol band as well as with the *n*-butyl alcohol band. The separation of an aqueous aldehydic mixture using column No. 1 is shown in Fig. 2. On all columns which are recommended for aldehydes (*cf.* Table 1), formaldehyde travels more slowly than acetaldehyde in spite of its lower boiling point. Obviously the hydrogen bonding forces are responsible for this effect. Examples of the separation of aqueous mixtures of ketones and aqueous mixtures of acids using polyethylene glycols as stationary phases are given in Figs. 3 and 4. The separation of the components in technical diethyl ether using 1 m of column No. 1 and 1 m of column No. 2 in series is shown in Fig. 5. Since this work was completed, Bodnar and Mayeux [4] have reported the use of

Table 1. Columns for the gas chromatographic analysis of aqueous mixtures.

Column No.	Stationary phase (wt %)	Column length (m)	Carrier	The column is recommended for the determination of water in	Approximate elution time (min) of water at 100°C and a flow of 50 ml He/min.
1	Polyethylene glycol (aver. mol. wt 200) (20 %)	1	Celite 545 *	Alcohols, ethers, aldehydes, ketones, fatty acids, amines	3.5
2	Polyethylene glycol (aver. mol.wt 400) (20 %)	1	Celite 545 *	The same compounds as for column No. 1	2
3	Glycerol (10 %) plus tricresyl phosphate (10 %)	1	Silocel, C22 **	Alcohols, ethers, aldehydes, ketones	6.5
4	Diglycerol (20 %) $[CH_2(OH)CH(OH)CH_2]_2O$	1	Celite 545 *	The same compounds as for column No. 3	5
5	Hexantriol (2,4-dihydroxy-3-hydroxymethylpentane) (20 %)	1	Celite 545 *	The same compounds as for column No. 3	3
6	Triethanolamine (20 %)	1	Celite 545 *	Alcohols, ethers, formaldehyde, ketones, amines	5.5
7	Tween 80 (polyoxyethylene sorbitan monooleate) (20 %)	1	Celite 545 *	Alcohols, ethers, ketones, fatty acids	1.5
8	Tween 80 (5 %)	2	Celite 545 *	Fatty acids	1 ***
9	Glycerol monooleate (20 %)	1	Chromosorb**	Fatty acids	1.5 ***

* 60—100 mesh.
** 30—60 mesh.
*** 100°C, 100 ml He/min.

polyethylene glycol 400 and triethylene glycol for the analysis of aqueous mixtures.

Glycerol/tricresyl phosphate. Browning and Watts [3] recommended the use of a stationary phase containing 40 % by weight of glycerol and 26 % by weight of tricresyl phosphate on 34 % by weight of Celite for the analysis of aqueous mixtures. This composition was tried in this laboratory but it was found to be not specially satisfactory. The high liquid content makes the carrier greasy and difficult to pack evenly in the column. Furthermore, high gas velocities are necessary in order to complete the analysis in a reasonable time. However, by diminishing the liquid content to 10 % by weight of glycerol and 10 % by weight of tricresyl phosphate applied on 80 % by weight

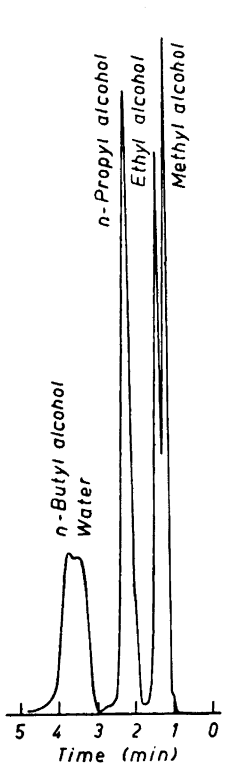


Fig. 1. Water-alcohol mixture. Column No. 1. Temperature 100°C. Flow of He 50 ml/min.

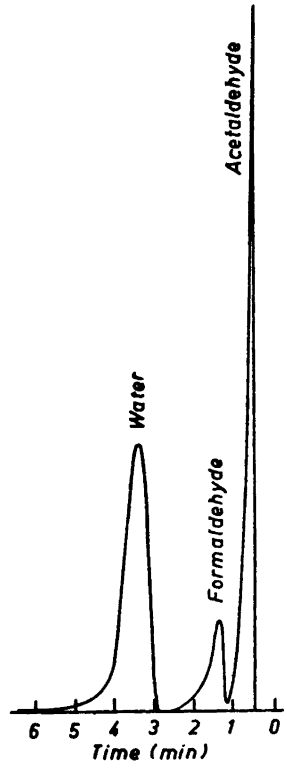


Fig. 2. Water-aldehyde mixture. Column No 1. Temperature 100°C. Flow of He 50 ml/min.

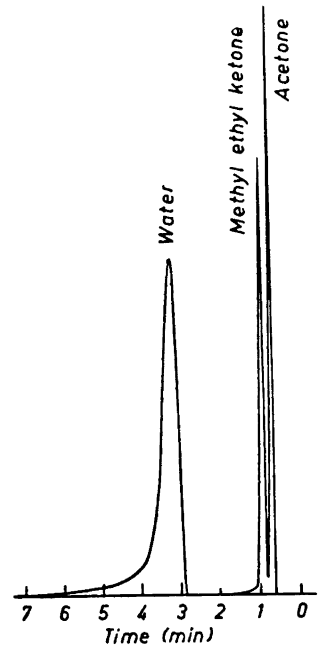


Fig. 3. Water-ketone mixture. Column No. 1. Temperature 100°C. Flow of He 50 ml/min.

of Silocel, an easily packed, dry powder with a good separating power was obtained. Because of the relatively long retention time for water on this column (No. 3), it can be used to determine water in all the lower alcohols, including all the C_4-alcohols.

Diglycerol. The performance of column No. 4 was similar to that of No. 3. The lower alcohols appeared a good distance before the water band. The selectivity of this column for the lower alcohols was low. Thus, methyl, ethyl and *n*-propyl alcohol were not separated but gave instead only one peak. The good thermostability and low volatility of this stationary phase make it very suitable for use in series analyses extending over long periods.

Hexantriol. Water and *n*-propyl alcohol appeared in the same place when using this column.

Triethanolamine. According to Harvey and Chalkley [5], triethanolamine is unsuitable as a stationary phase for the analysis of aqueous mixtures. This

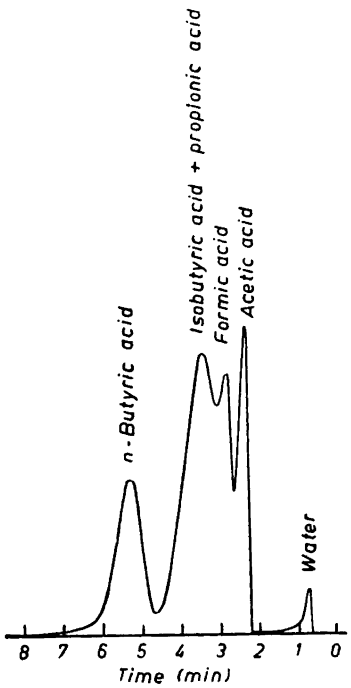


Fig. 4. Water-fatty acid mixture. Column No. 2. Temperature 128°C. Flow of He 60 ml/min.

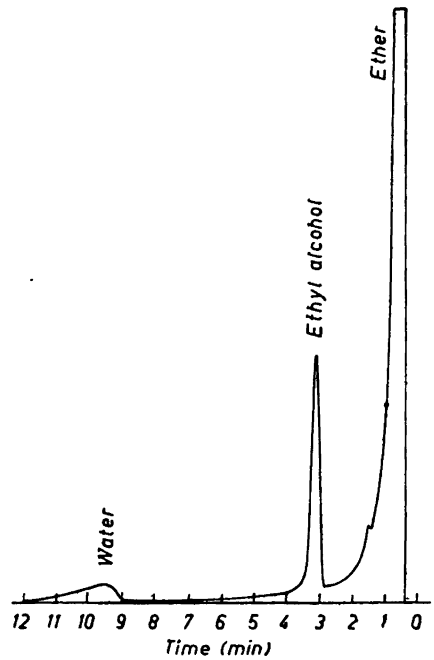


Fig. 5. Industrial diethyl ether. Column No. 1 plus column No. 2. Temperature 75°C. Flow of He 100 ml/min.

point of view seems to result from the use of too large a percentage of liquid on the carrier and the use of conditions favouring long retention times. By using 20 % by weight of liquid on Celite and changing the operating conditions, it was found that triethanolamine could be used for the determination of water in all the lower alcohols and in ketones, amines and ethers.

Tween 80. Water was eluted rapidly from column No. 7 (*cf.* Table 1). This column seems to be well suited for the determination of small amounts of low boiling alcohols or ketones in aqueous solution (*cf.* Fig. 6). Column No. 8, where the liquid content is diminished to 5 % by weight and the length of the column increased to 2 m, is suitable for the determination of water in the lower fatty acids (*cf.* Fig. 7). If the separation of formic and acetic acid is unnecessary, then a 1 m column is sufficient.

Glycerol monooleate. This substance may be used for the analysis of aqueous mixture of fatty acids in the same manner as Tween 80. Only a partial separation of formic and acetic acids can be obtained using column No. 9.

Columns with hydrophobic stationary phases have also sometimes been used. Thus Haskin *et al.*[6] utilized a column packed with di(2-ethylhexyl) phthalate for the analysis of an aqueous mixture of ethanol, acetonitrile and triethyla-

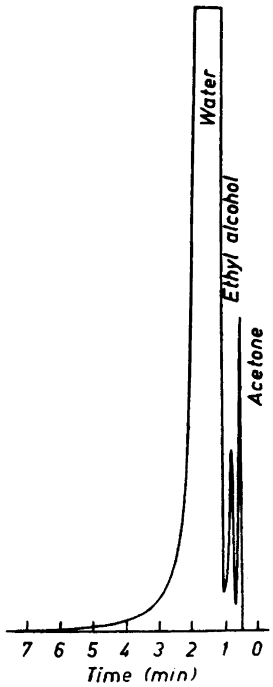


Fig. 6. Small amounts of ethyl alcohol and acetone in water. Column No. 7. Temperature 100°C. Flow of He 50 ml/min.

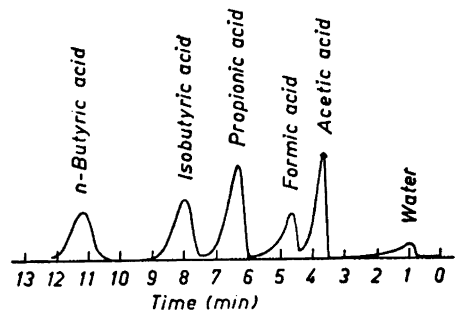


Fig. 7. Water-fatty acid mixture. Column No. 8. Temperature 100°C. Flow of He 100 ml/min.

amine. On this column, water is eluted first as a relatively narrow band with a steep front and a tail. We have tried to determine small amounts of water in ethanol by means of a 2 m column containing 30 % by weight of dioctyl phthalate on Celite. It was found, however, that the retention time for water varied with the amount of water in the mixture and increased as the water content decreased. While a good separation was obtained for mixtures containing several per cent water, the separation became bad, thus making quantitative measurements impossible, when the water content was decreased to a few tenths of a per cent.

In the foregoing, a few examples of separations of aqueous mixtures have been given. It should be pointed out, however, that, by varying the column length and using different columns in series and choosing suitable operating variables, aqueous mixtures of almost any kind can be treated successfully.

Quantitative analysis. The internal normalization method is very applicable for the quantitative analysis of water in aqueous mixtures. Although it is best suited for the assay of moderate amounts of water, even relatively small amounts of water can be determined by making proper use of the sensitivity switch of the gas chromatograph. In Fig. 8, the chromatogram, using column

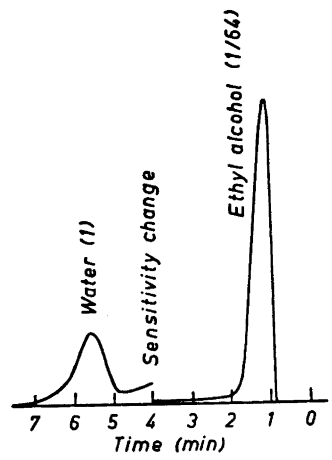


Fig. 8. Absolute ethyl alcohol. Column No. 6. Temperature 100°C. Flow of He 50 ml/min. Recorder attenuation shown in brackets.

No. 6, for absolute ethyl alcohol containing about 0.6 % of water is given. The water peak was run at full detector sensitivity and the alcohol peak at 1/64 of its maximum output. By this procedure, the areas under the two peaks are kept similar in size in spite of the large difference in the contents of the two components.

In the internal normalization method, the areas under the peaks are measured. In order to get a reasonably accurate value for the area, the peak should not be too narrow. Several methods are available for broadening a narrow peak, *e.g.* by increasing the paper speed of the recorder, by decreasing the flow of the carrier gas or by decreasing the temperature. It is also possible to increase the length of the column. It has been recommended when using this last method not to add a length of column containing the same stationary phase as the original column but instead to employ one containing a hydrophobic stationary phase, *e.g.* paraffin. In this way, the retention time for the organic component is increased while that for water is changed only to a minor extent. The water peak may, however, become distorted by this procedure.

A convenient and much used method for calculating the area under a peak is to multiply the height of the peak by the width of the peak at half the peak height (half band width). To get a correct value in this way, the peak should not be distorted. To investigate the influence of the tailing of the water peak, often encountered in GLPC-analysis of aqueous mixtures, the following experiment was made. Mixtures of water and ethyl alcohol were made up and analyzed using column No. 6. The area under each peak was measured by two methods. In the first one, the area was obtained by calculating the peak height times half the band width while, in the second, the area was planimetered. The per cent water peak area was calculated and the figures obtained placed in a diagram to give the relationship between the weight per cent of water in the mixture and the per cent area under the water peak. It was found

that, when the area was calculated by multiplying the peak height by half the band width, the deviation from linearity was not greater, than when the area was planimetered. The same result was obtained with mixtures of acetone and water. Thus it does not seem to be necessary to planimeter the water peak in the GLPC-analysis of aqueous mixtures of alcohols and ketones using triethanolamine as the stationary phase. Since the symmetry of the water peaks obtained when using columns Nos. 1—5 was nearly as good as when using column No. 6, it may be concluded that the simpler area calculation method can also be used in these cases. It should, however, be pointed out that, to get accurate values, it is necessary to make a calibration curve. Any deviation from a linear relationship is then revealed. It is also advisable to check the curve from time to time because the column may change its properties during use, *e.g.* by evaporation of the stationary phase or in other ways. For semi-quantitative work, the per cent area under the water peak may be put equal to the weight per cent of water in the mixture.

Instead of applying the internal normalization method, it is often best to base the quantitative measurements on the peak height or peak area of one or a few of the components in the mixture using either the internal standard or fixed volume method. If the latter method is employed, it is necessary to add exactly the same volume every time. The Agla micrometer syringe or capillary pipettes may be used. Recently a method for injecting exact volumes by means of ordinary syringes has also been described [7]. To exemplify the use of these methods, their application to the analysis of a water-acetone mixture will be described. In the fixed volume method, an exact volume of the mixture is injected and separated on, for example, column No. 1 (*cf.* Fig. 3). The height of the acetone peak is measured. To get the best accuracy, the procedure is repeated several times and the mean value of the acetone peak heights is taken. The per cent acetone in the mixture is read from a calibration curve obtained by injecting the same volume of mixtures containing known amounts of water and acetone using the same experimental conditions and measuring the heights of the acetone peaks. The water content is obtained by difference.

If the internal standard method is applied, a known amount of methyl ethyl ketone is added to the mixture of unknown composition as an internal standard and the mixture is run as before on column No. 1 (*cf.* Fig. 3). The height of the two ketone peaks is measured and the ratio calculated. From this ratio, the per cent acetone is obtained using a calibration curve giving the ratio of per cent acetone to per cent methyl ethyl ketone as a function of the ratio of the peak height of acetone to the peak height of methyl ethyl ketone. This method is somewhat more laborious than the former one but it generally gives the most accurate values since minor fluctuations in the operating variables are compensated for. For comparison, the analysis values for a mixture of acetone and water using the internal normalization method, fixed volume method and internal standard method using column Nos. 6 and 1 are given in Table 2. It is obvious that the fixed volume method is inferior in accuracy to the other two in this case. However, the accuracy of the fixed volume method increases when the acetone content decreases since the relative injection error decreases when larger volumes can be injected.

Table 2. Analysis values for a water-acetone mixture obtained by various methods.

Method	Acetone (wt %)		Water (wt %)		Column No.
	True value	Observed	True value	Observed or calculated	
Internal normalization	11.1	11.2 11.2 11.4 } 11.3	88.9	88.8 88.8 88.6 } 88.7	6
Internal * standard	11.1	11.0 11.1 11.2 } 11.1	88.9	88.9 **	7
Fixed volume ***	11.1	10.9 11.8 11.4 } 11.4	88.9	88.6 **	7

* Internal standard methyl ethyl ketone.
** Calculated as difference from the acetone value.
*** Injected volume 6 μl.

For trace analyses, the internal standard method or fixed volume method must be used. The measurement is in this case always based on the trace component or components. Either the peak height or peak area is used. It should be pointed out that, when using the peak height, peak height maxima are generally not seriously affected by a certain amount of overlap. As the water peak is generally rather flat, measurements of trace amounts of water must be based on the area of the water peak which impairs the accuracy and sensitivity somewhat.

EXPERIMENTAL

The analyses were performed using a Perkin Elmer Vapour Fractometer Model 154. The columns were made from aluminum tubing of 4 mm internal diameter.

The liquids used as stationary phases were heated in vacuum in a stream of nitrogen to remove low boiling impurities. Diglycerol was obtained from May and Baker Ltd, Dagenham, England, and hexantriol, Tween 80 and glycerol monooleate from T. Schuchardt, München, Germany. The other compounds used as stationary phases may be obtained from any chemical firm.

REFERENCES

1. Adlard, E. R. in Desty, D. H. *Vapour Phase Chromatography*, Butterworths Scientific Publications, London 1957, p. 98.
2. Whitham, B. T. in Desty, D. H. *Vapour Phase Chromatography*, Butterworths Scientific Publications, London 1957, p. 395.

3. Browning, L. C. and Watts, J. O. *Anal. Chem.* **29** (1957) 24.
4. Bodnar, S. J. and Mayeux, S. J. *Anal. Chem.* **30** (1958) 1384.
5. Harvey, D. and Chalkley, D. E. *Fuel* **34** (1955) 191.
6. Haskin, J. F., Warren, G. W., Priestley, Jr., L. J. and Yarborough, V. A. *Anal. Chem.* **30** (1958) 217.
7. Gruber, K. *Holzforschung und Holzverwertung* **9** (1957) 104.

Received December 20, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 489—496

Ammonium Salts as Nitrogen Source in the Synthesis of Protein by the Ruminant

HARING LAND and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Two cows have been fed on a diet containing an ammonium salt labelled with ^{15}N. Milk protein has been shown to contain this heavy isotope to an extent corresponding to incorporation of 17—25 % of the ammonium nitrogen into proteins. It is made plausible that the maximum incorporation into both milk and body proteins may be some 40 %. The milk collected about 14 hours after administration of the labelled salt shows the highest content of ^{15}N in the amides, followed by glutamic acid, aspartic acid and alanine. After 24 hours the labelling of most of the protein components has already become comparatively even. Histidine shows an especially low content of ^{15}N; this may be attributed to incapability of the rumen bacteria to synthesize the imidazole ring. Cystine, too, is relatively low in ^{15}N content.

An extensive literature has been published in the last three decades concerning the synthesis of protein by the ruminants, the role displayed in this synthesis by the rumen bacteria and the significance of non-protein nitrogen compounds as substitutes for proteins in the diet of the ruminants. A survey of this literature has been given by Chalmers and Synge [1]. The results of the experimental work show considerable divergence among the various authors as to the extent to which non-protein nitrogen, in the form of urea (in most of the cases), ammonium salts, ammoniated molasses, ammoniated straw, ammoniated sugarbeet pulp, dicyanamide, is effectively used in the process of protein synthesis. This difference of opinion arises mainly from the difficulty, inherent to all experimental work on nutrition problems, to start from welldefined uniform basic diets, and from the lack of a common standard of comparison. Little doubt, however, exists about the reality of the utilization of non-protein nitrogen by ruminants. A vast number of indirect experiments has been performed in which the extent of the utilization of the non-protein compound fed is inferred from either protein synthesis in an artificial rumen [2,3] or from changes in body weight or milk production of the animal under observation. Direct results, however, are rather rarely met in literature. To the

authors' knowledge the only direct evidence of protein synthesis from non-protein nitrogen by ruminants is presented in a paper by Watson *et al.*[4] They fed urea labelled with ^{15}N to sheep and were able to detect this isotope in proteins from blood, liver and kidneys in excess over the ^{15}N content of proteins of sheep fed with unlabelled urea.

The present paper gives the results of an investigation based on feeding experiments in which ammonium salt labelled with ^{15}N was fed to two cows. The experiments were conducted on the farm of one of the authors (A.I.V.). The cows received AIV-fodder, coarsely ground oats and some hay. According to the principle of the AIV preservation method the acidity of the fresh fodder is increased so that the pH of the ensilaged material does not exceed the critical value 4. In the preparation of the AIV-fodder used in these experiments, instead of strong mineral acids 1 kg of ammonium bisulphate had been added per 100 kg of fresh greenfodder in order to reach this acidity; the pH of the finished product was 3.8—3.9. The preserved material consisted of a mixture of about 70 % of red clover and 30 % of timothy, blown into the silo by means of a fan equipped with cutting blades.

When the AIV-method was developed in the years before 1930, some attempts were already made to reach the necessary acidity in the fodder mass by means of acid salts, but the quantity of salt required for non-chopped clover-rich fodder was so high (1.8—2.0 kg per 100 kg of fresh fodder) that the health of the cattle might be impaired. The amount of acid to be added decreased, however, when choppers came into use. In recent years (1953—1958) new series of preservation trials have been conducted in this laboratory by Virtanen and Kreula [5] and their results indicate that application of ammonium bisulphate or of mixtures of ammonium and sodium bisulphate is entirely feasible also in the preservation of clover. For fodderplants poorer in protein much smaller quantities of these salts suffice. Therewith the question of the utilization of ammonium nitrogen in the synthesis of amino acids and proteins in the rumen has become actual also when an effective method for preservation of fresh fodder precluding the formation of ammonia from the fodder proteins, is used.

The purpose of the present investigation was to establish the value of ammonium salts in the synthesis of proteins in the ruminant. Apart from this directly important information, knowledge of more details of the actual synthetic process might be obtained by tracing the heavy isotope in the various fractions and different amino acids of the milk proteins.

EXPERIMENTAL

Two experiments have been performed so far. During the first one, started on March 21 at approximately 4.30 P.M., the test cow Latsi with a production of about 10 kg of milk (4.3 % fat) per day was fed on a diet administered in two portions (in the morning and in the evening) each consisting of 3.4 kg of coarsely ground oats, 9.2 kg of AIV-fodder and 1 kg of hay. This corresponds to 10 Scandinavian fodder units and 1 040 g of digestible raw protein (NH_4-N subtracted) per day. The overfeeding both in fodder units and in protein was fairly high, because the food requirement corresponding to the milk production was about 8 fodder units and 850 g of digestible protein. This diet was given for 2 1/2 days before the beginning of the experiment proper; then 0.9993 g of ammonium nitrate containing 61 atom % of ^{15}N in the ammonium part was added as a fairly concentrated solution in a small volume of water to the oats. For another 5 days after the addition of the labelled ammonium salt the cow received the same diet as mentioned, and after this period returned to her normal diet. Analytical details of the diet are given in Table 1.

Table 1. Analyses of fodders used in the experiments.

	% total N	% dry matter	% NH_4—N	kg fed	g total N	g NH_4—N
	1.54	87.5	0.020			
Oats	1.53	87.6	0.020	3.4	52	0.7
	1.53	87.6	0.019			
	0.70	25.5	0.131			
AIV-fodder	0.63	26.1	0.135	9.2	60	12.1
	0.61	25.4	0.131			
	1.10	91.1	0.042			
Hay	1.22	90.9	0.044	1.0	12	0.4
	—	91.6	0.043			

According to Table 1 the NH_4-N was 10.7 % of the total N. 0.9993 g of NH_4NO_3 with 61 atom % ^{15}N in the ammonium group contains 0.181 g of NH_4-N, that is, 1.37 % of the total NH_4-N of the fodders. The amount of excess ^{15}N in it is 7.52 mg-atoms.

In the second experiment beginning on May 14 at about 4.00 P.M., another cow (Hieno) with a daily production of about 16 kg of milk (4.2 % fat) was fed on substantially the same diet; this could provide her with the required food units (10 f.u.) per day. The digestible protein in the diet was already somewhat lower than the need (1 040 g and 1 100 g, respectively). After the same preparatory feeding period as in the first experiment, 1.8953 g of ammonium sulphate containing 90.1 atom % ^{15}N was added to the food, *i.e.* 0.443 g of NH_4-N which amounts to about 3.5 % of the total NH_4-N of the diet. The excess ^{15}N administered in this case is 25.42 mg-atoms. After 5 days on the diet as mentioned before, the cow received again the customary rations, and on May 23 was brought out to pasture.

Table 4. Excess ^{15}N in milk protein and in its amino acids in the second experiment (cow Hieno).

Milk a: collected 15.5.58, at 7.10 A.M.
b: » 15.5.58, » 5.15 P.M.

Protein precipitated with 15 % trichloroacetic acid; precipitate washed with water, dialyzed; lyophilized; extracted with ether; hydrolyzed with 6 N HCl.

Substance	At % excess ^{15}N in: (a)	(b)
Total protein	0.039	0.032
Amide-N	0.071	0.039
Glu	0.054	0.030
Asp	0.047	0.037
Tyr	0.042	0.036
Ala	0.047	0.038
Ser + Thr	0.038	0.031
Gly	0.044	0.033
Val	0.032	0.032
Pro	0.026	0.023
Lys	0.029	0.031
Met + Leu + Ileu	0.033	0.029
His	0.008	0.009
Phe	0.027	0.027
Arg	0.022	0.022
Cys	—	0.018

The cows were milked twice daily. In the first experiment the milk was weighed for 12 days, in the second for 20 days. Urine and feces were not collected quantitatively. From samples of milk the ^{15}N content was determined in the total N, in the protein and the non-protein fractions separated according to Rowland [6], and in the amide-N fraction split off from the precipitated protein fraction [7]. The total-N content of the milk of the first experiment, estimated on a number of samples from various days, had an average value of 0.468 %, and a non-protein N content of 0.035 %. In addition, the ^{15}N content was estimated in samples of urine and feces, and in a few cases in the urine ammonia distilled according to Vickery *et al.* [7]; no analyses were performed on urine and feces to assess the total amount of nitrogen excreted.

From samples of milk, urine and feces collected during the second experiment similar estimations were made; the total-N content of the milk was here 0.430 % (average of 8 determinations); the non-protein N content was not determined. The amide-N content was estimated according to Vickery [7] in the protein of the milk collected on May 15 at 7.10 A.M. and at 5.15 P.M.; it amounted to 8.9 % of the total protein-N content in both cases.

From these same milk samples the protein fractions obtained by precipitation with 15 % trichloroacetic acid, were dialyzed and lyophilized. After extraction with ether to remove fatty substances the dry protein was hydrolyzed with 6 N HCl in a sealed tube at 105° for 24 h, and the hydrolysate was separated into various amino acid fractions according to Hirs, Moore and Stein [8]; their procedure was in so far modified that it permitted the separation of rather large quantities of the amino acids. Details of the separation have been published elsewhere [9]. A rigorous separation of all amino acids was not aimed at; hence methionine, *iso*leucine and leucine have been taken together, so have serine and threonine. The yield of cystine from the first protein sample was too low for further treatment. Phenylalanine and arginine, emerging together from the ion exchange column, have been separated on a cellulose column in order to keep a distinction between basic, acidic and neutral amino acids.

The various fractions of the purified amino acids were subjected to Kjeldahl digestion and the ^{15}N content was estimated in the ammonia obtained. The measurements were performed with a CEC mass spectrometer model 21−401, at an ionizing current of 90 μA and an auxiliary amplifier output of 20 V. Corrections for the presence of air have not been applied, since in no nitrogen sample the quantity of air present as indicated by the argon peak, exceeded 0.1 %. Measurements were performed on either three or four samples, each sample allowing at least four readings; the cystine sample from the second protein fraction only allowed four measurements altogether to be made. The standard deviation did not exceed 0.1 % in the most unfavourable case and was usually of the order of 0.05 %.

The results of the measurements are given in Tables 2, 3 and 4.

In Tables 2 and 3 the time given in column 2 is the time of milking; the samples of urine and feces mentioned behind the same time, were collected within one hour after that time.

DISCUSSION

From Tables 2 and 3 the high rate of uptake of ammonia into the milk becomes evident: within one hour the milk is labelled, although in the first experiment the protein fraction does not yet contain any ^{15}N, and in the second experiment the labelling of the proteins is very weak. The non-protein fraction has not been subjected to a closer investigation whether the ^{15}N occurs in the form of the ammonium ion or as amide or low-molecular peptide. Analysis of the milk collected after about 14 h after the feeding of the labelled fodder shows a high content of ^{15}N in the amide-N (0.071 atom % excess in the second experiment), whereas the total-protein N contains 0.039 atom %. Since amide-N makes up 8.9 % of the protein-N, its ^{15}N content accounts for only 15 % of the total amount of ^{15}N present in the total N. This percentage

Table 2. First experiment started on March 21 at approximately 4.30 P.M. (cow Latsi). Administered 7.52 mg-atom excess of ^{15}N as NH_4NO_3 in the NH_4; total quantity of NH_4-N in diet 13.4 g. Total N in milk: 0.468 %; non-protein N: 0.035 %.

Date	Time	kg milk	tot.N in milk (mg-at)	At% excess ^{15}N in milk			urine		feces	Excess ^{15}N in milk (mg-at)	% ^{15}N recovered from milk
				tot.N	prot.N	N.P.N	tot.N	NH_4-N			
21.3	5.30 P.M.	—	—	0.004	0.000	0.036	—	—	—	—	—
22.3	7.00 A.M.	5.0	1670	0.012	0.012	0.023	0.019	0.044	0.021	0.200	2.66
22.3	5.30 P.M.	4.2	1400	0.011	0.010	0.016	0.016	0.031	0.018	0.154	2.05
23.3	7.00 A.M.	5.4	1810	0.008	0.009	0.012	0.011	0.020	0.013	0.145	1.93
23.3	5.30 P.M.	4.4	1470	0.007	0.007	0.010	0.005	—	0.009	0.103	1.37
24.3	7.00 A.M.	5.9	1970	0.006	0.006	0.008	0.003	0.021	0.008	0.118	1.57
24.3	6.15 P.M.	4.4	1470	0.005	0.005	0.007	—	—	0.004	0.074	0.99
25.3	6.50 A.M.	5.4	1810	0.003	—	—	0.001	—	0.004	0.054	0.72
25.3	6.00 P.M.	4.6	1540	0.001	—	—	—	—	—	0.015	0.20
26.3	7.00 A.M.	4.3	1440	0.002	—	—	0.001	—	—	0.029	0.39
26.3	6.00 P.M.	4.3	1440	0.002	—	—	—	—	—	0.029	0.39
27.3	6.45 A.M.	5.6	1870	0.002	—	—	0.000	—	—	0.037	0.49
27.3	6.00 P.M.	4.4	1470	0.002	—	—	—	—	—	0.029	0.39
28.3	7.00 A.M.	3.7	1240	0.003	—	—	0.000	—	—	0.037	0.49
28.3	5.30 P.M.	4.3	1440	0.003	—	—	—	—	—	0.043	0.57
29.3	6.30 A.M.	4.8	1610	0.002						0.032	0.43
29.3	5.40 P.M.	4.2	1400	0.002						0.028	0.37
30.3	7.00 A.M.	5.0	1670	0.002						0.033	0.44
30.3	5.40 P.M.	3.8	1270	0.002						0.025	0.33
31.3	6.30 A.M.	5.2	1740	0.001						0.017	0.23
31.3	5.40 P.M.	4.0	1340	0.002						0.027	0.36
1.4	6.45 A.M.	5.0	1670	0.002						0.033	0.44
1.4	6.30 P.M.	4.0	1340	0.001						0.013	0.17
2.4	7.45 A.M.	5.0	1670	0.001						0.017	0.23
									Total:		17.21
13.4	7.00 A.M.			0.001							
13.4	6.40 P.M.			0.001							
14.4	7.00 A.M.			0.001							
6.5	7.00 A.M.			0.001							

Amide-N of milk protein of 22.3, 7.00 A.M.: 0.026 atom% excess ^{15}N.
» » » » » 22.3, 5.30 P.M.: 0.012 » » »

decreases quickly to 11 % in the following milk sample. After this it approaches the value found for the total-N. The incorporation of the ammonium-N into the milk proteins seems to proceed primarily *via* the amide, followed by glutamic acid (Table 4). Aspartic acid does not play a role comparable to that of glutamic acid, for the labelling of the latter compound decreases with 45 % within 10 h, of the former with 21 %; the decrease for the total N is 18 %. With the other amino acids there is a general downward trend in the ^{15}N content, more pronounced for the smaller molecules; the basic amino acids, however, show rather an increase. Especially histidine contains little ^{15}N; if labelling is assumed to occur in the amino-N only, the atom %

Table 3. Second experiment started on May 14 at approximately 4.00 P.M. (cow Hieno). Administered 25.42 mg-atom excess of ^{15}N as $(NH_4)_2SO_4$. Total N in milk: 0.430 %; amide-N 8.9 % of protein-N.

Date	Time	kg milk	tot. N in milk (mg-at)	At % excess ^{15}N in: milk: tot. N	prot.N	N.P.N	Amide-N	urine	feces	Excess % ^{15}N in milk (mg-at)	% ^{15}N recovered from milk
14.4	4.45 P.M.	6.4	1970	0.005	0.002	0.050	0.003	0.101	0.002	0.098	0.37
15.5	7.10 A.M.	9.1	2800	0.039	0.039	0.053	0.071	0.077	0.018	1.092	4.29
15.5	5.15 P.M.	7.2	2210	0.032	0.032	0.038	0.039	0.050	0.084	0.707	2.78
16.5	7.30 A.M.	8.8	2700	0.022	0.024	0.030	0.025	0.032	0.036	0.594	2.34
16.5	5.15 P.M.	6.9	2120	0.018	0.017	0.024	0.017	0.015	0.028	0.382	1.50
17.5	6.30 A.M.	9.0	2760	0.014	0.013	0.021	0.012	0.012	0.016	0.386	1.52
17.5	6.00 P.M.	7.9	2430	0.011	0.010	0.014	0.008	0.008	0.012	0.267	1.05
18.5	6.40 A.M.	8.7	2680	0.009	0.008	0.012	0.006	0.007	—	0.241	0.95
18.5	5.15 P.M.	7.0	2150	0.009	—	—	—	0.006	0.007	0.194	0.76
19.5	6.45 A.M.	8.5	2610	0.008	—	—	—	0.005	0.005	0.209	0.82
19.5	5.40 P.M.	6.2	1910	0.007	0.007	0.005	—	0.005	0.004	0.134	0.53
20.5	6.25 A.M.	7.8	2400	0.006	0.007	0.004	—	0.004	—	0.144	0.57
20.5	5.00 P.M.	6.1	1880	0.006	—	—	—	—	0.003	0.113	0.44
21.5	7.10 A.M.	6.7	2060	0.006	0.005	0.003	0.004	0.004	0.002	0.124	0.49
22.5	7.10 A.M.	6.0	1840	0.005	0.005	0.002	0.003	0.002	0.002	0.092	0.36
23.5	7.10 A.M.	5.7	1750	0.005				0.002		0.088	0.35
24.5	6.45 A.M.	5.5	1690	0.004				0.000		0.068	0.27
25.5	6.55 A.M.	6.5	2000	0.004						0.080	0.31
26.5	7.30 A.M.	7.0	2150	0.004				0.000		0.086	0.34
27.5	5.30 P.M.	6.2	1910	0.004						0.076	0.30
28.5	6.45 A.M.	7.6	2320	0.003						0.070	0.28
29.5	7.00 A.M.	8.4	2580	0.002					0.001	0.052	0.20
30.5	7.40 A.M.	8.5	2610	0.002						0.052	0.20
31.5	8.00 A.M.	9.0	2760	0.002						0.055	0.22
1.6	7.30 A.M.	9.3	2860	0.002						0.057	0.22
2.6	6.30 A.M.	8.8	2700	0.002						0.054	0.21
4.6	5.30 P.M.	7.5	2300	0.001						0.023	0.09

excess of ^{15}N in this group will be three times higher than the value observed. This new value (in histidine a and *b*, respectively, 0.0024 and 0.0027) is in fair agreement with the values found for the other amino acids. Thus the low content of ^{15}N in histidine may be interpreted as incapability of the rumen bacteria to synthesize the imidazole ring. The relatively low content in cystine is an interesting point, too.

The last columns in Tables 2 and 3 give the percentage of the initially added amount of ^{15}N that was recovered from the milk. No more labelling can be detected in urine and feces after about one week, but some six weeks after the start of Expt. 1 there was still a detectable trace of ^{15}N in the milk; in Expt. 2 the measurements have not been extended to so long a period. The first cow is seen to produce a recovery value of 17.2 % in ten days; the

trailing over another four weeks has not been considered since the excess of ^{15}N is so minute by that time that it approximates to the accuracy of the measurements. The recovery value for the second cow, having a considerably higher milk production (79.3 kg over a period of five days, compared with 47.9 kg over the same period for the first cow) with a slightly lower protein content (0.430 % *versus* 0.468 %), and being fed on approximately the same rations, is evidently higher. For its calculation it must be taken into account that the values after May 20 have to be doubled, since only one sample of milk per day was investigated. Thus the total recovery found over a period of 17 days amounts to 25.4 %, the yield after this period having again been neglected. The overfeeding with protein is probably the reason for the lower utilization of ammonium nitrogen in the first experiment. In the second experiment the protein in the diet was somewhat lower than the calculated requirement, and the ammonium nitrogen utilization is seen to be about 40 % higher than in the first experiment. More experimental material is needed to elucidate the influence of the composition of the food on the utilization of non-protein nitrogen.

On the basis of the milk yields and the content of ^{15}N one arrives, for this particular diet and these particular cows, at a utilization of about 20 % for the ammonium nitrogen in the formation of milk protein. This value presents a minimum for the total utilization because the uptake into body protein has been left out of consideration. Since in these experiments no quantitative data were collected regarding the amount of nitrogen excreted in the urine and the feces, a rigorous treatment of the total utilization of the ammonium-N cannot be given. It is, however, possible to derive an approximate value along the following lines.

On the assumption that the digestibility of the total protein of oats is 80 %, of AIV-fodder 70 % and of hay 60 %, the ration fed leads to an excretion of 33 g of nitrogen in the feces per 12 h period (*cf.* Table 1 for total N fed). The urine production may be estimated at 11 kg (containing 0.6 % total N) per 10 kg of milk; for the cow of the first experiment, producing slightly less than 10 kg of milk per day during the course of the experiment, the production of urine-N amounts to another 33 g of nitrogen, *i.e.* 2 360 mg-

Table 5. Estimation of the amount of ^{15}N excreted in urine and feces in Expt. 1. Administered 7.52 mg atoms of ^{15}N. Production (per 12 h period following the time mentioned) of urine-N and of feces-N: 2 360 mg-atoms.

Date	Time	Atom % excess in urine	Atom % excess in feces	Excess ^{15}N (mg-at.) in urine + feces	% ^{15}N recoveed
21.3	17.30	0.024	0.026	1.18	15.7
22.3	7.00	0.019	0.021	0.94	12.5
22.3	17.30	0.016	0.018	0.80	10.6
23.3	7.00	0.011	0.013	0.57	7.6
23.3	17.30	0.005	0.009	0.33	4.4
24.3	7.00	0.003	0.008	0.26	3.5
24.3	18.15	0.002	0.004	0.14	1.9
25.3	6.50	0.001	0.004	0.12	1.6
					57.8

atoms per 12 h. Extrapolation of the values for the atom % excess of ^{15}N in urine and feces to the first 12 h following the adminstration of the labelled ammonium salt gives the values 0.024 and 0.026, respectively, with a reasonable trustworthiness. In Table 5 the results are combined.

The further yield of ^{15}N after March 25 will still amount to a few percent, but has not been taken into consideration since measurements on urine and feces were only occasionally performed after that date. It has been found that the total percentage of ^{15}N found in urine and feces amounts to roughly 60 %. If loss of nitrogen through respiration is regarded as negligible, this would mean that from the ration fed, in which 10.8 % of the total N is present as NH_4-N, possibly 40 % of the NH_4-N is utilized for protein synthesis.

REFERENCES

1. Chalmers, M. I. and Synge, R. L. M. *Advances in Protein Chem.* **9** (1954) 93.
2. Pearson, R. M. and Smith, J. A. B. *Biochem. J.* **37** (1943) 153.
3. Bohstedt, G., Booth, A. N., Hart, E. B. and Wegner, M. I. *J. Dairy Sci.* **23** (1940) 1123.
4. Davidson, W. M., Kennedy, J. W., Muir, G. W., Robinson, C. H. and Watson, C. J. *Sci. Agr.* **29** (1949) 185.
5. *cf.* Virtanen, A. I. *Karjatalous* **34** (1958) 132.
6. Rowland, S. J. *J. Dairy Research* **9** (1938) 42.
7. Leavenworth, C. S., Pucher, G. W. and Vickery, H. B. *Ind. Eng. Chem. Anal. Ed.* **7** (1935) 152.
8. Hirs, C. H. W., Moore, S. and Stein, W. H. *J. Am. Chem. Soc.* **76** (1954) 6063.
9. Wahlroos, Ö. and Land, H. *Acta Chem. Scand.* **13** (1959) 595.

Received December 1, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 497—501

Phosphate Compounds in Respiring Mycelium of *Merulius lacrymans* (Jacq.) Fr.

JOSTEIN GOKSØYR

Botanical Laboratory, University of Bergen, and

LEIV KLUNGSØYR

Pharmacological Institute, University of Bergen, Norway

Incubation experiments with ^{32}P labelled orthophosphate have been carried out with the Cartwright strain of *Merulius lacrymans*, grown as shake cultures. Phosphate compounds found to be labelled with ^{32}P in experiments lasting up to two hours, were: pyrophosphate, ADP, ATP, GTP and UDP. On paper chromatograms, several unidentified radioactive spots were also seen. Poly-metaphosphate was identified in the residue after TCA extraction. In the barium insoluble fraction a metabolically inert phosphate compound occurred, which was believed to be tri- or tetraphosphate. This overlapped the ATP elution peak on column fractionations. Time-course experiments showed that pyrophosphate became more rapidly labelled than ATP. Both respiration and ^{32}P incorporation were strongly inhibited by 10^{-4} M 2,4-dinitrophenol.

Little information seems to be available regarding the occurrence of adenosine triphosphate (ATP) or other phosphate compounds in the Basidiomycete group of fungi. Nord and Sciarini [1] studied the presence of phosphorylation processes in *Merulius niveus* and concluded that "phosphorylation does not constitute an integrate part of the phase sequence of carbohydrate degradation by wood-rotting fungi".

As part of an investigation of the biochemistry and physiology of the wood-decomposing Basidiomycete *Merulius lacrymans*, it was therefore considered of interest to study the presence of phosphate compounds and their metabolic activity.

MATERIALS AND METHODS

The Cartwright strain of *M. lacrymans* from *Centraalbureau voor Schimmelcultures*, Baarn, Holland, has been used throughout these investigations. The fungus was grown submerged in 200 ml portions of medium in 500 ml Erlenmeyer flasks, on a reciprocal shaker at room temperature (18—22°C). The medium consisted of: glucose, 20 g; peptone,

10 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7\ H_2O$, 1.5 g; thiamine, 1 mg per liter. The flasks were covered with aluminium foil, and were inoculated from a surface culture or a previous shake culture which had been disintegrated in a Waring blendor running with low speed for less than 1 min. The cultures were harvested after 8—10 days of growth. pH in the medium had then fallen to about 2.5. Inoculation on agar plates revealed no presence of bacteria. The mycelium was separated from the substrate by filtering through folded filter paper. The filter was washed thoroughly by a mixture of 0.01 M KCl and 0.01 M $MgCl_2$, and the mycelium finally suspended in the same solution. pH in the suspension was about 4.5. Respiration measurements were carried out either by gas analysis, utilizing a Scholander 1/2 ml Gas Analysis Apparatus, or by Warburg technique.

For isolation and identification of phosphate compounds, the suspension of washed mycelium was incubated with carrier-free ^{32}P-labelled orthophosphate with vigorous shaking in order to obtain a high rate of respiration. To the respiring suspension, trichloroacetic acid (TCA) was added to a final concentration of 5 % and the shaking continued for a couple of minutes. The suspension was then cooled and centrifuged and the extract treated with barium acetate. The barium insoluble compounds were separated by centrifugation, treated with Dowex 50 in hydrogen form until dissolved, neutralized with KOH, centrifuged with high speed to remove mucous material and fractionated on a Dowex 1,X8 formate column according to Hurlbert *et al.*[2] Metaphosphate was isolated from the residue after the TCA precipitation as described by Klungsøyr *et al.*[3] Paper chromatography was carried out on Whatman No. 1 filter paper washed with 1 % oxalic acid. The solvent systems used, were: *n*-propanol, NH_3, H_2O (6:3:1) and *tert*-butanol, formic acid, H_2O (8:3:4). The papers were developed with the phosphate reagent of Hanes and Isherwood[4]. Radioautography of two-dimensional chromatography was carried out using Kodak X-ray film.

RESULTS

Separation and identification of phosphate compounds. A considerable number of phosphate compounds became labelled by incubation of the mycelial suspension with ^{32}P labelled orthophosphate. Fig. 1 shows a paper chromatogram of the TCA extract from an experiment when the incubation time was 2 h. Five hundred ml suspension was here incubated with 800 μC ^{32}P. Ten ml of the TCA extract was freeze-dried after removal of the TCA with ether in a continous liquid-liquid extraction apparatus, and the residue dissolved in 1 ml water. Ten μl of this solution was placed on the paper. Only a few

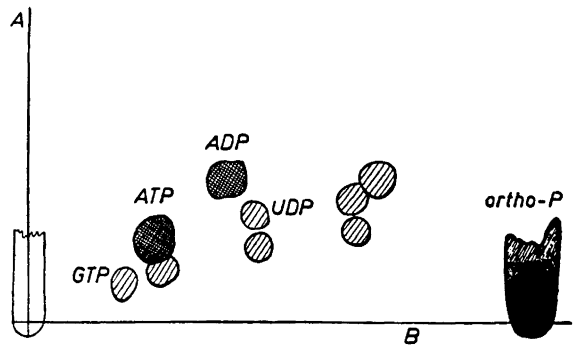


Fig. 1. Paper chromatogram of a TCA extract of *Merulius* mycelium, incubated with radioactive orthophosphate for 2 h. The degree of shading indicates the amount of radioactivity in the spots. Solvent systems: A, *n*-propanol, ammonia, water (6:3:1) and B, *tert.*-butanol, formic acid, water (8:3:4).

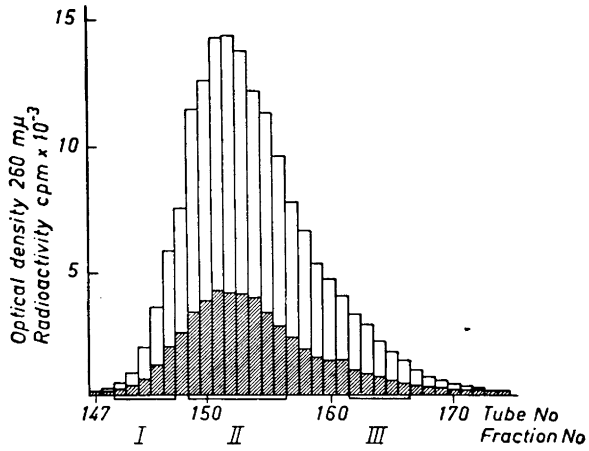


Fig. 2. Rechromatography of ATP from *Merulius* mycelium. The fractions marked I, II and III were lyophilized and analysed separately. Open columns: radioactivity in counts per min × 10^{-3}. Shaded columns: optical density at 260 mμ.

of the compounds have been identified, but there are definite radioactive spots for ATP, ADP, GTP and UDP.

From the main portion of the extract, the Ba insoluble phosphates were fractionated on an ion exchange column. Here fractions were obtained containing pyrophosphate and ADP. A fraction containing radioactivity and adenine like material absorbing at 260 mμ was obtained at the elution volume characteristic for ATP. The whole fraction was pooled, lypohilized and analyzed. The molar ratios were 4, 1 and 1, for acid labile phosphate, ribose and adenine, respectively. The substance was rechromatographed on the formate column, Fig. 2, and the tubes containing radioactivity and absorbing at 260 mμ were pooled in 3 fractions, lypohilized and analyzed separately for purine, acid labile P and radioactivity. Each fraction was examined by paper chromatography. The paper chromatography showed the presence of ATP in fractions 1 and 2, and of GTP in fraction 3, all radioactive. All three fractions gave a spot at the starting point, trailing in the direction of the ammoniacal system.

Table 1. Analysis of the three fractions of the rechromatographed triphosphate peak shown in Fig. 2.

Fraction number	I	II	III
Optical density ratio 260 mμ/275 mμ	0.428	0.422	0.676
Radioactivity Cpm/μ mole purine	9 220	9 100	8 250
Acid labile P/purine	4.7	3.05	9.15

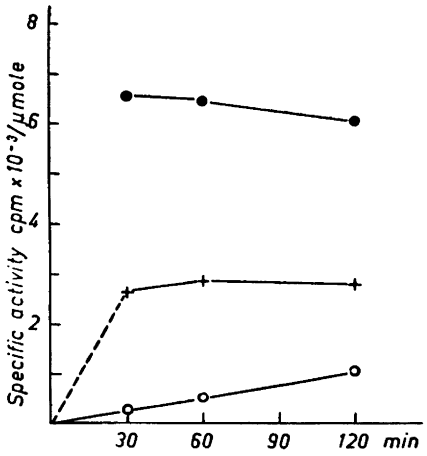


Fig. 3. The specific radioactivity in orthophosphate, pyrophosphate and ATP as a function of incubation time. The values are given as counts per min per μmole phosphorus in orthophosphate and pyrophosphate, and per 2 × μmole purine in ATP. The third phosphate in ATP is probably somewhat radioactive, so that the specific activities calculated for this compound are maximal values. ● : orthophosphate, + : pyrophosphate, ○ : ATP.

This spot contained no radioactivity, and is probably given by the phosphate compound(s) that overlaps the ATP and GTP peaks from the column. The amounts of acid labile P per μmole of purine differed widely in the three fractions (Table 1), while the radioactivities per μmole purine were the same in all of them. These results demonstrate the presence of acid labile, ribose and purine free phosphate contaminants in the isolated ATP and GTP. These metabolically inert phosphate compounds might be low molecular polyphosphates, for instance triphosphate or tetraphosphate.

About three μmoles acid labile phosphate per μmole adenin has been obtained repeatedly on the most highly purified ATP from different batches of mycelium in this fungus. This lead to the tentative conclusion that the fungus contained adenosine tetraphosphate (AQP) instead of ATP. Paper chromatography demonstrated, however, that this was not the case. Spots corresponding with ATP were consistently found, while spots corresponding with AQP (a preparation from Sigma Chem. Corp. was used as reference sample) could not be detected.

Metaphosphate. A phosphate-containing compound was isolated from the TCA insoluble residue, which was not dialyzable, gave a purple to pink colour with toluidine blue and was converted to orthophosphate by hydrolysis

Table 2. The effect of 2,4-dinitrophenol on respiration and radioactive phosphate incorporation in *Merulius* mycelium. Experimental time one hour.

	Respiration rate μl O_2 per mg dry weight and hour	^{32}P incorp., %
Control	5.5	1.10
10^{-4} M DNP	1.8	<0.01

with normal sulfuric acid at 100°C for 15 min. This polymetaphosphate was present in the fungus in comparatively large amounts. The incorporation during respiration, of ^{32}P labelled orthophosphate into the TCA insoluble fraction of the cells was negligible.

The time course of the incorporation process. An experiment was carried out in which samples of mycelium incubated with ^{32}P labelled orthophosphate were analyzed after 30, 60 and 120 min of respiration. The barium insoluble fractions of the TCA extracts were fractionated as usual, and the radioactivity in the fractions determined. The results are shown in Fig. 3. It will be seen that the specific activity in pyrophosphate increases rapidly, while that in ATP does so more slowly, and remains during the experimental period lower than that of pyrophosphate.

The effect of dinitrophenol on the incorporation. 2,4-Dinitrophenol is a potent inhibitor of oxygen uptake in *M. lacrymans*, and was also found to inhibit phosphate incorporation. Results from one experiment are shown in Table 2. The experiment was carried out in Warburg flasks, and the cells killed by adding TCA from the side arm at the end of the experimental period, which lasted one hour.

DISCUSSION

The chemical identification of ATP in *M. lacrymans* met with some difficulties. Whereas paper chromatography gave definite evidence to the presence of ATP, the column fractionation technique used obviously did not separate between ATP and some metabolically inactive phosphate which we believe to be tri- or tetraphosphate. For this reason too high values for acid labile and total phosphate were found in the ATP preparations.

The time course of ^{32}P incorporations from orthophosphate follows closely that earlier described by Klungsøyr *et al.*[3] for *Acetobacter suboxydans*. The only difference seems to be that the reaction goes considerably more slowly in *M. lacrymans;* something which probably is connected with its metabolic activity as a whole.

As far as these investigations go, they show that *M. lacrymans* contains a system for generating energy-rich phosphate bonds operating during active respiration and with ATP as the main store for such bonds. The phosphate metabolism does not seem to differ much from that of other microorganisms.

The work has been supported by grants from *A/S Norsk Varekrigsforsikrings Fond* and *Norges almenvitenskapelige forskningsråd.*

REFERENCES

1. Nord, F. F. and Sciarini, L. J. *Arch. Biochem.* **9** (1946) 419.
2. Hurlbert, R. B., Schmitz, H., Brumm, A. F. and Potter, V. R. *J. Biol. Chem.* **209** (1954) 23.
3. Klungsøyr, L., King, T. E. and Cheldelin, V. H. *J. Biol. Chem.* **227** (1957) 135.
4. Miettinen, J. K. *Second United Nations International Conference on the Peaceful Uses of Atomic Energy, Geneva* 1958. A/Conf. 15/P/1102 Finland.
5. Hanes, C. S. and Isherwood, F. A. *Nature* **164** (1949) 1107.

Received December 12, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 502—504

Some Reactions of 3-Reteneacetic Acid

EYLIF DYNESEN

Chemical Institute of the University of Århus, Denmark

3-Reteneacetic acid has been characterized through simple functional derivatives.

β-(3-Retene-ethanol), 1,3-dimethyl-7-*iso*propylphenanthrene, α-(3-retene)-*o*-nitrocinnamic acid and 3-(*o*-nitrostyryl)-retene have further been prepared by suitable reactions.

3-Reteneacetic acid [1] (I) has been subjected to a series of reactions. The methyl and ethyl-esters and the amide were prepared by conventional methods. Reduction of the ethyl ester(II) by lithium aluminium hydride gave β-(3-retene)-ethanol(III) in good yield. 1,3-Dimethyl-7-*iso*propylphenanthrene(V), previously prepared by total synthesis [2], could be prepared in 84 % yield by decarboxylation of I with soda lime.

In connection with other work α-(3-retene)-*o*-aminocinnamic acid was required, wherefore the corresponding nitro compound VI was prepared by condensation of *o*-nitrobenzaldehyde with 3-reteneacetic acid. The Perkin reaction in the Bakunin and Peccerillo modification [3] was applied. In this modification the free arylacetic acid is condensed with the substituted benzaldehyde by means of acetic anhydride in the presence of a tertiary amine as catalyst. The yield of VI was 40—45 %.

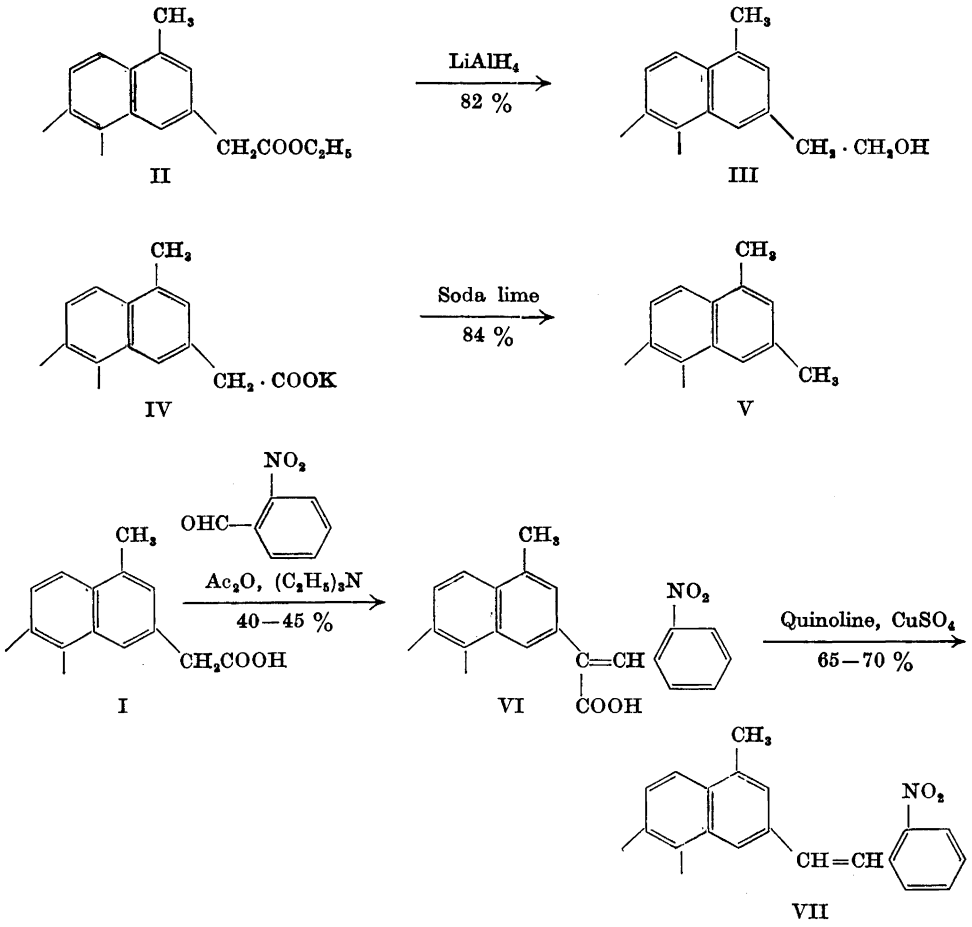
Decarboxylation of VI in quinoline with anhydrous copper sulfate according to a modification [4] of the method of Galimberti [5] gave 3-(*o*-nitrostyryl)-retene(VII) in good yield. The substance presumably was contaminated with the geometrical isomer, since the melting point was unsharp; recrystallization gave, however, a pure compound.

EXPERIMENTAL

3-Reteneacetic acid was prepared by the Willgerodt-Kindler reaction as previously reported [1].

Methyl-3-reteneacetate. A mixture of 3-reteneacetic acid (2.9 g, 0.01 mole), methanol (80 ml) and concentrated sulfuric acid (0.4 ml) was refluxed for 3 h. Yield after recrystallization from alcohol 2.7 g; m. p. 118—119.5°. (Found: C 82.2; H 7.27. Calc. for $C_{21}H_{22}O_2$ (306.4): C 82.3; H 7.24.)

Ethyl-3-reteneacetate. Prepared similarly, refluxing time 7 h. Yield after recrystallization from methanol 2.8 g; m. p. 67—68°. (Found: C 82.4; H 7.51. Calc. for $C_{22}H_{24}O_2$ (320.4): C 82.5; H 7.55.)



3-Reteneacetamide. 3-Reteneacetic acid (2.34 g, 0.008 mole) was mixed with phosphorus pentachloride (1.77 g, 10 % excess) and dry benzene (20 ml) was added. After spontaneous initial reaction the mixture was kept at 50—60° for half an hour and a clear solution was obtained. Benzene and phosphorus oxychloride were distilled off at diminished pressure. In order to remove all phosphorus oxychloride some benzene was added to the crystalline residue and evaporated to dryness and this procedure repeated. The crystals were dissolved in hot benzene and after cooling treated with concentrated ammonia water (10 ml). The mixture was left overnight. The precipitate was washed with ammonia water and recrystallized from *n*-propanol. Colourless crystals (2.0 g) m. p. 240—241.5° (subl.). (Found: C 82.5; H 7.25; N 4.82. Calc. for $C_{20}H_{21}NO$ (291.4): C 82.4; H 7.27; N 4.81.)

β-(3-Retene)-ethanol (III). Ethyl-3-reteneacetate (9.6 g, 0.03 mole) dissolved in dry ether (100 ml) was added with stirring in the course of 25 min to a suspension of lithium aluminium hydride (0.7 g) in dry ether (60 ml). The ether refluxed gently during this time and was kept refluxing for further 45 min on a water bath. With external cooling were then added successively water (25 ml) and 4 N sulfuric acid (30 ml). The ether layer was washed with sodium carbonate solution (2 %) and a saturated sodium chloride

solution and dried. After evaporation of the solvent the residue was recrystallized from benzene-hexane; yield 6.8 g (82 %) of colourless crystals with m. p. 117—118.5°. A second recrystallization brought the m. p. to 118—119°. (Found: C 85.9; H 8.00. Calc. for $C_{20}H_{22}O$ (278.4): C 86.3; H 7.97.)

Acetate of III. A mixture of III (0.6 g), acetic anhydride (5 ml) and pyridine (5 ml) was refluxed for 2.5 h and poured in water. The crystalline precipitate was recrystallized from methanol. Yield 0.4 g of white crystals, m. p. 66—67°. (Found: C 82.5; H 7.61; Calc. for $C_{22}H_{24}O_2$ (320.4): C 82.5; H 7.55.)

p-Nitrobenzoate of III. A mixture of III (2.0 g), *p*-nitrobenzoylchloride (1.5 g, 20 % excess), pyridine (10 ml) and dry benzene (20 ml) was heated on a boiling water bath for half an hour, the solution washed with water, hydrochloric acid (2 N) and sodium hydroxide (2 N). After drying the solvent was evaporated and the residue recrystallized twice from *n*-propanol. Yield 1.7 g, light yellow crystals with m. p. 122—123.5°. (Found: C 75.7; H 5.92; N 3.29. Calc. for $C_{27}H_{25}NO_4$ (427.5): C 75.9; H 5.90; N 3.28.)

1,3-Dimethyl-7-isopropylphenanthrene (V). Potassium-3-reteneacetate (IV) was prepared by neutralizing 3-reteneacetic acid(I) in hot *n*-propanol with the calculated amount of alcoholic potassium hydroxide. IV separated on cooling. IV (5 g) was intimately mixed with soda lime (10 g) and heated in a two bulb distilling apparatus with a wide side-arm. The apparatus was evacuated by means of an oil pump. At 0.5 mm pressure and a bath temperature of 325—380° the decarboxylation product distilled and the solid distillate was recrystallized from alcohol. 3.2 g (84 %) of colourless crystals, m. p. 76—77°, were obtained. M. p. reported [2] 73—76°. (Found: C 91.9; H 8.05. Calc. for $C_{19}H_{20}$ (248.4): C 91.9; H 8.12.) The picrate had m. p. 143.5—144.5°, previously reported [2] 145—146°.

α-(3-Retene)-o-nitrocinnamic acid (VI). A mixture of 3-reteneacetic acid (14.6 g, 0,05 mole), *o*-nitrobenzaldehyde (8.3 g, 0.055 mole), acetic anhydride (70 ml), and triethylamine (7.0 ml, 0.05 mole) was heated at 95—100° for 21 h. The excess acetic anhydride was decomposed by adding water (40 ml) and heating for further 1.5 h; the reaction mixture was then poured into 5 % hydrochloric acid (500 ml). The yellow product was filtered off, washed with water and dissolved in a hot solution of sodium carbonate. After cooling the rather dark coloured solution was extracted several times with ether and afterwards boiled with charcoal. On acidification of the filtrate a yellow precipitate separated. The product was recrystallized twice from glacial acetic acid. The yield was 9.7 g (45 %), yellow crystals with m. p. 257—258.5°. (Found: Equiv.wt. 422; C 75.8; H 5.40; N 3.27. Calc. for $C_{27}H_{23}NO_4$ (425.5): C 76.2; H 5.45; N 3.29.)

3-(o-Nitrostyryl)-retene (VII). A mixture of VI (0.5 g), anhydrous copper sulfate (0.15 g) and quinoline (12 ml) was heated at 215—220° for 25 min. After cooling the reaction mixture was diluted with benzene and the solution extracted several times with hydrochloric acid (2 N). Some dark coloured material, which had separated in the organic layer, was filtered off and washed with benzene. The filtrate was washed with sodium carbonate (2 %) and dried. After evaporation of the solvent the residue was dissolved in hot *n*-propanol, the solution treated with charcoal and filtered. On cooling yellow crystalline material separated. The yield was 0.3 g (68 %), m. p. 170—176°. The substance was recrystallized from glacial acetic acid, weight 0.23 g, m. p. 177.5—179°. (Found: C 81.4; H 6.10; N 3.63. Calc. for $C_{26}H_{23}O_2N$ (381.5): C 81.9; H 6.08; N 3.67.)

The analyses were performed in this laboratory by miss B. Møller.

REFERENCES

1. Dynesen, E. *Acta Chem. Scand.* **13** (1959) 360.
2. Huebner, C. F. and Jacobs, W. A. *J. Biol. Chem.* **169** (1947) 211.
3. Bakunin, M. and Peccerillo, D. *Gazz. chim. ital.* **65** (1935) 1145.
4. Rapoport, H., Williams, A. R. and Cisney, M. E. *J. Am. Chem. Soc.* **73** (1951) 1414.
5. Galimberti, L. *Boll. sci. fac. chim. ind. Bologna* **1940** 351.

Received December 19, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 505—519

Periodate Oxidation of Phenols

I. Monoethers of Pyrocatechol and Hydroquinone

ERICH ADLER and RUNE MAGNUSSON

Institutionen för organisk kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

Treatment of monoalkyl, monobenzyl, and monophenyl ethers of pyrocatechol or hydroquinone with sodium metaperiodate leads mainly to oxidative removal of the ether substituent, with the formation of *o*- or *p*-quinone and the corresponding alcohol (or phenol). In the same way, 1- and 4-hydroxy-dibenzofuran undergo oxidative cleavage at the ring oxygen. These reactions presumably proceed *via* a quinol-like hemiketal intermediate.

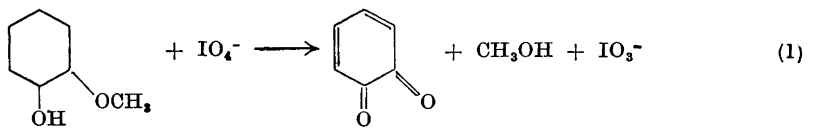
The monophenyl ether of hydroquinone gave the cleavage products (*p*-quinone and phenol), and in addition a compound, (2-hydroxyphenyl)-*p*-benzoquinone, obviously formed as a result of rearrangement and further oxidation of the intermediate.

The action of periodate on pyrocatechol and hydroquinone monoethers is discussed in comparison with that of some other specific oxidants, *e.g.*, sodium bismuthate, lead tetraacetate, and potassium nitrosodisulphonate.

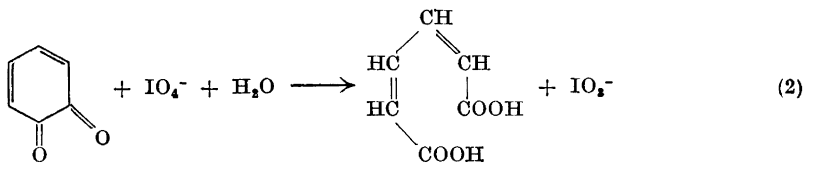
PYROCATECHOL MONOETHERS

In a previous communication [1] it was reported that the treatment of guaiacol (I) with sodium metaperiodate in aqueous or dilute acetic acid solution resulted in the rapid liberation of nearly one mole of methanol per mole of phenol. A method of estimation of the number of guaiacyl residues with a free phenolic hydroxyl group in lignin was based on this result [2].

The formation of methanol was found to be complete in a few minutes. During this time about one mole of periodate per mole of guaiacol was consumed, and the solution acquired a dark-red colour indicating the formation of *o*-benzoquinone (II). This initial reaction was followed by further periodate consumption at a lower rate and *cis, cis*-muconic acid (III) was isolated [1] from the brownish solution finally obtained. These observations indicated the following as the main reactions in the rapid initial step (1) and the subsequent slower phase (2):



I II (1)



II III (2)

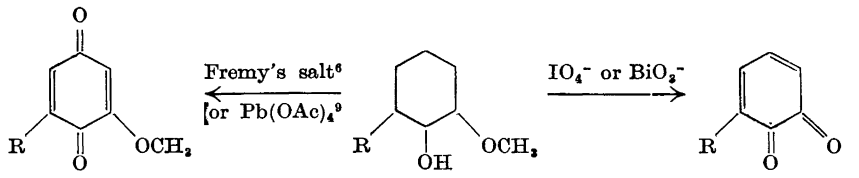
In the aqueous solvents used the oxidative cleavage by reaction (2) is accompanied by a competitive decomposition (polymerisation) of the *o*-benzoquinone primarily formed. It has been possible, however, to isolate *o*-benzoquinone in good yield by rapidly extracting the freshly prepared, dark-red aqueous mixture of guaiacol and periodate with methylene chloride. This proves the correctness of eqn. (1) for the initial step of the periodate oxidation of guaiacol.

Since oxidative demethoxylation with periodate proceeds readily with a great variety of guaiacol compounds [1], it appears to open a new way for the preparation of *o*-quinones. 4-Methyl-*o*-benzoquinone [3] was obtained in 70 % yield after short treatment of 4-methylguaiacol (creosol) with periodate in aqueous solution. This periodate method can be considered complementary to the method of Teuber [4,5], which involves *ortho*-oxidation of *p*-substituted phenols by Fremy's salt (potassium nitrosodisulphonate, $ON(SO_3K)_2$). Unlike periodate, Fremy's salt attacks the free *o*-position rather than the methoxyl group of *p*-substituted guaiacols, 4-methylguaiacol, for instance, being converted into 3-methoxy-5-methyl-*o*-benzoquinone [5].

The two oxidants mentioned also differ with respect to their behaviour towards guaiacols with a free *p*-position. The action of Fremy's salt on such phenols gives the corresponding *p*-quinones [6]; guaiacol, for instance, gives methoxy-*p*-benzoquinone (IV) in 90 % yield. In the reaction between guaiacol and periodate, however, the oxidation takes place almost exclusively at the 2-position, that is, at the ring carbon atom carrying the methoxyl group, with the formation of *o*-benzoquinone. As indicated by the formation of about 90 % of the calculated quantity of methanol, only about 10 % of the guaiacol is oxidised at the 4- and 6-positions.

The periodate method can therefore also be expected to be suitable for the preparation of *o*-quinones carrying a substituent in the 3-position and unsubstituted in the 5-position (II, R = substituent), from 6-substituted guaiacols (I, R = substituent). As yet, the action of periodate upon pyrogallol-1,3-dimethyl ether (V) has been examined and found to yield, under certain conditions, 3-methoxy-*o*-quinone (VI) as a major product.

Potassium bismuthate ($KBiO_3$), which was introduced by Rigby [7] as a glycol-splitting agent similar to periodate and lead tetraacetate, was found [8] to oxidise guaiacol in the same way as periodate, as shown in the reaction scheme below:



IV. R = H (90 %[6] or 57 %[9])
VII. R = CH_3O

I. R = H
V. R = CH_3O

II. R = H
VI. R = CH_3O

On the other hand, lead tetraacetate, although being largely equivalent to both periodate and bismuthate as a glycol-splitting agent, differs from the two latter oxidants but resembles Fremy's salt with regard to its action on guaiacols. Thus, similar to Fremy's salt [6], lead tetraacetate oxidises guaiacol and 2,6-dimethoxyphenol to give methoxy-*p*-quinone (IV) and 2,6-dimethoxy-*p*-quinone (VII) [9], respectively, without attacking the methoxyl groups.

In polar solvents such as acetone and methylene chloride, 3-methoxy-*o*-benzoquinone (VI) gave the hitherto unknown dimer, m.p. 139—140°. By the action of periodate this dimeric *o*-quinone was readily converted into 3,8-dimethoxy-1,2-naphthoquinone. Similarly, periodate oxidation of the recently described dimeric *o*-benzoquinone [20] yielded 1,2-naphthoquinone. These reactions, as well as the periodate oxidation of V, will be further discussed in following communications.

Pyrocatechol and its homologues are also rapidly oxidised by periodate, giving the corresponding *o*-quinones. The action of periodate on 4-methyl-pyrocatechol, for instance, produced 4-methyl-*o*-quinone in high yield.

The oxidative cleavage of pyrocatechol monoethers is a general reaction. An investigation was made of its application to the following ethers: 1) guaethol (VIII) (the monoethyl ether of pyrocatechol); 2) 2-hydroxydiphenyl ether (IX) (as an example of pyrocatechol monoaryl ethers); 3) 4-hydroxy-dibenzofuran (X) (as an example of a cyclic monoaryl ether of pyrocatechol).

Guaethol (VIII), in aqueous solution or in 80 % acetic acid, consumed about the same amount of periodate at about the same rate as guaiacol (Fig. 1). Ethanol was detected by gas phase chromatography in the distillate obtained from an aqueous guaethol-periodate reaction mixture after removal of excess periodate and iodate by precipitation with lead nitrate.

The initial attack of periodate on 2-hydroxydiphenyl ether (IX) in 80 % acetic acid solution was appreciably slower than on the pyrocatechol monoalkyl ethers, and there was no distinct break in the periodate consumption curve after the consumption of one mole of periodate per mole of substance (Fig. 1). As shown by the formation of phenol, the reaction was, however, similar to that with the monoalkyl ethers.

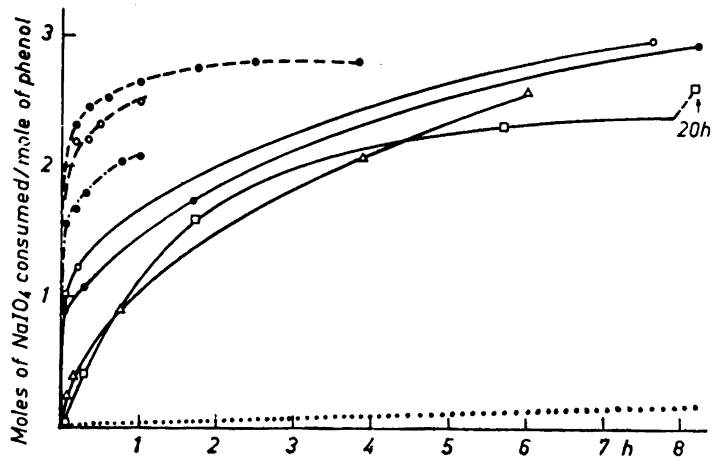
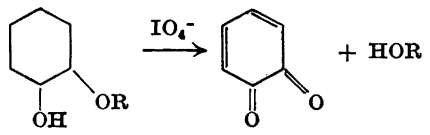


Fig. 1. Consumption of periodate by pyrocatechol monoethers. The reaction mixtures contained 10 mmole of the phenol and 70 mmole of $NaIO_4$ per litre.

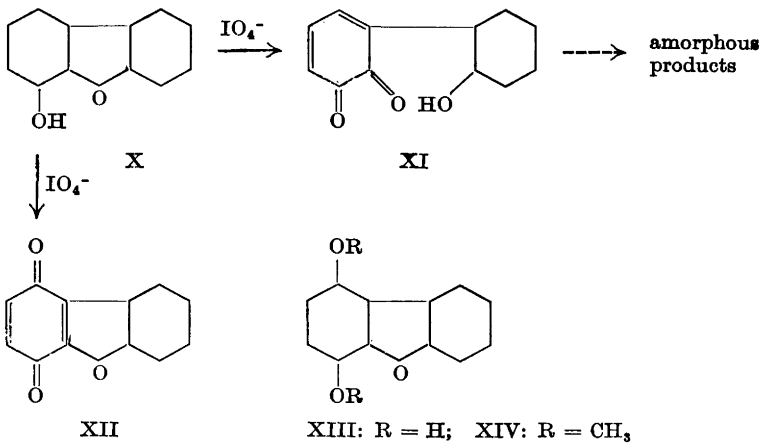
●—·—·—● Guaiacol in water, 4°; ●— — —● 25°; ●——● in 80 % acetic acid, 25°. ○— — —○ Guaethol (VIII) in water, 25°; ○——○ in 80 % acetic acid, 25°. △——△ 2-Hydroxydiphenyl ether (IX); □——□ 4-Hydroxydibenzofuran (X); ..... Phenol; in 80 % acetic acid, 25°.



VIII: R = C_2H_5
IX: R = C_6H_5

Although excess periodate was present, phenol could be detected even after a reaction time of 48 h. Phenol, unlike pyrocatechol and its monoethers, thus reacts only very slowly with periodate (*cf.* Fig. 1). In this respect periodate differs from Fremy's salt which readily oxidises phenol to *p*-benzoquinone [6]. As will be described in a forthcoming communication, the homologues of phenol, with alkyl substituents at the *o*- or *p*-positions or at both, exhibit strongly enhanced reactivity towards periodate.

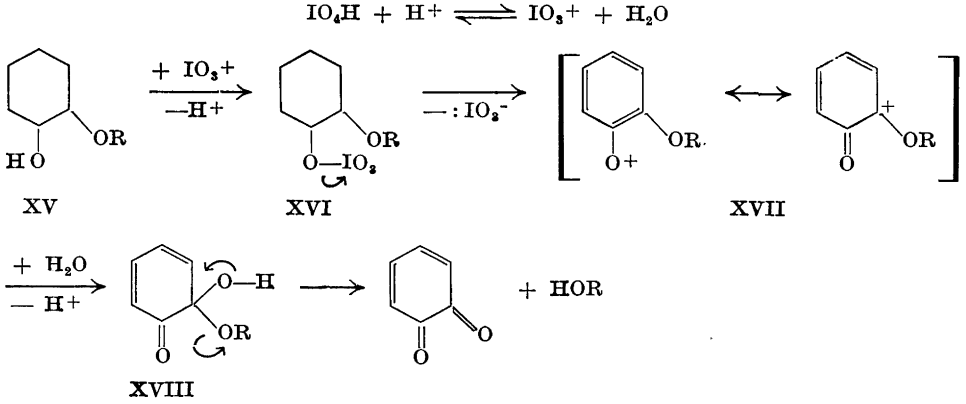
The periodate consumption curve of 4-hydroxydibenzofuran (X) (Fig. 1) was similar to that of IX, and the bulk of the material was converted into a brown, amorphous product. This may indicate that the reaction proceeded mainly in the normal way, *i.e.*, by *o*-oxidation, with the opening of the furan ring and the intermediate formation of the *o*-quinone XI, followed by polymerisation or by further periodate oxidation. A side-reaction, with *p*-oxidation of X to dibenzofuran-1,4-quinone (XII), m.p. 187—188°, occurred to an extent of about 15 %.



The structure of XII was demonstrated by reduction to the previously known 1,4-dihydroxy-dibenzofuran (XIII), m.p. 217—218°[10], and methylation of this compound to the dimethyl ether (XIV), m.p. 76—78°[10].

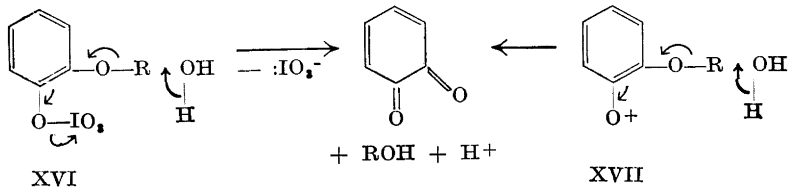
The quinone XII was regenerated in quantitative yield from the hydroquinone XIII when the latter was treated with periodate. Obviously, in compound XIII the dehydrogenation of the hydroquinone structure is more favoured than the oxidative ether cleavage (*cf.* also p. 515).

It was tentatively suggested in a previous communication [1] that the oxidative cleavage of pyrocatechol monoethers by periodate followed a radical mechanism involving two successive one-electron transfers. However, according to Levitt [11] a great variety of oxidants used in organic chemistry, including periodic acid, probably react by a two-electron transfer, thus giving rise to ionic mechanisms. Criegee [12] has adopted the views of Levitt for the mechanism of lead tetraacetate oxidation. Analogously, the main reaction between pyrocatechol monoethers and periodate can be represented as follows.



According to Levitt, the active species in periodate oxidation is the periodonium cation, IO_3^+, (or its hydrates), formed in a reversible reaction from periodic acid by the loss of OH^-. The cation would attack the pyrocatechol monoether (XV) at the site of the greatest electron density, *i.e.* at the phenolic hydroxyl group, yielding the periodic acid aryl ester XVI, the phenolic hydrogen atom being released as a proton. The ester XVI can then decompose forming an iodate anion ("two-electron transfer") and the mesomeric aroxyl cation XVII. Addition of a molecule of water to XVII would then give the *o*-quinol-like hemiketal XVIII and a proton and, finally, spontaneous decomposition of the hemiketal would yield the *o*-quinone and the alcohol HOR.

Alternatively, the ester XVI or the cation XVII may decompose directly, without the intermediate formation of the hemiketal XVIII, in a concerted reaction with a molecule of water as an electron donor:



XVI XVII

The remarkable ease of oxidative cleavage of the pyrocatechol monoethers could suggest some specific relationship between the periodate molecule and the two *ortho* oxygen atoms of the pyrocatechol component, possibly involving the initial formation of a highly reactive periodic acid *di*ester-like complex. Such an assumption, however, seems superfluous, since, as described in the following section, hydroquinone monoethers, where such a complex is not possible, are split by periodate as readily as the pyrocatechol analogues.

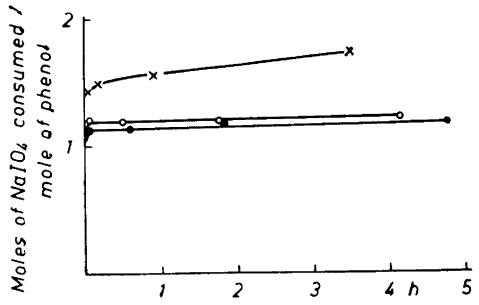


Fig. 2. Consumption of periodate by hydroquinone monoethers in 80 % acetic acid at 25°. Concentrations, same as given in Fig. 1.

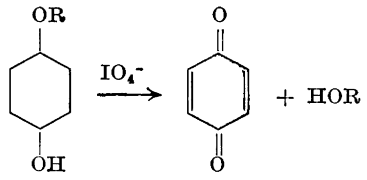
●——● Hydroquinone monomethyl ether (XIX).
O——O Hydroquinone monobenzyl ether (XX).
×——× Hydroquinone monophenyl ether (XXI).

HYDROQUINONE MONOETHERS

Hydroquinone itself is instantaneously oxidised by periodate (*cf.* also Ref.[13]) giving *p*-benzoquinone which, unlike *o*-benzoquinone, is stable in the presence of periodate. Four different monoethers of hydroquinone (XIX, XX, XXI, and XXIV) were examined.

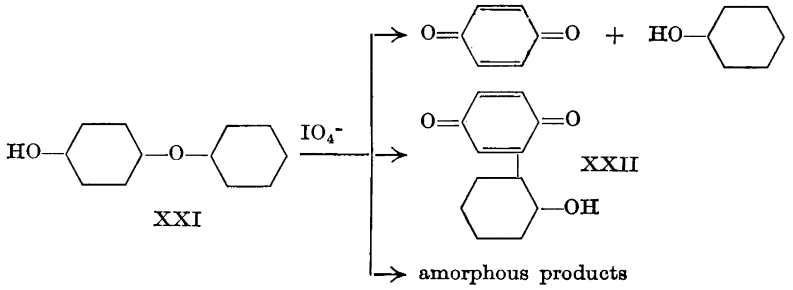
Hydroquinone monomethyl ether (XIX), when treated with excess periodate in aqueous or aqueous acetic acid solution (80 % HOAc), consumed very rapidly slightly more than one mole of periodate (Fig. 2), yielding *p*-benzoquinone and 93—94 % of the calculated amount of methanol.

Hydroquinone monobenzyl ether (XX), in 80 % acetic acid, behaved similarly (Fig. 2). Benzyl alcohol was isolated as the 3,5-dinitrobenzoate.



XIX : R = CH_3
XX : R = $CH_2C_6H_5$

The oxidation of hydroquinone monophenyl ether (XXI) was more complicated. In 80 % acetic acid it rapidly consumed about 1.4 mole of periodate. Dilution of the reaction mixture (after a reaction time of 30 min) yielded a brown, amorphous precipitate (about 14 % of the weight of compound XXI used). The expected reaction products, *p*-benzoquinone and phenol, were found in the reaction mixture; the yields of these products indicated that about 60 % of the starting material had undergone the "normal" ether cleavage reaction.



A brick-red crystalline substance, $C_{12}H_8O_3$, m.p. 196°, was also isolated from the solution in about 15 % yield. It was found to be identical with a product obtained by Grundmann [14] by the oxidation of 2,2'-dihydroxydiphenyl (XXIII) with peracetic acid. On the basis of the analytical composition and the chemical properties of the red product Grundmann considered it to be (2-hydroxyphenyl)-*p*-benzoquinone (XXII). This structure has now been

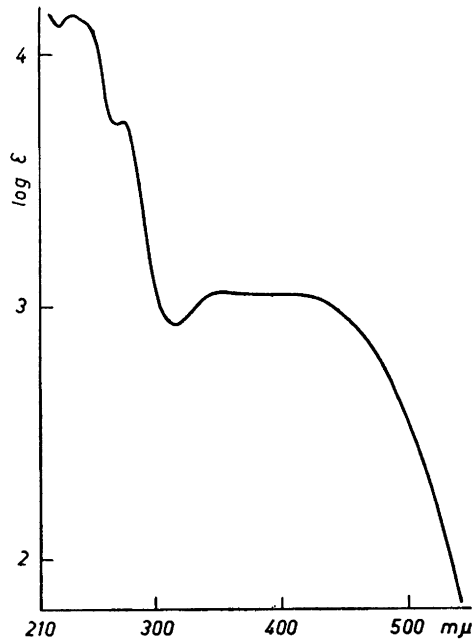
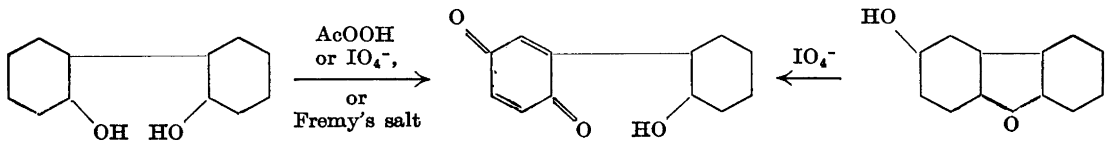


Fig. 3. Light absorption of (2-hydroxyphenyl)-*p*-benzoquinone (XXII). Solvent: 95 % ethanol.

confirmed by the following observations. The red substance (light absorption curve, Fig. 3) was readily reduced by zinc dust in acetic acid to the hitherto unknown 2,2',5'-trihydroxydiphenyl (XXVII, see p. 513), obtained as a distillable colourless glass (b.p. 150°/0.001 mm Hg). Treatment of the latter product with periodate regenerated substance XXII. In harmony with the view that XXII is a *p*-quinone, it could be titrated with sodium thiosulphate [15]. The result of the titration indicated a molecular weight of 199 (calc. for $C_{12}H_8O_3$: 200). Oxidative degradation of XXII with hydrogen peroxide yielded salicylic acid, clearly indicating the presence of the 2-hydroxyphenyl residue.

The quinone XXII was formed from 2,2'-dihydroxydiphenyl (XXIII) not only by peracetic acid according to Grundmann but also — although in a lower yield — on treatment with sodium periodate. The formation of a red compound was also observed by Teuber and Rau [6] when XXIII was oxidised with Fremy's salt. The latter authors, however, could not decide whether the reaction product was XXII or an addition product of di-*p*-quinone and

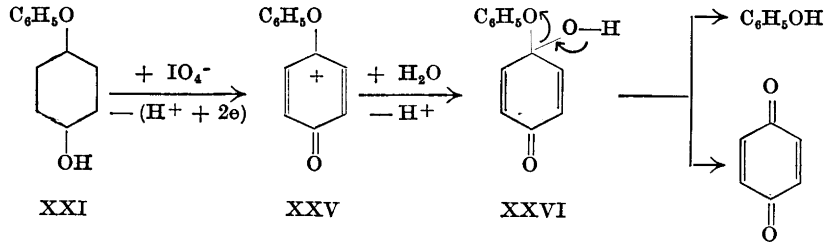


XXIII XXII XXIV

XXIII. It has now been established that the product prepared according to Teuber and Rau is identical with XXII, obtained by one of the methods mentioned above.

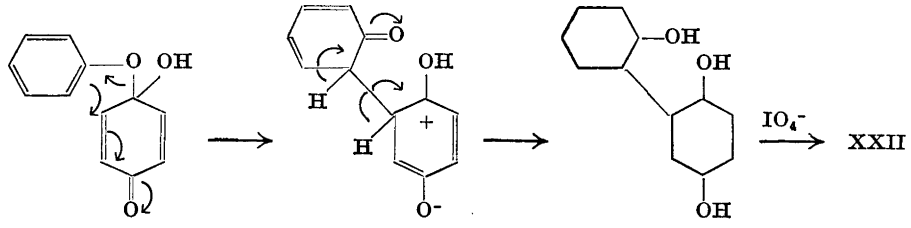
Treatment of a ring-closed analogue of XXI, 2-hydroxydibenzofuran (XXIV), with periodate resulted in cleavage of the cyclic ether bond and gave the (2-hydroxyphenyl)-*p*-benzoquinone XXII in good yield.

The mechanism of the oxidative cleavage of hydroquinone monoethers should be similar to that discussed above for the corresponding reaction of pyrocatechol monoethers. The oxidation of hydroquinone monophenyl ether (XXI), for instance, would then involve the intermediate steps of the mesomeric cation XXV and the *p*-quinol-like hemiketal XXVI, which would decompose to yield the major reaction products, phenol and *p*-quinone. The same result would be obtained by assuming concerted reactions similar to those given for the pyrocatechol series (p. 510).



XXI XXV XXVI

The side-reaction giving XXII that occurs in the oxidation of XXI, can be easily understood as the result of the rearrangement of the hypothetical *o*-quinol-like intermediate XXVI. This would yield the aromatic 2,2',5'-trihydroxydiphenyl (XXVII) which, in the presence of periodate, would be oxidised to XXII, as decribed above (p. 512). The rearrangement XXVI ⟶ XXVII is comparable to that undergone

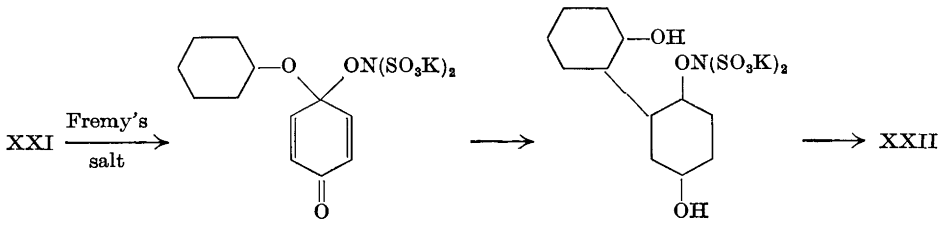


XXVI XXVII

by ordinary *p*-quinols, although here this reaction requires the presence of a strong acid (*cf.* Bamberger [16], Witkop [17]).

No systematic comparison has yet been made of the action of periodate and other specific oxidants on hydroquinone monoethers, but some preliminary conclusions can be drawn from the experimental material available. As in the case of the pyrocatechol monoethers, the action of sodium bismuthate is

quite similar to that of periodate, the oxidative cleavage of the hydroquinone monoethers into *p*-quinone and the corresponding alcohol being the main reaction [8]. It has been reported that lead tetraacetate reacts with hydroquinone monomethyl ether; however, the reaction products have not been isolated [9]. Fremy's radical does not affect the ether groupings of the monomethyl and monobenzyl ethers of hydroquinone (XIX, XX), but gives high yields of 4-methoxy and 4-benzyloxy-*o*-quinone, respectively [5]. This is analogous to the reaction between Fremy's salt and 4-alkylphenols, and is also in accordance with the production of methoxy-*p*-quinone (IV) from guaiacol [6] by the same oxidant. Surprisingly enough, it has now been found that hydroquinone monophenyl ether (XXI), when treated with Fremy's salt, is converted into (2-hydroxyphenyl)-*p*-quinone (XXII), which is also obtained as a by-product in the reaction between periodate and XXI. Since *p*-benzoquinone and phenol are not formed in the reaction between Fremy's salt and XXI it seems that the latter reaction does not proceed *via* the quinol-like intermediate XXVI, which has been assumed to be the precursor, in the periodate reaction, of the cleavage products (*p*-quinone and phenol) as well as of the rearrangement product XXII. The result of the oxidation of XXI by Fremy's salt suggests, that the primary intermediate could be the quinitrol-like substance XXVIII, which would undergo direct rearrangement to XXIX, followed by conversion of the latter into XXII.

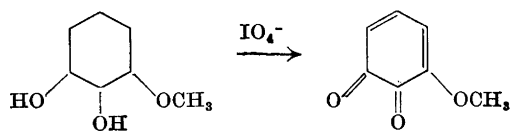


XXVIII XXIX

Quinitrol-like substances of type XXVIII have been obtained by Teuber and Götz [18], by the oxidation of phenanthrols and naphthols with Fremy's salt.

At the same time as the investigations described in the preceding sections, an examination was also made of the periodate oxidation of pyrogallol-1-methyl ether (XXX), methoxyhydroquinone (XXXI), 2,5-dimethoxyhydroquinone (XXXII), and 2,4-dimethoxyphenol (XXXIII).

Oxidation of XXX with an equimolar amount of periodate yielded 3-methoxy-*o*-quinone (VI) in a yield of 57 %. As XXX contains a free "pyro-

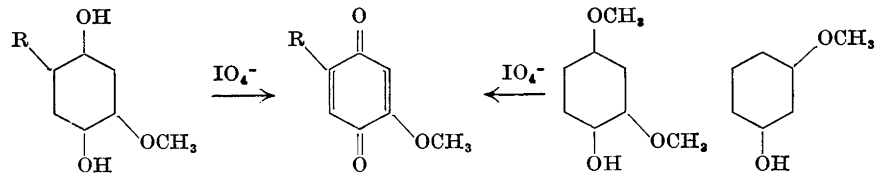


XXX VI

catechol" as well as a' "pyrocatechol monoether" grouping, the result indicates that the former grouping is more easily attacked than the latter one.

When XXX was treated with excess periodate, a 58 % yield of VI could be isolated after 2 min. About 1.5 mole of periodate was consumed in this time; on more prolonged oxidation there was further consumption of periodate. The quinone VI also consumed appreciable amounts of the oxidant (about 2 mole of periodate after 20 h). In both cases, almost one mole of methanol was liberated. This periodate degradation of 3-methoxy-*o*-quinone (VI) is being further investigated.

The hydroquinone groupings in methoxyhydroquinone (XXXI) and in 2,5-dimethoxyhydroquinone (XXXII) were so rapidly oxidised that the methoxyl groups of the "pyrocatechol monoether" groupings also present in these compounds remained unaffected (*cf.* also the oxidation of XIII, p. 509). The corresponding *p*-quinones (IV and XXXIV) were produced in practically quantitative yields; unlike the 3-methoxy-*o*-quinone (VI), they were not further attacked by periodate.



XXXI: R = H
XXXII: R = CH_3O

IV: R = H
XXXIV: R = CH_3O

XXXIII

XXXV

The preparation of *p*-quinones from the corresponding hydroquinones, by periodate oxidation, seems to be widely applicable. In addition to the examples reported above, a quantitative conversion of tetrabromohydroquinone into bromanil has also been made.

2,4-Dimethoxyphenol (XXXIII), when treated with an excess of periodate, yielded one mole of methanol and methoxy-*p*-quinone (IV). Thus, of the "pyrocatechol monoether" and the "hydroquinone monoether" groupings, both of which are present in XXXIII, the latter grouping was attacked preferentially.

A few experiments with resorcinol monomethyl ether (XXXV) confirmed the expectation that the rapid ether cleavage reaction is confined to the monoethers of the pyrocatechol and hydroquinone series. The resorcinol ether consumed periodate comparatively slowly, although appreciably faster than phenol (*cf.* Fig. 1). After a reaction time of 16 h, 0.11 mole of methanol had been liberated and a small quantity of methoxy-*p*-quinone (IV) could be isolated. Obviously, there was a slow oxidation in the positions *ortho* and *para* to the phenolic hydroxyl group, giving methoxy-*p*-quinone and methoxy-*o*-quinone. The latter appears to be the source of the methanol formed (*cf.* above).

Some of the experiments reported in the present work have been carried out by civilingenjör Britt Berggren and civilingenjör C.-G. Setterquist.

Financial support from *Statens Naturvetenskapliga Forskningsråd* is gratefully acknowledged.

EXPERIMENTAL

Periodate consumption. For oxidation in an aqueous medium a 0.02 M solution of the phenol was mixed with an equal volume of 0.14 M sodium periodate. The mixture was kept in a thermostat at 25° (in one case, *cf.* Fig. 1, at 4°) protected from direct daylight. The amount of periodate present was determined on 5 ml samples by the arsenite method.

In most cases 80 % acetic acid solutions were used, and the residual periodate was determined by a potassium iodide-thiosulphate method. As the quinones formed would consume thiosulphate, they had to be removed. The following procedure was used.

The reaction mixture sample (5 ml) was added to a separatory funnel containing 20 ml of water, and the mixture was extracted with three 10 ml portions of methylene chloride. The aqueous layer was added to a mixture of 0.1 N sodium thiosulphate (30 ml) and 10 % potassium iodide (15 ml). After the addition of a few millilitres of 2 N sulphuric acid, the unconsumed thiosulphate was determined by iodine titration. A blank determination was made on a solution without phenol, and the amount of periodate consumed was obtained by difference.

Methanol was determined as described previously [1].

Oxidation of guaiacol. a) To an ice-cold aqueous solution (150 ml) of guaiacol (3.0 g = 24.2 mmole) a similarly cooled solution of sodium metaperiodate (10.5 g = 49.1 mmole) in water (75 ml) was added at once, and the resulting dark-red mixture immediately extracted with methylene chloride (100 ml). The extract was shortly dried over anhydrous sodium sulphate, filtered, and rapidly concentrated to a volume of about 20 ml by means of a rotating vacuum evaporator. After the addition of hexane the solution deposited dark-red crystals of *o-benzoquinone*, no sharp m.p., decomposition beginning at 60—70°, as described by Willstätter and Pfannenstiel [19]; yield 64 %.

b) In another experiment the methylene chloride solution obtained as above was brought to dryness under reduced pressure, and the dark-red, crystalline residue (2.5 g) immediately treated with aqueous sulphur dioxide. From the resulting solution a very small amount of undissolved material (identified as dimeric *o*-benzoquinone, see below) was removed by filtration. Continuous extraction of the solution with ether yielded *pyrocatechol* (56 %, based on the amount of guaiacol used), m.p. 103—104° after recrystallisation from benzene-hexane.

c) If the methylene chloride solution of *o*-benzoquinone obtained according to a) was kept in the refrigerator for several hours, it deposited a brown crystalline product, which became yellow on washing with acetone. Yield 45 %. It was identified by m.p., mixed m.p., and infra-red absorption spectrum with the *dimeric o-benzoquinone* which was recently obtained [20] from acetone solutions of *o*-benzoquinone.

4-Methyl-o-benzoquinone (*homo-o-quinone*). a) Creosol (4-methylguaiacol, 414 mg = 3 mmole) dissolved in ether (5 ml), was added to an aqueous solution (100 ml) of sodium metaperiodate (706 mg = 3.3 mmole) which was cooled by ice-water. After 15 min, the orange-red reaction mixture was extracted four times with methylene chloride, the red-green dichroic extract was dried over anhydrous calcium sulphate and the solvent was removed under reduced pressure. The dark-red crystalline residue was dissolved in ether; on cooling to −60° the solution deposited red prismatic needles, m.p. 75°; yield 70 %. Recrystallisation from ether or ether-petroleum ether yielded 4-methyl-*o*-quinone, either the red modification, m.p. 80°, or the yellowish-pink modification, m.p. 65—67° [3,5]. Admixture of similar samples, obtained from *p*-cresol according to Teuber and Staiger [5], gave no depression of the m.p. The infra-red absorption spectra of the two modifications, obtained by either method, were identical.

b) 4-Methyl-*o*-quinone was obtained in a similar way by oxidation of 4-methyl-pyrocatechol (see below) with periodate. The yield of the crude product, m.p. 75°, was 95 %.

4-Methyl-pyrocatechol (*homopyrocatechol*). The orange-red aqueous solution obtained by periodate oxidation of creosol as described above was saturated with sulphur dioxide and, after addition of sodium chloride, extracted with ether. The ethereal solution was dried and evaporated, and the slightly brown, oily residue was extracted several times with boiling hexane. The combined hexane solutions yielded 4-methyl-pyrocatechol, colourless thin plates, m.p. 66—67°; yield 62 %.

Oxidation of 4-hydroxydibenzofuran (*X*). a) 4-Hydroxydibenzofuran (2.0 g = 10.9 mmole) was added in portions to a solution of sodium metaperiodate (12.0 g = 56 mmole)

in 60 % aqueous acetic acid (400 ml). The reaction mixture was shielded from daylight and kept at 25° for 15 h. The addition of water (400 ml) to the red-brown solution produced a flocculent brown precipitate which was filtered off. The filtrate was extracted with methylene chloride and the extract was washed with water, dried over anhydrous sodium sulphate and evaporated under reduced pressure. Sublimation of the residue (bath temperature 100°, 3 mm Hg) and recrystallisation of the sublimate from hexane yielded *dibenzofuran-1,4-quinone* (XII) as yellow needles, m.p. 187—188°; yield 108 mg (5 %). (Found: C 72.13, 72.12; H 3.36, 3.21. Calc. for $C_{12}H_6O_3$: C 72.73; H 3.05.)

Reduction of XII (10 mg) with zinc dust in glacial acetic acid, followed by dilution with water and extraction with ether yielded the known *1,4-dihydroxydibenzofuran* (XIII), needles, m.p. 217—219° (217—218° [10]) after recrystallisation from water.

b) 4-Hydroxydibenzofuran (XII) (3.0 g) was oxidised with periodate as above. The solid residue obtained after evaporation of the methylene chloride solution (crude quinone XII) was reduced with zinc in acetic acid and the reaction mixture was diluted with water, heated and filtered. The filtrate deposited 0.50 g of the hydroquinone XIII; yield 15 %.

Methylation of XIII with dimethyl sulphate yielded 1,4-dimethoxydibenzofuran (XIV), m.p. 76—78° (78.5° [10]) after recrystallisation from hexane.

A solution of 1,4-dihydroxydibenzofuran (XIII) (10 mg) in glacial acetic acid (1 ml) was mixed with a solution of sodium metaperiodate (60 mg) in 60 % acetic acid (2 ml). Dilution with water and extraction with methylene chloride gave the quinone XII in quantitative yield.

Oxidation of hydroquinone monomethyl ether (*XIX*). Hydroquinone monomethyl ether (1.0 g = 8.06 mmole), dissolved in water (100 ml), was oxidised with sodium metaperiodate (5.17 g = 24.2 mmole) in aqueous solution (75 ml). Extraction with methylene chloride after a reaction time of 10 min yielded *p-benzoquinone;* yield, after sublimation, 80 %.

Solutions of hydroquinone monomethyl ether (5 mmole) and sodium metaperiodate (35 mmole) in water (25 ml) were cooled to 4° and mixed. After 5 min, 15 % lead nitrate solution (25 ml) was added. The methanol formed was determined as described by Adler and Hernestam [1]. The *p*-quinone in the filtrate was irreversibly adsorbed during the filtration through the anion exchange resin. The yield of *methanol* was 15.1 mg (94 %). In a similar experiment with a reaction time of 19 h the yield of methanol was 93 %.

Oxidation of hydroquinone monobenzyl ether (*XX*). A solution of sodium metaperiodate (8.0 g) in 60 % acetic acid (50 ml) was added to a solution of XX (4.0 g) in glacial acetic acid (250 ml). After 15 min the mixture was diluted with water and extracted with methylene chloride. The methylene chloride solution was washed with sodium hydroxide solution to remove *p*-quinone and then with water, dried over anhydrous sodium sulphate and evaporated. Distillation of the liquid residue gave *benzyl alcohol* (b.p. 200—205°, about 2 ml), which yielded a 3,5-dinitrobenzoate, m.p. 112°, undepressed on admixture with authentic benzyl-3,5-dinitrobenzoate (m.p. 112°).

p-Quinone was isolated from a second experiment.

Oxidation of hydroquinone monophenyl ether (*XXI*). a) A solution of XXI (1.0 g = 5.38 mmole) in glacial acetic acid (10 ml) was mixed with sodium periodate (3.0 g = 14.0 mmole) dissolved in 60 % acetic acid (100 ml). After 30 min the mixture was diluted with water to a volume of 1 l, the brown amorphous precipitate formed was removed by filtration, and the filtrate was extracted with methylene chloride. The methylene chloride solution was washed with water and evaporated, and the residue was steam-distilled. The yellow distillate was made up to a volume of 250 ml. The ultra-violet absorption showed that the solution contained 0.21 g of *p*-quinone (yield 35 %; control experiments showed that steam distillation of *p*-quinone involves appreciable loss of material). The solution was extracted with ether. Careful evaporation of the yellow ether extract gave a red residue recrystallised from hexane to give red needles, m.p. 70°, identical with »*phenoquinone*», the addition compound of phenol (2 mole) and *p*-quinone (1 mole).

The phenoquinone isolated was combined with the residue from evaporation of the hexane mother liquor and the combined products were dissolved in aqueous sodium hydroxide and shaken with air until the quinone odour had disappeared. The mixture was then neutralised with carbon dioxide and extracted with ether. The ether extract was evaporated and the residue dissolved in water. Addition of bromine yielded *tri-*

bromophenol (0.86 g), m.p. 89°, after recrystallisation and sublimation m.p. 93.5°, undepressed by an authentic sample; yield 54 %.

b) A solution of XXI (1.62 g = 8.72 mmole) in glacial acetic acid (25 ml) was treated with sodium periodate (4.20 g = 19.6 mmole) dissolved in 60 % acetic acid (75 ml). After 30 min the mixture was diluted with water (500 ml) and the amorphous precipitate formed was collected, washed with water, and dried. Yield 13.5 % of the weight of the starting material. The filtrate was extracted several times with methylene chloride and the extract was washed with water, dried over anhydrous sodium sulphate, and evaporated. The volatile phenoquinone was removed by sublimation (0.1 mm Hg) for 2 h. Recrystallisation of the residual product from ethyl acetate gave red prisms (0.18 g), m.p. 196°. Admixture of *(2-hydroxyphenyl)-p-quinone* (XXII) prepared according to Grundmann [14], m.p. 196° after recrystallisation from ethyl acetate, did not depress the m.p. Yield 10 %. (Found: C 71.97, 72.14; H 4.37, 4.33. Calc. for $C_{12}H_8O_3$: C 71.99; H 4.03.)

Substance XXII (98.60 mg), partially dissolved in a mixture of acetic acid and water, was slowly titrated with 0.1000 N sodium thiosulphate [15]; towards the end of the titration the mixture was heated to 40°. The substance gradually dissolved, yielding a colourless solution. Thiosulphate consumed: 4.94 ml, indicating a molecular weight of 199. Calc. for $C_{12}H_8O_3$: 200.18.

2,2',5'-Trihydroxydiphenyl (XXVII). Substance XXII (166 mg), dissolved in glacial acetic acid, was reduced with zinc dust. The solution obtained was diluted with water, extracted with ether, and the ethereal solution was washed with water and aqueous sodium bicarbonate, dried over anhydrous sodium sulphate, and evaporated giving a viscous oil which distilled at 150°/0.001 mm Hg. At room temperature the distillate (106 mg) was a colourless glass. (Found: C 69.96; H 5.13. Calc. for $C_{12}H_{10}O_3$: C 71.28; H 4.99.) Attempts to obtain crystalline derivatives (acetate, methyl ether, aryloxyacetic acid) were unsuccessful.

Substance XXVII (0.1 g), dissolved in glacial acetic acid (10 ml), was added to a solution of sodium metaperiodate (0.3 g) in 60 % acetic acid (10 ml). After 5 min the mixture was diluted with water, and the red quinone XXII was extracted with ether. It was recrystallised from ethyl acetate, m.p. and mixed m.p. 196°; yield nearly quantitative.

Similar oxidation of 2,2'-dihydroxydiphenyl (XXIII) with periodate yielded the quinone XXII in 5 % yield.

Oxidative degradation of (2-hydroxyphenyl)-p-quinone (XXII). Treatment of XXII (115 mg) with alkaline hydrogen peroxide, following the procedure used by Kögl, Erxleben, and Jänecke [21] for the degradation of 4,2',5'-trihydroxydiphenyl yielded *salicylic acid*, m.p. 157—159°, undepressed by an authentic sample. Yield 23 mg (26 %).

Oxidation of 2-hydroxydibenzofuran (XXIV). Substance XXIV (0.468 mmole), dissolved in glacial acetic acid (15 ml) was oxidised with sodium metaperiodate (2.10 mmole) dissolved in 60 % acetic acid (15 ml). The quinone XXII (45 mg) was obtained; yield 49 %.

Oxidation of hydroquinone monophenyl ether (XXI) with Fremy's salt. Substance XXI (0.5 g = 2.69 mmole) dissolved in methanol (12.5 ml) was added to an aqueous solution (150 ml) of Fremy's salt (3.0 g = 11.2 mmole). A red precipitate formed instantly, m.p. 180—185°. After recrystallisation from ethyl acetate the m.p. was 195—196°, undepressed on admixture of quinone XXII. Yield 63 %.

Oxidation of pyrogallol-1-methyl ether (XXX). a) Aqueous solutions of XXX (250 ml, containing 0.35 g = 2.5 mmole) and sodium metaperiodate (250 ml, containing 0.535 g = 2.5 mmole), cooled with ice-water, were mixed, and, after 2 min, the resulting dark-red solution was repeatedly extracted with methylene chloride. The extract was shortly dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The residue was dissolved in boiling ether, and the ethereal solution, after filtration and cooling to −80°, deposited red-brown crystals of *3-methoxy-o-quinone* (VI) (yield 57 %), m.p. 106—109°, undepressed on admixture of VI prepared according to Willstätter and Müller [3], m.p. 107—110°. The occurrence of a weak carbonyl band at 5.75 μ in the infrared absorption curve of the periodate product indicated the presence, in the latter product, of small amounts of the dimeric 3-methoxy-*o*-quinone, which will be described in a forthcoming communication.

b) In a similar experiment aqueous solutions (25 ml) of 73.5 mg of XXX (= 0.53 mmole) and 750 mg of periodate (= 3.5 mmole) (molar ratio about 1 : 7) were mixed. The yield of 3-methoxy-*o*-quinone was 58 %.

c) In an oxidation experiment according to b) the reaction time was increased to 24 h. The reaction mixture was found to contain 14.72 mg of methanol; yield 96 %.

Oxidation of methoxyhydroquinone (XXXI). Methoxyhydroquinone (67 mg = 0.62 mmole) was added to an aqueous solution (15 ml) of sodium metaperiodate (450 mg = 2.10 mmole), and the mixture was shaken for 1 h in a separatory funnel. The resultant yellow solution contained yellow crystalline material; it was extracted with methylene chloride. The extract yielded *methoxy-p-quinone* (IV) (65.4 mg), m.p. 142—146°, undepressed by authentic material. Yield 99 %.

Oxidation of 2,5-dimethoxyhydroquinone (XXXII). An aqueous solution (15 ml) of sodium metaperiodate (450 mg = 2.10 mmole) was added to a solution of the hydroquinone (131 mg = 0.77 mmole) in water (10 ml). After 30 min the crystalline precipitate formed (120 mg) was collected; extraction of the filtrate with methylene chloride yielded 5 mg of the same product, *2,5-dimethoxy-p-quinone (XXXIV).* The substance darkens at 310° without melting. Yield 96 %. At a bath-temperature of 160—180° and 0.1 mm Hg the material sublimed almost completely.

Reduction with zinc dust in glacial acetic acid regenerated the hydroquinone XXXII, m.p. 167—70°.

Oxidation of 2,4-dimethoxyphenol (XXXIII). a) Aqueous solutions of 2,4-dimethoxyphenol (50 ml containing 168 mg = 1.1 mmole of the phenol) and of sodium metaperiodate (1.5 g = 7.0 mmole in 100 ml) were mixed; after 15 min the yellow solution was extracted with methylene chloride. The solvent was evaporated and the residue was sublimed (100°, 20 mm Hg) giving *methoxy-p-quinone* (91 mg), identified by m.p. and mixed m.p. Yield 60 %.

b) Two reaction mixtures were prepared in the same way as in a) and kept at 4°; an excess of sodium pyrosulphite was added after reaction times of 15 and 120 min, respectively. From each solution 100 millilitres were distilled off, the distillate was made alkaline and redistilled, and the amount of methanol present in the final distillate was determined [1]. Both experiments gave 0.90 mole of methanol per mole of 2,4-dimethoxyphenol.

REFERENCES

1. Adler, E. and Hernestam, S. *Acta Chem. Scand.* **9** (1955) 319.
2. Adler, E., Hernestam, S. and Walldén, I. *Svensk Papperstidn.* **61** (1958) 641.
3. Willstätter, R. and Müller, W. *Ber.* **44** (1911) 2171.
4. Teuber, H. J. and Jellinek, G. *Chem. Ber.* **87** (1954) 1841.
5. Teuber, H. J. and Staiger, G. *Chem. Ber.* **88** (1955) 802.
6. Teuber, H. J. and Rau, W. *Chem. Ber.* **86** (1953) 1036.
7. Rigby, W. *Nature* **164** (1949) 185; *J. Chem. Soc.* **1950** 1907.
8. Adler, E. and Lindberg, C. B. *To be published.*
9. Wessely, F. and Kotlan, J. *Monatsh.* **84** (1953) 291.
10. Gilman, H. and van Ess, P. R. *J. Am. Chem. Soc.* **61** (1939) 1365.
11. Levitt, L. *J. Org. Chem.* **20** (1955) 1297.
12. Criegee, R. *Angew. Chem.* **70** (1958) 173.
13. Pennington, D. E. and Ritter, D. M. *J. Am. Chem. Soc.* **69** (1947) 187.
14. Grundmann, C. *Ber.* **69** (1936) 1755.
15. Rzymkowski, J. *Z. Elektrochem.* **31** (1925) 371.
16. Bamberger, E. *Ber.* **33** (1901) 3600; *Ann.* **390** (1912) 164.
17. Goodwin, S. and Witkop, B. *J. Am. Chem. Soc.* **79** (1957) 179.
18. Teuber, H. J. and Götz, N. *Chem. Ber.* **89** (1956) 2654.
19. Willstätter, R. and Pfannenstiel, A. *Ber.* **37** (1905) 4744.
20. Patchett, A. A. and Witkop, B. *J. Org. Chem.* **22** (1957) 1477; Harley-Mason, J. and Laird, A. H. *J. Chem. Soc.* **1958** 1718.
21. Kögl, F., Erxleben, H. and Jänecke, L. *Ann.* **482** (1930) 105.

Received November 18, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 520—524

On the Connection between Electrical Potential and Free Energy for Concentration Cells with Transference

T. FÖRLAND

N.T.N.F.'s Institutt for Silikatforskning, N.T.H., Trondheim, Norway, and

J. KROGH-MOE

Svenska Silikatforskningsinstitutet, Göteborg, Sweden

From knowledge about the transport numbers the chemical changes have been calculated in a concentration cell when an electrical charge is transported through the cell. For the case that the electrolyte is a good conductor, so changes in dipol layers can be neglected, a rigorous correlation between electromotive force and free energy of the components has been derived.

In the classical treatment of concentration cells with transference, the transport number of the cations or anions appears in the equation for the potential. *E.g.*, for a concentration cell

$$\mathrm{Ag;\ AgNO_3\ in\ H_2O(I):\ AgNO_3\ in\ H_2O(II);\ Ag}$$

where the two electrolytes are silver nitrate-water solutions of different concentrations, the E.M.F. of the cell is given by:

$$E = (t_{\mathrm{NO_3^-}})\ \frac{RT}{F}\ \ln\frac{a_{\mathrm{II}}}{a_{\mathrm{I}}}$$

Here $t_{\mathrm{NO_3^-}}$ is the transport number of the nitrate ion, R is the gass constant, T the absolute temperature, F Faraday's number, and a_{I} and a_{II} are the activities of silver nitrate in the two solutions.

It has been shown by Sundheim [1] that for a system of fused salts absolute transport numbers have no meaning. Whether the electrical charge is transported by the cations or by the anions is not a property of the fused salt, but of the experimental arrangement. As was pointed out by Förland and Krogh-Moe [2], this also applies to water solutions where hydroxyl ions take

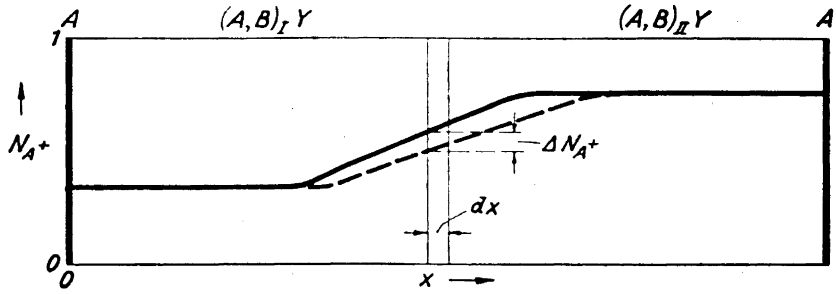


Fig. 1. The content of the component AY as a function of the distance from one side for the concentration cell A; $(A, B)_I Y : (A,B)_{II} Y$; A.

part in the electrical transport, and thus the water cannot be considered as a neutral reference frame.

The question now arises how to formulate the equation of the potential of a concentration cell when the electrolyte is a fused salt. As the fused salt lacks a reference frame, an equation like eqn. (1) obviously cannot be used.

It is also of interest to know under what conditions the liquid junction potential will be of importance, and if it is significant, how it may be calculated. Concentration cells are often experimentally simple, and it would therefore be of importance to eliminate the uncertainty connected with the liquid junction potential.

We will consider the following concentration cell

$$A; (A,B)_I Y : (A,B)_{II} Y; A$$

where the electrolytes consist of a fused mixture of two salts AY and BY (A^+ and B^+ forming cations and Y^- forming the anion) the A^+/B^+ ratio being different on the two sides of the cell. Further the electrodes are reversible only to the metal A.

In Fig. 1 is plotted the ionic fraction N_{A+} and N_{B+} of the two cations as a function of the distance x from one side of the cell. The ionic fractions are defined as

$$N_{A+} = \frac{n_{A+}}{n_{A+} + n_{B+}} \text{ and } N_{B+} = \frac{n_{B+}}{n_{A+} + n_{B+}}$$

where n with subscript indicate the number of moles of an ion. With a cell arrangement as indicated, we have $t_{Y^-} = 0$. (In a thermodynamic treatment we are not interested in whether the transport of the cations is a highly coupled process or not.)

We will assume that the transport number of A^+ is a function of the ionic fraction of A^+:

$$t_{A+} = t_{(N_{A+})} \qquad (2)$$

We may first calculate the changes taking place in the region of the concentration gradient of the cell, when an electrical charge (Q) is transported through the cell.

The transport number of A^+ at a distance x from one side of the cell we denote by $t_{A+\,(x)}$. At a distance $x + dx$ the transport number is

$$t_{A+\,(x+dx)} = t_{A+\,(x)} + \frac{\partial\, t_{A+}}{\partial\, x}\, dx = t_{A+\,(x)} + t'_{(N_{A+})}\, \frac{\partial\, N_{A+}}{\partial\, x}\, dx \qquad (3)$$

The change in the content of A^+ in the volume of thickness dx by the transport of electrical charge will be:

$$\frac{\partial\, n_{A+}}{\partial\, Q} = \frac{1}{F}\,(t_{A+\,(x)} - t_{A+\,(x+dx)}) \qquad (4)$$

and as $n_{A+} = \frac{dx \cdot q}{V} \cdot N_{A+}$ where q is the crossection of the electrolyte and V is the volume of one mole of (A,B)Y, the change in the ionic fraction of A^+ will be:

$$\frac{\partial\, N_{A+}}{\partial\, Q} = -\frac{V}{q \cdot F}\, t'_{(N_{A+})}\, \frac{\partial\, N_{A+}}{\partial\, x} \qquad (5)$$

One may notice that if $\partial N_{A+}/\partial Q$ is proportional to the concentration gradient $\partial N_{A+}/\partial x$, then the curve showing N_{A+} as a function of x will not change its slope by the transport of electricity. It will only be shifted in a parallel way towards right. From eqn. (5) one can see that this will be the case when

$$t'_{(N_{A+})} = \text{constant} \qquad (6)$$

in the concentration interval in question, or if the transport number can be expressed by an equation

$$t_{A+} = a\, N_{A+} + b \qquad (7)$$

where a and b are constants. One may further notice that the shift of the concentration gradient is equal to:

$$\left(\frac{\partial\, x}{\partial\, Q}\right)_{N_{A+}\,=\,\text{const.}} = -\frac{V}{q \cdot F}\, t'_{(N_{A+})} \qquad (8)$$

which shows that it is independent of the gradient $\partial N_{A+}/\partial x$.

One may now calculate all changes taking place in the cell by the transfer of a small electrical charge ΔQ.

At the left side electrode $\Delta Q/F$ mole of A^+ ions will form and $t_{A+\,(I)} \cdot \Delta Q/F$ mole of A^+ ions and $t_{B+\,(I)} \cdot \Delta Q/F$ mole of B^+ ions will leave this side of the cell. This change is equivalent to the introduction of $\frac{t_{B+\,(I)}}{N_{B+\,(I)}} \cdot \Delta Q/F$ mole of AY, and the simultaneous removal of $\frac{t_{B+\,(I)}}{N_{B+\,(I)}} \cdot \Delta Q/F$ mole of $(A,B)_I Y$ with the same A/B ratio as the electrolyte on this side of the cell.

The change at the right side of the cell is equivalent to removal of $\frac{t_{B+\,(II)}}{N_{B+\,(II)}} \cdot \Delta Q/F$ mole of AY and the simultaneous introduction of $\frac{t_{B+\,(II)}}{N_{B+\,(II)}} \cdot \Delta Q/F$ mole of $(A, B)_{II}$ Y with the same A/B ratio as the electrolyte on the right side of the cell.

At the same time the concentration gradient will be shifted according to eqn. (7). The changes taking place are schematically shown in Fig. 1.

The free energy change taking place in the region of the concentration gradient, when a charge ΔQ is transported through the cell, is equal to

$$\Delta F_1 = \int \frac{q \cdot dx}{V} \cdot \frac{d\Delta F}{dN_{A+}} \cdot \Delta N_{A+} \tag{9}$$

where ΔF is the free energy of mixing per one mole of the mixture AY—BY, and ΔN_{A+} is the change in the ionic fraction of A^+:

$$\Delta N_{A+} = \frac{\partial N_{A+}}{\partial Q} \cdot \Delta Q = -\frac{V \cdot \Delta Q}{q \cdot F} t'_{(N_{A+})} \frac{\partial N_{A+}}{\partial x} \tag{10}$$

Introducing this into eqn. (9) one has:

$$\Delta F_1 = -\frac{\Delta Q}{F} \int_{N_{A+\,(I)}}^{N_{A+\,(II)}} \frac{d\Delta F}{dN_{A+}} \cdot t'_{(N_{A+})}\, dN_{A+} \tag{11}$$

The total change in free energy associated with the transport of the charge ΔQ is equal to

$$\Delta F_{total} = \left[\frac{t_{B+\,(I)}}{N_{B+\,(I)}} (\Delta \bar{F}_{AY(I)} - \Delta F_{(I)}) - \frac{t_{B+(II)}}{N_{B+(II)}} (\Delta \bar{F}_{AY(II)} - \Delta F_{(II)}) - \int_{N_{A+\,(I)}}^{N_{A+\,(II)}} \frac{d\Delta F}{dN_{A+}} \cdot t'_{(N_{A+})}\, dN_{A+} \right] \frac{\Delta Q}{F} \tag{12}$$

where $\Delta F_{(II)}$ and $\Delta F_{(I)}$ are the free energy changes by mixing AY and BY to give one mole of the two electrolytes $(A,B)_{II}Y$ and $(A,B)_{I}Y$, respectively.

The liquid junction may form a dipol layer whose energy is changed by the cell reaction. However, in a system of fused salts it is reasonable to assume that this energy change is insignificant, and it will be omitted here.

Since the transfer of the charge ΔQ is not connected with any other changes in the cell than what is expressed by eqn. (12) (it can not depend on any time-dependent process as the transfer of the charge is time-independent), the E.M.F. of the cell is given by

$$E \cdot \Delta Q = \Delta F_{total}$$

or

$$E \cdot F = \frac{t_{B+\,(I)}}{N_{B+\,(I)}} \Delta\overline{F}_{AY(I)} - \frac{t_{B+\,(II)}}{N_{B+\,(II)}} \Delta\overline{F}_{AY(II)}$$

$$+ \frac{t_{B+\,(II)}}{N_{B+\,(II)}} \Delta F_{(II)} - \frac{t_{B+\,(I)}}{N_{B+\,(I)}} \Delta F_{(I)} - \int_{N_{A+\,(I)}}^{N_{A+\,(II)}} \frac{d\Delta F}{dN_{A+}} \cdot t'_{(N_{A+})} dN_{A+} \quad (13)$$

The correlation between E.M.F. of the cell and the partial free energy of one component is thus rather complicated in the general case.

For the special case when $t_{A+} = N_{A+}$ and $t_{B+} = N_{B+}$ over the concentration interval in question one obtains

$$E \cdot F = \Delta\overline{F}_{AY\,(I)} - \Delta\overline{F}_{AY\,(II)}$$

Further one will see that the E.M.F. is zero if $t_{B+} = 0$.

If the electrodes were reversible to Y, the E.M.F. would be zero if the relative transport numbers of the cations were proportional to their ionic fractions ($t_{A+} = N_{A+}$, $t_{B+} = N_{B+}$ and $t_{Y^-} = 0$ equivalent to $t_{A+} = 0$, $t_{B+} = 0$ and $t_{Y^-} = 1$). If the electrodes are reversible to Y, and $t_{B+} = -t_{Y^-} = -1$ and $t_{A+} = 1$, E.M.F. will be given by the equation

$$E \cdot F = \Delta\overline{F}_{AY\,(II)} - \Delta\overline{F}_{AY\,(I)}$$

An electrolyte of sodium silicate can formally be described as consisting of the ions Na^+, Si^{4+} and O^{2-}. A cell with two oxygen electrodes and an electrolyte of an acid sodium silicate would thus be of the latter type.

It has come to our knowledge that Sundheim [3,4] has recently derived an expression for the liquid junction potential. His derivation is based on the equations of the thermodynamics of irreversible processes, and the result is in a form different from that given above.

Acknowledgement is given to sivilingeniör Jorulf Brynestad for valuable discussions, and to *Norges Teknisk-Naturvitenskapelige Forskningsråd* for financial support.

REFERENCES

1. Sundheim, B. R. *J. Phys. Chem.* **60** (1956) 1381.
2. Förland, T. and Krogh-Moe, J. *Ibid.* **61** (1957) 511.
3. Sundheim, B. R. *Ibid.* **61** (1957) 485.
4. Sundheim, B. R. *J. Chem. Phys.* **27** (1957) 791.

Received December 17, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 525—532

Colorimetric Determination of Thiosulphate in the Presence of Polythionates and Hydrogen Sulphite

NILS-HERMAN SCHÖÖN

Department of Engineering Chemistry, Chalmers Tekniska Högskola, Göteborg, Sweden

The influence of pentathionate, tetrathionate, trithionate, and hydrogen sulphite upon the colorimetric determination of thiosulphate according to the silver thiocyanate method has been studied. Furthermore, a method for thiosulphate determination based on the reaction between thiosulphate and *p*-benzoquinone, has been worked out. Under proper conditions these additions have only a slight influence.

Colorimetric methods for the determination of thiosulphate have been reported by Utsumi [1,2], Sörbo [3], Straub and Kiss [4], Budd and Bewick [5], and Stolyarov [6].

The first method given by Utsumi (the silver thiocyanate method) is based upon the reaction between solid silver thiocyanate and thiosulphate, which can be written:

$$m\ AgSCN_{(s)} + n\ S_2O_3^{2-} \rightleftharpoons Ag_m(S_2O_3)_n^{(2n-m)-} + m\ SCN^-$$

After separating the solid phase, the amount of thiocyanate can be determined colorimetrically as iron(III)thiocyanate complexes.

In the second method reported by Utsumi (the mercury(II)thiocyanate method) the determination of thiosulphate is based on the formation of thiocyanate in the reaction between thiosulphate and mercury(II) thiocyanate. The reaction may be written:

$$2\ S_2O_3^{2-} + 3\ Hg(SCN)_2 + 2\ H_2O \rightarrow Hg(SCN)_2 \cdot 2\ HgS_{(s)} + 4\ H^+ + 4\ SCN^- + 2\ SO_4^{2-}$$

Since mercury(II)thiocyanate is very slightly dissociated, it is possible to determine directly the thiocyanate ions formed in this reaction. The determination is made colorimetrically after the addition of iron(III)nitrate.

In the method reported by Sörbo (the cyanide method) thiosulphate reacts with an excess of cyanide at room temperature giving thiocyanate according to the reaction:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$$

Copper(II)salt is added as a catalyst to the solution. The thiocyanate formed is determined as in the other methods. The cyanide method has also been studied by Straub and Kiss.

In the method given by Budd and Bewick thiosulphate is reduced by tin(II)chloride and aluminium to sulphide. The hydrogen sulphide driven off from the solution is determined colorimetrically by formation of methylene blue with *p*-aminodimethylaniline.

The method given by Stolyarov is based upon oxidizing the thiosulphate by cerium(IV). The amount of thiosulphate is determined indirectly by colorimetrically measuring the decrease of the concentration of cerium(IV). The determination is performed at 365 mμ where cerium(III) does not influence.

The mercury(II)thiocyanate method, the cyanide method, and the method reported by Budd and Bewick, are not applicable in the presence of polythionates since these ions react in a similar way to thiosulphate *. Sulphite also reacts with mercury(II)thiocyanate giving thiocyanate ions. In the presence of sulphite, too high values are also obtained by the silver thiocyanate method [7]. This is due to the formation of complexes containing silver and sulphite ions. Hydrogen sulphite, on the other hand, does not influence this determination, provided that the thiosulphate concentration is higher than 0.5 mM. Sulphite also influences the determination of thiosulphate according to the method by Budd and Bewick, and the method by Stolyarov.

In the present work, the influence of polythionates upon the determination of thiosulphate according to the silver thiocyanate method has been studied. The determination of thiosulphate at extremely low concentrations in the presence of hydrogen sulphite will also be dealt with. A colorimetric method, based on the reaction between thiosulphate and *p*-benzoquinone (the quinone method), is given, and the influence of polythionates and hydrogen sulphite has been studied. The investigation only includes three polythionates: trithionate, tetrathionate, and pentathionate.

I. THE SILVER THIOCYANATE METHOD

Experimental

The shaking- and filtration procedures were performed as described in a previous paper [7]. In the experiments with polythionates and thiosulphate given in Tables 2 and 3, 70 mg solid silver thiocyanate was added to 30 ml of the solution. The thiocyanate dissolved was determined colorimetrically. Ten ml iron(III)nitrate solution, 0.5 M iron(III)-nitrate and 0.5 M nitric acid were added to 15 ml of the filtered test solution. In the other experimental series, 15—20 mg radioactive silver thiocyanate (^{110}Ag being used as a tracer) was added to 30 ml of the solution.

Radioactive silver thiocyanate was used in these experiments in order to make it possible to determine the concentration of silver in the filtrate. The radioactivity measurements were performed in the same way as that described in the previous paper. The colorimetric determination was performed after addition of 5 ml 1.2 M formaldehyde and 5 ml 0.5 M iron(III) nitrate solution to 15 ml of the test solution. This large excess of formaldehyde must be added before the colorimetric determination in order to obtain a stable colour in the solution containing hydrogen sulphite. — A calibration was performed

* The colorimetric determination of polythionates according to the cyanide method and the mercury (II) thiocyanate method will be described in a forthcoming paper.

with potassium thiocyanate in order to determine the relationship between the absorbance and the concentration of thiocyanate. This calibration gave the absorbance $0.262/10^{-4}$ M thiocyanate. It may be observed that this concentration as well as the concentrations of thiosulphate and polythionates given in Tables 1—3 refer to the solution before the addition of iron(III) nitrate and formaldehyde.

The absorbance was measured at the wave-length 460 mμ by means of a spectrophotometer Beckman Model DU. The slit width was 0.02—0.03 mm and 1 cm cells were used.

In all cases, freshly boiled distilled water was used to obtain stable solutions. Trithionate and tetrathionate were prepared according to Kurtenacker and Matejka [8]. Pentathionate was prepared according to Stamm, Siepold and Goehring [9]. All the salts were more than 97 % pure.

Results

The influence of hydrogen sulphite on the determination of thiosulphate is given in Table 1. The results indicate a decrease in the dissolution of silver thiocyanate which is only to a certain extent compensated for by adding an excess of formaldehyde. In one experiment, the time of shaking was increased from 15 min to 40 min. Despite this increase in the time of reaction, no increase in the dissolution was obtained. This shows that the lower dissolution obtained for solutions containing hydrogen sulphite is not due to a lowered rate of dissolution.

In all experiments given in Table 1, the silver content of the filtrates was also measured in order to make sure that the decreased absorbance really is due to a decreased dissolution of silver thiocyanate. The results show that this explanation holds true although the decrease in dissolution found by the radiometric method is somewhat lower than the effect observed colorimetrically.

In Table 2 the interference of polythionates in the silver thiocyanate method is shown. In these experiments the time of reaction was 10 min and 16 h. For the shorter time of reaction, the dissolution of silver thiocyanate in the polythionate solutions is less than 5 % of the dissolution obtained for thiosulphate. A higher dissolution is obtained, especially for trithionate, upon increasing the time of reaction. After 16 h, the dissolution of silver thiocyanate

Table 1. Influence of hydrogen sulphite upon the determination of thiosulphate according to the silver thiocyanate method.

	Absorbance at the thiosulphate concentration			Silver content, mM at the thiosulphate concentration		
	0.1 mM	0.2 mM	0.3 mM	0.1 mM	0.2 mM	0.3 mM
—	0.207	0.389	0.530	0.078	0.142	0.202
10 mM HSO_3^-	0.174, 0.170*	0.333	0.495	0.070	0.134	0.190
10 mM HSO_3^- + 0.12 M HCHO	0.193	0.352	0.501	0.074	0.138	0.197

* Reaction time: 40 min.

Table 2. Interference of polythionates in the silver thiocyanate method.

Species of ion	Time of reaction	Absorbance at the concentration		
		0.1 mM	0.15 mM	0.20 mM
$S_2O_3^{2-}$	10 min	0.211	0.290	0.392
	16 h	0.518	0.783	—
$S_3O_6^{2-}$	10 min	0.007	0.007	0.008
	16 h	0.079	—	0.098
$S_4O_6^{2-}$	10 min	0.004	0.011	0.012
	16 h	0.018	—	0.020
$S_5O_6^{2-}$	10 min	0.004	0.013	0.016
	16 h	0.022	—	0.034

in 0.1 mM trithionate amounts to 15 % of the value obtained in 0.1 mM thiosulphate. The increased dissolution is probably due to the decomposition of the polythionates and the thiosulphate giving silver sulphide among other things. A short time of reaction must therefore be used for the determination of thiosulphate according to this method.

The dissolution of silver thiocyanate in mixtures of polythionates and thiosulphate has also been studied. The results given in Table 3 show that the increase in the dissolution in the presence of polythionates is less than 5%.

II. THE QUINONE METHOD

Rzymkowski [10] has investigated a method for the determination of *p*-benzoquinone by titration with thiosulphate in acidic solution. The reaction between *p*-benzoquinone and thiosulphate proceeds, according to Rzymkowski, in three steps.

In the first step the uncoloured hydroquinone monothiosulphuric acid is formed, which in the next step reacts with quinone giving hydroquinone and the yellow quinone monothiosulphuric acid. This reaction was shown to be

Table 3. Influence of polythionates upon the determination of thiosulphate according to the silver thiocyanate method.

Test solution	Absorbance
0.1 mM $S_2O_3^{2-}$	0.211
0.1 mM $S_2O_3^{2-}$ + 0.1 mM $S_3O_6^{2-}$	0.211
0.1 mM $S_2O_3^{2-}$ + 0.1 mM $S_4O_6^{2-}$	0.215
0.1 mM $S_2O_3^{2-}$ + 0.1 mM $S_5O_6^{2-}$	0.220
0.1 mM $S_2O_3^{2-}$ + 0.1 mM $S_3O_6^{2-}$ + +0.1 mM $S_4O_6^{2-}$ + 0.1 mM $S_5O_6^{2-}$	0.215

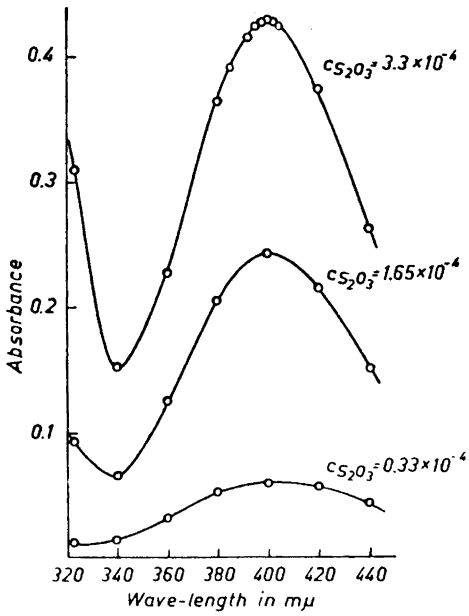


Fig. 1. Absorbance as a function of the wave-length.

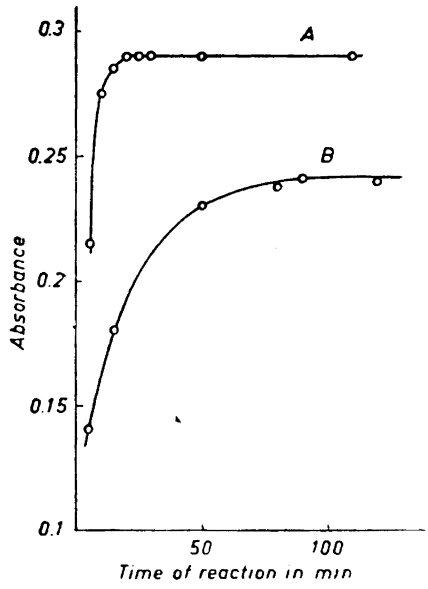


Fig. 2. Absorbance as a function of the time of reaction.

Curve A: 33 mM quinone
Curve B: 3.3 mM quinone

reversible. In the last step, quinone monothiosulphuric acid reacts with thiosulphate giving hydroquinone-2,3-dithiosulphuric acid which is colourless. With excess quinone, this compound is probably further oxidized giving the yellow-red quinone dithiosulphuric acid. With excess quinone the solution is yellow while, with excess thiosulphate, the yellow colour disappears. The reaction has also been studied by Brauer and Staude [11] and Sandved and Holte [12].

The possibility of applying this reaction for the colorimetric determination of thiosulphate at low concentrations has been studied in the present work. The investigation reported here comprises the measurement of absorbance curves and a study of the influence of polythionates, hydrogen sulphite, and formaldehyde on the reaction. The stability of the yellow colour was also studied.

Experimental

For the measurement of the absorbance curves, 10 ml 1.7 M acetic acid and 10 ml 10 mM *p*-benzoquinone were added to 10 ml of 1, 0.5, and 0.1 mM thiosulphate. In the reference solution, the thiosulphate was replaced by 10 ml water. In the other experiments, the addition of acetic acid and *p*-benzoquinone was the same as that above. The slit width was 0.06 mm and 1 cm cells were used in all measurements. The *p*-benzoquinone was of analytical grade, from Dr. T. Schuchardt, München.

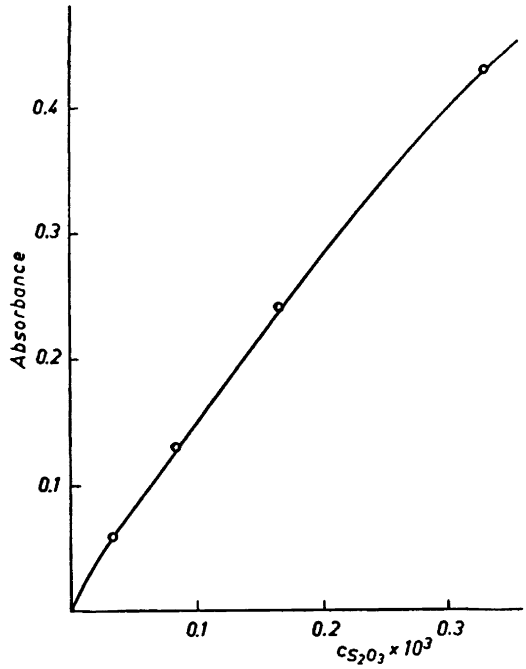


Fig. 3. Absorbance as a function of the concentration of thiosulphate.

Results

In Fig. 1, the absorbance is given as a function of the wave-length in the interval 320—440 mμ. As seen from the figure, the absorbance reaches a maximum at the wave-length 400 mμ.

All the measurements were made 2 h after the addition of the quinone solution since it was found (Fig. 2) that the absorbance increases rapidly until about 90 min after the addition of quinone solution. After this time, the absorbance is constant for at least one hour. The absorbance then decreases by only 6 % during the following 24 h. By increasing the concentration of quinone ten times, *viz.* from 3.3 to 33 mM, a stable absorbance was obtained after only 25 min of reaction. After 24 h, the absorbance was about 7 % less. For this concentrated solution, the absorbance was about 20 % higher than that for 3.3 mM quinone.

From Fig. 3 it is seen that, in the concentration interval 0.33—0.033 mM, the absorbance is not proportional to the thiosulphate content of the solution.

The behaviour of polythionates towards quinone was investigated in the same way as that described for thiosulphate. The absorbance was measured for solutions containing 0.56 M acetic acid, 3.3 mM *p*-benzoquinone, and 0.33 mM polythionate or 0.33 mM polythionate together with 0.165 mM thiosulphate.

Table 4. Influence of polythionates upon the determination of thiosulphate according to the quinone method.

Test solution	Absorbance at 400 mμ
0.33 mM $S_3O_6^{2-}$	0.002
0.33 mM $S_4O_6^{2-}$	0.005
0.33 mM $S_5O_6^{2-}$	0.000
0.165 mM $S_2O_3^{2-}$	0.241
0.165 mM $S_2O_3^{2-}$ + 0.33 mM $S_3O_6^{2-}$	0.242
0.165 mM $S_2O_3^{2-}$ + 0.33 mM $S_4O_6^{2-}$	0.240
0.165 mM $S_2O_3^{2-}$ + 0.33 mM $S_5O_6^{2-}$	0.240

The results in Table 4 show that the polythionates have no effect on the formation of the quinone thiosulphuric acids. As may be seen from Table 5, a considerable influence was obtained when hydrogen sulphite was added. The influence of hydrogen sulphite can, however, be eliminated by the addition of excess formaldehyde. Formaldehyde was in this case also added to the reference solution. At the highest concentration of hydrogen sulphite the absorbance is about 3 % too low despite the addition of formaldehyde. A corresponding decrease in the absorbance is also obtained for solutions containing only formaldehyde and thiosulphate, which shows that the decrease is not due to the presence of hydrogen sulphite. The formation of formaldehyde thiosulphuric acid [13] may to some extent explain the decrease in the absorbance. No experiments have been performed to investigate whether the intermediate compounds formed in the reaction between quinone and thiosulphate react with formaldehyde. The difference in the absorbances obtained for the experiments given in Table 4 and Table 5 may be explained by the fact that different samples of *p*-benzoquinone were used in the two series of experiments.

Table 5. Influence of hydrogen sulphite and formaldehyde upon the determination of thiosulphate according to the quinone method.

$c_{S_2O_3} \times 10^3$	$c_{SO_3} \times 10^3$	$c_{HCHO} \times 10^3$	Absorbance at 400 mμ
0.165	—	—	0.232
0.165	0.165	—	0.202
0.165	0.33	—	0.175
0.165	0.33	4	0.233
0.165	3.3	40	0.233
0.165	33	400	0.225
0.165	—	4	0.232
0.165	—	40	0.230
0.165	—	400	0.222

DISCUSSION

A comparison between the two methods for the determination of thiosulphate shows that the silver thiocyanate method is the more sensitive one, giving about twice the absorbance while, on the other hand, the quinone method is the more specific one since it is not at all influenced by polythionates. In both methods, hydrogen sulphite interferes. This interference is completely eliminated in the quinone method by the addition of formaldehyde while, in the silver thiocyanate method, it can be eliminated only to a certain extent by the addition of formaldehyde. In both methods, the relation between the absorbance and the concentration of thiosulphate is non-linear.

The financial support of *Statens Tekniska Forskningsråd* is gratefully acknowledged.

REFERENCES

1. Utsumi, S. *J. Chem. Soc. Japan, Pure Chem. Sect.* **74** (1953) 358.
2. Utsumi, S. *Ibid.* **74** (1953) 526.
3. Sörbo, B. H. *Biochim. et Biophys. Acta* **23** (1957) 412.
4. Straub, G. and Kiss, S. A. *Magyar Kém. Folyóirat* **61** (1955) 43.
5. Budd, M. S. and Bewick, H. A. *Anal. Chem.* **24** (1952) 1536.
6. Stolyarov, K. P. *Zhur. Anal. Khim.* **9** (1954) 141.
7. Schöön, N.-H. *Acta Chem. Scand.* **12** (1958) 1730.
8. Kurtenacker, A. and Matejka, K. *Z. anorg. u. allgem. Chem.* **193** (1930) 367.
9. Stamm, H., Siepold, O. and Goehring, M. *Z. anorg. u. allgem. Chem.* **247** (1941) 277.
10. Rzymkowski, J. *Z. Elektrochem.* **31** (1925) 371.
11. Brauer, E. and Staude, H. *Z. wiss. Phot.* **48** (1953) 16.
12. Sandved, K. and Holte, J. B. *Kgl. Norske Videnskab. Selskab, Forh.* **13** (1940) 9.
13. Schmidt, O. *Ber.* **40** (1907) 865.

Received December 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 533—550

Studies on the Hydrolysis of Metal Ions 22

Equilibrium Studies in Self-medium; Application to the Hydrolysis of Th^{4+}

SIRKKA HIETANEN and LARS GUNNAR SILLÉN

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

It is suggested that added information on polynuclear complexes, A_pB_q, between the reagents, A and B, may be obtained by working with an ionic medium where one of the reagents, say B, forms the major part of the ions of one charge. This is termed the "self-medium method", as distinguished from the "inert medium method", *e. g.* with $NaClO_4$, in current use. The self-medium method favors complexes A_pB_q with low values for p/q so that information may be obtained about such complexes with a greater certainty than from work with inert media.

The self-medium method has been applied to the hydrolysis of Th^{4+}. The data indicate the two reactions $2Th^{4+} + 2H_2O \rightleftharpoons Th_2(OH)_2^{6+} + 2H^+$ (equil. const. β_{22}) and $2Th^{4+} + H_2O \rightleftharpoons Th_2OH^{7+} + H^+$ (β_{12}). In the medium 0.5 Th, 2.2 (Na)Cl, $\log \beta_{22} = -5.02 \pm 0.04$, $\log \beta_{12} = -2.8 \pm 0.2$. In 0.7 Th, 3.0 (Na)Cl, $\log \beta_{22} = -5.09 \pm 0.04$, $\log \beta_{12} = -2.9 \pm 0.2$. Temperature 25°C.

Symbols

A, B = reactants, here OH^- ($-H^+$) and Th^{4+}; a = concentration of free A, here $a = h^{-1}$, eqn. (17); b = concentration of free B; B = total concentration of B; E = electromotive force of cell (16); E_0 = constant in eqn (18); $f = 2u^2 + 2ku$, eqn (12); $F(h) = BZ + \Delta$, eqn (19); $F'(h)$, theoretical expression for $F(h)$; $h = [H^+]$; H = analytical excess of H^+, often negative; j = constant in term for liquid junction potential, eqn (18); k = parameter defined by eqn (11); p, q = coefficients in formula of complex A_pB_q; K'_p = equilibrium constant for formation of the "A_p group" of complexes, eqn (4); P, Q = coefficients in formula of single complex; u = normalized variable, corresponding to a, eqns (7) or (11); Z = average number of A (here OH^-) bound per B (here Th^{4+}); β_{pq} = equilibrium constant for formation of A_pB_q, eqn (1); Δ = analytical error in H, eqn (19).

In the equations, equilibrium constants, and figures, the unit M (Mole/liter) is used throughout. However, in some of the tables, and in the text, the more convenient unit mM, mmole/liter, is sometimes used.

The inert medium method

In accurate work on complicated equilibria where charged species appear, say, equilibria between a metal ion, B, and a ligand, A, it has been increasingly common, since the work of Leden [1], to add to the solvent an "inert medium", *i.e.* an excess of ions like Na^+ and ClO_4^-, which are supposed not to take part in the reactions. In the studies of the hydrolysis of cations [2] and anions [3] in this laboratory, an inert medium has practically always been used, usually 3 M $(Na)ClO_4$ for cations, and 3 M $Na(ClO_4)$ for anions.

It is assumed that the activity factors keep constant when an inert medium is used; they are conventionally set = 1. Biedermann and Sillén [4] have given some experimental evidence for this assumption, and also for the assumption that the liquid junction potential is a (linear) function of $[H^+]$ only, at least as the method is applied in this laboratory. Forthcoming papers by Biedermann [5] will make use of a large amount of experimental evidence to indicate the range of applicability of the method, and the deviations obtained when the concentrations of the reactants, A and B, cease to be negligible in comparison with those of the medium ions.

The great advantage of the inert medium method is that the calculations are much simpler and much more reliable than if the variation of the activity factors must be considered. Emf measurements give the concentrations of free ions directly, and concentrations may be used in the law of mass action. One may indeed say that only by using the inert medium method has it been possible to draw reliable conclusions on the formulas of the species formed in many complicated systems with several products, especially in those where polynuclear species appear.

The available range in B, the total concentration of B, is limited by the decreasing experimental accuracy for low values of B, and by the deviations of the activity factors at high values of B. Using 3 M $(Na)ClO_4$ as inert medium, it has been thought advisable not to go much beyond 0.1 or 0.2 M in B for polyvalent cations.

Self-medium method

We would suggest that additional information on the species formed may be obtained by what we are going to term the "self-medium" method, which implies that the ionic medium is made to consist chiefly of the ion B to be studied, with an "inert" counter ion. In the present paper on Th^{4+} hydrolysis, 0.5 M $ThCl_4$, or 0.7 M $ThCl_4$ has been used as the main constituent; for anion hydrolysis one might think of, say, 2 M Na_2CrO_4 (or $Na_2Cr_2O_7$), 4 M $NaAs(OH)_4$ *etc.* In order not to change the ionic medium, and the activity factors, too much, one must keep the added amount of the ligand A, and thus Z, fairly low.

To make possible the necessary variations in Z, one must in general either change the concentration of the counter ion slightly, or add a small amount of an inert ion of the same sign of charge as B. Both ways have been used in the present work; the second seems preferable.

Let us assume that the two reactants, A and B, form a number of complexes, A_pB_q with the formation constants β_{pq}. For each set (p, q) we have

$$[A_pB_q] = \beta_{pq}a^pb^q \tag{1}$$

From the definitions, it follows for B (the total concentration of B) and BZ (moles of A bound to B per liter):

$$B = [B] + \sum_p \sum_q q[A_pB_q] = b + \sum_p \sum_q q\beta_{pq}a^pb^q \tag{2}$$

$$BZ = \sum_p \sum_q p[A_pB_q] = \sum_p \sum_q p\beta_{pq}a^pb^q \tag{3}$$

If Z is kept at such low values that practically $b \approx B$, we have

$$BZ = \sum_p pK'_pa^p; \quad K'_p = \sum_q \beta_{pq}B^q \tag{4}$$

From data $Z(\log a)$, measured in self-medium, it would be possible to compute the constants, K'_p in (4), for instance using curve-fitting (Sillén [6]). One might formally describe K'_p as the equilibrium constant for the association reaction, $pA \rightleftharpoons A_p$, in the medium in question. We may conventionally write A_p for the whole set of complexes A_pB_q, with varying q but the same p, since self-medium data will give practically no direct information on q. Even in those cases where a small correction for B—b is necessary, the accuracy does not allow a decision on the value for q.

Information on species

It might be held as a disadvantage that the information on the formulas of the species which is obtained with the self-medium method is thus still more restricted than that with the inert medium method, which is again more restricted than that obtained from dilute solutions. On the other hand, even the "restricted" information might have been unobtainable by the other methods.

To take an example, from equilibrium studies in *dilute* borate *solutions* one may conclude that there is a mononuclear species containing one B atom and having the charge —1; from the equilibrium data alone it is, however, not possible to decide between formulas with varying amounts of the solvent, H_2O: BO_2^-, $H_2BO_3^-$, $B(OH)_4^-$. Only the last formula is, by the way, supported by structural work.

From equilibrium studies in an *inert ionic medium* such as $Na^+ClO_4^-$, one may in addition deduce the formulas and equilibrium constants of polynuclear complexes: $B_3O_3(OH)_4^-$, and $B_3O_3(OH)_5^{2-}$, or $B_4O_5(OH)_4^{2-}$, information which could not have been obtained from studies in dilute solutions (Ingri *et al.*[3]).

On the other hand, it is not possible to discern between species that differ by containing various amounts of water, and of the medium ions: "$[B_3O_3(OH)_4^-]$" is thus really = $\Sigma[B_3O_3(OH)_4(H_2O)_x(Na)_y(ClO_4)_z^{y-1-z}]$.

In this special case, there may have been no strong reason to suppose that the medium ions would play any important role; it has been tested [7] that the formulas of the species are the same, and the equilibrium constants are not much effected on changing to a different inert medium, like Li^+Br^-, or Na^+Br^-.

In other cases, this consideration may be more serious. For instance, by equilibrium studies in the medium $NH_4^+NO_3^-$, it would not be possible to distinguish between $Hg(NH_3)_2^{2+}$, $HgNH_2^+$, $Hg(OH)_2$, and other species that could briefly be written as "HgH_{-2}" (Sillén [8]). In such a case it is very important to make equilibrium studies in several different media, or to add structural evidence.

With the *self-medium* method, the information obtained would seem still more restricted since it would not be possible to distinguish between species with different numbers, q, of the reagent B; on the other hand, one might hope in this way to obtain information not available in other ways.

In the example of the borate equilibria, one might choose $Na^+B(OH)_4^-$ as self-medium (reagents A = H^+, B = $B(OH)_4^-$), and would then group together species such as AB = $B(OH)_3$ and $AB_3 = B_3O_3(OH)_5^{2-}$ in the A_1-group, and species like $A_2B_3 = B_3O_3(OH)_4^-$ and $A_2B_4 = B_4O_5(OH)_4^{2-}$ in the A_2-group. (Note that the present choice of A and B differs from that in Ref.[3]).

Similarly, in the present study on the hydrolysis of Th^{4+} with mainly $ThCl_4$ as self-medium, there is evidence for a species with a deficit of two protons, for which the formula $Th_2(OH)_2^{6+}$ seems plausible. Really, however, it would not be possible to distinguish between formulas with varying amounts of solvent and medium ions:

$$"[Th_2(OH)_2^{6+}]" = \Sigma\Sigma\Sigma[Th_qH_{-2}(H_2O)_xCl_y^{(4q-2-y)}]$$

One might thus, slightly more correctly, refer to this species as "H_{-2}" or "$(OH)_2$".

In the cases that have hitherto been studied, this limitation in the self-medium method has not proved very serious. In general the numbers of ligands have come out low, 1 or 2, and it has been possible to deduce the most important value for q fairly reliably by comparing the equlibrium constant obtained in the self-medium with data from inert media. Structural considerations may also give some guidance.

Which species are favored?

One might ask which types of complexes are favored by measurements in self-medium as compared with measurements in an inert medium with lower values of B. It is plausible to suggest that the accuracy of the experiments with different values of B will be comparable at identical values of BZ.

Suppose that there are only two complexes with the sets $(p'q')$ and $(p''q'')$, and concentrations $c' = \beta' a^{p'} B^{q'}$, $c'' = \beta'' a^{p''} B^{q''}$, since $b \approx B$ (1). If $BZ = p'c' + p''c''$ (3) is kept constant, one may easily deduce

$$\left(\frac{\partial \ln(c'/c'')}{\partial \ln B}\right)_{BZ} = \frac{(p'c' + p''c'')}{(p'p'c' + p''p''c'')} (q'p'' - q''p') \qquad (5)$$

In the expression to the right in (5), the fraction is certainly positive, provided BZ is positive, so the sign of the derivative is determined by $(q'p''—q''p')$. If all coefficients p and q are positive (we omit the case where one of p' and p'' is negative), c'/c'' will increase with B, provided $q'/p' > q''/p''$. In general, increasing B (*e.g.* using the self-medium method) will favor complexes with high values for q/p. So it might be hoped that the self-medium method would give more certain evidence for some complexes with high ratios q/p than can be obtained using an inert medium.

Some arguments might be given for comparing, instead, points of equal Z. For instance, in self-media of different B, the uncertainty from the activity factors may set a limit at roughly the same Z. If $\ln(c'/c'')$ is differentiated with respect to $\ln B$ at constant Z, the third term to the right in (5) becomes $((q'—1)p''—(q''—1)p')$. Hence, complexes with high values for $(q—1)/p$ are favored, which will in general work in the same direction.

Sometimes, the limit of the experimental range will be set by the precipitation of a solid, say $A_NB(s)$, at low Z before the uncertainty from the activity factors becomes serious. $A_NB(s)$ may, of course, also contain solvent and inert ions, but these are left out as usual. For any complex we would have at the point of precipitation, and thus at equilibrium with $A_NB(s)$

$$[A_pB_q] = \text{constant} \cdot b^{q-pN^{-1}} \approx \text{constant} \cdot B^{q-pN^{-1}}$$

Increase in B would then give a wider concentration range to be studied, and thus more information for any single complex with $p/q < N$. Of two complexes, the one with the higher value for $(Nq—p)$ will be favored.

The equilibrium constants in a self-medium may be expected to differ somewhat from those in an inert medium of the same concentration of inert counter ion. However, our experience hitherto has shown that the differences are only of the order of 0.1 in the logarithm of the formation constant.

Correcting for decrease in b

In the present work it was desirable to extend the measurements up to Z-values of around 0.10. Here, the difference between the total concentration, B, and the free concentration, b, is no longer negligible and a correction must be made.

Single complex. If the whole effect measured is due to a single complex, A_PB_Q, then from (2) and (3)

$$B = b + Q\beta_{PQ}a^P b^Q \; ; \; BZ = P\beta_{PQ}a^P b^Q \qquad (6)$$

Eliminating $b = B(1-ZQP^{-1})$, we find

$$Z(1-ZQP^{-1})^{-Q} = a^P P\beta_{PQ} B^{Q-1} = u^P \tag{7}$$

Here, u is a normalized variable corresponding to a. We may use eqn (7) to calculate a normalized curve $Z(\log u)$ and move it along the experimental data, $Z(\log a)$. If a fit is obtained for a certain set (P, Q), one may obtain β_{PQ} from the constant difference

$$\log u - \log a = P^{-1}(\log P + \log \beta_{PQ} + (Q-1) \log B) \tag{8}$$

In actual fact the shape of the curve $Z(\log u)$ is mainly determined by P. Any reasonable value of Q will only cause a minor change, in the upper part of the Z range, in comparison with the curve obtained from $Z = u^P$. This is what eqn (7) reduces to if one puts $Q = 0$ that is neglects the difference between b and B.

Two complexes. If two complexes must be considered, the experimental data $Z(\log a)$ must be compared with a family of curves $Z(\log u)_k$, where k is a parameter containing the formation constants for the two complexes. The procedure will be illustrated for the combination of two complexes AB_2 and A_2B_2, which will be useful in this paper, and in a following one on the hydrolysis of UO_2^{2+}.

From (2) and (3) we have

$$B = b + 2\beta_{12}ab^2 + 2\beta_{22}a^2b^2 \tag{9}$$

$$BZ = \beta_{12}ab^2 + 2\beta_{22}a^2b^2 \tag{10}$$

We shall define the normalized variable u (corresponding to a), the parameter k, and the useful abbreviation f:

$$u^2 = 2\beta_{22}a^2B \; ; \; ku = 2\beta_{12}aB \tag{11}$$

$$f = 2u^2 + 2ku \tag{12}$$

Eliminating b, we obtain finally from (9 to 12)

$$Z = \frac{f - ku}{1+f+\sqrt{1+2f}} = (f - ku)f^{-2}(1+f-\sqrt{1+2f}) \tag{13}$$

Using eqn (13) one may calculate a family of curve $Z(\log u)_k$, for a number of values of k, and compare them with the experimental curve $Z(\log a)$. If a fit is obtained for a certain value of k, one may notice the difference of coordinates $(\log u - \log a)$ and obtain the equilibrium constants from:

$$\log \beta_{22} = 2(\log u - \log a) - \log 2B \tag{14}$$

$$\log \beta_{12} = \log k + (\log u - \log a) - \log 2B \tag{15}$$

which are easily deduced from (11).

HYDROLYSIS OF Th^{4+} IN $ThCl_4$ SELF-MEDIUM

Composition of solutions

It was decided to use Cl^- as the inert anion, which has some advantages since practically the same solution can then be used in the reference electrode.

Our first work on the hydrolysis of thorium (Hietanen[9]) was made in 1 M (Na)ClO_4 medium, like the work of Kraus and Holmberg[10]. In the meantime, studies with 3 M (Na)Cl as inert ionic medium have been performed, which will shortly be published[11].

The total concentration, B, of Th was chosen as 0.5 or 0.7 M, and kept constant in each series. The analytical hydrogen excess, H, was varied either by varying $[Cl^-]$ or by adding Na^+, at constant $[Cl^-]$. The solutions studied had the following compositions:

Medium	B	H	$[Na^+]$	$[Cl^-]$
0.5 Th(Cl)	0.5	H	—	$2 + H$
0.5 Th, 2.2 (Na)Cl	0.5	H	$0.2 - H$	2.2
0.7 Th, 3.0 (Na)Cl	0.7	H	$0.2 - H$	3.0

The solutions were mixed, as usual in this laboratory, by a titration procedure, starting with around 40 ml of a certain solution S, and adding from a buret increasing amounts of another solution T. The value of B was the same in both solutions S and T. Usually, one of them had a positive value for H (H^+ excess), and the other a negative value. The compositions of S and T used are given in Table 1.

In our titrations with 0.5 Th(Cl), S was 0.5 M $ThCl_4$ with a certain excess of HCl, whereas T was partly hydrolysed thorium chloride with 0.5 M Th but a deficiency in Cl^-. With the others, the solution with negative H could be obtained simply by mixing $ThCl_4$, NaCl(HCl), and NaOH($NaHCO_3$) solutions in calculated amounts.

Reagents

Thorium chloride, p.a. (from Lindsay Chemical Company, West Chicago, Illinois) contained a small amount of Fe, which was removed by extraction with *iso*propyl ether from 6 M HCl solution. The HCl was removed by evaporation under a heating lamp; in general, this resulted in solutions that were partly hydrolyzed, thus had less than 4 Cl per Th, which was rather convenient for our studies.

In the first experiments, the thorium chloride solutions were prepared by evaporating thorium nitrate (puriss, Kebo) repeatedly with concentrated HCl, extracting the iron, and then evaporating again.

The solutions were analysed for Th by precipitating with oxine, igniting the precipitate, and weighing as ThO_2. The total amount of Cl^- was determined by passing the solution through a H^+ saturated ion exchanger (Wofatite KPS-200) and titrating the eluate with standard NaOH. These data gave the analytical H^+ excess of the solution, $H = [Cl^-] - 4\ [Th]_{total}$.

The sodium chloride (Merck p. a., or Baker p. a.) contained a small amount of alkaline impurity, probably carbonate, which was neutralized with HCl. For instance, 3 M NaCl needed an addition of 0.02 mM HCl.

Hydrochloric acid (p.a.) was standardized against solid $KHCO_3$, and standard NaOH. *Sodium hydrogen carbonate* (Merck p.a.) was analysed against HCl. A 50 % *sodium hydr-*

oxide solution was prepared in a polythene bottle from commercial p. a. material. The clear solution was pipetted out and diluted with de-aerated water, and the dilute solution standardized against hydrazine sulfate, and against HCl; the determinations with the two methods agreed within less than 0.1 %.

Apparatus

The reference half-cell was

$$RE\| = Ag,\ AgCl|B\ M\ ThCl_4,\ \text{equilibrated with}\ AgCl|B\ M\ ThCl_4$$

with $B = 0.5$ or 0.7 M, as in the solution to be studied.

The Ag, AgCl electrodes were prepared according to Brown [12]. The $ThCl_4$ solution in the reference electrode was equilibrated with AgCl, either by shaking with solid AgCl in a thermostated room, or by passing it twice through a tube, filled with AgCl. The solubility of AgCl in these solutions is of the order of a few mM. If the reference electrode solution is not saturated with AgCl, the AgCl may be dissolved off the solid electrode; in any case the half-cell will finally give a steady potential. The equilibration was more carefully done with 0.7 M Th, which may account for the smaller variation of E_0 (Table 1).

The hydrogen ion concentration, h, was measured in a cell

$$-Pt,H_2(1\ atm)|\text{equilibrium solution}\|RE+ \qquad (16)$$

The lightly platinized Pt electrodes were prepared approximately as described by Bates [13]. The electrode vessel was of the usual "Wilhelm" type [14], and the emfs were measured by means of a Vernier potentiometer. Our experiments were carried out in a thermostated room at 25°C, and with the cell immer-

Table 1. Survey of titrations. All concentrations, including B and H, are given in the unit mM.

		S			T				
Symbol	B	Na+	H	Cl⁻	Na+	H	Cl⁻	H-range	Corr mV
□	500	—	200	2 200	—	−145.3	1 854.7	200→−38	0.3
◩	500	—	200	2 200	—	−145.3	1 854.7	200→−42	—
+	500	—	201.2	2 201.2	—	−473.1	1 527	201→−91	0.5
○	500	—	200	2 200	345.3	−145.3	2 200	200→−42	—
◇	500	345.3	−145.3	2 200	—	200	2 200	−145→135	+0.5
□	500	—	210.2	2 210.2	392.2	−192.2	2 200	205→9	−0.2 *
△	500	641.5	−441.5	2 200	—	210.2	2 210.2	−197→−8	−1.3
●	700	—	200	3 000	400	−200	3 000	200→0	—
◒	700	400	−200	3 000	—	200	3 000	−172→0	—
◒	700	—	200	3 000	400	−200	3 000	200→0	—
◒	700	400	−200	3 000	—	200	3 000	−200→0	—
◐	700	350	−150	3 000	—	200	3 000	−150→87	—
◑	700	—	200	3 000	350	−150	3 000	200→13	−1.5

* Correction in H: −5 mM.

sed in a paraffin oil thermostat at 25.0°C. As usual with the hydrogen electrode, one often had to wait 15—20 min after each addition of T from the buret to get a constant E. There was no indication of slowness in the Th^{4+} equilibria, and titrations with increasing and decreasing Z gave the same results.

Calculations

As in the other studies of cation hydrolysis equilibria [15], we shall find it convenient to write the hydrolysed complexes as $A_pB_q = Th_q(OH)_p^{(4q-p)+}$, to count formally a proton deficiency, $-H^+$, as the ligand A, and to set

$$a = h^{-1} \tag{17}$$

The primary experimental data were E, measured emf in the cell (16), (corrected for the barometric pressure, and the vapor pressure of water), and H, the analytical excess of H^+; in many of our solutions, H was negative. If the data from different experiments with the same medium were plotted in a diagram $E(H)$, these were seen to be small shifts. Now, the following equations should hold for E of cell (16), and H:

$$E = E_o - 59.15 \log h + jh \tag{18}$$

$$H = h - BZ - \Delta = h - F(h) \tag{19}$$

In eqn (18), E_o can be assumed to be a constant, when short periods like one or two days are considered. However, E_o is known to vary slightly between different reference electrodes, and also to change slightly over longer periods for one and the same reference electrode. The term jh stands for the liquid junction potential [4]; j is a constant which may be assumed to be the same in all experiments with the same medium.

In eqn (19), Δ is the possible error in the analytically determined H, which is the difference between two large numbers. BZ is a function of h, which is defined by eqns (2 and 3), or, under more special assumptions, by (4), by (6), or by (9 and 10), remembering that $a = h^{-1}$ (17). It is convenient, in the discussion, to treat the sum $BZ + \Delta$ as a single function, $F(h)$.

The fact that the curves $E(H)$ for different titrations do not coincide exactly might be ascribed to small differences in E_o, or to small differences in Δ. In the former case, there would be parallel shifts along the E-axis, in the second along the H-axis.

In order to decide whether a certain curve I can be made to coincide with another curve II, one may take a set of points from curve I, make each of these points in turn coincide with the origin of a transparent coordinate paper, and then draw on the paper the segment of curve II that falls in the nearest quadrant. If the curves I and II can be made to coincide, the segments should intersect in one point, which is the vector head of the necessary displacement.

Such plots were made, taking arbitrarily one titration as the standard for each medium. In all cases but one, the vector of displacement proved to be parallel with the E-axis: the curves could be made to coincide by a shift in

Table 2. Medium 0.5 Th(Cl). Experimental data H and E (corrected) for three titrations. Every other point given, log h calculated from E with $E_0 + 3 \cdot 59.15 = 384.1$ mV and $j = 22$ mV M^{-1}; Z from $h - H = BZ$.

H	E	log h	Z	H	E	log h	Z
symbol ■				symbol ◩ contd			
0.2000	252.2	−0.692	0.006	−0.0158	312.5	−1.788	0.064
0.1916	253.1	−0.711	0.006	−0.0374	321.0	−1.933	0.098
0.1616	256.8	−0.784	0.006	symbol +			
0.1308	261.4	−0.875	0.005	0.2012	252.1	−0.689	0.007
0.0847	270.6	−1.047	0.010	0.1591	257.2	−0.792	0.005
0.0518	280.3	−1.225	0.016	0.1399	260.0	−0.846	0.005
0.0271	290.5	−1.406	0.024	0.1307	261.0	−0.868	0.010
0.0024	303.4	−1.629	0.042	0.1132	264.6	−0.936	0.005
−0.0223	315.4	−1.837	0.074	0.0888	269.6	−1.031	0.009
−0.0377	321.4	−1.940	0.098	0.0592	277.7	−1.178	0.014
symbol ◩				0.0456	282.6	−1.266	0.017
0.2000	252.1	−0.689	0.009	0.0326	287.8	−1.358	0.022
0.1953	252.7	−0.701	0.002	0.0203	293.7	−1.462	0.028
0.1759	254.9	−0.752	0.003	0.0085	299.9	−1.568	0.037
0.1308	261.5	−0.877	0.004	−0.0134	310.9	−1.759	0.061
0.0849	270.6	−1.047	0.009	−0.0333	319.1	−1.899	0.092
0.0520	280.1	−1.220	0.016	−0.0517	325.0	−2.001	0.123
0.0274	290.3	−1.402	0.024	−0.0685	329.4	−2.075	0.153
0.0027	303.2	−1.627	0.042	−0.0914	334.7	−2.165	0.196

Table 3. Medium 0.5 Th, 2.2 (Na)Cl. Experimental data H and E (corrected) for four titrations; log h calculated from E with $E_0 + 3 \cdot 59.15 = 380.5$ mV, $j = 39$ mV M^{-1}; Z from $BZ = h - H$.

H	E	log h	Z	H	E	log h	Z
symbol ○				symbol □			
0.2000	251.9	−0.694	0.004	0.2052	251.5	−0.684	0.004
0.1916	252.8	−0.717	0.000	0.2002	252.0	−0.697	0.001
0.1485	257.7	−0.826	0.001	0.1771	254.3	−0.750	0.002
0.1202	261.8	−0.912	0.004	0.1453	258.0	−0.833	0.003
0.0847	268.4	−1.046	0.011	0.1183	261.9	−0.916	0.006
0.0518	277.6	−1.222	0.016	0.0955	266.0	−0.998	0.010
0.0271	287.5	−1.401	0.025	0.0709	271.9	−1.113	0.012
0.0024	300.1	−1.624	0.043	0.0502	278.1	−1.230	0.017
−0.0161	309.2	−1.783	0.065	0.0293	286.5	−1.382	0.024
−0.0305	315.1	−1.884	0.087	0.0092	296.7	−1.563	0.036
symbol ◆				symbol △			
−0.1453	340.6	−2.320	0.300	−0.1167	335.9	−2.241	0.245
−0.1325	338.6	−2.187	0.275	−0.1012	333.2	−2.195	0.215
−0.0896	330.9	−2.156	0.193	−0.0807	329.0	−2.123	0.176
−0.0641	325.5	−2.063	0.145	−0.0626	324.9	−2.053	0.143
−0.0363	317.3	−1.921	0.096	−0.0518	322.0	−2.003	0.123
−0.0088	306.0	−1.727	0.055	−0.0281	314.6	−1.876	0.083
0.0204	290.7	−1.458	0.029	−0.0084	306.1	−1.729	0.054
0.0397	282.0	−1.301	0.021				
0.0653	273.4	−1.145	0.013				
0.0838	268.6	−1.051	0.010				
0.1089	263.3	−0.946	0.009				
0.1251	261.2	−0.900	0.000				

Table 4. Medium 0.7 Th, 3.0 (Na)Cl. Experimental data H and E (corrected), points fo rsix titrations; log h calculated from E with $E_0 + 3 \cdot 59.15 = 359.7$ mV, $j = 22$ mV M^{-1}, Z from $BZ = h - H$.

H	E	log h	Z	H	E	log h	Z
symbol ●				symbol ◓			
0.2000	227.9	−0.697	0.001	−0.2000	317.8	−2.290	0.293
0.1628	232.0	−0.780	0.004	−0.1721	314.3	−2.231	0.254
0.1200	238.6	−0.907	0.006	−0.1404	310.1	−2.160	0.210
0.0909	244.1	−1.010	0.010	−0.1137	305.9	−2.087	0.174
0.0462	255.9	−1.223	0.020	−0.0909	301.7	−2.016	0.143
0.0133	269.6	−1.465	0.030	−0.0667	296.4	−1.925	0.112
				−0.0286	286.7	−1.757	0.066
symbol ◒				0.0000	275.4	−1.565	0.039
−0.1721	313.9	−2.224	0.254	symbol ◐			
−0.1333	308.5	−2.133	0.201	−0.1500	311.0	−2.175	0.225
−0.0909	301.5	−2.013	0.143	−0.1224	307.0	−2.107	0.186
−0.0712	297.2	−1.949	0.118	−0.1062	304.5	−2.063	0.164
−0.0462	291.5	−1.842	0.087	−0.0722	298.0	−1.954	0.119
−0.0133	280.3	−1.649	0.051	−0.0227	283.9	−1.712	0.060
0.0000	274.6	−1.551	0.040				
symbol ◒				0.0085	271.1	−1.490	0.034
0.2000	227.4	−0.689	0.007	0.0465	255.8	−1.222	0.019
0.1809	229.9	−0.738	0.003	0.0770	246.7	−1.059	0.015
0.1404	234.8	−0.834	0.009	0.0867	245.2	−1.029	0.010
0.1200	238.6	−0.907	0.006	symbol ◑			
0.0909	243.9	−1.007	0.011	0.2000	227.7	−0.694	0.003
0.0462	256.0	−1.225	0.019	0.1641	232.1	−0.782	0.002
0.0133	269.3	−1.460	0.031	0.1222	238.1	−0.899	0.006
0.0000	275.3	−1.563	0.039				
				0.0727	248.0	−1.083	0.014
				0.0385	258.7	−1.276	0.021
				0.0133	268.5	−1.446	0.032

E (last column in Table 1). In one case, however (marked in Table 1), a displacement of 5 mM along the H-axis was also necessary. This corresponds to an error of 0.25 % in the analysis for acid or thorium, or in the mixing of the solutions, and was a warning that unexpected errors of this order of magnitude might occur.

After these small shifts, the data obtained with each of the self-media fell on a smooth curve $E(H)$. These three sets of data were then treated separately by successive approximations, based on eqns (18) and (19). The data are given in Tables 2, 3, and 4, for every other experimental point; the Figures give every point. Points with very high Z values (> 0.25) have been left out.

For an ion which begins to hydrolyse at lower acidities than Th^{4+}, it might have been possible to determine Δ straight-forwardly by a Gran plot [16], and then E_0 and j directly from the region where $h \approx H$ by plotting $E + 59.15 \log H$ as a function of H. In the present work, the strong hydrolysis of Th^{4+} caused both plots to be curved, and so the somewhat more laborious method of successive approximations had to be used.

The aim was to find a theoretical function $F'(h)$, and two constants E_0 and j such that a) if h' is calculated from H with F' and (19), a plot of

(E + 59.15 log h') *versus* h' according to (18) will give a linear plot with the constants E_o and j, and b) if h is calculated from E, with E_o, j, and (18), then the $F(h)$ calculated from (19), $F(h) = H - h$, will give good agreement with $F'(h)$.

The linear part of the plot (E + 59.15 log H) *versus* H at high acidities was used to obtain the first approximate values E_{o1} and j_1. Using these constants and eqn (18), an auxiliary function $j_1 h(E)$ was calculated. It was then possible from the various E-values and (18) to calculate a first set of approximate values h_1. Inserting these into (19), a first approximate function $F_1(h_1) = h_1 - H$ was calculated. This function was plotted as F_1 (log h_1) and compared with various theoretical curves, and from the best fit a first theoretical function $F_1'(h)$ was chosen. The function $h_1'(H)$, defined by $h_1' - F_1'(h_1') = H$, was calculated for each experimental point. From a plot of (E + 59.15 log h_1') *versus* log h_1' and eqn (18), improved approximations E_{o2} and j_2 were obtained.

As before, with the auxiliary function $j_2 h(E)$, a new approximation h_2 was calculated from $-59.15 \log h_2 = E - E_{o2} - j_2 h_2$; h_2 was in turn used for calculating $F_2(h_2) = h_2 - H$. If the F_2 calculated from the data agreed well with the theoretical F_1' used in the calculations, an acceptable solution had been found. Otherwise, another theoretical function F_2', a better approximation to F_2, would have been adopted and a new cycle of approximations made.

In applying this procedure to the present data for thorium hydrolysis, it was first assumed that there was no analytical error, thus $\Delta = 0$. The data covered a range up to $Z \sim 0.25$. Because of the increasing uncertainty of activity factors, little attention was however paid to data with $Z > 0.1$.

The first approximation curves, F_1 (log h_1), were first compared with the theoretical curves, $F' = BZ$ *versus* log h, calculated for the single complexes A_1, A_2, or A_3, using (7) with $Q = 0$, and $P = 1$, 2, and 3. Between $Z = 0.05$ and 0.10, there was fair agreement with the A_2 curve. At lower values for Z, there were deviations and the shape approached that of the curve for the complex A_1. This would indicate that, under the present experimental conditions, the most important complexes formed are those containing 1 or 2 OH-groups, and that neither can be neglected.

In correcting for the change in b, it was assumed that in both cases the binuclear complexes predominated, thus $Th_2(OH)_2^{6+}$, and Th_2OH^{7+} — of course, possibly with extra H_2O and Cl^- attached. This assumption will be justified by arguments given in the discussion below.

A comparison with theoretical curves $F' = BZ$ *versus* log u calculated from (7) with $Q = 2$ and $P = 1$ or 2 gave the same result as before; the curves are intermediate between those for complexes with 1 and 2 A.

Next, theoretical curves were tried assuming two complexes, AB_2 and A_2B_2 (*viz.* Th_2OH^{7+} and $Th_2(OH)_2^{6+}$). Using (13), families of curves $F' = BZ$ (log u)$_k$ were calculated for a series of values of k. Successive approximations (two steps sufficed) gave the values for the parameters, and hence β_{22} and β_{12} given in Table 5, for the three sets of data ("assumption $\Delta = 0$").

The values for $Z(= F/B)$ and h, given in Tables 2—4, were calculated with E_o and j from Table 5. The corresponding plots of Z *versus* log h, compared with the theoretical curve, and of (E + 59.15 log h) *versus* h, are given in Figs 1 and 6 for 0.5 Th(Cl); in Figs 2, 4 and 7 for 0.5 Th, 2.2 (Na)Cl; and in

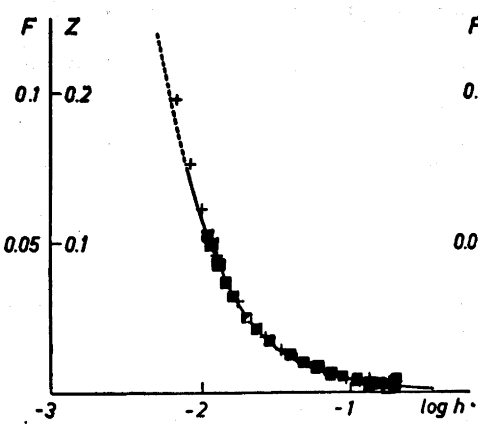


Fig. 1. Hydrolysis of Th^{4+}, medium 0.5 Th(Cl). $F = BZ$ *versus* log h. Points: calculated from experimental data $E(H)$, using E_0 and j from Table 5. Curve: calculated F', assuming $\Delta = 0$ and values for β_{22} and β_{12} in Table 5.

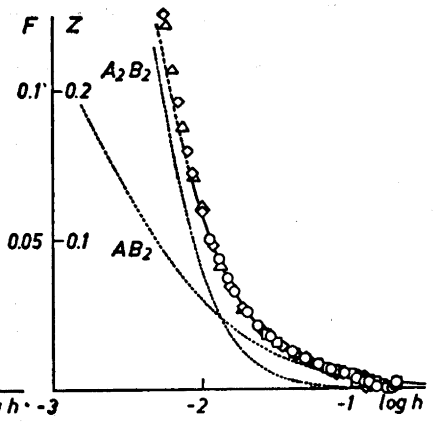


Fig. 2. Hydrolysis of Th^{4+}, medium 0.5 Th, 2.2 (Na)Cl. Experimental points and fulldrawn curve obtained as in Fig. 1. Dotted curves calculated assuming only one of the complexes present and with the same formation constant as in the full-drawn curve. See also Fig. 4.

Figs 3, and 5 for 0.7 Th, 3.0 (Na)Cl. In Figs 2, and 4 for comparison, are given also two (dotted) curves which have been calculated neglecting either AB_2 or A_2B_2, to indicate the contribution of each.

The occurrence of a single experiment with a deviation in H of 5 mM (0.01 in Z) was, however, a warning that systematic errors might exist in the analytical

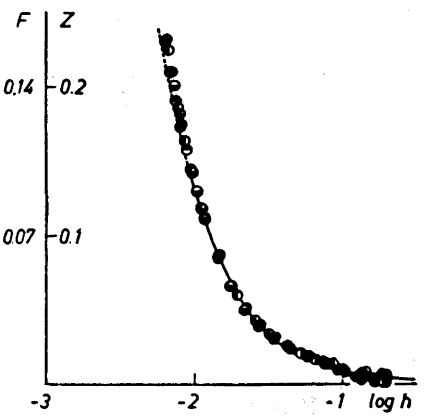


Fig. 3. Hydrolysis of Th^{4+}, medium 0.7 Th, 3.0 (Na)Cl. Experimental points and curve obtained as in Fig. 1. See also Fig. 5.

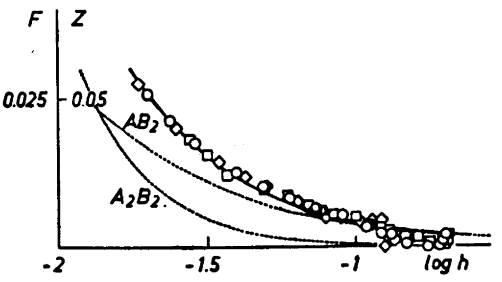


Fig. 4. Medium 0.5 Th, 2.2 (Na)Cl. Part of Fig. 2 on a larger scale.

Table 5. Equilibrium constants *etc*, obtained by successive approximations from the experimental data $E(H)$. Emfs in mV, concentrations in M.

Assumption	Quantity	0.5 Th(Cl)	0.5 Th,2.2(Na)Cl	0.7 Th,3.0(Na)Cl
$\Delta = 0$	log k	−0.3	−0.3	−0.3
	log u − log a	−2.52	−2.51	−2.47
	log β_{22}	−5.04	−5.02	−5.09
	log β_{12}	−2.82	−2.81	−2.92
	j	22	39	22
	$E_0 + 3 \cdot 59.15$	384.1	380.5	359.7
$\beta_{12} = 0$	Δ	0.009	0.009	0.011
	log u − log a	−2.42	−2.43	−2.475
	log β_{22}	−4.84	−4.86	−4.95
	j	24	38	25
	$E_0 + 3 \cdot 59.15$	384.7	381.3	360.0

Estimated uncertainty, approximately ±0.2 in log k and log β_{12}, ±0.02 in log u − log a, ±0.04 in log β_{22}, under the assumption given.

H. Now, with a large enough correction, Δ, it might be possible to fit the data to the curves for the single complex $Th_2(OH)_2^{6+} = A_2B_2$. To test, how certain the evidence for AB_2 is, another series of successive approximations was made, where it was assumed that only the complex A_2B_2 existed, thus $\beta_{12} = 0$, and an attempt was made to get a good fit by adjusting Δ and β_{22}.

The values for Δ and log β_{22} thus obtained are also given in Table 5, "assumption $\beta_{12} = 0$". The corresponding best plots for 0.5 Th, 2.2 (Na)Cl are given in Figs 8 and 9. The plot for E_0 is seen to be less linear than that obtained assuming $\Delta = 0$ (Fig 7). The E_0 plot is at any rate very sensitive to small changes in F'. However, assuming $\beta_{12} = 0$, it seemed inevitable to get two bends in the plot, whatever changes were made in F'.

Moreover, a large analytical error must be assumed to make $\beta_{12} = 0$: a value for Δ of 9 mM would correspond to an error of around 0.45 % in the analysis for Th (weighing ThO_2) or Cl^- (really a H^+ titration). Analytical errors of this order of magnitude do not seem likely, and even if β_{12} may be

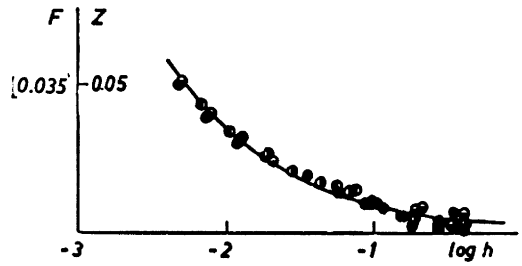


Fig. 5. Medium 0.7 Th, 3.0 (Na)Cl. Part of Fig. 3 on a larger scale.

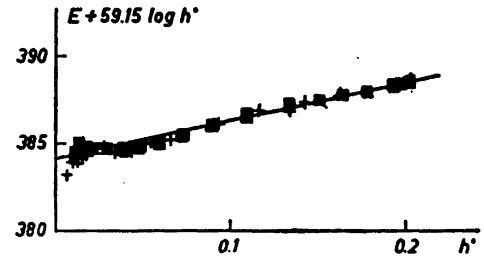


Fig. 6. Medium 0.5 Th(Cl). Abscissa: h', M, calculated from H and F' using (19) and values for β_{12} and β_{22} in Table 5. Ordinate: $E + 59.15$ (log $h' + 3$) mV. Straight line: given by values of j and E_0 in Table 5, $\Delta = 0$.

Added in proof: In Figs. 6, 7, and 9, the ordinate is mislabeled: (log $h' + 3$) was really used.

slightly in error, we think there is good evidence for the existence of the complex Th_2OH^{7+}, and for an equilibrium constant of the order of magnitude given.

We would thus prefer the values for $\Delta = 0$ and propose the following set of equilibrium constants:

Medium	log β_{12}	log β_{22}
0.5 Th, 2.2 (Na)Cl	-2.8 ± 0.2	-5.02 ± 0.04
0.7 Th, 3.0 (Na)Cl	-2.9 ± 0.2	-5.09 ± 0.04

With the medium 0.5 Th(Cl), practically the same equilibrium constants were obtained as with 0.5 Th, 2.2 (Na)Cl. The equilibrium constants thus do not seem to be very sensitive to small changes in the ionic medium. However, the constants for 0.5 Th(Cl) are left out since it seems preferable to leave the anion medium unchanged when the reactions studied are between cations;

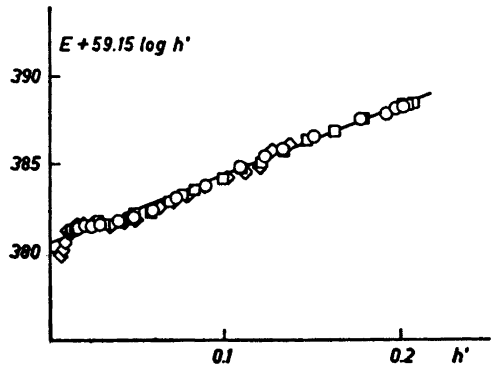


Fig. 7. Medium 0.5 Th, 2.2 (Na)Cl. $E + 59.15$ (log $h' + 3$) mV *versus* h', M, calculated as in Fig. 6.

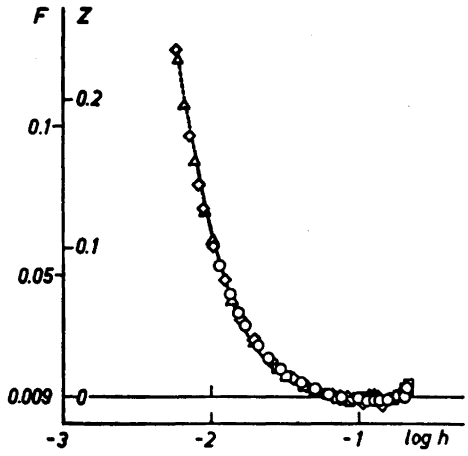


Fig. 8. Medium 0.5 Th, 2.2 (Na)Cl. Best fit assuming $\beta_{12} = 0$. $F = BZ + \Delta$ *versus* log h. Points: calculated from $E(H)$ with E_0 and j from Table 5. Curve: F' calculated with values for Δ and β_{22} from Table 5.

it has been found [4] that activity factors of cations are much more influenced by changes in the anion medium than by changes in the cation medium.

The agreement between the experimental $F(h)$ and the theoretical $F'(h)$ in Figs 1, 2 (4), and 3 (5) is very satisfactory up to beyond $Z = 0.10$. At higher values of Z, a considerable part of the ionic medium has been exchanged, and deviations are observed, especially in Fig 1, where the anionic medium changes.

The differences in E_0 and j in Table 5 are natural, considering the differences in the media.

DISCUSSION

The hydrolysis of Th^{4+} has been studied in inert medium, especially in Stockholm and Oakridge. Using 1(Na)ClO_4, Hietanen [9] found evidence for complexes $Th((OH)_3Th)_n^{(4+n)+}$, with log $\beta_{3n,n+1} \approx -7.50$ n: according to Rossotti, Rossotti and Sillén [17], the data are still better described with log $\beta_{3n,n+1} = 0.06 - 7.53\ n$.

Using 1($NaClO_4$) medium and a glass electrode, Kraus and Holmberg [10], who studied particularly the region of low Z, claimed evidence for mononuclear $ThOH^{3+}$, $Th(OH)_2^{2+}$ and dinuclear $Th_2(OH)_2^{6+}$, with the formation constants log $\beta_{11} = -4.3$, log $\beta_{21} = -7.7$, and log $\beta_{22} = -4.7$. Using measurements with 0.5 M $NaClO_4$ medium, Pan and Hseu [18] calculated, for infinite dilution, log $\beta_{11} = -3.89$ and log $\beta_{21} = -8.09$ (only mononuclear constants).

Literature data, presumably those of Hietanen, have later on been recalculated by Lefevbre [19], who has decided on a different set of complexes, with log $\beta_{21} = -7.42$, log $\beta_{22} = -4.56$, log $\beta_{12,5} = -29.5$; he also claims evidence for $Th_7(OH)_x$, with $x = 18$ to 21.

Later work in this laboratory using 3 M (Na)Cl medium [11] and studying lower concentrations, has given evidence for, in addition to the complexes

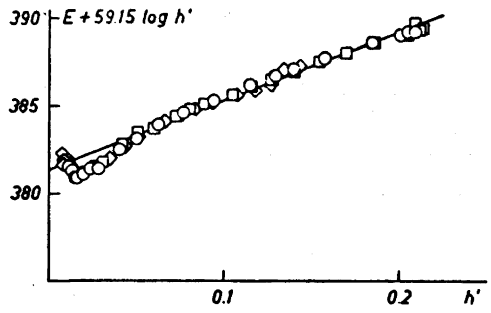


Fig. 9. Medium 0.5 Th, 2.2 (Na)Cl. Best fit assuming $\beta_{12} = 0$. $E + 59.15$ (log $h' + 3$) *versus* h', calculated as in Fig. 6.

$Th_{n+1}(OH)_{3n}^{(4+n)+}$, the complex $Th_2(OH)_2^{6+}$ with the equilibrium constant log $\beta_{22} \approx -4.7$.

We may now ask whether the formulas assigned to our complexes, $Th_2(OH)_2^{6+}$ and Th_2OH^{7+}, are plausible or not. As mentioned, a change in the number of Th-atoms would only mean a small correction in the F' curves so, strictly speaking, the only information obtainable from the data is that there are one or more complexes with 2 OH, and one or more with 1 OH; the equilibrium constants interpreted as β_{12} and β_{22} are really the over-all constants K_1' and K_2' of eqn (4).

The value for log β_{22} obtained is in good agreement with those obtained from work in inert media of comparable anionic composition [10,11]. This, in itself, would favor the assumption of 2 Th in the complex.

The idea that any of these complexes are mononuclear can, we think, easily be ruled out. If the constants interpreted here as log $\beta_{12} \approx -2.9$ and log $\beta_{22} \approx -5.0$ had really been the mononuclear log β_{11} and log β_{21}, they would have been much higher than the values deduced by earlier workers [10,18]. Moreover, if there had existed mononuclear complexes with formation constants of this order of magnitude, hydrolysis would have started at much higher h than observed in earlier experiments [9,10,11], and the "mononuclear wall" would have been pratically the only Z curve obtained.

On the other hand, a formulation with three or more Th-atoms sharing 2 or 1 OH^- would seem rather awkward geometrically, and would moreover give compositions still farther removed from the $Th_{n+1}(OH)_{3n}^{(4+n)+}$ concluded for higher degrees of hydrolysis.

So it seems to us that the formulas $Th_2(OH)_2^{6+}$ and Th_2OH^{7+}, of course probably with some H_2O and Cl^- attached, are the only plausible ones.

Acknowledgements. We are indebted to *Atomkommittén* and *Statens Naturvetenskapliga Forskningsråd (The Swedish Atomic Energy Commission*, and *The Swedish Natural Science Research Council)* for supporting this investigation. We would also like to thank Mr Frans Ericson for valuable technical assistance, and Dr Helen S. Dunsmore for correcting the English text.

REFERENCES

1. Leden, I. *Z. physik. Chem.* **A 188** (1941) 160; Dissertation, Lund 1943.
2. Tobias, R. S. *Acta Chem. Scand.* **12** (1958) 198 (Cations, part 21).
3. Ingri, N., Lagerström, G., Frydman, M. and Sillén, L. G. *Acta Chem. Scand.* **11** (1957) 1034 (Anions, part 2).
4. Biedermann, G. and Sillén, L. G. *Arkiv Kemi* **5** (1953) 425.
5. Biedermann, G. *To be published.*
6. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 186.
7. Ingri, N. *et al. To be published.*
8. Sillén, L. G. *J. Inorg. Nuclear Chem.* **8** (1958) 176.
9. Hietanen, S. *Acta Chem. Scand.* **8** (1954) 1626.
10. Kraus, K. A. and Holmberg, R. W. *J. Phys. Chem.* **58** (1954) 325.
11. Hietanen, S. and Sillén, L. G. *To be published.*
12. Brown, A. S. *J. Am. Chem. Soc.* **56** (1934) 646.
13. Bates, R. G. *Electrometric pH determinations*, New York & London 1954, p. 166.
14. Forsling, W., Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **6** (1952) 901.
15. Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 1607.
16. Gran, G. *Analyst* **77** (1952) 661.
17. Rossotti, F. J. C., Rossotti, H. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 203.
18. Pan, K. and Hseu, T. M. *Bull. Chem. Soc. Japan* **28** (1955) 162.
19. Lefevbre, J. *J. chim. phys.* **55** (1958) 227.

Received November 28, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 551—560

The Crystal Structure of $Ni_{12}P_5$

STIG RUNDQVIST and EGON LARSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The crystal structure of $Ni_{12}P_5$ has been determined with single-crystal methods. The unit cell, containing 2 formula units, is tetragonal, $a = 8.646$ Å; $c = 5.070$ Å. The space group is $I4/m-(C_{4h}^5)$ with 16 Ni in 16(i): $x = 0.116_6$; $y = 0.182_2$; $z = 0.248$; 8 Ni in 8(h): $x = 0.368$; $y = 0.060$; 8 P in 8(h): $x = 0.195$; $y = 0.415$; and 2 P in 2(a). A comparison of $Ni_{12}P_5$ with related structures is made. Some phase-analytical observations in the Ni-P system are reported. NiP_3 is cubic, $a = 7.819$ Å; and isostructural with CoP_3, $a = 7.706$ Å. Both NiP_3 and CoP_3 belong to the $D2$ ($CoAs_3$) structure type.

Although the nickel phosphides have been the object of several investigations, the knowledge of them is still very incomplete. Therefore, an X-ray investigation of the Ni-P system has been started at this institute in order to establish the existence and compositions of the intermediate phases and to determine the crystal structures. The present paper gives an account of the crystal structure determination of the phase $Ni_{12}P_5$, which has not earlier been properly characterized. In addition, some preliminary phase-analytical observations in the Ni-P system are reported.

EXPERIMENTAL

Alloys were prepared by heating mixtures of nickel powder (99.8 %, kindly donated by Mond Nickel Co., Ltd.) and red phosphorus (99 %) in evacuated and sealed silica tubes at temperatures between 700°C and 900°C. No appreciable attack on the silica tubes was detected. The reaction products were investigated with X-ray powder methods using Guinier-type cameras with Cr$K\alpha$ and Cu$K\alpha$ radiations. Unit cell dimensions were determined using CaF_2 as internal calibration standard on each powder film. The lattice parameter values have an accuracy better than 0.05 %. For the single-crystal work, a small, roughly spherical crystal fragment was selected from a crushed $Ni_{2.4}P$ alloy. The intensity material was recorded in an ordinary Weissenberg camera with MoK radiation using the multiple-film technique with thin iron foils between successive films. The intensities were visually estimated with the aid of a standard intensity scale. No absorption correction was applied. Fourier summations were made with the Hägg-Laurent machine and with Beevers-Lipson strips.

THE Ni-P SYSTEM

Previous work on the Ni-P system is summarized in Ref.[1] As the result of a thermal and microscopical investigation in the range 0—35 at.% P, Konstantinow [2] reported the existence of the phases Ni_3P, Ni_5P_2 and Ni_2P. Ni_5P_2 was stated to undergo a polymorphic transformation. Nowotny and Henglein [3], who made an X-ray investigation in the same range of composition, found four phases with compositions close to Ni_3P, Ni_5P_2, Ni_7P_3 and Ni_2P. Ni_3P was stated to be isostructural with Fe_3P and Ni_2P isostructural with Fe_2P. The Ni_3P structure was later determined by Aronsson [4]. Using mainly tensimetric methods, Heimbrecht and Biltz [5] studied the Ni-P system and reported the existence of the following phases: $Ni_{0.83}P$, NiP_2 and NiP_3.

Our phase-analytical work with the X-ray powder method is still in progress and only a few observations will be mentioned here. In the range Ni-Ni_2P four phases have been found with compositions close to Ni_3P, $Ni_{2.5}P$, $Ni_{2.4}P$ and Ni_2P, respectively. No transformations in the solid state were observed in the range 700—900°C *. For Ni_3P we determined the tetragonal unit cell dimensions as $a = 8.954$ Å; $c = 4.387$ Å in very close agreement with the values given by Aronsson [4]. The powder pattern of $Ni_{2.5}P$ is complicated and has not yet been interpreted. The powder pattern of $Ni_{2.4}P$ (which is presumably the same phase as Nowotny and Henglein's "Ni_7P_3") could be indexed with the body-centered tetragonal unit cell $a = 8.646$ Å; $c = 5.070$ Å. Lattice parameter variations indicating an extended homogeneity range were not observed. Nowotny and Henglein indexed their "Ni_7P_3" powder pattern with a cubic unit cell ($a = 8.63$ Å), but additional weak lines indicated lower symmetry in their opinion. For Ni_2P we found the hexagonal unit cell dimensions $a = 5.864$ Å; $c = 3.385$ Å. Ni_2P belongs to the revised *C* 22 structure type [6].

In the region Ni_2P—NiP the system seems to be rather complicated, and transformations in the solid state probably occur. It was mentioned by Biltz, Heimbrecht and Meisel [7], that the X-ray powder pattern of NiP_3 was similar to that of CoP_3. This was indeed found to be the case, since our powder photographs of CoP_3 and NiP_3 could be indexed with cubic unit cells: for NiP_3 $a = 7.819$ Å and for CoP_3 $a = 7.706$ Å. These two phosphides are isostructural with $CoAs_3$ (*D* 2 type), the structure of which was determined by Oftedal [8].

DETERMINATION OF THE $Ni_{12}P_5$ CRYSTAL STRUCTURE

From powder and single-crystal data, the unit cell of the phase $Ni_{2.4}P$ was determined to be tetragonal: $a = 8.646$ Å; $c = 5.070$ Å; $c/a = 0.5864$; $U = 379.0$ Å^3. The measured density 7.44 g·cm^{-3} indicated a unit cell content of 24 Ni atoms and 10 P atoms, giving the calculated density value 7.53 g·cm^{-3}. The formula of the $Ni_{2.4}P$ phase should therefore be written $Ni_{12}P_5$.

* Professor T. Rosenqvist, Norges Tekniske Høgskole, Trondheim, Norway, has kindly informed us that X-ray powder investigations made at his institute in the range Ni-Ni_2P confirm our results. Our interpretation of the $Ni_{12}P_5$ powder pattern is in agreement with his findings.

For the structure determination, the intensity material from the layer lines 0—4 around the *c*-axis was used. The intensities from the layer lines with $l > 4$ were very difficult to estimate and were not included in the refinements.

Only reflexions with $h + k + l = 2n$ were observed. Since $|F(hkl)|$ was different from $|F(\bar{h}kl)|$ the possible space groups were $I4$, $I\bar{4}$ and $I4/m$. Using the entire intensity material, the Patterson section $P(xy0)$ was calculated. The Patterson function had a large peak at $(\frac{1}{2}, \frac{1}{2}, 0)$ [equivalent to a peak in $(0,0,\frac{1}{2})$], whose height, being roughly 2/3 of the origin peak height, showed that about 2/3 of the Ni atoms must lie in pairs with the same x and y parameters and spaced about $c/2$ apart. This indicated strongly that the space group was $I4/m$ with 16 Ni in 16(i) having a z parameter close to 1/4. Further analysis showed that the heights and positions of all large peaks in $P(x\,y\,0)$ could be interpreted by assuming the space group to be $I4/m$ with 16 Ni_I in a 16(i) position and 8 Ni_{II} in an 8(h) position.

Starting with the rough parameter values read from the Patterson map, signs were calculated for the observed $F(hk0)$ values and the electron density projection $\varrho\,(xy)$ was computed. Ni maxima were found at the expected positions and, in addition, P maxima were found at the origin and in an 8(h) position. The different x and y parameters were refined by successive difference syntheses. The scattering factors for Ni were interpolated from tables given by Thomas and Umeda [9] and those of P from Tomiie and Stam [10]. After the final refinement cycle, the R-value 8.0 % for the observed independant $hk0$ reflexions was obtained, when an empirical temperature factor with $B = 0.49$ Å^2 was applied. The following parameters were found: Ni_I in 16(i): $x = 0.116_0$; $y = 0.182_2$; Ni_{II} in 8(h): $x = 0.368$; $y = 0.060$; P_I in 8(h): $x = 0.195$; $y = 0.415$; P_{II} in 2(a) or 2(b) ($x = y = 0$). Since the observed $|F(hk0)|$ and $|F(hk4)|$ values were almost equal (apart from the normal decline) and the same was true for the $|F(hk1)|$ and $|(F(hk3)|$ values, the z parameter of the Ni_I atoms could not be very far from 1/4. With these parameters, a reasonable structure with normal interatomic distances could be visualized. Since there was apparently enough space for a P atom in both (0,0,0) and $(0,0,\frac{1}{2})$, it could not be immediately decided whether P_{II} was situated in 2(a) or in 2(b). However, a comparison of calculated and observed $|F(hk1)|$ values proved beyond doubt that P_{II} was situated in 2(a). With P_{II} in 2(a), the R-value for the recorded $|F(hk1)|$ values was 9.0 %, whereas with P_{II} in 2(b), the R-value was larger than 40 %.

It still remained to be settled whether the z_I parameter of Ni_I was appreciably different from 1/4 or not. The $F(hkl)$ values with $l = 2n$ should hardly be affected by a small shift, but the $F(hkl)$ values with $l = 2n + 1$ should be more sensitive. Starting with the z_I parameter exactly 1/4, the $F(hk3)$ values were calculated, and the R-value obtained, was 10.9 %. It was then found that the agreement was somewhat improved with a z_I-value slightly less than 1/4. The best agreement was obtained with $z_I = 0.248$, giving the R-value 10.1 %. The $F(hk1)$ values were recalculated using this z_I value and the R-value for these reflexions dropped from 9.0 % to 8.8 %. Finally the remaining $F(hk2)$ and $F(hk4)$ values were calculated, and the R-value 8.7 % for all measured reflexions was obtained after applying an overall temperature factor with

Table 1.

h k l	F_o	F_c	*h k l*	F_o	F_c
0 0 0	—	822	1 7 0	142.0	−142.7
2 0 0	37.8	− 34.2	3 7 0	181.1	−173.3
4 0 0	238.1	−241.0	5 7 0	163.3	−147.4
6 0 0	57.5	39.2	7 7 0	58.4	41.0
8 0 0	28.6	− 32.0	9 7 0	—	20.2
10 0 0	64.1	71.7	11 7 0	—	− 11.4
12 0 0	—	− 44.0	13 7 0	—	− 14.4
14 0 0	37.1	− 48.0	15 7 0	79.3	96.5
16 0 0	98.1	103.2	2 8 0	107.5	− 90.1
1 1 0	—	− 6.2	4 8 0	201.3	189.5
3 1 0	148.8	−147.1	6 8 0	39.9	44.3
5 1 0	171.3	183.3	8 8 0	148.3	−162.9
7 1 0	191.7	200.4	10 8 0	—	− 4.5
9 1 0	80.5	− 84.5	12 8 0	—	39.1
11 1 0	—	− 16.1	14 8 0	—	22.2
13 1 0	—	− 5.3	1 9 0	142.0	118.6
15 1 0	—	− 26.6	3 9 0	—	− 18.8
2 2 0	62.9	− 49.7	5 9 0	69.9	74.9
4 2 0	218.2	−230.4	7 9 0	37.1	30.3
6 2 0	190.0	188.5	9 9 0	—	− 44.8
8 2 0	54.9	47.4	11 9 0	—	76.0
10 2 0	95.3	− 92.2	13 9 0	—	− 26.8
12 2 0	—	66.0	2 10 0	38.5	45.3
14 2 0	—	31.0	4 10 0	46.9	− 62.6
16 2 0	—	47.5	6 10 0	—	− 24.0
1 3 0	—	− 0.3	8 10 0	—	− 43.6
3 3 0	234.6	236.5	10 10 0	65.0	63.0
5 3 0	49.7	46.6	12 10 0	95.3	100.1
7 3 0	—	− 10.0	1 11 0	193.6	191.9
9 3 0	119.4	−128.6	3 11 0	—	9.9
11 3 0	—	− 22.2	5 11 0	135.1	−123.8
13 3 0	120.1	137.7	7 11 0	77.9	85.0
15 3 0	—	− 42.5	9 11 0	—	4.9
2 4 0	332.2	381.0	11 11 0	—	− 43.5
4 4 0	44.6	33.9	2 12 0	53.0	− 50.9
6 4 0	163.8	−164.3	4 12 0	—	− 39.5
8 4 0	100.4	97.8	6 12 0	104.2	98.8
10 4 0	57.0	50.0	8 12 0	102.1	98.3
12 4 0	—	45.0	10 12 0	—	− 55.1
14 4 0	—	− 14.2	1 13 0	61.7	− 66.6
16 4 0	57.0	− 65.5	3 13 0	68.0	58.1
1 5 0	—	− 14.5	5 13 0	60.8	− 53.9
3 5 0	—	26.9	7 13 0	—	− 5.9
5 5 0	120.1	−119.8	9 13 0	—	25.8
7 5 0	45.8	− 44.7	11 13 0	—	− 34.4
9 5 0	117.3	126.2	13 13 0	56.6	57.8
11 5 0	—	− 40.0	2 14 0	—	30.6
13 5 0	—	− 0.4	4 14 0	106.1	106.1
15 5 0	—	44.3	6 14 0	—	− 0.2
2 6 0	—	7.6	8 14 0	—	− 35.8
4 6 0	78.1	67.8	1 15 0	67.6	− 55.9
6 6 0	85.2	− 80.3	3 15 0	73.4	70.9
8 6 0	102.5	111.3	5 15 0	—	22.5
10 6 0	55.1	62.7	7 15 0	63.8	− 73.5
12 6 0	116.6	−116.6	1 0 1	—	26.9
14 6 0	—	− 19.7	3 0 1	93.7	85.0

h k l	F_o	F_c	h k l	F_o	F_c
5 0 1	89.6	96.1	1 12 1	50.0	38.8
7 0 1	80.2	— 79.8	3 12 1	—	21.3
9 0 1	42.2	— 51.5	2 13 1	—	— 36.8
11 0 1	—	19.2	1 14 1	32.2	43.0
13 0 1	—	— 7.5			
2 1 1	37.3	32.6	0 0 2	—	— 34.4
4 1 1	27.7	— 27.5	2 0 2	136.6	148.9
6 1 1	47.4	38.2	4 0 2	102.3	94.0
8 1 1	125.7	127.7	6 0 2	86.9	— 63.2
10 1 1	—	18.2	8 0 2	—	— 16.9
12 1 1	—	— 36.9	10 0 2	74.6	— 80.0
1 2 1	32.2	— 22.1	12 0 2	—	6.0
3 2 1	56.4	— 69.3	14 0 2	116.1	119.6
5 2 1	—	15.7	16 0 2	—	— 19.5
7 2 1	43.3	— 45.0	1 1 2	—	—207.0
9 2 1	43.5	— 45.3	3 1 2	391.2	428.0
11 2 1	89.2	84.8	5 1 2	77.0	76.0
13 2 1	62.5	62.1	7 1 2	138.9	—131.8
2 3 1	151.1	145.9	9 1 2	65.2	61.7
4 3 1	81.6	83.8	11 1 2	—	12.6
6 3 1	38.5	— 34.9	13 1 2	—	37.7
8 3 1	—	20.8	15 1 2	—	— 38.2
10 3 1	—	— 16.5	2 2 2	—	3.9
12 3 1	65.6	— 68.0	4 2 2	68.2	— 63.6
1 4 1	110.3	—107.0	6 2 2	158.5	—173.7
3 4 1	65.8	— 68.1	8 2 2	65.9	65.5
5 4 1	70.5	65.6	10 2 2	178.3	186.4
7 4 1	107.2	122.2	12 2 2	62.7	— 58.5
9 4 1	—	12.3	14 2 2	—	— 26.9
11 4 1	—	22.1	16 2 2	—	27.8
2 5 1	56.2	53.9	1 3 2	—	4.4
4 5 1	26.4	— 19.9	3 3 2	107.1	— 83.3
6 5 1	99.6	—114.1	5 3 2	94.7	93.5
8 5 1	39.4	— 41.2	7 3 2	64.1	— 60.7
10 5 1	73.8	73.2	9 3 2	61.5	63.3
1 6 1	48.2	32.5	11 3 2	—	— 6.3
3 6 1	66.8	63.3	13 3 2	75.6	— 76.4
5 6 1	67.0	70.0	15 3 2	—	44.2
7 6 1	60.3	72.4	2 4 2	125.0	—132.4
9 6 1	43.3	— 28.2	4 4 2	38.7	— 29.8
11 6 1	63.6	— 66.7	6 4 2	167.3	169.5
2 7 1	43.3	— 42.3	8 4 2	—	15.5
4 7 1	—	8.7	10 4 2	—	25.8
6 7 1	36.5	27.7	12 4 2	—	— 21.1
8 7 1	—	14.9	14 4 2	—	— 37.3
10 7 1	72.8	78.4	16 4 2	71.2	74.5
1 8 1	65.4	66.8	1 5 2	68.4	— 59.0
3 8 1	—	— 13.1	3 5 2	205.1	—189.3
5 8 1	55.6	— 66.2	5 5 2	193.3	196.0
7 8 1	—	— 10.0	7 5 2	56.0	47.7
2 9 1	62.9	59.4	9 5 2	158.3	—159.9
4 9 1	83.0	83.6	11 5 2	—	23.5
6 9 1	45.9	55.3	13 5 2	66.1	63.2
8 9 1	—	— 26.0	15 5 2	—	52.3
1 10 1	—	— 11.7	2 6 2	216.1	216.5
3 10 1	49.6	— 46.2	4 6 2	50.7	53.5
5 10 1	—	— 22.6	6 6 2	—	24.2
7 10 1	—	21.2	8 6 2	53.9	— 56.8
9 10 1	59.9	61.2	10 6 2	—	— 28.4

$h\,k\,l$	F_o	F_c	$h\,k\,l$	F_o	F_c
12 6 2	91.2	100.9	5 0 3	84.3	70.9
14 6 2	—	− 54.5	7 0 3	63.6	− 68.4
1 7 2	174.9	170.2	9 0 3	36.4	− 37.7
3 7 2	—	− 2.3	11 0 3	—	11.3
5 7 2	103.9	−113.0	13 0 3	—	− 6.3
7 7 2	64.7	56.5	2 1 3	25.6	28.8
9 7 2	—	12.6	4 1 3	—	− 19.5
11 7 2	55.5	54.0	6 1 3	—	23.2
13 7 2	57.1	53.5	8 1 3	115.8	117.7
15 7 2	—	− 48.1	10 1 3	—	16.2
2 8 2	138.2	151.0	12 1 3	—	− 38.5
4 8 2	—	− 26.0	1 2 3	—	− 14.7
6 8 2	130.9	−121.2	3 2 3	40.2	− 44.4
8 8 2	74.2	75.5	5 2 3	—	16.8
10 8 2	—	− 9.7	7 2 3	32.8	− 38.4
12 8 2	—	− 13.6	9 2 3	35.0	− 40.6
14 8 2	—	9.0	11 2 3	78.7	76.1
1 9 2	—	− 4.0	13 2 3	58.0	56.2
3 9 2	—	− 16.9	2 3 3	96.2	102.9
5 9 2	56.7	− 56.1	4 3 3	78.0	67.8
7 9 2	132.0	138.7	6 3 3	—	− 19.3
9 9 2	88.2	97.5	8 3 3	—	10.5
11 9 2	91.7	−105.7	10 3 3	—	− 14.0
13 9 2	—	− 37.6	12 3 3	61.8	− 56.9
2 10 2	143.5	−140.5	1 4 3	79.3	− 82.8
4 10 2	104.8	100.6	3 4 3	58.4	− 53.0
6 10 2	—	− 12.2	5 4 3	60.0	59.8
8 10 2	—	− 7.7	7 4 3	97.6	106.2
10 10 2	—	14.2	9 4 3	—	6.0
12 10 2	—	− 4.9	11 4 3	—	24.4
1 11 2	40.3	− 30.8	2 5 3	47.9	50.0
3 11 2	105.8	105.1	4 5 3	—	− 15.5
5 11 2	68.7	68.7	6 5 3	81.6	− 94.6
7 11 2	76.5	− 74.0	8 5 3	—	− 33.6
9 11 2	—	− 24.2	10 5 3	65.4	64.7
11 11 2	—	− 14.2	1 6 3	35.3	25.4
2 12 2	67.0	− 62.0	3 6 3	77.3	65.3
4 12 2	89.2	86.5	5 6 3	51.5	51.6
6 12 2	53.7	48.1	7 6 3	47.0	64.5
8 12 2	67.5	− 64.3	9 6 3	—	− 19.7
10 12 2	55.1	70.0	11 6 3	52.6	− 66.0
1 13 2	91.2	97.3	2 7 3	32.8	− 33.8
3 13 2	—	− 26.8	4 7 3	—	7.5
5 13 2	63.4	− 64.5	6 7 3	27.2	21.3
7 13 2	46.3	− 52.7	8 7 3	—	17.4
9 13 2	47.7	39.2	10 7 3	63.8	68.3
11 13 2	79.0	82.6	1 8 3	49.2	58.5
13 13 2	—	− 27.8	3 8 3	—	− 16.7
2 14 2	—	14.9	5 8 3	50.3	− 50.1
4 14 2	68.0	− 66.7	7 8 3	—	− 9.7
6 14 2	74.6	83.8	2 9 3	53.5	45.0
8 14 2	—	4.4	4 9 3	77.8	75.0
1 15 2	—	22.2	6 9 3	60.2	54.7
3 15 2	47.2	− 38.3	8 9 3	—	− 29.6
5 15 2	—	13.4	1 10 3	—	− 10.5
7 15 2	68.2	74.7	3 10 3	41.4	− 42.7
1 0 3	—	11.4	5 10 3	—	− 19.2
3 0 3	76.9	78.7	7 10 3	—	17.4

$h\,k\,l$	F_o	F_c
9 10 3	—	54.0
1 12 3	56.4	34.9
3 12 3	—	23.5
2 13 3	—	− 33.3
1 14 3	—	37.6
0 0 4	—	−244.3
2 0 4	—	− 19.3
4 0 4	153.9	−155.0
6 0 4	43.4	27.9
8 0 4	—	− 24.2
10 0 4	74.7	56.5
12 0 4	—	− 36.4
14 0 4	52.2	− 40.9
16 0 4	75.8	87.7
1 1 4	—	− 3.3
3 1 4	98.8	− 86.5
5 1 4	101.3	126.5
7 1 4	138.3	148.7
9 1 4	71.0	− 66.0
11 1 4	—	− 12.2
13 1 4	—	− 5.0
15 1 4	—	− 23.2
2 2 4	—	− 30.0
4 2 4	133.0	−154.0
6 2 4	159.4	139.0
8 2 4	35.8	36.5
10 2 4	91.9	− 74.3
12 2 4	58.8	54.1
14 2 4	—	27.0
16 2 4	—	40.1
1 3 4	—	− 0.1
3 3 4	156.5	157.0
5 3 4	—	32.3
7 3 4	—	− 7.9
9 3 4	80.0	−101.6
11 3 4	—	− 19.1
13 3 4	110.1	114.2
15 3 4	—	− 36.4
2 4 4	259.2	268.1
4 4 4	36.8	24.3
6 4 4	117.9	−121.2
8 4 4	74.4	76.1
10 4 4	—	41.1
12 4 4	—	36.4
14 4 4	—	− 12.6
16 4 4	46.6	− 49.5
1 5 4	—	− 11.0
3 5 4	—	18.7
5 5 4	79.3	− 87.1
7 5 4	43.2	− 34.4
9 5 4	115.8	100.9
11 5 4	—	− 32.8
13 5 4	—	− 0.3
15 5 4	—	37.0
2 6 4	—	6.4
4 6 4	34.5	50.0
6 6 4	70.3	− 61.6
8 6 4	98.1	88.0
10 6 4	52.4	50.9
12 6 4	89.6	− 98.0
14 6 4	—	− 16.7
1 7 4	89.6	−103.4
3 7 4	108.7	−130.0
5 7 4	120.2	114.3
7 7 4	—	32.7
9 7 4	—	17.0
11 7 4	—	− 9.9
13 7 4	—	− 12.5
15 7 4	75.6	82.5
2 8 4	85.5	− 69.0
4 8 4	150.5	147.5
6 8 4	58.1	35.3
8 8 4	118.3	−133.6
10 8 4	—	− 3.6
12 8 4	—	33.8
14 8 4	—	18.6
1 9 4	101.3	92.6
3 9 4	—	− 14.5
5 9 4	54.2	61.0
7 9 4	—	23.2
9 9 4	—	− 37.2
11 9 4	58.6	63.5
13 9 4	—	− 22.7
2 10 4	58.1	36.3
4 10 4	52.2	− 51.7
6 10 4	—	− 19.0
8 10 4	43.2	− 36.0
10 10 4	49.2	52.0
12 10 4	63.6	85.3
1 11 4	146.6	159.3
3 11 4	—	6.5
5 11 4	108.2	−101.5
7 11 4	73.1	70.0
9 11 4	—	4.6
11 11 4	—	− 36.8
2 12 4	45.0	− 41.2
4 12 4	42.7	− 32.8
6 12 4	74.4	81.8
8 12 4	83.2	83.2
10 12 4	—	− 47.8
1 13 4	68.7	− 55.2
3 13 4	51.0	47.8
5 13 4	50.0	− 44.7
7 13 4	—	− 5.0
9 13 4	—	21.3
11 13 4	—	− 29.1
13 13 4	44.1	49.5
2 14 4	—	24.7
4 14 4	87.5	87.9
6 14 4	—	− 0.8
8 14 4	—	− 30.3
1 15 4	46.2	− 56.0
3 15 4	56.5	60.2
5 15 4	—	18.2
7 15 4	56.8	− 63.2

Table 2. Interatomic distances in $Ni_{12}P_5$ in Å. (Distances shorter than 3.25 Å are listed.)

Ni_I	— 5 Ni_I	: 2.45, 2.51, 2.56, 2.64 (2).
	— 3 Ni_{II}	: 2.54, 2.58, 2.73.
	— 3 P_I	: 2.24, 2.41, 2.47.
	— 1 P_{II}	: 2.25.
Ni_{II}	— 6 Ni_I	: 2.54 (2), 2.58 (2), 2.73 (2).
	— 5 Ni_{II}	: 2.51, 3.09 (4).
	— 4 P_I	: 2.22 (2), 2.60 (2).
	— 1 P_{II}	: 3.22.
P_I	— 6 Ni_I	: 2.24 (2), 2.41 (2), 2.47 (2).
	— 4 Ni_{II}	: 2.22 (2), 2.60 (2).
P_{II}	— 8 Ni_I	: 2.25 (8).
	— 4 Ni_{II}	: 3.22 (4).

$B = 0.49$ Å^2. No further refinement was considered worthwhile. Observed and calculated structure factors are given in Table 1. The final structure of $Ni_{12}P_5$ is the following:

Space group $I4/m$ — $(C_{4h}^5 h)$; $Z = 2$

16 Ni_I	in 16(i)	with	$x = 0.116_0$;	$y = 0.182_2$;	$z = 0.248$;
8 Ni_{III}	in 8(h)	with	$x = 0.368$;	$y = 0.060$;	
8 P_I	in 8(h)	with	$x = 0.195$;	$y = 0.415$;	
2 P_{II}	in 2(a).				

Interatomic distances are listed in Table 2.

DESCRIPTION OF THE STRUCTURE

A projection of the $Ni_{12}P_5$ structure on the basal plane is shown in Fig. 1. As a comparison, an analogous projection of the Ni_3P structure (according to Aronsson [4]) is shown in Fig. 2. The structure of Ni_3P is based on space group $I\bar{4}$ and the unit cell contains 24 Ni atoms (as in $Ni_{12}P_5$) and 8 P atoms. All atoms are situated in 8(g) positions with z parameters close to 0, 1/4, 1/2 and 3/4. From Figs. 1 and 2, the similarities between the two structures are easily seen. If, for instance, the Ni_I and Ni_{III} atoms in Ni_3P are moved together (indicated by arrows in Fig. 2) so as to lie directly above each other at $z = 1/4$ and $z = 3/4$, a metal atom lattice of the $Ni_{12}P_5$ type is created. In fact, the mean value of the x parameters for Ni_I and Ni_{III} in Ni_3P corresponds closely to the x parameter for Ni_I in $Ni_{12}P_5$, and the same is true for the y-parameters. The positions of the Ni_{II} and P atoms in Ni_3P correspond rather closely to the positions of Ni_{II} and P_I in $Ni_{12}P_5$.

In many transition metal phosphides, the P atoms are surrounded by nine metal atoms in a more or less regular tetrakaidecahedral arrangement. This coordination is, *e.g.*, found in the Fe_2P [6], the Co_2P [11] and in the Fe_3P [4] type structures. In $Ni_{12}P_5$, the P_I atoms have 10 near Ni neighbours, whereas the P_{II} atoms have only 8 near Ni neighbours in an almost cubic arrangement.

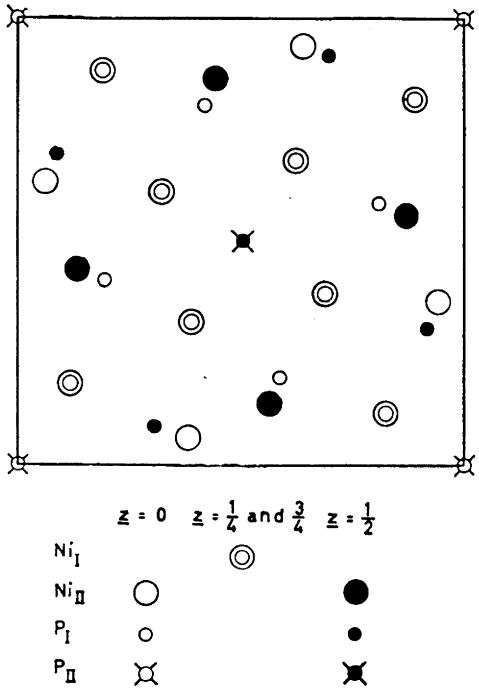


Fig. 1. The unit cell of $Ni_{12}P_5$ projected on the basal plane.

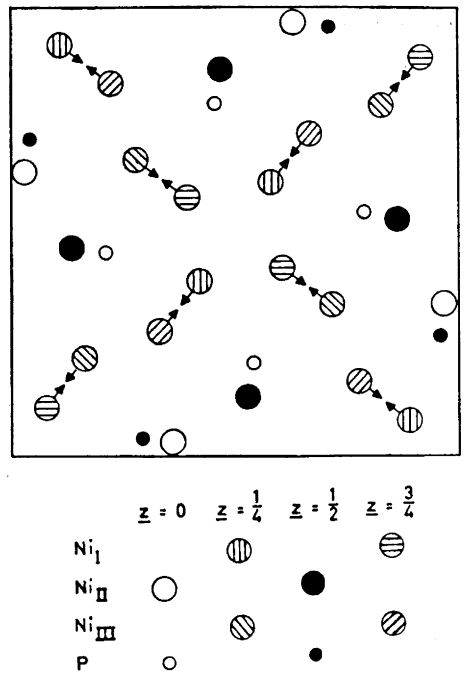


Fig. 2. The unit cell of Ni_3P projected on the basal plane. Arrows on the Ni_I and Ni_{III} atoms indicate imagined displacements towards the Ni atom arrangement in $Ni_{12}P_5$.

The tendency for higher metal atom coordination than 8 around the P_{II} atoms is perhaps indicated by the fact that P_{II} is situated in 2(*a*) and not in 2(*b*). The cubic Ni_I environment is the same for both 2(*a*) and 2(*b*) positions, but a P atom in 2(*a*) has 4 additional Ni_{II} neighbours in the same plane at a distance of 3.22 Å, whereas a hypothetical P atom in 2(*b*) has 4 neighbouring P_I atoms in the same plane at a distance of 2.73 Å.

Acknowledgements. This work has been financially supported by the *Statens Tekniska Forskningsråd* and by the *European Commission of Air Research and Development Command* under Contract No. AF 61(052)-40. One of us (S.R.) wishes to express his gratitude for a generous scholarship from the *Statens Tekniska Forskningsråd.*

The authors wish to thank Professor G. Hägg for his kind interest. Thanks are also due Dr. B. Aronsson for valuable suggestions and discussions.

REFERENCES

1. Hansen, M. *Constitution of Binary Alloys,* 2nd ed., (1958) 1027.
2. Konstantinow, N. *Z. anorg. Chem.* **60** (1908) 405.
3. Nowotny, H. and Henglein, E. *Z. physik. Chem.* **B 40** (1938) 281.

4. Aronsson, B. *Acta Chem. Scand.* **9** (1955) 137.
5. Biltz, W. and Heimbrecht, M. *Z. anorg. Chem.* **237** (1938) 132.
6. Rundqvist, S. and Jellinek, F. *Acta Chem. Scand.* **13** (1959) 425.
7. Biltz, W., Heimbrecht, M. and Meisel, K. *Z. anorg. Chem.* **241** (1939) 349.
8. Oftedal, I. *Z. Krist.* **66** (1928) 517.
9. Thomas, L. H. and Umeda, K. *J. Chem. Phys.* **26** (1957) 293.
10. Tomiie, Y. and Stam, C. H. *Acta Cryst.* **11** (1958) 126.
11. Nowotny, H. *Z. anorg. Chem.* **254** (1947) 31.

Received December 5, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 561—570

Some Reactions between Uranium(VI) and Di-*n*-butyl Phosphate(DBP) in Chloroform and Methyl *Iso*butyl Ketone (Hexone)

DAVID DYRSSEN and FRANC KRAŠOVEC

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The distribution of U(VI) between solutions of di-*n*-butyl phosphate (DBP = HA) in chloroform or hexone (methyl *iso*butyl ketone) and 1 M $HClO_4$—$NaClO_4$ solutions has been investigated at 25°C.

The data demonstrate the presence of a complex $UO_2(HA)_2A_2$ with four molecules of DBP per uranyl ion in both solvents at low concentrations of uranium. The constants for the equilibrium

UO_2^{2+} (aq) + 2 H_2A_2(org) ⇌ $UO_2(HA)_2A_2$ (org) + 2 H^+ (aq)

are $10^{3.58}$ (chloroform) and $10^{4.50}$ (hexone).

In addition to this complex an uranyl complex UO_2A_2 with only two molecules of DBP was found in hexone at lower concentrations of DBP. The equilibrium constant for the reaction

$$UO_2A_2 + 2\ HA \rightleftharpoons UO_2(HA)_2A_2$$

in the hexone phase was $10^{4.78}$.

With increasing uranium concentration in the chloroform phase the ratio $[U]_{org}/[DBP]_{org}$ approaches 0.5 thus indicating the composition $(UO_2)_nA_{2n}$ of the extractable uranyl complex. No definite conclusions about the degree of polymerization can be drawn from the distribution experiments, but isopiestic data of Baes *et al.*[5] indicate that n increases to 6 as $[U]_{org}/[DBP]_{org}$ approaches 0.5.

Dialkyl phosphates, $(RO)_2PO_2H$, have proved to be very useful as liquid-extracting agents for lanthanide and actinide ions of various oxidation states. Extensive work on the extraction of U(VI) from acidic sulfate-process liquors has been carried out at Oak Ridge[1] (Dapex process) and several radiochemical separation procedures have been worked out at Argonne[2]. Di-*n*-butyl phosphate (DBP) has been used at FOA[3] for the separation of ^{90}Sr and ^{90}Y.

The reactions between metal ions and the dialkyl phosphates can be correctly understood only if one takes into account the very pronounced tendency of the dialkyl phosphates to dimerize. Although Kosolapoff and Powell[4] have reported dimerization of dialkyl phosphinic acids, R_2PO_2H,

no studies on the dialkyl phosphoric acids have been published in the open literature until recently [5–7]. It was shown by Dyrssen [6] that the dimerization constant of DBP in chloroform is much larger than for carboxylic acids, and that dimerization also takes place in oxygen-containing solvents like hexone and water.

The aim of the present investigation was to study the extraction of the uranyl ion from 1 M $HClO_4$—$NaClO_4$ solutions with DBP in two different solvents, choloroform and hexone. Calculations on the distribution data throw some light on the reactions that can take place in the organic phase.

EXPERIMENTAL

Reagents. The DBP, $(C_4H_9O)_2PO_2H$, was kindly supplied by Albright and Wilson Ltd., London. Potentiometric titration with alkali and analysis for C and H showed the compound to be at least 99 % pure. The remaining non-acidic impurities are probably tri-butyl phosphate and butanol. The chloroform was washed three times with twice its volume of water to remove the 1 % alcohol. The hexone (methyl *iso*butyl ketone) was washed with alkali, acid, and finally water. The 1 M $HClO_4$-$NaClO_4$ solutions were prepared from analytical grade reagents. The solutions of U(VI) in 0.1 M $HClO_4$ + 0.9 M $NaClO_4$ were prepared from "nuclear grade" UO_3. The ^{233}U was purchased from AERE, Harwell, England. The solution contained 5 μC of ^{233}U per mg of U.

Distribution measurements. Equal volumes (5 or 10 ml) of a solution of DBP in chloroform or hexone and a 1 M $HClO_4$-$NaClO_4$ solution containing U(VI) were shaken for 2 h. The two liquid phases were then separated by centrifugation.

In the concentration range below 0.0025 M the uranium was analyzed by alpha-counting of 1 ml of the organic phase after evaporation of the solvent and removal of most of the DBP at 200°C. The distribution ratio, q, could then be calculated as

$$q = \frac{A}{C-A}$$

A = α-disintegrations per min and ml of the organic phase
C = total activity (cpm per ml) added.

For the determination of larger q-values part of the aqueous phase was shaken with an equal volume of a fresh solution of DBP (with the same concentration as in the first extraction). The distribution ratio is then calculated as

$$q = \frac{A-B}{B}$$

B = α-disintegrations per min and ml of the organic phase after the second extraction.

The alpha-activity was measured in a Tracerlab SC-16 windowless proportional counter.

Concentrations of uranium larger than 0.0025 M were analyzed spectrophotometrically at 375 mμ using a thiocyanate method [8]: The sample +1 ml of 10 % $SnCl_2$ + 0.5 ml of conc. H_2SO_4 + 15 ml of saturated NH_4SCN in acetone was diluted with water to 25 ml and measured 3 min after addition of the reagent.

The following constants, from Ref.[6], are used for the calculation of $[H_2A_2]_{org}$, $[HA]_{org}$, [HA] and $[A^-]$ (ionic strength = 1 M $HClO_4$-$NaClO_4$):

$[H^+]\,[A^-]\,[HA]^{-1} = 10^{-1.00}$
$[HA]_{org}\,[HA]^{-1} = 10^{0.34}$ (chloroform), $10^{1.49}$ (hexone)
$[H_2A_2]_{org}\,[HA]_{org}^{-2} = 10^{4.48}$ (chloroform), $10^{1.19}$ (hexone).

C_A is the initial concentration of DBP in the organic phase. [ ]$_{org}$ and [ ] denote concentrations in the organic and aqueous phases, respectively.

Table 1. The distribution of ^{233}U between chloroform and 1 M perchlorate solutions as a function of C_A and [H+]. The initial conc. of U(VI) in the aqueous phase was 2.69×10^{-6} M.

C_A	A	B	$q = \frac{\log \frac{A-B}{B}}{}$	$q = \frac{\log \frac{A}{C-A}}{}$	log [HA]
[H+] = 0.1 M					
0.5	6 419	5.0	+3.11		−2.89
0.2	8 137	14.7	+2.74		−3.09
0.1	7 419	25.8	+2.46		−3.24
0.08	7 712	33	+2.37		−3.29
0.05	8 165	57	+2.15		−3.39
0.03	7 529	126	+1.77		−3.50
0.02	8 282	211	+1.58		−3.60
0.015	7 344	410	+1.23		−3.66
0.01	6 733			+0.78	−3.75
0.004	4 294			+0.08	−3.96
0.002	1 581			−0.60	−4.12
0.001	423			−1.25	−4.28
[H+] = 0.31 M					
0.05	8 333	322	+1.40		−3.39
0.01	2 896			−0.23	−3.75
0.004	916			−0.88	−3.96
0.002	218			−1.54	−4.12
0.001	48			−2.21	−4.27
[H+] = 0.91 M					
0.05	6 110	1 449	+0.51		−3.39
0.01	720			−1.00	−3.75
0.004	113			−1.84	−3.96
0.002	36			−2.34	−4.12
0.001	6.0			−3.12	−4.27

RESULTS

A. Experiments with low concentrations of U(VI). The results obtained with ^{233}U are given in Tables 1 (chloroform) and 2 (hexone). If these data are plotted as log q against log $C_A[H^+]^{-1}$, a marked difference between the two solvents is revealed (Fig. 1). The chloroform values lie on a straight line with a slope of 2. The deviation from this line at higher values of $C_A[H^+]^{-1}$ ($> 10^{-0.2}$) may be ascribed to the formation of the complexes UO_2A^+ and UO_2A_2 in the aqueous phase [9]. The values from the distribution experiments with hexone, however, lie approximately on a line with a slope equal to 2.72. The deviation from this line at higher values of q is very abrupt and may be caused by an experimental error due to the fact that the aqueous phase may be slightly contaminated with the highly radioactive and lighter hexone phase when part of the aqueous bottom-layer is drawn off for the second extraction (and determination of B). This error will have a marked influence on the measurements only at high q-values.

Since the extraction of uranium by chloroform is proportional to $C_A^2[H^+]^{-2}$ and since it is known [6] that the dimerization of DBP in chloroform is almost

Table 2. The distribution of ^{233}U between hexone and 0.1 M $HClO_4$ + 0.9 M $NaClO_4$ as a function of the initial conc. of DBP in the hexone phase, C_A. The initial conc. of U(VI) in the aqueous phase was $2.69 \cdot 10^{-6}$ M.

C_A	A	B	$\log q = \frac{A-B}{B}$	$\log q = \frac{A}{C-A}$	log [HA] (=log [A^-])
0.5	5 358	12.0	+2.65		−2.44
0.2	7 091	14.7	+2.68		−2.68
0.1	7 726	13.3	+2.76		−2.87
0.1	5 777	13.3	+2.65		−2.87
0.07	6 455	12.0	+2.73		−2.97
0.05	7 519	21.3	+2.55		−3.07
0.04	6 516	28	+2.36		−3.14
0.04	7 262	31	+2.37		−3.14
0.03	6 129	78	+1.89		−3.22
0.02	6 349	203	+1.48		−3.36
0.015	6 527	508	+1.07		−3.45
0.012	5 899	865	+0.76		−3.54
0.009	5 401	1 644	+0.36		−3.65
0.008	5 203	1 943	+0.22		−3.69
0.007	4 114			+0.17	−3.74
0.006	2 788			−0.17	−3.80
0.005	1 973			−0.40	−3.87
0.004	1 377			−0.60	−3.96
0.003	729			−0.93	−4.07
0.002	318			−1.32	−4.25
0.0015	158			−1.63	−4.37
0.001	87			−1.89	−4.55

complete at concentrations ≥ 0.001 M, the following reaction will describe the data in Fig. 1 when $C_A[H^+]^{-1} < 10^{-0.2}$:

$$UO_2^{2+}(aq) + 2\ H_2A_2(org) = UO_2(HA)_2A_2(org) + 2\ H^+(aq)$$

The equilibrium constant may approximately ($C_A/2 = [H_2A_2]_{org}$) be calculated as

$$\log K = \log q + 2 \log [H^+] - 2 \log C_A/2 = 3.46$$

The same principle reaction was found in a study of the extraction of U(VI) with dioctyl phosphate in hexane from 2 M acid perchlorate solutions (log $K = 4.60 \pm 0.04$) recently published by Baes *et al.*[5] It has thus been demonstrated that the uranyl ion reacts with 4 molecules of dialkyl phosphates in inert solvents. Similar results have been found with other metal ions [3,10], *e.g.* $Y(HA)_3A_3$.

The results with hexone can be explained if one takes into account the existence of the DBP monomer and the uranyl complex UO_2A_2 due to reactions between these species and the solvent. The reaction with DBP leads to a fairly low value of the dimerization constant k_2 in hexone and a higher value of the distribution constant of the monomer as compared with chloroform [6]. The difference between the behavior of DBP in chloroform (fulldrawn curve)

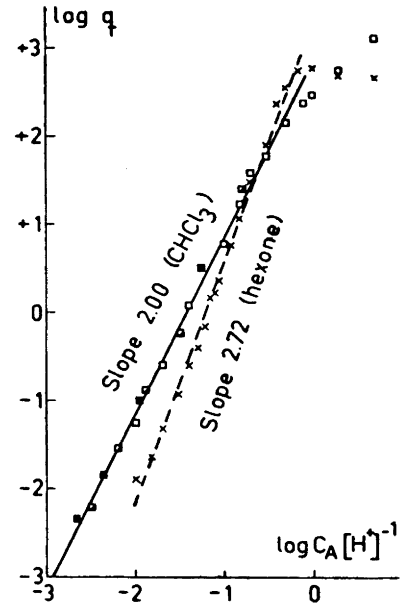


Fig. 1. The distribution of ^{233}U between a solution of di-*n*-butyl phosphate (DBP) in chloroform or hexone and 1 M $HClO_4$-$NaClO_4$. Chloroform data: □ 0.1 M H^+, ◪ 0.31 M H^+, ■ 0.91 M H^+. Hexone data: × 0.1 M H^+. q = distribution ratio $[U]_{org}/[U]_{aq}$. C_A = initial conc. of DBP in the organic phase. The data are given in Tables 1 and 2.

and hexone (dashed curve) is shown in Fig. 2 and may be explained by the fact that the oxygen atom in hexone is a good acceptor for the hydrogen atom in DBP.

Some conclusions on the reactions between UO_2A_2 and HA in hexone may be drawn from a plot of log $q[A^-]^{-2}$ against log [HA] (or log $[HA]_{org}$). The

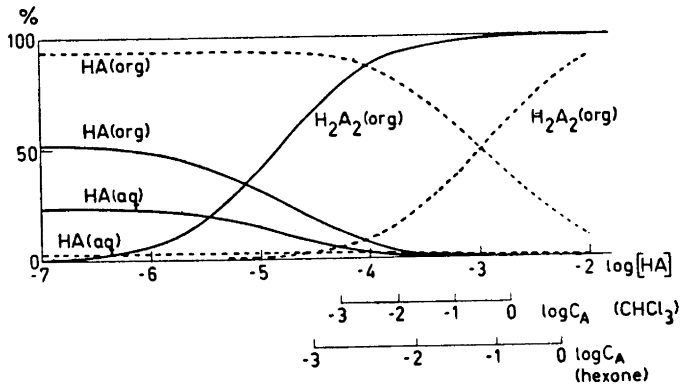


Fig. 2. The percentage of different DBP species in the two-phase systems chloroform/0.1 M $HClO_4$ + 0.9 M $NaClO_4$ (fulldrawn curves) and hexone/0.1 M $HClO_4$ + 0.9 M $NaClO_4$ (dashed curves) as a function of the conc. of HA (DBP monomer) in the aqueous phase. The volumes of the two phases are equal. $[H^+] = k_a$ and $[HA] = [A^-]$. The diagram is based on data given in the text.

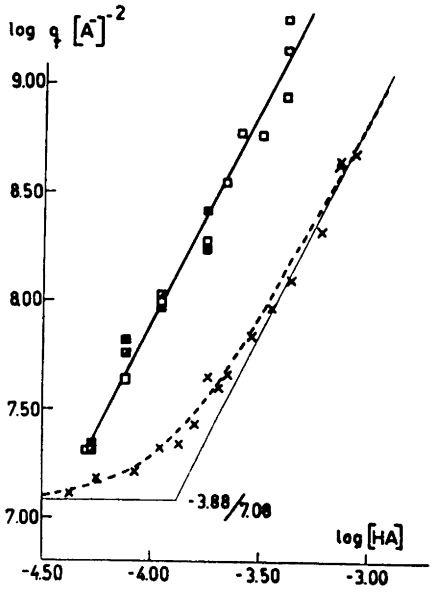


Fig. 3. Variation of $q[A^-]^{-2}$ with [HA]. These functions are calculated from data given in Tables 1 and 2. Fulldrawn line: chloroform-DBP system. Dashed curve: hexone-DBP system. The symbols and experimental points are the same as in Fig. 1. The horizontal asymptote to the hexone curve corresponds to $q[A^-]^{-2} = \lambda_2\beta_2$ and the equation for the asymptote with slope 2 is $q[A^-]^{-2} = \varkappa\lambda_2\beta_2[HA]^2$; the constants λ_2, β_2 and $\varkappa$ are defined in the text.

data in Fig. 1 (except those for $\log C_A[H^+]^{-1} > -0.2$) are therefore replotted in Fig. 3. Again the values for chloroform lie on a straight line with a slope of 2 demonstrating the presence of the complex $UO_2(HA)_2A_2$. The distribution with hexone is proportional to $[A^-]^2(1 + [HA]^2)$, which demonstrates the presence of both UO_2A_2 and $UO_2(HA)_2A_2$ in the hexone phase. The expression for the distribution ratio thus contains an additional term:

$$q = \frac{[UO_2A_2]_{org} + [UO_2(HA)_2A_2]_{org}}{[UO_2^{2+}]}$$

or, if we introduce the complexity and distribution constants β_2 and λ_2

$$\beta_2 = [UO_2A_2][UO_2^{2+}]^{-1}[A^-]^{-2};\ \lambda_2 = [UO_2A_2]_{org}[UO_2A_2]^{-1}$$

and the constant $\varkappa$ of the equilibrium

$$UO_2A_2(org) + 2\ HA(aq) = UO_2(HA)_2A_2(org)$$

$$q[A^-]^{-2} = \lambda_2\beta_2 + \varkappa\lambda_2\beta_2[HA]^2$$

The values of $\log \lambda_2\beta_2 = 7.08$ and $\log \varkappa = 7.76$ may be calculated from Fig. 3. The data give no evidence for the existence of an intermediate complex $UO_2(HA)A_2$ in appreciable amounts. Using the constants given above for the dissociation, distribution and dimerization of DBP the values of $\log K$ for the reaction

$$UO_2^{2+}(aq) + 2\ H_2A_2(org) = UO_2(HA)_2A_2(org) + 2\ H^+(aq)$$

may be calculated as 3.58 (chloroform) and 4.50 (hexone).

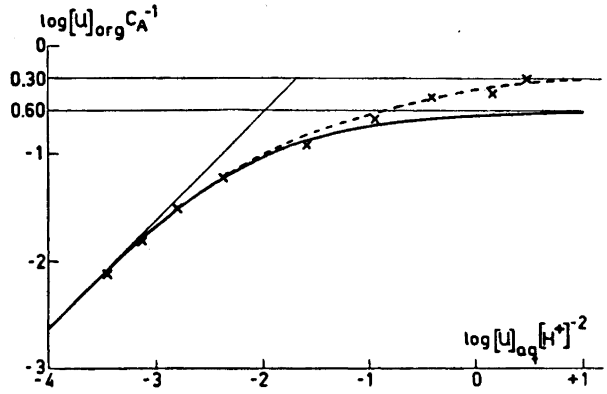


Fig. 4. The effect of increasing uranium concentration on the extraction of U(VI) by a solution of 0.04 M DBP in chloroform. The data are plotted as log $[U]_{org}C_A^{-1}$ against log $[U]_{aq}[H^+]^{-2}$. The fulldrawn curve is calculated assuming only the complex $UO_2(HA)_2A_2$ in the chloroform phase. The dashed curve is calculated assuming that, in addition to $UO_2(HA)_2A_2$, the dinuclear complex $(UO_2)_2A_4$ is formed in the chloroform phase.

Although such experiments as these cannot decide whether a complex is formed between hexone and UO_2A_2, the presence of UO_2A_2 in hexone and not in chloroform is very likely due to such a complex formation. Reactions between UO_2A_2 and hexone might be studied by distribution experiments with solutions of hexone in chloroform. Calculations on such data presuppose, however, a corresponding study of DBP in order to be able to calculate the variation of [HA] with $[\text{hexone}]_{CHCl_3}$.

It is thus possible to explain that the hexone data in Fig. 1 do not follow a straight line with a slope 2 by assuming the presence of HA and UO_2A_2 in the hexone phase. Similar values of the slope, that are higher than 2 have been reported by Stewart and Hicks [11]. However, their results are probably further complicated by the fact that they use nitric acid. It is therefore quite

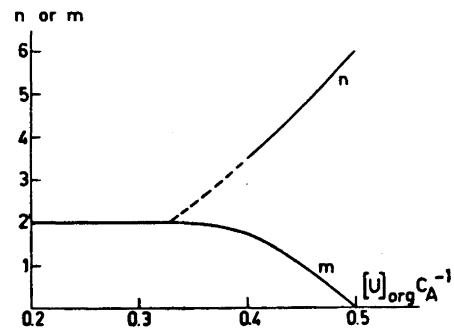


Fig. 5. Calculated mean values of n and m of the complex $(UO_2)_n(HA)_mA_{2n}$ in di-octyl phosphate-hexane solutions as a function of $[U]_{org}C_A^{-1}$. The calculations (see text) are based on data given by Baes *et al.*[5], Fig. 2.

Table 3. The distribution of U(VI) between a solution of 0.04 M DBP in chloroform and 0.1 M $HClO_4$ with increasing conc. of uranium. The volumes of the two phases are equal.

$[UO_2^{2+}]_{aq}$ [a] M	q	$[H^+]$ [b] M	$\log \frac{[U]_{aq}}{[H^+]^2}$	$\log \frac{[U]_{org}}{0.04}$
0.08	0.332	0.1399	+0.49	−0.30
0.04	0.595	0.1298	+0.17	−0.43
0.02	2.09	0.1270	−0.40	−0.47
0.01	5.32	0.1168	−0.94	−0.68
0.005	15.0	0.1094	−1.58	−0.93
0.0025	54.4	0.1048	−2.38	−1.22
0.00125	76.2	0.1024	−2.81	−1.51
0.0006277	81.4	0.1012	−3.13	−1.81
0.0003152	88.3	0.1006	−3.46	−2.11
0 (extrapol.)	95.5	0.1000	—	—
2.69×10^{-6}	95.5 [c]	0.1000	—	—

[a]) Initial conc. of U (VI) in the aqueous phase.
[b]) Calculated from $0.1000 + 2[U]_{org}$.
[c]) Interpolated from values in Table 1.

possible that some uranium was extracted as uranyl nitrate into the organic phase (butyl ether or hexane), DBP acting like a tri-alkyl phosphate (*e.g.* TBP). Other oxygen-containing solvents such as TBP, ethers, esters, and alcohols may react in a similar way to hexone.

B. Experiments with increasing uranium concentration. In order to study the possible formation of UO_2A_2 from $UO_2(HA)_2A_2$ at higher concentrations of uranium a separate investigation was carried out with 0.04 M DBP in chloroform. The data are given in Table 3 and plotted in Fig. 4. A similar plot has been published by Baes *et al.*[5], Fig. 3 for the dioctyl phosphate-hexane system. It is seen from Fig. 4 that the ratio $[U]_{org}/C_A$ is increased above 0.25 to a limiting value of 0.5 The simple equilibria

$$UO_2^{2+}(aq) + 2\ H_2A_2(org) = UO_2(HA)_2A_2(org) + 2\ H^+(aq)$$

can thus not explain the whole extraction process.

The lower full-drawn curve in Fig. 4 is based on the reaction above using the value of $10^{3.38}$ at $C_A = 0.04$ M for the equilibrium constant, K, and calculating the free conc. of H_2A_2 in the chloroform phase as $C_A/2 - 2\ [U]_{org}$. The equation for this curve is

$$[U]_{org} = K[U]_{aq}[H^+]^{-2}(C_A/2 - 2\ [U]_{org})^2$$

This equation seems to be valid up to $[U]_{org}C_A^{-1} \sim 0.1$. The same result was found by Baes *et al.*[5] for the dioctyl phosphate-hexane system.

In order to explain the increase of $[U]_{org}C_A^{-1}$ above 0.25 several simple reactions may be considered. One might assume the formation of only mononuclear complexes, $UO_2(HA)A_2$ and UO_2A_2, or the formation of dinuclear com-

plexes such as $(UO_2)_2(HA)_2A_4$ and $(UO_2)_2A_4$. With constants for the formation of two complexes in addition to the formation constant of $UO_2(HA)_2A_2$ (K) it should be possible to explain the data in Fig. 4 very well. In fact the dashed curve in Fig. 4 has been derived assuming only the reaction (equilibrium constant = 2.25 K)

$$2\,UO_2^{2+}(aq) + 2\,H_2A_2(org) = (UO_2)_2A_4(org) + 4\,H^+(aq)$$

in addition to reaction given above (eq. const. = K). Also there might be complexes such as UO_2A^+ and UO_2A_2 formed in the aqueous phase. It is therefore not possible to draw any definite conclusions from the distribution data about the reactions in the chloroform phase at higher concentrations of uranium.

Baes *et al.*[5] have published, for solutions of U(VI) and dioctyl phosphate in *n*-hexane, some isopiestic results which indicate the formation of polymers with more than two uranyl ions. Calculations * on Fig. 2 in the work of Baes *et al.*[5] allow an approximate determination of the mean values of n and m in $(UO_2)_n(HA)_mA_{2n}$ as a function of $[U]_{org}C_A^{-1}$. The curves obtained are shown in Fig. 5. It may be seen that no change in m (= 2) seems to occur at values of $[U]_{org}C_A^{-1}$ below 0.33. It seems as if Baes *et al.*[5] have not considered a decrease in m. Viscosity measurements have lead them assume the presence of complexes with considerably higher values of n than 6. However, the viscosity properties of the dioctyl phosphate with its longer hydrocarbon groups may differ considerably from DBP (*i.e.*.. micelles may be formed much more readily).

Acknowledgement. The present investigation has been supported by Atomkommittén *(Swedish Atomic Energy Council)*, and by a grant from the *Inštitut Jozef Stefan*, Ljubljana, Yugoslavia. The authors also wish to thank Mr. H. A. C. McKay, AERE, Harwell, England, for support in obtaining the uranium-233, and Dr. H. S. Dunsmore for revising the English text. Sincere thanks are due to Professor Lars Gunnar Sillén for his advice and his criticism of the manuscript.

* For the calculation of the curves in Fig. 5 we have used the following approximate equations:

$$\begin{aligned} C_m &= [(UO_2)_n(HA)_mA_{2n}] + [H_2A_2] \\ [U]_{org} &= n[(UO_2)_n(HA)_mA_{2n}] \\ C_A &= (2n+m)[(UO_2)_n(HA)_mA_{2n}] + 2[H_2A_2] \\ C_A - 2\,C_m &= \frac{2n+m-2}{n}\cdot[U]_{org} \end{aligned}$$

If we apply this equation to the data of Baes *et al.* [5, Fig. 2], read from a smoothed curve, we obtain $\frac{2n+m-2}{n} = 2$ for $[U]_{org}C_A^{-1} < 0.33$; *i.e.*, $m = 2$; whereas n is undetermined. With decreasing $[U]_{org}C_A^{-1}$ the complex $UO_2(HA)_2A_2$ should finally dominate, *i.e.*, $n \to 1$. For values of $[U]_{org}C_A^{-1} > 0.4$ we might plausibly neglect $[H_2A_2]$ and then estimate n from $C_mC_A^{-1} = \frac{1}{2n+m}$ and $[U]_{org}C_A^{-1} = \frac{n}{2n+m}$.

REFERENCES

1. Brown, K. B. *et al.*, *Second Int. Conf. on the Peaceful Uses of Atomic Energy*, Geneva 1958, Paper 1550.
2. Peppard, D. F. *et. al. J. Inorg. & Nuclear Chem.* **4** (1957) 334, 344; **5** (1957) 141.
3. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1277.
4. Kosolopoff, G. M. and Powell, J. S. *J. Chem. Soc.* **1950** 3535.
5. Baes, C. F., Zingaro, R. A. and Coleman, C. F. *J. Phys. Chem.* **62** (1958) 129.
6. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1771.
7. Peppard, D. F., Ferraro, J. R. and Mason, G. W. *J. Inorg. & Nuclear Chem.* **4** (1957) 371.
8. Crouthamel, C. E. and Johnson, C. E. *Anal. Chem.* **24** (1952) 1780.
9. Dyrssen, D. and Sillén, L. G. *Acta Chem. Scand.* **7** (1953) 663.
10. Dyrssen, D. *Proc. Int. Symp. on the Chemistry of the Coordination Compounds*, Rome 1957.
11. Hyde, E. K. *Int. Conf. on Peaceful Uses of Atomic Energy*, Geneva 1955, Vol. **7**, p. 290.

Received December 31, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 571—592

Electrophoresis Studies on Blood Plasma Esterases

I. Mammalian Plasmata

KLAS-BERTIL AUGUSTINSSON

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

The esterase activity of mammalian plasmata was investigated using as substrates a series of aliphatic, aromatic, heterocyclic and choline esters. Electrophoresis on cellulose columns was used for separation of the various esterases present in each plasma. The distribution of esterase activity and total protein after electrophoretic separation are reported for the plasmata of man, monkey, horse, cow, reindeer, goat, swine, cat, dog, guinea-pig, rabbit, and rat. Each esterase component was further studied as to substrate specificity and sensitivity to certain selective inhibitors.

Three types of esterases were present in the plasmata studied. An arylesterase (aromatic esterase) migrated on electrophoresis in most cases together with or close to the albumins, an aliesterase ("lipase") in the α_1-globulin region, and a cholinesterase between the α_2- and β-globulins. Within each group of esterases there were species variations in the properties of the individual enzymes. All plasmata studied contained an arylesterase which hydrolysed phenyl acetate at a higher rate than phenyl butyrate. Two arylesterases were found in the plasma of reindeer and rabbit. Aliesterases hydrolysed aliphatic and aromatic esters but not choline esters; phenyl acetate was hydrolysed at a lower rate than phenyl butyrate. Such enzymes were not detected in human, monkey, and dog plasmata. The concentration and properties of the plasma cholinesterases varied greatly with species. The ruminant plasmata had very low or no cholinesterase activity. Butyrylcholinesterase was present in the man, horse, cat, dog, and guinea-pig; propionylcholinesterase was found in the cow, rabbit, and rat; and acetylcholinesterase was present in the goat. Yet another type of cholinesterase occurred in swine plasma.

Differentiation of blood plasma esterases has previously been based mainly on substrate specificity and inhibitor experiment with orginal plasma (serum) or crude enzyme preparations. In the present investigation these enzymes were separated electrophoretically, the properties of the esterase fractions obtained were studied, and the esterase patterns of various vertebrate plasmata were compared.

The results, briefly summarized in a previous communication [1], showed that vertebrate plasmata contain three types of esterases, the characteristic features of which are as follows:

Arylesterases: aromatic esterases which hydrolyse phenyl acetate at a higher rate than phenyl butyrate; aliphatic esters are normally not attacked. They are resistant to certain organophosphorus compounds and to physostigmine, and have, in most cases, the greatest electrophoretic mobility of the three esterase groups.

Aliesterases, including lipases: both aliphatic and aromatic esters are hydrolysed, but not choline esters. There exist acetyl-, propionyl- and butyryl-aliesterases. They are sensitive to 10^{-5} M of a great number of organophosphorus compounds, but resistant to 10^{-5} M physostigmine; some of them are sensitive to higher concentrations of the latter compound. In most cases, these enzymes move electrophoretically more slowly than the arylesterases, but more rapidly than cholinesterases.

Cholinesterases: choline esters are split at a higher rate than both aliphatic and aromatic esters, the latter usually being hydrolysed at a lower rate than aliphatic esters or not at all; the specificity regarding the acyl group varies greatly. These esterases are sensitive to many organophosphorus compounds 10^{-6} M or less and to physostigmine, the latter giving complete inhibition in 10^{-5} M or lower concentration. They have the lowest electrophoretic mobility of all plasma esterases.

In the present and following reports, describing the experimental results on which the above classification is based, these three types of enzymes will be referred to, for practical reasons, as A-, B- and C-esterases respectively.

MATERIAL AND METHODS

Plasmata. Plasma was prepared from heparinized blood (venous or heart) and was free from all cells, including thrombocytes. Serum instead of plasma was used in some cases; both had the same esterase activity when tested with various substrates.

Substrates. The aliphatic, aromatic and choline derivatives used were the acetyl (Ac), propionyl (Pr), and butyryl (Bu) esters of the following radicals: *iso*amyl (Am), glycerol (T; *e. g.*, tributyrin, TBu), phenyl (Ph) and choline (Ch); also employed were acetyl-β-methylcholine (MeCh), benzoylcholine (BzCh), succinylcholine (SuCh), the carbon analogue of acetylcholine (3,3-dimethylbutyl acetate), Tween 20 (polyoxyethylene sorbitan monolaurate), procaine hydrochloride, atropine sulphate, cocaine hydrochloride, and heroin. Choline esters were all used in the form of iodides. The substrates were dissolved in a bicarbonate-CO_2 buffer solution (pH 7.4) to give 0.01 M in the reaction mixture.

The activity of some plasma protein fractions was also tested against indoxyl acetate. One half ml of the fractions from electrophoresis were added to 1.0 ml of a 10^{-3} M substrate solution. The intensity of indigo-blue dye formed in 30 min at 22°C was determined in these preliminary studies.

Esterase inhibitors. Eight compounds frequently used as selective enzyme inhibitors in recent esterase studies [2] were employed: physostigmine salicylate, tetra-*iso*propylpyrophosphoramide (*iso*-OMPA), *bis*-mono*iso*propylamino fluorophosphine oxide (mipafox), 10-(1-diethylaminopropionyl) phenothiazine hydrochloride (Astra 1397), 1,5-*bis*(4-trimethylammoniumphenyl) pentane-3-one diiodide (62C47), *bis*-(piperidinomethyl coumaranyl-5) ketone (3318CT), atoxyl (sodium arsanilate), and quinine sulphate. The compounds were dissolved in distilled water, and the enzyme preparations were incubated at 25°C for 50 min with the inhibitor solution before addition of substrate. The efficiency of esterase inhibition was expressed in pI_{50} values, *i. e.* negative logarithm of molar inhi-

Table 1. Enzymic hydrolysis of various esters by mammalian plasmata. Samples from at least three animals of each species were analysed. The activity values (b_{30}) refer to initial hydrolysis rates with a representative sample and are expressed in μl CO_2 per 0.10 ml of plasma per 30 min, corrections made for nonenzymic hydrolysis. Where values are indicated in brackets, initial hydrolysis rate was determined by extrapolation. An asterisk denotes that the activity varies greatly from individual to individual. Code names are explained in the text.

Species	AcCh	PrCh	BuCh	MeCh	BzCh	SuCh	AmAc	AmPr	AmBu	TAc	TPr	TBu	PhAc	PhPr	PhBu
Man	135	310	360	2	50	3	10	15	50	5	12	55	3 300	1 000	260
Monkey (Macaque	22	44	71	1	9	1	2	3	6	2	6	10	655	200	50
Monkey Mangabey)	180	390	540	2	70	8	8	25	75	10	65	90	225	290	395
Horse	130	225	365	2	40	1	25	40	70	25	245	100	2 200	650	310
Cow	3	4	2	0	0	0	4	4	3	1	2	4	4 700	600	100
Reindeer	8	14	15	<1	2	0	1	2	4	2	5	4	250	100	50
Goat	8	7	2	2	<1	<1	2	1	2	2	4	2	5 600	590	160
Sheep	7	7	2	2	<1	<1	2	2	2	2	3	2	5 500	550	150
Swine	16	14	23	1	3	2	4	1	2	<1	4	3	450*	200*	25*
Cat	50	75	150	5	12	1	6	8	40	45	260	80	5 700	1 250	500
Dog	70	115	180	6	30	0	4	<1	6	3	10	12	2 800	600	240
Guinea-pig	50	130	170	5	20	0	(150)	(200)	(30)	200	530	530	1 700	2 130	1 200
Rabbit	16	16	10	4	2	0	4	6	15	20	40	50	7 500*	2 400*	650*
Rat	20	30	15	5	10	1	(50)	(85)	(90)	45	150	90	3 000	1 700	1 150

Table 2. Susceptibility of plasma esterases to various inhibitors. A, B and C are the esterase types referred to in the text. Values refer to negative logarithm of molar inhibitor concentration producing 50 % esterase inhibition. They were obtained in experiments with plasma and/or

Species	Physostigmine			*iso*-OMPA			Mipafox			Astra 1397		
	A	B	C	A	B	C	A	B	C	A	B	C
Man	<3	—	7.7	<3	—	6.3	<3	—	6.7	<3	—	5.6
Monkey *	<3		8.5	<3		8.0	<3		8.0			≈6
Horse	<3	<3	8.0	<3	<3.5	7.7	<3	5.7	7.5	<3.5	<3.5	5.7
Cow	<3	<3	7.3	<3	≈6	≈6	<3	≈6	≈6	<3	<3	<3.5
Reindeer	<3	—	7.2	<3	—	7.6	<3	—	7.5	<3	—	5.2
Goat	<3		7.5	<3		3.8	<3		<4	<3		<3
Swine **	<3.5		7.0	<3		6.5	<3.5		6.5	<3		4.1
Cat	<3	≈3.5	7.8	<3	≈7	7.2	<3.5	≈8	7.7	<3.5	<3.5	5.8
Dog ***	<3		8.5	<3		7.7	<3		7.5	<3		6.2
Guinea-pig ****	<3	<3	8.5	<3	5.2	7.0	<3	7.9	7.0	<3	<3	6.2
Rabbit	<3.5	<3	6.4	<3.5	6.9	<4	<3	5.6	4.2	<3.5	<3	<3.5
Rat	<3.5	<3	6.3	<3.5	5.8	6.7	<3	7.4	6.4	<3.5	<3	6.6

* Values obtained with a plasma sample from macaque monkey.
** Values obtained with a plasma having high activity (b_{30} 300) against phenyl acetate.

bitor concentration producing 50 % enzyme inhibition; these values were obtained by extrapolation from curves of inhibition (%)-inhibitor concentration (pI), where sufficient inhibitor was used at the higher pI levels to establish the point of complete inhibition. The active fractions obtained in electrophoresis experiments were dialysed against the bicarbonate buffer prior to testing their sensitivity to inhibitors.

Electrophoresis. Preparative column electrophoretic separation was performed with an apparatus described by Svensson [3] using cellulose powder. ("Munktell's cellulose powder", prepared according to the direction of Flodin and Kupke [4]). Electrophoretic runs were carried out in veronal buffer solution (pH 8.4, $I = 0.1$) at 5—11°C as maintained by a water-cooled jacket. Columns of two sizes were used, 3 cm × 40 cm and 1.5 cm × 40 cm (values in brackets refer in the following to the smaller column); in most cases the larger column was employed unless otherwise stated. For each run, 5 ml (1.5 ml) of buffered plasma (serum in some instances) was used. The material, previously dialysed against the buffer solution, was introduced into the column and the zone allowed to move 2—3 cm below the surface of the cellulose. The electrode vessels were filled with buffer solution and the electrophoresis started. The current was 60 mA (30 mA), provided by an applied voltage of 260 V (340 V), and the duration of runs 30 h (16 h). After the completion of electrophoresis, the liquid in the column was displaced at a rate of 10 ml per hour in 2.8 ml (1.5 ml) fractions. The protein concentration of each fraction was estimated by a modified Folin procedure [5].

Assay of esterase activity. The esterase activity was determined manometrically by the Warburg technique [2] at 25°C in a bicarbonate-CO_2 buffer solution, pH 7.4, with 2.00 ml total liquid volume per flask. Results are expressed in b_{30} values, *i. e.*, the μl of CO_2 evolved during 30 min, after correction for non-enzymic hydrolysis of the substrates. The activities reported refer to initial hydrolysis rates for 0.10 ml of plasma or esterase fraction, unless otherwise stated.

RESULTS

The hydrolysis rates (values obtained with a representative sample selected from at least three animals) of various substrates by original plasma samples

separated esterase fractions using various esters as substrates. The enzyme preparation was incubated for 50 min with the inhibitor prior to addition of substrate. Code names are explained in the text.

3318CT			62C47			Atoxyl			Quinine sulphate		
A	B	C	A	B	C	A	B	C	A	B	C
<3	—	≈5	<3	—	4.8	<3	—	5.8	<3	—	5.0
		5.2			4.1			5.0			4.5
<3.5	<3	5.4	<3.5	<3.5	4.2	<3.5	<3.5	4.5	<3.5	<3.5	4.5
<3.5	<3.5	≈7	<3	<3	<5.5	<3.5	<3.5		<3		
<3	—	5.4	<3	—	4.2		—	4.1		—	4.1
<3		7.3	<3		5.9			<4			<3.5
<3		4.0	<3		3.4	<3.5		3.3	<4		4.5
<3	<3	5.2	<3	<3.5	4.7	<3.5	≈7	4.0	<3.5	<3.5	3.7
<3		4.8	<3		3.5	<3.5		<3.5	<4		4.0
<3	<3.5	4.5	<3	<3.5	3.9	<3	<3	4.3	<3	<3	<4
<3	<3	5.5	<3	<3	5.7	3.0	5.1	<2.5	4.0	<4	<3
<3.5	<3	4.4	<3	<3	4.1	<3	<3	<4	<4	<3.5	4.1

*** Values for C-esterase refer to the main peak fraction of the cholinesterase region after electrophoresis (Fig. 9).

**** Values for B-esterase refer to peak B1 (Fig. 10).

are summarized in Table 1. Activity values (b_{30} per 0.1 ml plasma) obtained with additional substrates are given in the text. The electrophoretic patterns presented in Figs. 1—12 show the rate of elution of the esterases in relation to various protein constituents, which are designated by the usual nomenclature [6]. In the legends to these figures are summarized in tabular form the relative substrate specificity and sensitivity to certain inhibitors of each esterase component. The peaks of esterase activity are designated A, B and C, corresponding to the three groups of esterases referred to in the introduction. In Table 2 the inhibitor studies are then summarized.

Human plasma (Fig. 1). The activity of human plasma in the hydrolysis of various esters has been extensively studied by many workers, and the results presented in Table 1 generally confirm previous observations, *e.g.*, that human plasma hydrolyses butyrylcholine at the highest rate of the choline esters, that the butyric acid esters of aliphatic alcohols are hydrolysed more rapidly than their other acyl homologues, and that phenyl butyrate is hydrolysed more slowly than the acetate and propionate.

On electrophoresis of human plasma two regions of high esterase activity appeared. The two peaks (A and C) are inferred to be due to the presence of at least two distinct enzymes of differing electrophoretic mobility: one (A) moved together with the albumin components and the other (C) was found between the α_2- and β-globulin fractions. A third esterase peak, demonstrated in the α-globulin region (between the peaks A and C) in most mammalian plasmata, was not detected in human plasma.

The A-esterase(s) hydrolysed phenyl acetate at a rate which was approximately 100 times greater than for phenyl butyrate. Neither the aliphatic

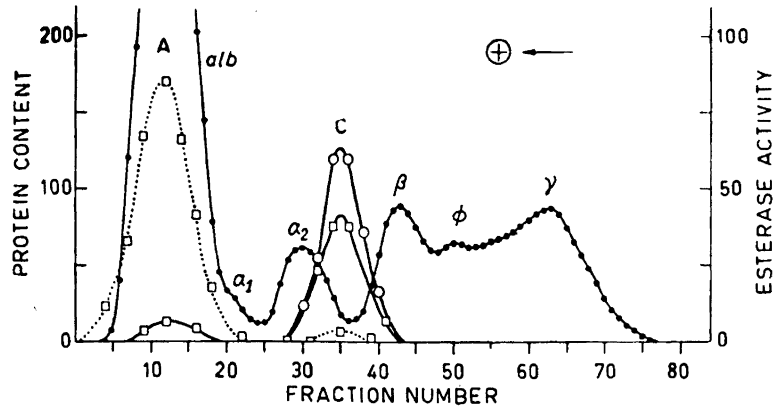


Fig. 1. Human plasma. Distribution of esterase activity and total protein after column electrophoresis (veronal buffer, pH 8.4; I = 0.1; 260 V; 60 mA; 30 h). The protein content is expressed as relative amount based on extinction values. Esterase activity is expressed in b_{30} values with the ml aliquots from each fraction as indicated below: ●——●, protein concentration; □.....□, PhAc (0.015); □——□, PhBu (0.10) and TBu (0.40); ○——○, BuCh (0.10).
Esterase activities (b_{30}/0.10 ml) against various substrates and esterase inhibition (pI_{50}) by physostigmine (Phys) and an organophosphorus compound of the peak fraction from each esterase component. Hydrolysis rates for indoxyl acetate expressed as relative amount (based on extinction values) of indigo blue formed after 30 min incubation with the peak fraction.

Esterase component	PhAc	PhBu	TBu	BuCh	BzCh	Indoxyl acetate	pI_{50}	
							Phys	Mipafox
A	560	7	0	0	0	8	<3	<3
C	24	40	10	64	7	80	7.7	6.7

nor the choline esters tested were hydrolysed by this enzyme which was resistant to all the inhibitors studied. Whether more than one arylesterase was involved in this esterase peak has not yet been established. It is possible that some human plasmata contain in low concentration an esterase running electrophoretically close to but more rapidly than the main peak fraction since the esterase peak was not uniform.

The C-esterase was a typical butyrylcholinesterase of low specificity. It also hydrolysed benzoylcholine, succinylcholine, and the butyrates of both phenol and aliphatic alcohols including glycerol. In addition, this enzyme hydrolysed procaine (b_{30} 0.5), atropine (b_{30} 0.3), Tween 20 (b_{30} 16) and the carbon analogue of acetylcholine (b_{30} 20). Cocaine and heroin hydrolyses were not detected with human plasma. The C-esterase was sensitive to all inhibitors studied (Table 2), with physostigmine being the most potent, and 62C47 and quinine the less potent compounds.

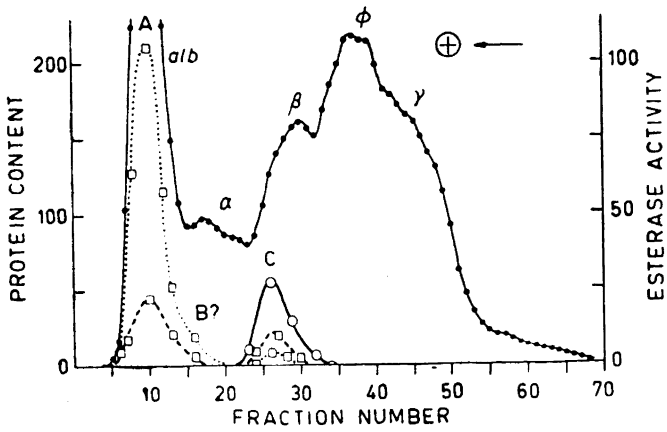


Fig. 2. Macaque-monkey plasma. Conditions as for Fig. 1. □.....□, PhAc (0.10); □— — — —□, PhPr (0.20); O———O, BuCh (0.20).

Species	Esterase component	PhAc	PhPr	PhBu	TBu	BuCh	pI_{50}	
							Phys	Mipafox
Macaque	A	105	11	1	0	0	<3	<3
	C	4	6	8	2—3	14	8.5	8.0
Mangabey	A or B	3	—	7	0	0	<3	>5
	C	50	—	95	25	150	8.4	8.0

Human plasma therefore contains two esterases. The arylesterase was responsible for 96 % of the hydrolysis of phenyl acetate and 14.5 % of that of phenylbutyrate, with the cholinesterase accounting for the remainder of the hydrolysis of these phenyl esters and for the entire activity against choline esters and triglycerides. In addition, preliminary experiments have shown that cholinesterase is responsible for more than 90 % of the hydrolysis of indoxylacetate by human plasma.

Monkey plasma (Fig. 2). Plasmata from the macaque monkey (*Macacus cynomolgus*) and the mangabey monkey (*Cercocebus torquatus*) were studied. The macaque plasma was similar to human plasma in pattern of esters hydrolysed, but the hydrolysis rates were much lower for the monkey plasma. The mangabey plasma hydrolysed choline esters at 10 times the rate of the macaque plasma, but hydrolysed the phenyl esters at a lower rate than either human or macaque plasma. Phenyl butyrate was hydrolysed more rapidly than the acetate.

The electrophoretic pattern of the macaque plasma is illustrated in Fig. 2; the legend of this figure also gives the results with the mangabey plasma. Similar to human plasma, the macaque plasma contained two distinct esterases. The A-esterase was eluted coincident with the albumin fraction and the

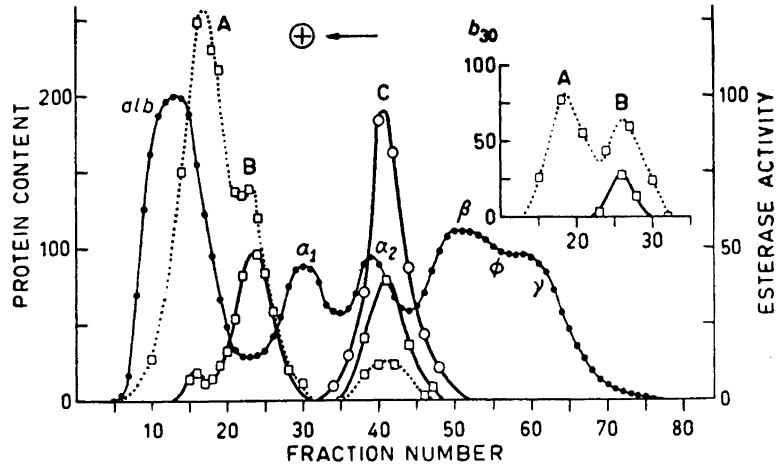


Fig. 3. Horse plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.05); □——□, PhBu (0.10); O——O, BuCh (0.10).

Esterase component	PhAc	PhBu	TPr	TBu	AmBu	PrCh	BuCh	BzCh	Indoxyl acetate	pI_{50}	
										Phys	Mipafox
A	260	9	0	0	0	0	0	0	2	<3	<3
B	140	49	41	20	18	0	0	0	20	<3	5.7
C	28	40	6	7	6	65	95	10	26	8.0	7.5

The insert is the electrophoretic pattern of a mixture of 3.8 ml of peak fraction A and 8.0 ml of peak fraction B (main diagram) showing clearer resolution of the enzymes. □ ..... □, PhAc (0.40); □——□, PhBu (0.40).

C-esterase between the α_2- and β-globulins. The first enzyme was a typical acetylarylesterase which was resistant to both physostigmine and certain organophosphorus compounds. Although aliesterase (B) was not detected in the macaque plasma, the activity may have been too low after electrophoresis to be determined by the techniques employed.

The butyrylcholinesterase (C) of the macaque plasma was present in low concentration compared with human and mangabey plasmata. This esterase was also responsible for the hydrolysis of tributyrin and for up to 65 % of the plasma activity against phenyl butyrate.

The mangabey plasma probably contained a B-esterase in comparatively low concentration. The low activity of the fractions did not allow assay of this enzyme for inhibition by organophosphorus compounds. Since phenyl butyrate was hydrolysed more rapidly than phenyl acetate and the esterase was eluted later than the A-esterase of macaque plasma, it appears likely that the mangabey esterase was a B-rather than an A-estrase. The butyrylcho-

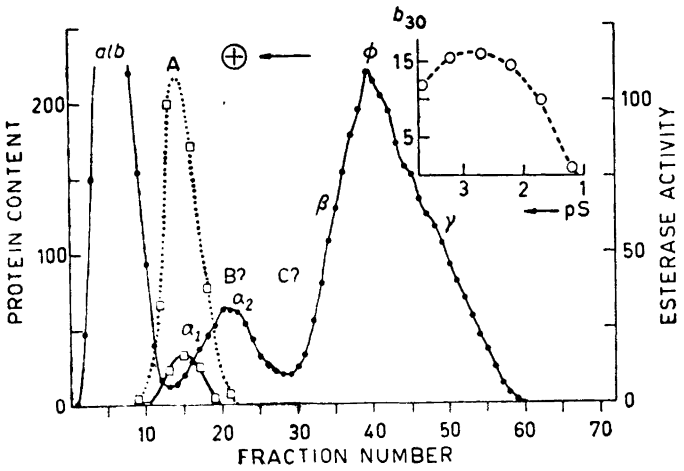


Fig. 4. Cow plasma. Results obtained with a sample from calf. Conditions as for Fig. 1. □.....□, PhAc (0.01); □——□, PhBu (0.40).

Esterase component	PhAc	PhBu	TBu	PrCh	pI_{50}	
					Phys	*iso*-OMPA
A	2 180	10	0	0	<3	<3
B	?	?	<1	0	<3	≈6
C	?	?	<1	<1	7.3	≈6

The insert is the activity-pS curve for the hydrolysis of propionylcholine by cow plasma (0.40 ml).

linesterase, present in high concentration, was responsible for the hydrolysis of tributyrin.

Horse plasma (Fig. 3). The pattern for hydrolysis of various esters by horse plasma was similar to that for human plasma except for the hydrolysis of the triglycerides, where tripropionin was optimal for horse plasma and tributyrin for human plasma.

Horse plasma contained three types of esterases resolved in three distinct peaks by electrophoresis. The A- and B-esterases migrated between the albumin and α_1-globulin components, and the C-esterase between the α_2- and β-globulins. The A-esterase was a typical acetylarylesterase, being resistant to all inhibitors studied. *p*-Nitrophenyl acetate was hydrolysed at a lower rate than phenyl acetate. The B-type esterase hydrolysed both aromatic and aliphatic esters, the propionates being split at a higher rate than the corresponding acetates and butyrates. This enzyme was also responsible for the hydrolysis of "Tween 20" (b_{30} 35). It was less sensitive to organophosphorus compounds than most B-esterases; *e.g.*, this esterase was resistant to *iso*-OMPA, while mipafox gave the same relatively low pI_{50} value (5.7), when this

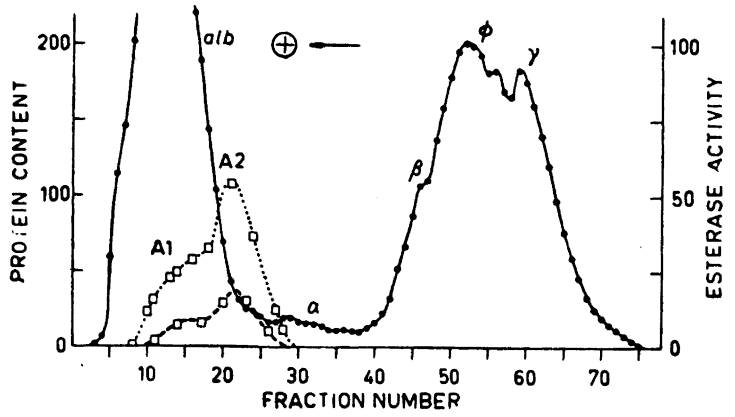


Fig. 5. Reindeer plasma. Conditions as for Fig. 1. □.....□, PhAc (0.40); □— — —□, PhPr (0.40). N.B.: the activity of the arylesterases was much lower than expected after electrophoresis (*cf.* Table 1).

Esterase component	PhAc	PhPr	PhBu	PrCh	pI_{50}	
					Phys	*iso*-OMPA
A1	7	2.5	?	0	<3	<3
A2	14	5	<1	0	<3	<3
C	?	?	<1	<1	7.2	7.6

B-esterase was assayed with a number of various substrates. Poor inhibitor efficiency was also observed with the quaternary ammonium compounds, the phenothiazine derivative (Astra 1397), atoxyl, and quinine sulphate. A better separation of the A-and B-esterases was accomplished by re-electrophoresis of the two peak fractions (inserted diagram of Fig. 3).

The C-esterase was a typical butyrylcholinesterase having the lowest specificity of the three esterases present. Its inhibition pattern was similar to that of human plasma cholinesterase. At least three enzymes in horse plasma hydrolyse aromatic esters since the cholinesterase was also active on these substrates. Indoxyl acetate was also hydrolysed by all three esterases, the C- and B-esterases being more active than the arylesterase.

Cow plasma (Fig. 4). Of the substrates tested, only the aromatic esters were hydrolysed at appreciable rates. The enzyme catalysing this reaction was a typical acetylarylesterase which had a lower electrophoretic mobility relative to that of the albumin components than was usually found for other mammalian plasma arylesterases.

Inhibitor experiments with the original cow plasma showed that tributyrin was hydrolysed by two esterases both present in very low concentration. Cholinesterase accounted for 80 % of the total hydrolysis of this ester with the remainder being hydrolysed by an esterase (probably a B-type esterase)

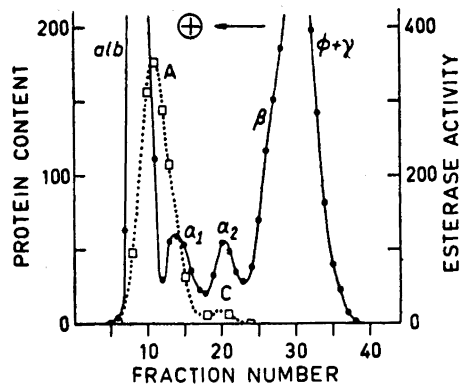


Fig. 6. Goat plasma. Electrophoresis was carried out on a small column. Otherwise, conditions were as for Fig. 1. □ ..... □, PhAc (0.10).

Esterase component	PhAc	AcCh	pI_{50}		
			Phys	Mipafox	3318 CT
A	360	0	<3	<3	<3
C	15	1	7.5	<4	7.3

resistant to physostigmine and sensitive to mipafox and *iso*-OMPA. The activities of the B- and C-esterases were too low to be detected in fractions after electrophoresis.

The proportion between the hydrolysis rates for phenyl acetate and phenyl butyrate was 47:1 for the whole plasma and 218:1 for the arylesterase peak fraction; thus the activity against the butyrate was reduced much more than that against the acetate by dialysis and electrophoresis. It appears therefore that the phenyl esters were hydrolysed by at least two enzymes of cow plasma. The sensitivity of these esterases to organophosphorus compounds was not successfully tested.

The physostigmine-sensitive esterase was a propionylcholinesterase which was inhibited by excess propionylcholine (see inserted graph of Fig. 4). This esterase was sensitive to those compounds (3318CT and 62C47) which are regarded as being selective inhibitors of acetylcholinesterase (of erythrocytes and nerve tissue), and was resistant to "Astra 1397". The cholinesterase of cow plasma therefore differs greatly from the cholinesterase present in most other mammalian plasmata.

Reindeer plasma (Fig. 5). As noted for other ruminant plasmata, the reindeer plasma hydrolysed most esters at a very low rate. Although the aromatic esters were attacked more readily than the other substrates even here the hydrolysis rates were comparatively low. Two esterase peaks were found after electrophoretic separation; they were due to the presence of two acetylarylesterases (A1 and A2), the properties of which were similar, in that both

enzymes were resistant to *iso*-OMPA and physostigmine. This plasma probably does not contain any B-type esterase.

The cholinesterase present in low concentration was a butyryl- (or propionyl-) cholinesterase, the activity of which was not inhibited by high substrate concentration. This enzyme was responsible for 20 % of the plasma activity against phenyl butyrate.

Goat plasma (Fig. 6) *and sheep plasma.* The goat plasma hydrolysed phenyl acetate at a very high rate due to the presence of an acetylarylesterase. A few per cent of the phenyl acetate hydrolysis was due to a second esterase. Inhibition experiments showed this second enzyme to be a cholinesterase which had the properties of an acetylcholinesterase. It was inhibited by high concentration of acetylcholine which explains why the peak fraction C hydrolysed phenyl acetate at a higher rate than acetylcholine with the substrate concentration used (1.0×10^{-2} M). The quaternary ammonium compounds were comparatively strong inhibitors and the phenothiazine derivative (Astra 1397) was not active as an inhibitor of the acetylcholine hydrolysis. Moreover, this enzyme was only sensitive to comparatively high concentration of the organophosphorus compounds, atoxyl and quinine (Fig. 6 and Table 2).

Sheep plasma showed much the same esterase pattern as goat plasma, both regarding hydrolysis rates of various esters (Table 1), observed esterase peaks of the electrophorogram, and esterase sensitivity to various inhibitors.

Swine plasma (Fig. 7). The arylesterase concentration of swine plasma exhibits considerable individual variation (b_{30} 20—900). The results obtained with a sample having a b_{30} of 450 will be considered here. More details on swine plasma esterases, including evidence for the inheritance of the plasma arylesterase have been reported in a previous paper [7].

The main esterase present in the plasma sample analysed was an acetylarylesterase. *Para*-substitution of phenyl acetate with a nitro group increased the enzymic hydrolysis rate about eight times. With other plasma arylesterases of mammals such a substitution in the aromatic ring usually greatly decreases the hydrolysis rate. The swine esterase was resistant to all inhibitors tested. By contrast, the hydrolysis of phenyl acetate by a plasma exhibiting low activity against this substrate was strongly inhibited by all those compounds which were strong inhibitors of the cholinesterase present in low concentration. This suggests that the hydrolysis of phenyl acetate by this type of plasma was due mainly to the plasma cholinesterase. The less active the plasma was in hydrolysing phenyl acetate, the more closely did the pattern of inhibition, when assayed with phenyl acetate, resemble that of cholinesterase.

The arylesterase migrated on electrophoresis together with the albumin components. The activities against phenyl acetate and its *p*-nitro derivative moved coincident with each other. No B-esterase or other arylesterases were detected.

Due to the low activity of swine plasma on choline esters, a cholinesterase could not be traced accurately in the fractions obtained in these electrophoresis experiments. However, by running the electrophoresis on smaller columns it was possible to find this type of esterase between the α_2- and β-globulins. Experiments with whole plasma revealed that this esterase was a butyryl-

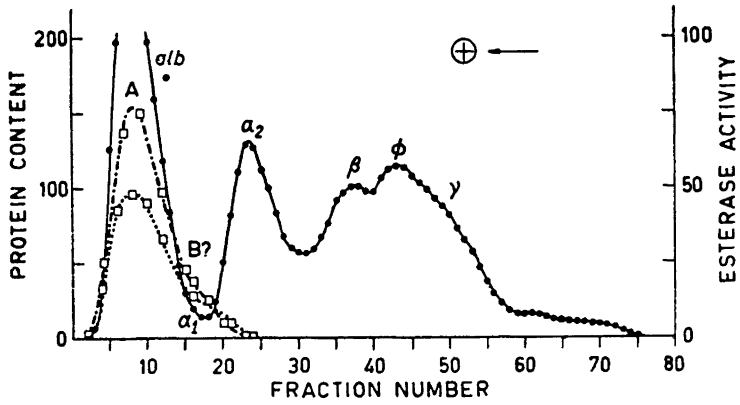


Fig. 7. Swine plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.10); □ —.—.□, *p*-nitrophenyl acetate (0.02).

Esterase component	PhAc	*p*-Nitro-PhAc	BuCh	pI_{50}	
				Phys	Mipafox
A	98	775	0	<3	<3
B?	?	?	0	<3	?
C	<1	?	<1	7.0	6.5

cholinesterase, the specificity of which differed from those of all other vertebrate plasmata by hydrolysing acetylcholine more rapidly than propionylcholine confirming previous results [8,9]. Benzoylcholine and succinylcholine were also hydrolysed by this enzyme. The hydrolysis of succinylcholine was unusual in that the swine plasma hydrolysed it more rapidly (comparable protein concentrations) that any other animal tissue so far studied. The hydrolysis rate of the carbon analogue of acetylcholine (3,3-dimethylbutyl acetate) was 10 % of the hydrolysis rate of the choline ester. Acetyl- β-methylcholine was not split. The swine cholinesterase also hydrolysed phenyl acetate, the hydrolysis rate being approximately the same as for butyrylcholine. In newborn animals, which irrespective of the plasma activities of the parents had low arylesterase activity, more than 97 % of the hydrolysis rate of phenyl acetate was due to a butyrylcholinesterase.

The inhibition experiments performed with swine plasma esterases have been previously reported [7].

Cat plasma (Fig. 8). The high hydrolysis rate for aromatic esters by this plasma was due to an arylesterase (probably an acetylarylesterase, in contrast to previous statements [1,10]) and a B-type esterase. The latter enzyme hydrolysed a variety of esters, the hydrolysis rates being greatly dependent on the configuration of the alcoholic group. Tripropionin was hydrolysed at a higher rate than tributyrin, the reverse being true for the phenyl and *iso*amyl derivatives. The most characteristic property of this enzyme was its high sensitivity

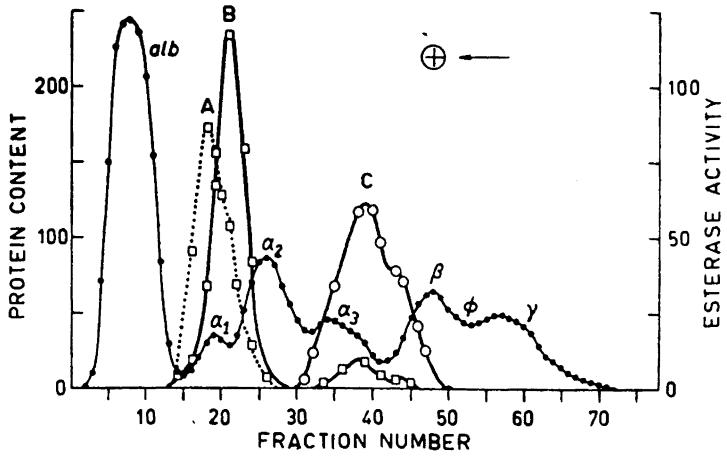


Fig. 8. Cat plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.01); □———□, PhBu (0.10); ○———○, BuCh (0.40), PrCh (0.80).

Esterase component	PhAc	PhBu	TPr	PrCh	BuCh	pI_{50}	
						Phys	*iso*-OMPA
A	870	≈10	0	0	0	<3	<3
B	<100	117	54	0	0	≈3.5	≈7
C	≈5	10	0	8	16	7.75	7.2

to atoxyl (pI_{50} 7), not found with any other plasma esterases studied in this series. Due to the unusually high mobility of the albumin component [6], the A-esterase fractions were all free from these proteins.

The cholinesterase of cat plasma was similar to other mammalian butyrylcholinesterases. It was responsible for approximately 10 % of the plasma activity against phenyl butyrate and for less than 1 % of that against phenyl acetate. The activity was found after electrophoresis between the α_3- and β-globulins and was not distributed in an uniform esterase peak. The enzyme protein seemed to move partly together with other protein constituents (possibly by complex formation). No evidence could be found by inhibition experiments and specificity studies with various fractions forming the peak or peaks that more than one cholinesterase was present. The same observations were made with three various plasma samples subjected to electrophoretic separation.

Dog plasma (Fig. 9). The esterase pattern of this plasma resembled that of human plasma in the hydrolysis of choline and aromatic esters. In comparison with human plasma the aliphatic esters were hydrolysed at a comparatively low rate by dog plasma. The main esterases present were an acetylarylesterase and a butyrylcholineasterase. Beside the main arylesterase

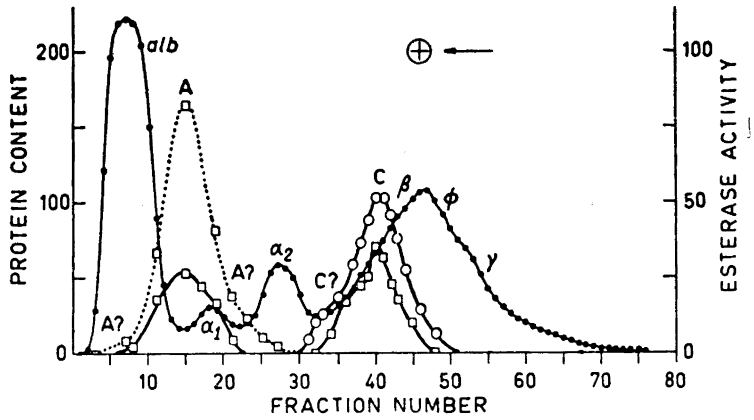


Fig. 9. Dog plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.025); □——□ PhBu (0.20); O——O, BuCh (0.20).

Esterase component	PhAc	PhBu	TPr	PrCh	BuCh	pI_{50}	
						Phys	*iso*-OMPA
A	248	14	0	0	0	<3	<3
C	<1	18	2	17	25	8.5	7.7

peak. fractions migrating on electrophoresis at a higher rate (together with the albumins) and at a lower rate (close to the α_2-globulins) were active in hydrolysing phenyl acetate. The esterases of both these "extra" fractions were resistant to *iso*-OMPA. Whether these activities were due to separate enzymes or merely due to the presence of traces of the arylesterase has not been further investigated.

The butyrylcholinesterase was found between the α_2- and β-globulins. The esterase peak was not uniform. Part of the enzyme(s) (C?) possibly moved together with other protein constituents. The specificity and sensitivity to inhibitors were the same for the active esterase constituents present in the main peak fraction and in the more rapid-moving fractions. In the cholinesterase region a B-type esterase might well have been present because the enzymic hydrolysis of phenyl butyrate by some fractions (B?) was inhibited by physostigmine only when this agent was present in higher concentration (10^{-4} M) than was necessary to inhibit completely the hydrolysis of a choline ester. If was found that 20—30 % of the phenyl butyrate hydrolysis by dog plasma was due to the cholinesterase which displayed the maximum activity against tripropionin. Before the complexity of the esterase fractions between the α_2- and β-globulins has been further investigated, it cannot be stated whether dog plasma contains a B-type esterases and more than one cholinesterase.

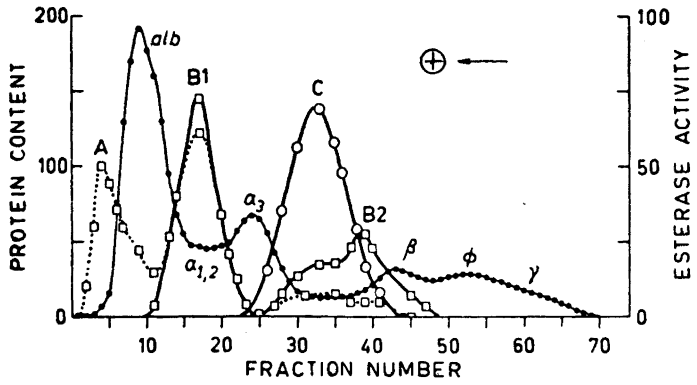


Fig. 10. Guinea-pig plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.05); □———□, PhBu (0.05); O———O, BuCh (0.20).

Esterase component	PhAc	PhBu	AmPr	AmBu	TPr	TBu	Tween	BuCh	pI_{50}	
									Phys	*iso*-OMPA
A	100	<1	0	0	0	0	0	0	<3	<3
B1	120	145	15	3	110	108	22	0	<3	5.0
B2	10	55	10	2	8	8	0	0	<3	5.9
C	15	35	0	0	10	12	0	35	8.5	7.0

Guinea-pig plasma (Fig. 10). In the series of aromatic and aliphatic esters tested the propionates were hydrolysed at the highest rate. Phenyl acetate and propionate were split by at least four enzymes. The first esterase fraction eluted following electrophoresis was a propionylarylesterase which migrated more rapidly than the albumins and was responsible for about 40 % of the total hydrolysis of phenyl acetate; the assay method was not sensitive enough to measure the hydrolysis rate of phenyl butyrate in these fractions. Two esterases resistant to physostigmine but sensitive to organophosphorus compounds were separated. One (Bl) was found in the α-globulin region and hydrolysed the propionates and butyrates at a higher rate than the acetates of the aliphatic and aromatic esters. This esterase was responsible for 50 % of the hydrolysis of phenyl acetate and 60—70 % of that of phenyl butyrate. The hydrolysis of ethyl acetate and ethyl butyrate as well as of "Tween 20", the rate of which was comparatively high (b_{30} 116) with this plasma, was also catalysed by this enzyme. The second B-type esterase (B2) migrated on electrophoresis at a higher rate than cholinesterase. The *iso*amyl esters were hydrolysed by both the B-esterases but not by the C-type esterase, which had the properties of a butyrylcholinesterase. In addition to the hydrolysis of choline esters, including benzoylcholine, this C-esterase was responsible for about 15 % of the plasma activity against phenyl butyrate and 5—10 % of that against phenyl acetate.

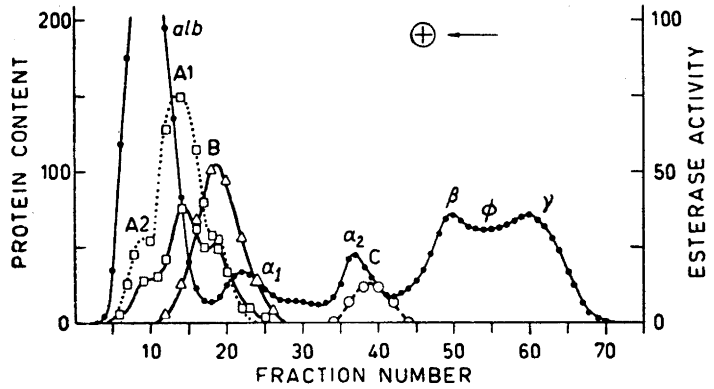


Fig. 11. Rabbit plasma. Conditions as for Fig. 1. □ ..... □, PhAc (0.02); □——□ PhBu (0.10); △——△, TBu (0.40); ○——○, PrCh (0.40).

Esterase component	PhAc	PhBu	TBu	PrCh	Indoxyl acetate	pI_{50}		
						Phys	*iso*-OMPA	62C47
Al	375	40	?	0	20	<3	<3	<3
A2	135	15	0	0	?	<3	<3	<3
B	?	25	12	0	45	<3	6.9	<3
C	<1	<1	<1	4	30	6.4	<4	5.7

Guinea-pig plasma was highly active in hydrolysing the carbon analogue of acetylcholine (b_{30} 150). It was characteristic of the reaction that the velocity fell off greatly with time. The enzyme responsible was the physostigmine resistant B-type esterase (B1). Heroin was also hydrolysed at a comparatively high rate by this plasma (b_{30} 12).

Rabbit plasma (Fig. 11). The esterase activity of rabbit plasma varied considerably from animal to animal, especially when aromatic esters were used as substrates. The activity of the enzymes responsible for the hydrolysis of these esters were reduced in varying degrees by dialysis against the veronal buffer. The electrophoretic pattern was in most instances difficult to interprete. There were at least three enzymes in the albumin and α_1-globulin regions which hydrolysed aromatic esters. The main esterase peak (A1) was due to an acetylarylesterase. Active fractions (A2) moving together with the albumins had esterase properties similar to those of the fractions of the main esterase peak (A1), but whether the two peaks were due to the same or different enzymes has not been further investigated.

The B-type esterase hydrolysed aliphatic and aromatic propionates and butyrates at higher rates than the acetates. It was much more sensitive to the organophosphorus compounds and to atoxyl than was the C-type esterase of this plasma. Great individual variation occurred in the concentration of this esterase.

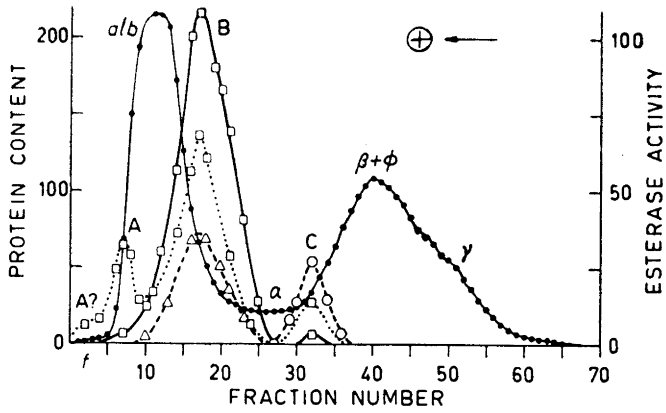


Fig. 12. Rat plasma. Conditions as for Fig. 1. □ . . . . . □, PhAc (0.05); □———□, PhBu (0.05); △— — —△, TPr (0.10); ○— — —○, PrCh (0.40).

Esterase component	PhAc	PhBu	AmAc	AmBu	TPr	Tween	PrCh	BuCh	pI_{50}	
									Phys	*iso*-OMPA
A	60	6	0	0	0	0	0	0	<3	<3
B	140	220	11	20	35	13	0	0	<3	5.8
C	4	1	0	0	0	0	7	4	6.3	6.7

The cholinesterase present in comparatively low concentration has the properties of an acetylcholinesterase. The activity was inhibited by high concentrations of acetylcholine (and propionylcholine). The enzyme was resistant to "Astra 1397", atoxyl and quinine, and was less sensitive to *iso*-OMPA and mipafox than other plasma cholinesterases. On the other hand, the quaternary ammonium compounds were comparatively strong inhibitors. These observations indicate that the rabbit plasma cholinesterase is of a type characteristic of acetylcholinesterase present in erythrocytes and nervous tissue of various animal species.

Preliminary experiments showed that indoxyl acetate was hydrolysed by all three types of esterases present in rabbit plasma, with the B- and C-esterases being the most active ones. This plasma hydrolysed heroin (b_{30} 10) and cocaine (b_{30} 5) at a higher rate than most other mammalian plasmata.

Rat plasma. (Fig. 12). The acetylarylesterase moved in electrophoresis at a higher rate than the albumins. Its activity was reduced during dialysis against the veronal buffer to a much greater degree than with the other esterases of this plasma. In the small amount of protein (f-component), moving faster than the albumins, arylesterase was also present. The B-type esterase hydrolysed phenyl butyrate and *iso*amyl butyrate at a higher rate than the corresponding acetates and propionates, and tripropionin more rapidly than its other acyl homologues. The broad B-esterase peak obtained

after electrophoresis probably consisted of a single enzyme because the activities of all fractions therein showed the same sensitivity to *iso*-OMPA when various esters were used as substrates. This esterase together with cholinesterase was responsible for about 40 % of the plasma hydrolysis of phenyl acetate.

The rat was one of the few mammalian species studied in this series (cf. cow, above) which had a typical propionylcholinesterase in its plasma, confirming previous observations [8]. A similar enzyme was present in mouse plasma. The propionylcholinesterase of rat plasma differed from the butyrylcholinesterases present in other plasmata in respect to inhibitory effects of various compounds (Table 2) and hydrolysis rates of choline and non-choline esters; *e.g.*, acetyl-β-methylcholine and benzoylcholine as well as phenyl acetate and propionate were split at relatively high rates.

Arylesterases as thiol enzymes. All the arylesterases studied were resistant to the selective inhibitors used (Table 2). Introductory studies were therefore performed to find inhibitors of these enzymes. Of the three main types of agents reacting with thiol groups, *p*-hydroxymercuribenzoate, iodoacetamide and *o*-iodosobenzoate, the first compound was the only powerful inhibiting agent of four different plasma arylesterases (human, cow, swine, rabbit) (pI_{50} 5—6). This suggests the presence of an active SH-group at the enzyme surface of these esterases. Aliesterases and cholinesterases were resistant to the mercaptide-forming agent (*p*-hydroxymercuribenzoate) and the alkylating agent (iodoacetomide). The oxidizing agent (*o*-iodosobenzoate) was a comparatively strong inhibitor (pI_{50} 5.0) of the cholinesterases of human and horse plasmata. A full account of these studies will be reported later.

DISCUSSION

The nature of plasma esterases has been the subject of investigation and speculation for nearly forty years. These enzymes have usually been characterized based on their substrates and sensitivity to inhibitors. Differentiation was based mainly on work with original plasma (serum) or crude enzyme preparations. Homogeneous esterase preparations such as plasma cholinesterases free from other esterases, were only rarely employed. A classification of plasma esterases is difficult to deduce from such studies due to much overlapping of properties and species differences.

As a preliminary to the present study, various preparative methods were tested for separating the esterases of vertebrate plasmata. Fractionation with ammonium sulphate gave poor separation of the two esterases present in human plasma, and even less promising results with plasmata where a more complex esterase pattern was involved. Ethanol fractionation at low temperature resulted in good separation of the two human plasma esterases. This technique was therefore employed with a number of other plasmata *, but was inadequate for separating the aryl- and aliesterases.

Except for a few studies of plasma cholinesterases [11,12] and of A- and B-esterases [13], no attempt has been made so far to separate electrophoretically

* This investigation was carried out in collaboration with Ing. H. Nihlén of Forskningslaboratoriet LKB, Stockholm.

plasma esterases. Preparative electrophoresis on cellulose columns was choosen as the technique for the present investigation, because of its adaptability for the isolation of plasma proteins in sufficient amounts to enable a study of their properties [14,15]. Phosphate and borate were found to be unsuitable as buffers for electrophoresis since the arylesterases and the aliesterases in many plasmata were not separated in these buffers. The best separation of plasma esterases was obtained with veronal buffer (pH 8.4 and $I = 0.1$). However, the various types of esterases were influenced differently by dialysis against this buffer. Species as well as esterase differences were noted in this respect. For this reason the activities of separated enzymes are not always comparable with those calculated from activity values obtained with original plasma.

The esterase peaks after electrophoresis are shown in graphs giving their relative position to known protein constituents. The absolute electrophoretic mobilities of the proteins from various plasmata cannot be compared as the fraction numbers plotted as abscissae are not the same for all plasmata. However, all electrophoresis runs and elutions were carried out under identical conditions, with the exception of temperature which varied from 5°C to 11°C in various runs.

Three types of esterases were shown for mammalian plasmata, based on electrophoretic mobility relative to known protein constituents, substrate specificity, and susceptibility to selective inhibitors. Some plasmata contain all three types, others two and still others only one. Each group of enzymes has several types, and every animal species has its own typical set of plasma esterases. When designating these esterases, the nomenclature generally accepted by most authors in this field was followed as far as possible. Since the three types of esterases have different electrophoretic mobilities under the experimental conditions used, they could be conveniently studied when separated after electrophoresis. Cholinesterases had in most cases much lower electrophoretic mobility than the aryl- and ali-esterases and were therefore easily obtained free from other esterases. Aryl- and aliesterases migrated more closely, but in most cases they could be studied separately and not in mixtures.

The presence of an aromatic esterase in human plasma has been proposed by Mounter and Whittaker [16]. Aldridge [10] found two types of serum esterases of several species hydrolysing *p*-nitrophenyl acetate, propionate and butyrate. The enzyme not inhibited by paraoxon (diethyl *p*-nitrophenyl phospate) and hydrolysing acetate at a higher rate than butyrate was called A-esterase, and the sensitive enzyme B-esterase. It turned out in the present investigation that the A-esterase is the enzyme which has the greatest electrophoretic mobility, and is resistant not only to paraoxon but to a number of other organophosphorus compounds and to physostigmine. Since this esterase hydrolyses various aromatic esters (in addition to phenyl esters, the acetates of α- and β-naphthol and indoxyl) but not aliphatic and choline esters, it was called *arylesterase*. Whether this type of esterase is also responsible for the hydrolysis of certain organophosphorus compounds will be the subject of a subsequent study. In contrast to other plasma esterases the arylesterases are highly sensitive to *p*-hydroxymercuribenzoate which suggests the presence of an active SH-group at the enzyme surface. All mammalian plasmata studied

contain this type of esterase in high concentration and it is the predominant esterase present in the plasmata of ruminants and swine.

In the earliest investigation on plasma esterases such simple esters as alkyl butyrates, fats and oils were used as substrates. "Simple esterases" having a substrate preference for short-chained fatty acid esters of simple alcohols were differentiated from "lipases" which were more active in hydrolysing esters of glycerol and fatty acids with long chains. Later it was observed that choline esters were also hydrolysed by the plasmata of several species, and that the enzyme responsible was different from "esterase" and "lipase". This led to a large amount of work on substrate specificity of plasma esterases. No clearcut line of differentiation between the various enzymes could be drawn, until Richter and Croft [17] made the observation that all enzymes classified as "cholinesterases" were completely inhibited by 10^{-5} M physostigmine, while "simple esterases" were resistant to this inhibitor dosage. These authors introduced the term *aliesterase* for the latter type of esterases. No evidence has been given so far as to the identity of this esterase type with the A- or B-esterases. It was demonstrated in the present investigation that the esterase migrating electrophoretically next to the arylesterase has the properties of a B-esterase and an aliesterase, being highly sensitive to certain organophosphorus compounds and resistant to 10^{-5} M physostigmine. This esterase hydrolyses both aliphatic and aromatic esters. Although the term aliesterase is not adequeate for this type of esterase, since some of them hydrolyse aromatic esters at a higher rate than the corresponding aliphatic ones, its use is recommended because the definition introduced by Richter and Croft is still valid. Triglycerides are also hydrolysed by this esterase, and since no special esterase hydrolysing only these esters has been detected in plasma, lipases are included in the aliesterases. Human, monkey, dog, swine and ruminant plasmata do not contain aliesterase. The hydrolysis of aliphatic esters by these plasmata is catalysed only by cholinesterases, which in these cases have a particularly low specificity for choline esters. The mammalian aliesterases of the plasmata of horse, cat, guinea-pig, rabbit and rat hydrolyse the butyrates and propionates at a higher rate than the acetates. Acetylaliesterases, however, exist in the plasmata of some lower vertebrates, as will be discussed more fully in the second paper of this series.

The specificity of *cholinesterases* present in the plasmata of various mammalian species has been studied extensively in previous investigations [8, 18, 19]. By ammonium sulphate fractionation the human plasma cholinesterase is precipitated with the albumins [18, 20, 21], but on electrophoresis it migrates between α_2- and β-globulins [11, 12]. It was demonstrated in the present investigation that this relatively low mobility under the experimental conditions used was valid also for the cholinesterases of other mammalian plasmata. These plasma esterases were therefore easily separated from the aryl- and aliesterases by electrophoresis, and for practical reasons they were designated "C-esterases" in the present study. The hydrolysis of choline esters and the high sensitivity to physostigmine are properties sufficiently distinct to regard the cholinesterases as a separate group of esterases. When only the sensitivity to certain organophosphorus compounds is considered, cholinesterases might be regarded as B-type esterases [22], but plasma cholinesterases are definitely distinct enzy-

mes differing electrophoretically and in their characteristics as esterases from the aliesterases as clearly as do the arylesterases.

Cholinesterases were present in all mammalian plasmata studied except in those of the ruminants where their activities were very low or too low for detection. Various types of plasma cholinesterases exist and overlapping of properties is very marked. This is especially true for the enzymes present in the plasmata of rabbit, swine, cow and reindeer. A butyrylcholinesterase is characteristic of several of the mammalian plasmata studied, and a propionylcholinesterase is present in rat plasma. As will be demonstrated in a second paper of this series, butyrylcholinesterase does not occur in the plasmata of lower animals, where propionyl- and acetylcholinesterases are the characteristic physostigmine-sensitive esterases instead.

This investigation has been supported by a grant from the *Swedish Natural Science Research Council*. The skilled technical assistance of Mrs. Toril Rode and Mrs. Gunnel Jonsson is gratefully acknowledged. My thanks are also due to Prof. John E. Casida who revised the English in the manuscript. A complete list of acknowledgements will be given in the second paper of this series.

REFERENCES

1. Augustinsson, K.-B. *Nature* **181** (1958) 1786.
2. Augustinsson, K.-B. *Methods of Biochem. Anal.* **5** (1957) 1.
3. Svensson, H. *IVA* **25** (1954) 252.
4. Flodin, P. and Kupke, D. W. *Biochim. et Biophys. Acta* **21** (1956) 368.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. *J. Biol. Chem.* **193** (1951) 265.
6. Deutsch, H. F. and Goodloe, M. B. *J. Biol. Chem.* **161** (1945) 1.
7. Augustinsson, K.-B. and Olsson, B. *Biochem. J.* **71** (1959) 477, 484.
8. Myers, D. K. *Biochem. J.* **55** (1953) 67.
9. Levin, M. G. and Suran, A. A. *Enzymologia* **15** (1951) 17.
10. Aldridge, W. N. *Biochem. J.* **53** (1953) 110.
11. Glick, D., Glaubach, S. and Moore, D. H. *J. Biol. Chem.* **144** (1942) 525.
12. Cohen, J. A. and Warringa, M. G. P. J. *Biochim. et Biophys. Acta* **25** (1957) 600.
13. Goutier, R. *Biochim. et Biophys. Acta* **19** (1956) 524.
14. Porath, J. *Biochim. et Biophys. Acta* **22** (1956) 151.
15. Jedin, H. I. and Porath, J. *Biochim. et Biophys. Acta* **26** (1957) 159.
16. Mounter, L. A. and Whittaker, V. P. *Biochem. J.* **54** (1953) 551.
17. Richter, D. and Croft, P. G. *Biochem. J.* **36** (1942) 746.
18. Augustinsson, K.-B. *Acta Physiol. Scand.* **15** (1948) Suppl. 52.
19. Whittaker, V. P. *Physiol. Revs.* **31** (1951) 312.
20. Faber, M. *Acta Med. Scand.* **114** (1943) 72.
21. Strelitz, F. *Biochem. J.* **38** (1944) 86.
22. Aldridge, W. N. *Personal communication.*

Received December 19, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 593—594

Synthesis of 4-*O*-Benzyl-D-Glucose

INGEMAR CROON and BENGT LINDBERG

Träkemiska avdelningen, Svenska Träforskningsinstitutet, Stockholm, Sweden

A new method for the benzylation of hydroxyl groups in carbohydrates has been developed. The method, by which alkali-labile groups, such as acetate ester groups, are not removed, has been applied for the synthesis of 4-*O*-benzyl-D-glucose.

4-*O*-Benzyl-D-glucose, which has not been prepared before, was of interest in this Department, and we therefore looked for a convenient route to this substance. 4-*O*-Methyl-D-glucose has recently been prepared [1] by methylation of methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside with methyl iodide and silver oxide in dimethyl formamide, taking advantage of the facile migration of an acetyl group from the 4- to the 6-position and subsequent deacetylation and hydrolysis. An analoguous route to the benzyl ether should be attractive. The benzylation of carbohydrates has recently been summarised by Mc Closkey [2]; it has always been done using strongly alkaline conditions, under which acyl groups should be split off. The benzylation of 1,2:5,6-di-*O*-*iso*propylidene-D-glucofuranose with benzyl bromide and silver oxide in dimethyl formamide was investigated first; after hydrolysis of the product a good yield of 3-*O*-benzyl-D-glucose was obtained. Methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside was then benzylated in the same way and the product was deacetylated and hydrolysed. Paper electrophoresis in borate buffer revealed the presence of three substances, with the mobilities expected [3] for glucose, and for 6-*O*- and 4-*O*-benzyl-D-glucose, of which the latter gave the strongest colour reaction. The first two substances were adsorbed on a column of Amberlite IR 400 in the borate state, a technique previously used by Richards and Lock [4] for the fractionation of methylated glucoses. This gave the presumed 4-*O*-benzyl-D-glucose in a crystalline state in 23 % yield.

The product analysed as a monobenzylated glucose and had an electrophoretic mobility of 0.17, relative to glucose, as would be expected for the 2-*O*- or 4-*O*-benzyl ether. To distinguish between these possibilities the substance was treated with aqueous calcium hydroxide, in which the former should be stable and the latter degraded, chiefly to *iso*saccharinic acid [5]. The substance was degraded to acidic material at a slower rate than that at which 3-*O*-benzyl-D-glucose was degraded in a parallel experiment. 2-*O*-Methyl-D-glucose was quite stable under these conditions. This indicated that the substance was the 4-*O*-benzyl ether and in agreement with this it

consumed 2.92 moles of periodate with the formation of 1.91 moles of formic acid. The 4-*O*-benzyl-D-glucose melted at 178—179° and showed an optical rotation of 96° → 52° in water, indicating that the crystaline material was the α-form.

EXPERIMENTAL

Melting points are corrected. Paper chromatography was carried out on Whatman No. 1 filter paper with the solvent system ethyl acetate-acetic acid-water(3:1:3).

Paper electrophoresis was carried out on Whatman No. 3 MM filter paper in borate buffer at pH 10.

3-O-Benzyl-D-glucose. 1,2:5,6-Di-*O-iso*propylidene-D-glucofuranose (3 g) was dissolved in dimethyl formamide (60 ml) and benzyl bromide (12 ml) was added. Silver oxide (12 g) was added over 60 min with vigorous stirring and external cooling with tap water. Stirring was continued for 16 h. The solids were removed by centrifuging and washed, first with dimethyl formamide (50 ml) and then with chloroform (50 ml). Potassium cyanide (1 %, 500 ml) was added to the combined solutions, which were then extracted with chloroform (3 × 100 ml). The combined chloroform solutions were washed with water (3 × 500 ml) and concentrated to a syrup. This was dissolved in a mixture of sulphuric acid (1 %, 70 ml) and ethanol (30 ml) and heated on the steam bath for 1 h after which the ethanol was distilled off and heating was continued for a further 3 h. Neutralisation with barium carbonate, filtration and concentration yielded a slightly yellow syrup (3.2 g). The syrup was fractionated on a cellulose column (80 × 4 cm), using butan-1-ol, saturated with water as solvent. The main fraction (2.6 g) gave chromatographically and electrophoretically pure 3-*O*-benzyl-D-glucose, which could be crystallised from acetone. The yield of crystalline product, m. p. 137—138°, was 2.08 g. The $R_{Gluc.}$-value was 5.5.

4-O-Benzyl-D-glucose. Methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (3 g) was dissolved in dimethyl formamide (30 ml) and benzylated with benzyl bromide (12 ml) and silver oxide (12 g) as described above. The resulting syrup was deacetylated with catalytic amounts of sodium ethoxide in ethanol, hydrolysed with 8 % sulphuric acid at 100°C for 4 h, neutralised with barium carbonate and then concentrated. The mixture of free sugars gave three spots on paper electrophoresis, with M_G values 0.17, 0.66 (elongated spot) and 1.0. Filtration through a column (30 × 1.2 cm) of Amberlite IR 400 in the borate state removed the two components with the higher M_G values. The component which passed through the column (0.90 g) was chromatographically and electrophoretically pure and could be crystallised from butan-2-one. Three crystallisations yielded the pure substance (0.62 g), m. p. 178—179°, $[\alpha]_D^{20}$ +52° (c = 2.0, in water). On periodate oxidation, using 0.04 N sodium metaperiodate at room temperature for 48 h, it consumed 2.92 moles of oxidant. In a parallel experiment, 1.91 moles of formic acid were formed. The $R_{Gluc.}$ value was 5.2. (Found: C 56.1; H 6.59. Calc. for $C_{13}H_{18}O_6$: C 57.6; H 6.68.)

2-*O*-Methyl-D-glucose, 3-*O*-benzyl-D-glucose and 4-*O*-benzyl-D-glucose were dissolved in saturated aqueous calcium hydroxide and kept at room temperature. The disappearance of sugar and the formation of acids was followed by paper electrophoresis in borate buffer (pH 10) and in acetate buffer (pH 4.5), using anisidine hydrogen chloride for the former and silver nitrate-sodium ethoxide for the latter as spraying reagents. The acids formed had the same electrophoretic mobilities as *meta*- and *iso*saccharinic acid, respectively, but were not further characterized. After 3 days most of the 3-*O*-benzyl ether and about half of the 4-*O*-benzyl ether were degraded into acids. After 5 days most of the latter was also degraded. The 2-*O*-methyl-D-glucose was quite stable.

REFERENCES

1. Bouveng, H., Lindberg, B. and Theander, O. *Acta Chem. Scand.* **11** (1957) 1788.
2. Mc Closkey, C. M. *Advances in Carbohydrate Chem.* **12** (1957) 137.
3. Foster, A. B. *Advances in Carbohydrate Chem.* **12** (1957) 81.
4. Lock, M. W. and Richards, G. N. *J. Chem. Soc.* **1955** 3024.
5. Kenner, J. and Richards, G. N. *J. Chem. Soc.* **1955** 1810.

Received December 23, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 595—626

Short Communications

Separation on a Preparative Scale of Amino Acids from a Protein Hydrolysate

ÖRN WAHLROOS and HARING LAND

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

In the course of a feeding experiment with cows, the ^{15}N content of amino acids of milk proteins had to be estimated [1]. Since the heavy isotope was expected to occur in low concentrations, it was intended to perform all measurements on relatively large amounts of nitrogen (approx. 2 mg). This quantity allows some 5 or 6 measurements to be made per sample with a high accuracy. For this purpose a separation of the amino acids was attempted on a preparative scale.

The milk protein precipitated with 15 % TCA was dialyzed, freeze-dried, extracted with ether in order to remove fatty material, and hydrolyzed with 6 N hydrochloric acid at 105°C for 24 h. Since the "humine" retained part of the amino acids, it was extracted twice with boiling water. The mixture was freed from HCl by adsorption on IR-120 and elution with piperidine [2].

The hydrolyzate from 7 g of protein, dissolved in 50 ml of 0.5 N acetic acid was poured onto a column of Dowex 1-X8 [3] with a cross-sectional area of 12.5 cm², and a height of 30 cm. The flow rate was 40 ml/h and fractions of a volume of 15 ml were collected (Fig. 1).

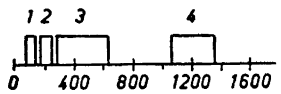


Fig. 1. Elution of the Dowex 1 column. (1) Neutral and basic amino acids; (2) Tyr; (3) Glu; (4) Asp.

The neutral and basic amino acids from the Dowex 1 column were rechromatographed on a Dowex 50-X4 column [3] with a cross-sectional area of 50 cm² and a height of 80 cm. First, the column was eluted with 4 800 ml of 1 N hydrochloric acid. Then gradient elution was applied, the gradient being of the shape [4]

$$C = C_2 - (C_2 - C_1)\,(1 - v)^{A_2/A_1}$$

where $A_1 = A_2$; A_1 and C_1 correspond to the cross-sectional area and the concentration of the mixing chamber, A_2 and C_2, respectively, to the vessel containing the hydrochloric acid of the higher concentration. The total volume of the system was 11 litres; C_1 = 1 N HCl, C_2 = 4 N HCl. In order to obtain an approximately constant gradient, A_2 and C_2 were changed to respectively $A_1/5.7$ and 5 N HCl when v/V had reached the value 0.8, v standing for the volume delivered, and V for the total volume of the system.

The column was closed with a rubber stopper through which a glass tube, adjustable in height, reached down to about 1 cm above the level of the resin. Since it was considered advisable to avoid another mixing of eluents of different strengths in the

Table 1. Yield of amino acids from 7 g of protein (in mg).

Glu	513	Val	163	His	98
Asp	346	Pro	243	Phe	91
Tyr	97	Lys	226	Arg	66
Ala	142	Cys	17		
Ser + Thr	374	Met + Ileu + Leu	492		
Gly	65				
			In total 2 933 mg		

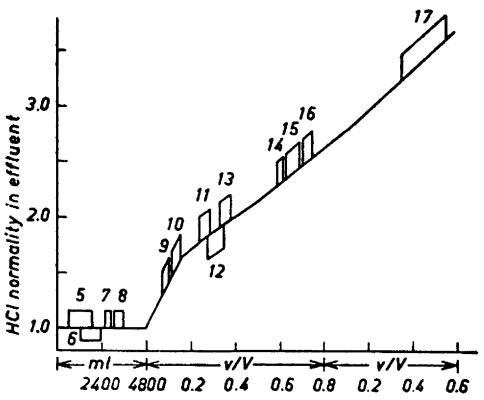


Fig. 2. Elution of the Dowex 50 column. (5) Ser; (6) Thr; (7) Gly; (8) Ala; (9) Val; (10) Pro; (11) Ileu; (12) Met; (13) Leu; (14) Cys; (15) Lys; (16) His; (17) Arg + Phe.

volume above the resin, this volume was kept at a minimum. This was achieved by application of a pressure equivalent to the difference in level between the mixing chamber and the top of the resin, *via* a side tube to the air space above the resin before the eluent was allowed to flow into the column. The flow rate was continuously kept at 108 ml/h by means of a capillary. The results are plotted in Fig. 2. Phenylalanine and arginine were separated on a cellulose column.

Purity of compounds rather than a high yield was the main aim in the separation. From Table 1 the total yield from 7 g of protein is seen to be about 40 %.

1. Land, H. and Virtanen, A. I. *Acta Chem. Scand.* **13** (1959) 489.
2. Buchanan, D. L. *Anal. Chem.* **29** (1957) 1877.
3. Hirs, C. H. W., Stein, W. H. and Moore, S. *J. Am. Chem. Soc.* **76** (1954) 1606.
4. Bock, R. M. and Ling, N.-S. *Anal. Chem.* **26** (1954) 1543.

Received December 15, 1958.

Crystalline Leghemoglobin

NILS ELLFOLK*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The red pigment from the soya bean root nodules was first demonstrated by Kubo [1] to have a hemoglobin nature. Ellfolk and Virtanen [2] showed that this pigment was not electrophoretically homogeneous after repeated ammonium sulphate fractionations, but contained two hemin components which seemed to occur in approximately equal concentrations. This was recently confirmed by Thorogood [3] who also found a third component in addition to the above two components and possibly a fourth.

Studying different techniques the present author has found that a cellulose ion exchange column was the most convenient one to separate these different hemin components. Diethylaminoethylcellulose according to Peterson and Sober [4] was used as adsorbent. The preparation of the individual components in this way was as follows:

After crushing the nodules, leghemoglobin was extracted twice into distilled water and fractionated once with ammonium sulphate between 55–80 % saturation (23°C). After dialysis against phosphate buffer (pH 8) and then against distilled water the protein solution was put on a cellulose ion exchange (DEAE-SF) column buffered to pH 5.2 with an acetate buffer of ionic strength 0.01 μ. All the hemin proteins remain on the top of the column as a sharp band. By elution with an acetate buffer (pH 5.2, μ = 0.01) one component moved down the column. This component was found to be the electrophoretically slowest one. Using buffer of this ionic strength the other components hardly moved at all and therefore, in order to increase the elution speed, the ionic strength of the acetate buffer was increased to 0.02 μ or 0.03 μ by addition of sodium chloride.

The second component was found to be the electrophoretically faster component. Between these two bands moved a faint, broad band which electrophoresis showed to be a mixture of several proteins with no

* Permanent address: Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland.

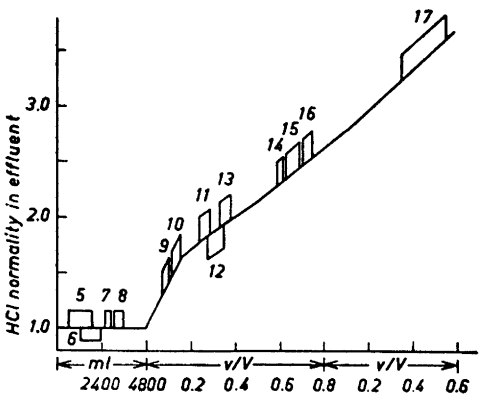


Fig. 2. Elution of the Dowex 50 column. (5) Ser; (6) Thr; (7) Gly; (8) Ala; (9) Val; (10) Pro; (11) Ileu; (12) Met; (13) Leu; (14) Cys; (15) Lys; (16) His; (17) Arg + Phe.

volume above the resin, this volume was kept at a minimum. This was achieved by application of a pressure equivalent to the difference in level between the mixing chamber and the top of the resin, *via* a side tube to the air space above the resin before the eluent was allowed to flow into the column. The flow rate was continuously kept at 108 ml/h by means of a capillary. The results are plotted in Fig. 2. Phenylalanine and arginine were separated on a cellulose column.

Purity of compounds rather than a high yield was the main aim in the separation. From Table 1 the total yield from 7 g of protein is seen to be about 40 %.

1. Land, H. and Virtanen, A. I. *Acta Chem. Scand.* **13** (1959) 489.
2. Buchanan, D. L. *Anal. Chem.* **29** (1957) 1877.
3. Hirs, C. H. W., Stein, W. H. and Moore, S. *J. Am. Chem. Soc.* **76** (1954) 1606.
4. Bock, R. M. and Ling, N.-S. *Anal. Chem.* **26** (1954) 1543.

Received December 15, 1958.

Crystalline Leghemoglobin

NILS ELLFOLK*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The red pigment from the soya bean root nodules was first demonstrated by Kubo [1] to have a hemoglobin nature. Ellfolk and Virtanen [2] showed that this pigment was not electrophoretically homogeneous after repeated ammonium sulphate fractionations, but contained two hemin components which seemed to occur in approximately equal concentrations. This was recently confirmed by Thorogood [3] who also found a third component in addition to the above two components and possibly a fourth.

Studying different techniques the present author has found that a cellulose ion exchange column was the most convenient one to separate these different hemin components. Diethylaminoethylcellulose according to Peterson and Sober [4] was used as adsorbent. The preparation of the individual components in this way was as follows:

After crushing the nodules, leghemoglobin was extracted twice into distilled water and fractionated once with ammonium sulphate between 55–80 % saturation (23°C). After dialysis against phosphate buffer (pH 8) and then against distilled water the protein solution was put on a cellulose ion exchange (DEAE-SF) column buffered to pH 5.2 with an acetate buffer of ionic strength 0.01 μ. All the hemin proteins remain on the top of the column as a sharp band. By elution with an acetate buffer (pH 5.2, μ = 0.01) one component moved down the column. This component was found to be the electrophoretically slowest one. Using buffer of this ionic strength the other components hardly moved at all and therefore, in order to increase the elution speed, the ionic strength of the acetate buffer was increased to 0.02 μ or 0.03 μ by addition of sodium chloride.

The second component was found to be the electrophoretically faster component. Between these two bands moved a faint, broad band which electrophoresis showed to be a mixture of several proteins with no

* Permanent address: Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland.

distinct peaks. It evidently contained parts of the first and second components and also some oxidized component (choleglobin). This component was too small to allow any decision whether it contained a distinct new fraction or not. After the second band a fourth small band was eluted, which separated distinctly and the mobility of which in electrophoresis was found to be slightly higher than that of the main fast component. All the spectra of these three components showed the acid hemiglobin nature of the components.

On the top of the column there still remained a brown band which did not move though the ionic strength was increased. It was partially eluted by 3 M NaCl and its spectrum did not show any maxima or minima but the absorption increased steadily toward the ultraviolet. This component was therefore assumed to be a denaturation product of the hemin proteins.

The two main components were homogeneous in the ultracentrifuge and their sedimentation constants were the same, about 1.9 *S*. They were also homogeneous in long run electrophoresis at two different pH values, *i. e.* pH 6.1 and 9.0. The iron content of the two fractions was slightly different, the electrophoretically faster one had an iron content of 0.320 % and the electrophoretically slower one a value of 0.287 % of iron per dry weight. The diffusion coefficient $D_{20°}$ of the fast component was found to be about 11.1 *F* and that of the slower one about 8.5 *F*. On the basis of iron content the fast component has a molecular weight of 17 500 and the slower one 19 500. It is evident that the faster component is not far from spherical in shape, whereas the slower one is somewhat assymetric or highly hydrated.

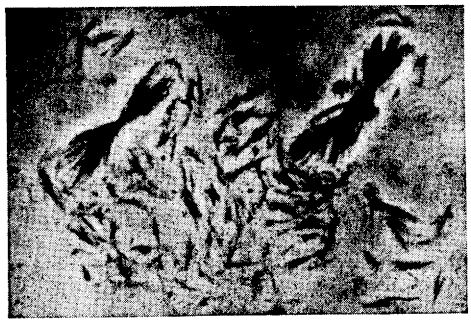


Fig. 1. Crystalline leghemoglobin, faster component, 1 130 ×.

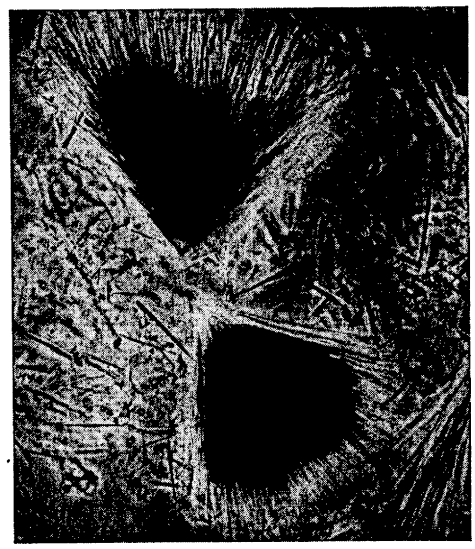


Fig. 2. Crystalline leghemoglobin, slower component, 230 ×.

Ellfolk and Virtanen [2] assumed that the slower component was not a homogeneous protein but probably an artefact of the faster component adsorbed to a neutral or basic protein. From these findings it is evident that this assumption was not correct.

Both the components were found to crystallize from ammonium sulphate solutions, 0.65 saturation, at slightly acid pH (4.8 and 4.7). Fig. 1 shows the crystals of the faster component and Fig. 2 those of the slower one.

A detailed report on these findings will be given in this journal.

1. Kubo, H. *Acta Phytochim.* **11** (1959) 195.
2. Ellfolk, N. and Virtanen, A. I. *Acta Chem. Scand.* **4** (1950) 1014.
3. Thorogood, E. *Science* **126** (1957) 1011.
4. Peterson, E. A. and Sober, A. *J. Am. Chem. Soc.* **78** (1956) 751.

Received February 16, 1959.

Isolation of Palustric Acid from Tall Oil Rosin by Partition Chromatography *

HENRIK H. BRUUN and STEIN GÅSLAND

The Institute of Wood Chemistry, Åbo Akademi, Åbo, Finland

In 1954 Loeblich, Baldwin and Lawrence isolated a new rosin acid of the abietic series from gum and wood rosins of two American pine species; the new acid they named palustric acid [1]. In this isolation the authors employed, with minor modifications, the chromatographic method developed by Ramsay and Patterson [2] for the separation of straight-chain fatty acids. We have applied the same method to isolate the components of Finnish tall oil rosin and have also found palustric acid among the rosin acids and in amounts of 14—15 % of the total rosin acids in the tall oil rosin. In the light of previous analytical data for tall oil rosins (see references in Ref.[3]), this finding is surprising and of significance from the standpoint of the industrial utilization of tall oil rosin.

By means of the chromatographic technique we were able to separate the components of artificial binary, ternary and quaternary mixtures of rosin acids in the following order (the volume given is peak volume of the eluant): tetrahydroabietic acid 95 ml, dihydroabietic acid 150 ml, palustric acid 190 ml, dextropimaric acid 225 ml, *iso*dextropimaric acid 225 ml, *l*-abietic acid 240 ml, levopimaric acid 285 ml, neoabietic acid 360 ml, dehydroabietic acid 490 ml. As will be noted, the hydrogenated rosin acids are eluted first, followed by palustric acid which is clearly separated from these acids and from dextro- and *iso*-dextropimaric acids and *l*-abietic acid, which are eluted next. It was found that when the abietic acid content was many times as large as the pimaric acid content, the peak for dextropimaric acid was not separated from the abietic acid peak but merged with the latter so that the peak for abietic acid was wider at the left face in the chromatogram. Likewise levopimaric acid expanded the right face of the peak for abietic acid when the levopimaric acid content was much lower than the abietic acid content. The next fraction eluted consists of neoabietic acid and this is clearly separated from dehydroabietic acid which follows it. These observations are in essential agreement with the findings of Loeblich, Baldwin and Lawrence [1].

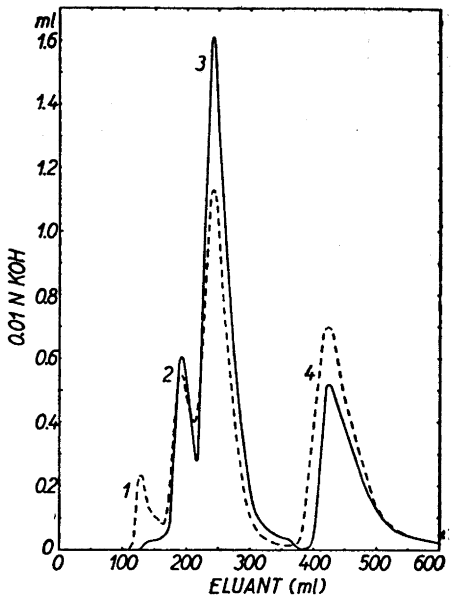


Fig. 1. Chromatogram of tall oil rosin showing the presence of palustric acid (peak 2). Ordinate: consumption of 0.0100 N alcoholic potassium hydroxide in titration of 5 ml eluant volumes (phenolphthalein as indicator). Sample *a* (———), tall oil rosin obtained by continuous distillation. Sample *b* (— — —), tall oil rosin obtained by batch distillation. For the other peaks, see text.

The chromatograms of two samples of crystalline tall oil rosins distilled by different methods are shown in Fig. 1. The samples have yielded chromatograms with four peaks. The first of these coincides with an eluant volume of 130 ml and according to the peak volumes given consists of hydrogenated rosin acids (which are present in only small quantities in sample *a*). The second peak at 190 ml lies at the peak volume for palustric acid. The third, the largest peak, undoubtedly refers to abietic acid and any small amounts of pimaric acids possibly present. We

* Paper read at the annual meeting of Finska Kemistsamfundet — Suomen Kemistiseura on December 8, 1958.

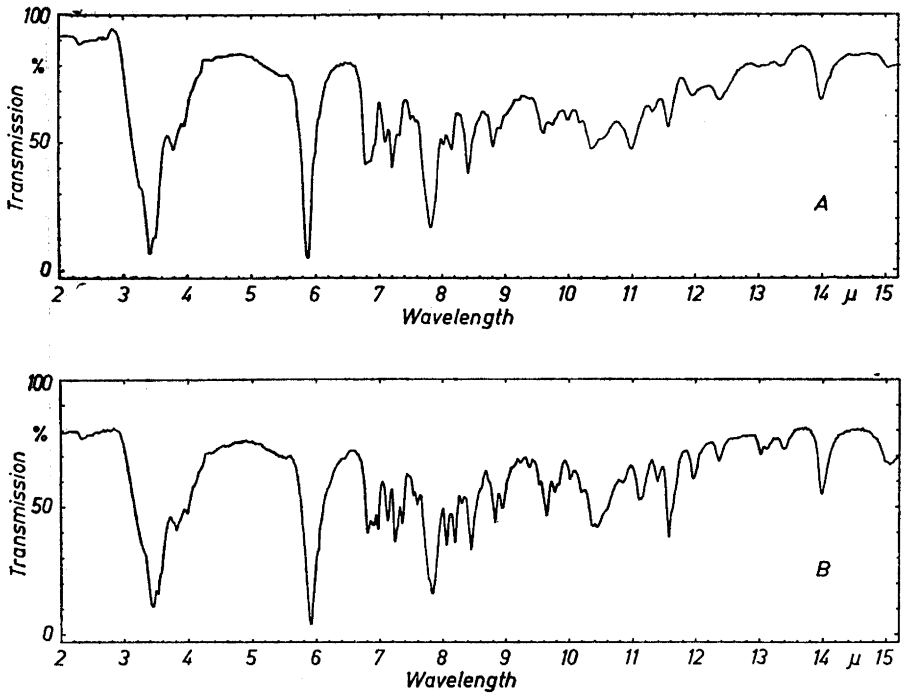


Fig. 2. Infrared absorption spectra of (A) palustric acid isolated from tall oil rosin sample *a* (peak 2 in curve for sample *a* in Fig. 1) and (B) authentic palustric acid.

have found that the addition of dextropimaric acid in an amount corresponding to 8 % of the abietic acid content to an artificial mixture of palustric and *l*-abietic acids in the proportions corresponding to those of the acids yielding peaks 2 and 3 in Fig. 1 leads to a filling up of the area between the peaks for palustric and *l*-abietic acids in the chromatogram. The dextropimaric acid contents of samples *a* and *b* are hence according to Fig. 1 in all probability less than 8 % of the abietic acid content. The shoulder at 360 ml in the chromatogram of sample *a* coincides with the peak volume of neoabietic acid. The fourth peak at 425 ml appears appreciably earlier than the peak for dehydroabietic acid in the chromatogram of the artificial mixture. The tall oil rosin thus evidently contains dehydrogenated rosin acids that differ in type from the dehydrogenated acids in the artificial mixture. The amounts of material in the peaks of the chromatogram are given in Table 1. The material of peak 2, which in view of its location in the chromatogram refers to palustric acid, represents 14—15 % of the rosin acids in both samples.

Table 1. Rosin acid contents (in g.equiv.) in peaks 1—4 in Fig. 1 as percentages of the total content of eluted rosin acids computed from the areas under the peaks.

Peak No.	Sample *a*	Sample *b*
1	< 1 %	5 %
2	14 »	15 »
3	58 »	41 »
4	27 »	39 »

In order to confirm the identity of the palustric acid (peak 2), chromatography of tall oil rosin (sample *b*) was carried out on a ten times larger scale than the above separations to obtain enough of the acid to permit the measurement of its infrared absorption. Although it was not possible

to carry out six recrystallizations from methanol as Loeblich, Baldwin and Lawrence [1] had done to obtain a constant specific rotation and melting point, but only one, the similarity of the recorded infrared absorption spectra reproduced in Fig. 2 clearly indicates that the isolated rosin acid of peak 2 consisted of palustric acid. It may be noted that the band at 12.37 μ is specific for palustric acid when the absorptions of the other rosin acids for which the eluant peak volumes were determined are taken into account (*cf.* Refs.[3-5]).

The infrared absorption spectra of rosin acid fractions of crystalline tall oil rosin (of the same origin as sample *b*) recorded by Bruun and Jalava [6] contained bands due to *l*-abietic and dehydroabietic acid but no bands characteristic of tetrahydroabietic, dihydroabietic and dextro- and *iso*dextropimaric acids (acc. to Refs.[3-4]). The chromatographic data obtained in the present study are in agreement with these observations. It may be difficult to determine from their infrared absorption the identity of the substances yielding peak 1 which amount to 5 % of the rosin acid mixtures and which possibly consist of isomeric rosin acids. This may be true also for dextropimaric acid when its content is as low as in the samples studied (according to the chromatograms of rosin acid mixtures, its content is less than 4 % of the total acids, and according to chromatograms of tall oil rosin samples from which the major part of the *l*-abietic acid was precipitated with diamylamine before chromatography, at the most 4—5 % of the total acids). — The infrared absorption spectrum of palustric acid, which spectrum was not known when the previous infrared absorption study was carried out, has its strongest bands at 8.46, 8.83, 9.65, 10.38 + 10.44, 11.11, 11.57, 11.97, 12.37 and 13.98 μ [5]. With the exception of the band at 12.37 μ, the strong and moderate bands are seen at or close to the same wavelengths as in the spectra of *l*-abietic and dehydroabietic acids, the two major components of tall oil rosin (*cf.* Refs. [3-4]). For this reason, it is difficult to detect from infrared spectra even relatively high contents of palustric acid in mixtures containing these acids. The content of 14—15 % of palustric acid deduced from the chromatographic analysis of the tall oil rosin is thus not necessarily at variance with the results of the infrared spectral studies of tall oil rosin acids [6].

Chromatographic data. Column diameter (inside): 11 mm. Adsorbent: 15.0 g of Mallinckrodt's silicic acid, A.R. Immobile solvent: 9 ml of a mixture of equal parts of furfuryl alcohol (B.D.H., technical, vacuum distilled after treatment with sodium hydroxide) and 2-aminopyridine (B.D.H., Laboratory Reagent). Eluting solvent: 2,2,4-trimethylpentane (B.D.H., Laboratory reagent): purified by distillation and passage through a column of aluminium oxide and saturated before use with the immobile solvent. The fraction volume was 5 ml. Data relating to the acid samples examined will be given in a forthcoming publication.

The authors are indebted to Mrs Karin Nilsson, Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala University, Uppsala, Sweden, for the recording of the infrared absorption curves and to Dr. E.L. Patton, Naval Stores Research Section, Olustee, U. S., for a sample of palustric acid. One of the authors (S.G.) has received a grant from the *Royal Norwegian Council for Science and Industrial Research*, which is gratefully acknowledged.

1. Loeblich, D., Baldwin, D. E. and Lawrence, R. V. *J. Am. Chem. Soc.* **77** (1955) 2823.
2. Ramsay, L. L. and Patterson, W. J. *J. Assoc. Offic. Agr. Chemists* **31** (1948) 441.
3. Bruun, H. H. *Paper and Timber* (Finland) **38** (1956) 577.
4. Bruun, H. H. *Paper and Timber* (Finland) **39** (1957) 221.
5. Bruun, H. H. *Acta Chem. Scand.* **11** (1957) 907.
6. Bruun, H. H. and Jalava, P. *Acta Chem. Scand.* **11** (1957) 401.

Received January 30, 1959.

Fractionation of Alginic Acid

ARNE HAUG

Norwegian Institute of Seaweed Research, Trondheim, Norway

Up to recently, it was generally accepted that alginic acid was a linear polymer of anhydro-β-D-mannuronic acid [1]. In 1955, however, Fischer and Dörfel [2] reported the presence of guluronic acid in hydrolysates of alginic acid. The proportion of the two uronic acids varied with the species. It has not been decided whether the alginic acid in a particular seaweed plant is a homogeneous compound, or whether it is a mixture of chain molecules of different uronic acid composition. Methods for investigation of the homogeneity of alginic acid are therefore of considerable interest.

Recently McDowell [3] reported that precipitation with manganous salts separated alginate into two fractions which not only differed markedly in molecular weight, but also seemed to differ with respect to calcium-sodium ion exchange properties. He did not investigate the uronic acid composition of the fractions.

During an investigation in this laboratory of the solubility of alginates it was observed that alginate could be separated into two fractions by salt solutions.

Materials and methods. All seaweed samples used contain at least 50 plants, collected at the same time, and from a small area. The plants are dried at room temperature and ground. Fucoidin and laminarin are removed by extraction overnight with 0.2 N sulphuric acid and the alginate is extracted by shaking the seaweed samples with 3 % sodium carbonate solution at room temperature. After filtration, the alginate is precipitated by addition of alcohol to about 30 %. The precipitated sodium alginate is washed with alcohol, and the excess alcohol pressed out. The alginate sample, which contains 60—80 % alcohol, is shaken with the salt solution for 2—3 h, left overnight, shaken again for about one hour, and then centrifuged. The volume of salt solution is chosen so that the alginate concentration is approximately 0.3 %. The gel brought down by centrifugation is washed with half the original amount of salt solution. The amounts of alginate in the gel and the solution are determined gravimetrically after precipitation with alcohol and washing with alcohol and ether.

The solubility of alginate from *Laminaria digitata* f. *stenophylla* (collected at Tarva, outside the Trondheimsfjord, 3/7 1957) in potassium chloride solutions of different strengths was investigated, and the results are given in Fig. 1. Approximately one third of the alginate is soluble and two thirds insoluble in solutions which contain between 0.6 and 1 N potassium chloride, and the shape of the curve gives evidence for the presence of two different compounds.

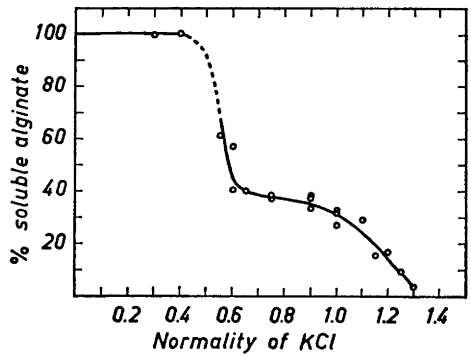


Fig. 1.

The soluble and insoluble fractions of the alginate were shaken with potassium and sodium chloride solutions of the same strength as those used in the original fractionation, and with a volume chosen to give the same alginate concentration. The results are given in Table 1 and show that there is a marked difference between the two fractions with respect to solubility in salt solutions.

Table 1.

Solvent	1st fractionation		2nd fractionation	
			Soluble	Insoluble
1 N NaCl	Soluble	29 %	84 %	16 %
	Insoluble	71 »	12 »	88 »
1 N KCl	Soluble	31 %	100 %	0 %
	Insoluble	69 »	3 »	97 »

Table 2.

	Precipitated, % 2.3 N KCl	Undissolved, % 0.75 N KCl	Undissolved, % 1.0 N KCl
L. digitata, Tarva 3/7—57	69	63	70
Ascophyllum nodosum, Lysøysund 17/9—58	58	55	60
L. hyperborea, Munkholmen 26/8—57			
Fronds	54	45	76
Stipes	—	10	27

The comparatively sharp fractionation makes it improbable that the separation is solely due to a difference in molecular weight. Determinations of viscosity gave an "apparent intrinsic viscosity" determined in a Höppler Viscometer [4] of 17.1, 18.5 and 19.3 $dl \cdot g^{-1}$ for the soluble fraction, the unfractionated alginate, and the insoluble fraction, respectively. A small part of the same *Laminaria digitata* sample as that used above was heated for 15 min to 120°C after the dry matter content had been adjusted to 60 %. The intrinsic viscosity of the alginate was thus reduced to 5.5 $dl \cdot g^{-1}$. In 1 N potassium chloride 45 % of this alginate was insoluble. These observations clearly indicate that the basis of the separation of the fractions in this way is not primarily one of molecular weight differences.

If potassium chloride was added to an alginate solution, no precipitation occurred at salt concentrations below 1.5 N. If, however, a 0.3 % solution of the *Laminaria digitata* alginate was rapidly mixed with equal volumes of saturated (4.65 N) potassium chloride, a gel-like precipitate was formed. The precipitate could be removed from the solution by centrifugation. In Table 2, the percentage of precipitated alginate is given, together with the percentage of alginate which was not dissolved in potassium chloride solution of two different strengths.

In stipes of *Laminaria hyperborea*, the gel that was formed could not be separated from the solution, even by prolonged centrifugation at 20 000 *g*. In the three other cases, the precipitated amount was approximately equal to the amount that was not dissolved in potassium chloride solution of a strength between 0.75 and 1 N. It was also shown that of the fraction that was precipitated only traces could be dissolved in 1 N potassium chloride solution, while the total amount dissolved of the fraction that was not precipitated. Fractionation by precipitation and by dissolution seems, therefore, to give the same fractions.

Alginate from *Laminaria digitata* was hydrolysed according to the method of Fischer and Dörfer [1]. Paper chromatograms of the uronic acids gave two spots with R_G values of 0.38 and 0.32, respectively, in pyridine:ethylacetate-acetic acid: water, 5:5:1:3. Chromatograms of the lactones gave two spots with R_F values 0.53 and 0.77 in pyridine:ethylacetate:water, 11:40:6. According to Fischer and Dörfel these correspond well with the movements of mannuronic and guluronic acids and their lactones. No attempts have been made to determine the uronic acids quantitatively, but on the basis of the size and intensity of the uronic acid spots, there appears to be about three times as much mannuronic as guluronic acid; this is as reported by Fischer and Dörfel.

The fractions separated by precipitation with potassium chloride solution, as described above, were hydrolysed and chromatographed. In both cases two spots with the same R_G values as those found for the unfractionated sample were observed on the uronic acid chromatograms. In the soluble fraction, however, the two spots were of approximately the same strength, while the spot corresponding to guluronic acid was weaker in the insoluble fraction than in the unfractionated sample. Even after five reprecipitations with potassium chloride solution a weak spot corresponding to guluronic acid could be observed in hydrolysates of the insoluble fraction.

The results show that alginic acid in *Laminaria digitata* is a heterogeneous compound, and can be separated into fractions which differ in solubility properties and in uronic acid composition.

The work is being continued and a detailed report will be published later.

1. Chanda, S. R., Hirst, E. L., Percival, E. G. V. and Ross, A. G. *J. Chem. Soc.* **1925** 1833.
2. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem. Hoppe-Seyler* **302** (1955) 186.
3. McDowell, R. H. Chem. & Ind. London **1958** 1401.
4. Haug, A. *Viscosity of Alginate Solutions.* In Report No. 20, Norwegian Institute of Seaweed Research, Trondheim 1958.

Received February 13, 1959.

Note on the Crystal Structure of Trivanadium Pentoxide

STIG ÅSBRINK, STIG FRIBERG and ARNE MAGNÉLI

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

and

GEORG ANDERSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The existence of a vanadium oxide V_3O_5 has been reported in a previous communication [1]. The compound was found to be monoclinic with the symmetry $C2/c$ or Cc and with the unit cell dimensions

$$a = 9.98 \text{ Å}, \quad b = 5.03 \text{ Å}, \quad c = 9.84 \text{ Å}, \quad \beta = 138.8°.$$

Slight deviations in the parameter values have been observed indicating that the range of composition of the oxide has a finite width.

The observed density of 4.6 is in accordance with a unit cell content of 4 units of V_3O_5 (calculated density 4.75).

The crystal structure has been derived on the basis of single-crystal data registered by one of us (G.A.) using MoK radiation. The following preliminary parameter values were thus obtained:

12 V and 20 O in 8 sets of point positions 4(a) of space-group Cc (No. 9): (0,0,0; $\frac{1}{2},\frac{1}{2},0$) + x,y,z; $x,\bar{y},\frac{1}{2}+z$.

4 V_1:	$x = 0.088$,	$y = 0.250$,	$z = 0.464$
4 V_2:	$x = 0.412$,	$y = 0.250$,	$z = 0.536$
4 V_3:	$x = 0.000$,	$y = 0.750$,	$z = 0.250$
4 O_1:	$x = 0.04_4$,	$y = 0.60$,	$z = 0.10_2$
4 O_2:	$x = 0.13_9$,	$y = 0.10$,	$z = 0.31_1$
4 O_3:	$x = 0.36_1$,	$y = 0.90$,	$z = 0.18_9$
4 O_4:	$x = 0.45_6$,	$y = 0.40$,	$z = 0.39_8$
4 O_5:	$x = 0.25_0$,	$y = 0.40$,	$z = 0.00_6$

The arrangement of the metal atoms is centrosymmetrical ($C2/c$) while that of the oxygen atoms (derived by S.Å.) is not.

The structure is built up of VO_6 octahedra which are mutually joined by sharing corners, edges and faces. By means of the latter kind of connection, "double-octahedra" (containing V_1 and V_2 atoms) result which are similar to those occurring in the corundum-type structures, *e. g.* V_2O_3. The "double-octahedra" are mutually joined by edges to form parallel rows extending infinitely through the structure. These rows are coupled together by "single-octahedra" (containing V_3 atoms). The V—V distance of octahedra joined by faces is 2.74 Å, while the V—V distances of octahedra sharing edges are 2.96 and 3.10 Å. The V—O and O—O distances, which so far are less accurately known, occur within the ranges 1.9—2.2 and 2.5—3.1 Å, respectively. Refinement of the structure is in progress.

X-Ray studies at temperatures up to 1 000°C did not show any indications of phase transformations in V_3O_5.

The structure of V_3O_5 is essentially different from those of low- and high-Ti_3O_5 [2]. The V_3O_5 structural type has, however, been found to be present in a phase $TiCr_2O_5$ obtained in low yield by melting a mixture of the pure oxides in an electric arc furnace under an inert atmosphere [3]. (This phase seems to be stabilized by minor contents of aluminium.)

This investigation has been sponsored in part by the *Swedish Natural Science Research Council* and in part by the *Office, Chief of Research and Development, U.S. Department of Army,* through its European Office.

1. Andersson, G. *Acta Chem. Scand.* 8 (1954) 1599.
2. Åsbrink, S. and Magnéli, A. *To be published.*
3. Åsbrink, S. *Unpublished results.*

Received February 14, 1959.

1. Chanda, S. R., Hirst, E. L., Percival, E. G. V. and Ross, A. G. *J. Chem. Soc.* **1925** 1833.
2. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem. Hoppe-Seyler* **302** (1955) 186.
3. McDowell, R. H. Chem. & Ind. London **1958** 1401.
4. Haug, A. *Viscosity of Alginate Solutions.* In Report No. 20, Norwegian Institute of Seaweed Research, Trondheim 1958.

Received February 13, 1959.

Note on the Crystal Structure of Trivanadium Pentoxide

STIG ÅSBRINK, STIG FRIBERG and ARNE MAGNÉLI

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

and

GEORG ANDERSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The existence of a vanadium oxide V_3O_5 has been reported in a previous communication [1]. The compound was found to be monoclinic with the symmetry $C2/c$ or Cc and with the unit cell dimensions

$$a = 9.98 \text{ Å}, \quad b = 5.03 \text{ Å}, \quad c = 9.84 \text{ Å}, \quad \beta = 138.8°.$$

Slight deviations in the parameter values have been observed indicating that the range of composition of the oxide has a finite width.

The observed density of 4.6 is in accordance with a unit cell content of 4 units of V_3O_5 (calculated density 4.75).

The crystal structure has been derived on the basis of single-crystal data registered by one of us (G.A.) using MoK radiation. The following preliminary parameter values were thus obtained:

12 V and 20 O in 8 sets of point positions 4(a) of space-group Cc (No. 9): (0,0,0; $\frac{1}{2},\frac{1}{2},0$) + x,y,z; $x,\bar{y},\frac{1}{2}+z$.

4 V_1:	$x = 0.088$,	$y = 0.250$,	$z = 0.464$
4 V_2:	$x = 0.412$,	$y = 0.250$,	$z = 0.536$
4 V_3:	$x = 0.000$,	$y = 0.750$,	$z = 0.250$
4 O_1:	$x = 0.04_4$,	$y = 0.60$,	$z = 0.10_2$
4 O_2:	$x = 0.13_9$,	$y = 0.10$,	$z = 0.31_1$
4 O_3:	$x = 0.36_1$,	$y = 0.90$,	$z = 0.18_9$
4 O_4:	$x = 0.45_6$,	$y = 0.40$,	$z = 0.39_8$
4 O_5:	$x = 0.25_0$,	$y = 0.40$,	$z = 0.00_6$

The arrangement of the metal atoms is centrosymmetrical ($C2/c$) while that of the oxygen atoms (derived by S.Å.) is not.

The structure is built up of VO_6 octahedra which are mutually joined by sharing corners, edges and faces. By means of the latter kind of connection, "double-octahedra" (containing V_1 and V_2 atoms) result which are similar to those occurring in the corundum-type structures, *e. g.* V_2O_3. The "double-octahedra" are mutually joined by edges to form parallel rows extending infinitely through the structure. These rows are coupled together by "single-octahedra" (containing V_3 atoms). The V—V distance of octahedra joined by faces is 2.74 Å, while the V—V distances of octahedra sharing edges are 2.96 and 3.10 Å. The V—O and O—O distances, which so far are less accurately known, occur within the ranges 1.9—2.2 and 2.5—3.1 Å, respectively. Refinement of the structure is in progress.

X-Ray studies at temperatures up to 1 000°C did not show any indications of phase transformations in V_3O_5.

The structure of V_3O_5 is essentially different from those of low- and high-Ti_3O_5 [2]. The V_3O_5 structural type has, however, been found to be present in a phase $TiCr_2O_5$ obtained in low yield by melting a mixture of the pure oxides in an electric arc furnace under an inert atmosphere [3]. (This phase seems to be stabilized by minor contents of aluminium.)

This investigation has been sponsored in part by the *Swedish Natural Science Research Council* and in part by the *Office, Chief of Research and Development, U.S. Department of Army,* through its European Office.

1. Andersson, G. *Acta Chem. Scand.* 8 (1954) 1599.
2. Åsbrink, S. and Magnéli, A. *To be published.*
3. Åsbrink, S. *Unpublished results.*

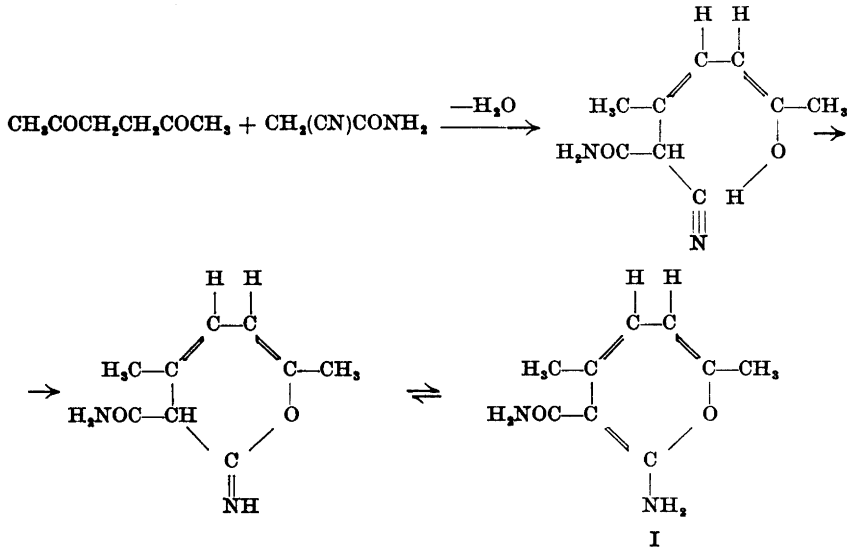
Received February 14, 1959.

The Preparation of an Oxa*cyclo*heptatriene

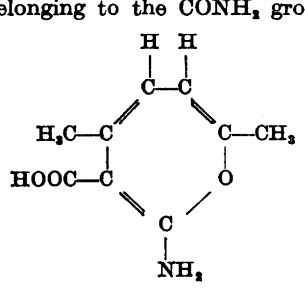
GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

In alkaline solution acetonylacetone reacts with cyanoacetamide with formation of a seven-membered ring, 2-amino-4,7-dimethyloxepine-3-carboxamide (I), according to the equation:



I is a weak base and can be titrated with perchloric acid in anhydrous medium. The structure I of the compound is supported by its IR spectrum, which has no nitrile band and only one C=O band at 1 670 cm^{-1}, belonging to the $CONH_2$ group.



II

When I is boiled with concentrated hydrochloric acid, the amide group is hydrolysed, the amino acid II being formed.

Experimental. Preparation of 2-amino-4,7-dimethyloxepine-3-carboxamide (I). Acetonylacetone (5.0 g) and cyanoacetamide (3.75 g) were dissolved in alkaline ethanol (10 ml of ethanol and 5 ml of 2.4 N sodium hydroxide solution) and allowed to stand overnight. The slightly yellow crystals formed were filtered and washed with 50 % ethanol. Yield 2.3 g. The product can be recrystallized from ethanol. M. p. about 222° (decomp.). (Found: C 60.0; H 6.7; N 15.4; O 17.7; equiv. wt. on titration with perchloric acid in acetic acid — chlorobenzene solution using methyl violet as indicator: 181. Calc. for $C_9H_{12}N_2O_2$: C 60.0; H 6.7; N 15.5; O 17.8; equiv. wt. 180.) UV absorption: $\lambda_{max.}$ = 233, 277 and 345 mμ.

Hydrolysis of I to form 2-amino-4,7-dimethyloxepine-3-carboxylic acid (II). A solution of I (5.0 g) in concentrated hydrochloric acid (75 ml) was boiled for 30 min. The acid was evaporated under reduced pressure and the dry residue was treated with water (75 ml). Sodium acetate solution was added until pH about 3 to make the precipitation of II complete. The precipitate was filtered and washed with water and ethanol. Yield 0.8 g. The product was purified by extraction of by-products with boiling ethanol for half a minute

and by recrystallization from ethanol (solution in excess of hot ethanol as rapidly as possible and evaporation of part of the solvent under reduced pressure to avoid decomposition by heat). M. p. 149° (decomp.). (Found: C 59.8; H 6.2; N 7.8; O 26.3; equiv.wt. by titration using thymolphthalein as indicator: 181. Calc. for $C_9H_{11}NO_3$: C 59.7; H 6.1; N 7.7; O 26.5; equiv.wt. 181.) The IR curve shows amino and carboxyl bands. UV absorption: $\lambda_{max.}$ = 235, 284 and 350 mμ.

The financial support of *Statens naturvetenskapliga forskningsråd* is gratefully acknowledged.

Received February 18, 1959.

o-Methoxybenzhydrol

GUST.-AD. HOLMBERG

Institutet för organisk kemi, Åbo Akademi, Åbo, Finland

o-Methoxybenzhydrol has been prepared by Stoermer and Friderici [1] by reacting *o*-methoxybenzaldehyde with phenylmagnesium iodide. Kahil and Nierenstein [2], who verified the results of Stoermer and Friderici, obtained the compound also by reduction of *o*-methoxybenzophenone. In both cases the melting point was reported to be 141°. Martynoff [3], who synthesized *o*-methoxybenzhydrol from *o*-methoxybenzaldehyde and phenylmagnesium iodide, did not report any melting point but used the crude product for the preparation of bis-*o*-methoxybenzhydryl ether.

When the experiment of Stoermer and Friderici was repeated in this laboratory with *o*-methoxybenzaldehyde and phenylmagnesium bromide the statements as to the melting point of *o*-methoxybenzhydrol could not be verified. Instead of a solid substance, an oil, b.p. 181—182°/8 mm, was obtained. The same substance was formed when *o*-methoxybenzophenone was reduced with zinc in alkaline ethanol. The identity of the oil with *o*-methoxybenzhydrol was established by preparing its 3,5-dinitrobenzoic acid by oxidizing it to *o*-methoxybenzophenone.

o-Methoxybenzhydrol can be heated in boiling xylene for several hours without any change. However, if a minute quantity of an acid is added to the solution, the substance is rapidly and quantitatively converted into bis-*o*-methoxybenzhydryl ether, m. p. 137—137.5°. This property seems to be the reason for the incorrect melting points reported previously.

When the products of the reaction between *o*-methoxybenzaldehyde and phenylmagnesium bromide were decomposed with ice and hydrochloric acid, a small quantity of bis-*o*-methoxybenzhydryl ether was isolated in spite of the fact that the reaction products were almost immediately treated with a potassium carbonate solution. If the decomposition was effected by adding ammonium chloride solution, the yield of the alcohol was slightly greater and no ether could be isolated. These observations demonstrate the instability of the alcohol towards acids.

Considering the difference in stability in the presence and absence of acids, it seems probable that the crude *o*-methoxybenzhydrol that Martynoff used in his synthesis of bis-*o*-methoxybenzhydryl ether contained a small quantity of acid.

Experimental. To a cooled Grignard reagent prepared from magnesium (3.60 g) and bromobenzene (23.55 g) in dry ether (60 ml), *o*-methoxybenzaldehyde (13.60 g) in dry ether (50 ml) was gradually added. After the mixture had been warmed in a water bath for 15 min, the products were decomposed by pouring them into a mixture of ice and dilute hydrochloric acid. The ether phase was thoroughly washed with water and a dilute potassium carbonate solution. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzhydrol, b. p. 181—182°/8 mm, was 77.5 % (16.61 g). (Found: C 78.37; H 6.45. Calc. for $C_{14}H_{14}O_2$: C 78.48; H 6.59.)

The residue in the distillation flask was dissolved in a small quantity of ethanol. After a while bis-*o*-methoxybenzhydryl ether (1.26 g) crystallized. It melted after recrystallization from ethanol at 137—137.5°.

When the decomposition of the reaction products was effected with ammonium chloride solution, the yield of the alcohol was almost 80 % (17.04 g) and no bis-*o*-methoxybenzhydryl ether could be isolated. In this case the washing with potassium carbonate solution was omitted.

A sample of *o*-methoxybenzhydrol was treated with the equivalent quantity of 3,5-dinitrobenzoyl chloride in benzene and pyridine. After the solution had stood for several hours, the precipitate was filtered off and the filtrate washed with water, dilute hydrochloric acid,

and by recrystallization from ethanol (solution in excess of hot ethanol as rapidly as possible and evaporation of part of the solvent under reduced pressure to avoid decomposition by heat). M. p. 149° (decomp.). (Found: C 59.8; H 6.2; N 7.8; O 26.3; equiv.wt. by titration using thymolphthalein as indicator: 181. Calc. for $C_9H_{11}NO_3$: C 59.7; H 6.1; N 7.7; O 26.5; equiv.wt. 181.) The IR curve shows amino and carboxyl bands. UV absorption: $\lambda_{max.}$ = 235, 284 and 350 mμ.

The financial support of *Statens naturvetenskapliga forskningsråd* is gratefully acknowledged.

Received February 18, 1959.

o-Methoxybenzhydrol

GUST.-AD. HOLMBERG

Institutet för organisk kemi, Åbo Akademi, Åbo, Finland

o-Methoxybenzhydrol has been prepared by Stoermer and Friderici [1] by reacting *o*-methoxybenzaldehyde with phenylmagnesium iodide. Kahil and Nierenstein [2], who verified the results of Stoermer and Friderici, obtained the compound also by reduction of *o*-methoxybenzophenone. In both cases the melting point was reported to be 141°. Martynoff [3], who synthesized *o*-methoxybenzhydrol from *o*-methoxybenzaldehyde and phenylmagnesium iodide, did not report any melting point but used the crude product for the preparation of bis-*o*-methoxybenzhydryl ether.

When the experiment of Stoermer and Friderici was repeated in this laboratory with *o*-methoxybenzaldehyde and phenylmagnesium bromide the statements as to the melting point of *o*-methoxybenzhydrol could not be verified. Instead of a solid substance, an oil, b.p. 181—182°/8 mm, was obtained. The same substance was formed when *o*-methoxybenzophenone was reduced with zinc in alkaline ethanol. The identity of the oil with *o*-methoxybenzhydrol was established by preparing its 3,5-dinitrobenzoic acid by oxidizing it to *o*-methoxybenzophenone.

o-Methoxybenzhydrol can be heated in boiling xylene for several hours without any change. However, if a minute quantity of an acid is added to the solution, the substance is rapidly and quantitatively converted into bis-*o*-methoxybenzhydryl ether, m. p. 137—137.5°. This property seems to be the reason for the incorrect melting points reported previously.

When the products of the reaction between *o*-methoxybenzaldehyde and phenylmagnesium bromide were decomposed with ice and hydrochloric acid, a small quantity of bis-*o*-methoxybenzhydryl ether was isolated in spite of the fact that the reaction products were almost immediately treated with a potassium carbonate solution. If the decomposition was effected by adding ammonium chloride solution, the yield of the alcohol was slightly greater and no ether could be isolated. These observations demonstrate the instability of the alcohol towards acids.

Considering the difference in stability in the presence and absence of acids, it seems probable that the crude *o*-methoxybenzhydrol that Martynoff used in his synthesis of bis-*o*-methoxybenzhydryl ether contained a small quantity of acid.

Experimental. To a cooled Grignard reagent prepared from magnesium (3.60 g) and bromobenzene (23.55 g) in dry ether (60 ml), *o*-methoxybenzaldehyde (13.60 g) in dry ether (50 ml) was gradually added. After the mixture had been warmed in a water bath for 15 min, the products were decomposed by pouring them into a mixture of ice and dilute hydrochloric acid. The ether phase was thoroughly washed with water and a dilute potassium carbonate solution. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzhydrol, b. p. 181—182°/8 mm, was 77.5 % (16.61 g). (Found: C 78.37; H 6.45. Calc. for $C_{14}H_{14}O_2$: C 78.48; H 6.59.)

The residue in the distillation flask was dissolved in a small quantity of ethanol. After a while bis-*o*-methoxybenzhydryl ether (1.26 g) crystallized. It melted after recrystallization from ethanol at 137—137.5°.

When the decomposition of the reaction products was effected with ammonium chloride solution, the yield of the alcohol was almost 80 % (17.04 g) and no bis-*o*-methoxybenzhydryl ether could be isolated. In this case the washing with potassium carbonate solution was omitted.

A sample of *o*-methoxybenzhydrol was treated with the equivalent quantity of 3,5-dinitrobenzoyl chloride in benzene and pyridine. After the solution had stood for several hours, the precipitate was filtered off and the filtrate washed with water, dilute hydrochloric acid,

and dilute potassium carbonate solution. After drying with sodium sulphate, most of the benzene was distilled off and the residue dissolved in ether. The solution was kept for some hours in a refrigerator and the pure crystals of 3,5-nitrobenzoic *o*-methoxybenzhydryl ester, m. p. 129—130°, that separated were isolated by filtration. (Found: N 6.71. Calc. for $C_{21}H_{16}N_2O_7$: 6.86.)

o-Methoxybenzophenone (10.36 g) was reduced with zinc (9.00 g) in a solution of potassium hydroxide (12.30 g) in water (6.4 ml) and ethanol (85 ml) according to the method previously used for the preparation of di-*o*-tolylcarbinol [4]. After the mixture had been boiled for 4 h, the excess of zinc was filtered off and the filtrate poured into a cold dilute sulphuric acid solution (4 l). The crude product was taken up in ether and the ether solution washed with dilute potassium carbonate solution. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzhydrol, b. p. 181—182°/8 mm, was 82 % (8.50 g). The identity of the substance was established by preparing its 3,5-dinitrobenzoic ester, which was identical with the above *o*-methoxybenzhydryl compound.

o-Methoxybenzhydrol (11.22 g) and a minute quantity of *p*-toluenesulphonic acid (0.02 g) were dissolved in xylene (100 ml). When the solution was heated in an apparatus for the determination of water, an almost quantitative amount of water distilled within 10 min. After cooling, the solution was washed with a dilute sodium hydroxide solution and the solvent was distilled off. The residue consisting of colourless crystals of crude bis-*o*-methoxybenzhydryl ether was treated with ethanol and filtered. After recrystallization from ethanol, the substance (10.14 g) melted at 137—137.5°.

When the experiment was repeated without adding *p*-toluenesulphonic acid, no water distilled within 6 h.

o-Methoxybenzhydrol (13.96 g) was stirred with a cold solution of potassium bichromate (17.7 g) and concentrated sulphuric acid (15 ml) in water (85 ml). When the exothermic reaction was over and the mixture had cooled, it was extracted with ether. The ether solution was washed twice with water, twice with dilute potassium hydroxide, and finally twice with water. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzophenone, b.p. 177—178°/12 mm, was 73 % (10.12 g). Its 2,4-dinitrophenylhydrazone, prepared according to Wild [5], melted at 173—174° or at the same temperature at which a previously obtained sample melted. By mistake the melting point of this substance was incorrectly reported previously [6].

1. Stoermer, R. and Friderici, E. *Ber.* **41** (1908) 324.
2. Kahil, A. I. M. and Nierenstein, M. *J. Am. Chem. Soc.* **46** (1924) 2556.
3. Martynoff, M. *Ann. Chim. (Paris)* [11] **7** (1937) 424.
4. Holmberg, G. A. *Acta Acad. Aboensis, Math. et Phys.* **16** (1949) No 6.
5. Wild, F. *Characterisation of organic compounds*, Cambridge University Press, Cambridge 1948, p. 114.
6. Holmberg, G. A. *Acta Chem. Scand.* **6** (1952) 1137.

Received February 7, 1959.

The Estimation of Iodine in Urine

GÖSTA WIDSTRÖM and KERSTIN CLAESON

Clinical Laboratories, Centrallasarettet, Danderyd, Sweden

Recently Rodger and Poole [1] have published an investigation on the determination of small amounts of iodine in urine and other biological materials. In order to get acceptable recoveries of iodine, they find it necessary to rely on a distillation method. With a dry ashing technique, slightly modified from that of Barker, Humphrey and Soley [2] for serum, we have obtained a recovery of 96 % of iodine in urine. Our modifications are founded on the experience that the losses of iodine will be smaller when K_2CO_3 is substituted for Na_2CO_3 in the incineration mixture [3], which must have a sufficient content of carboniferous material [4].

Technique: 1 ml 30 % $Zn(Ac)_2$ + 1 ml 0.5 N NaOH are mixed in the tubes used for incineration. The precipitates are allowed to stand about 30 min and centrifuged, the centrifugate is decanted, 1 or 2 ml of the urine and 2 ml of 4 N K_2CO_3 are added and thoroughly mixed with the precipitate.

The tubes are brought in an oven at 90°C in a declined position and their content is dry after 18—20 h. They are then incinerated in a furnace at 600°C for 3 h in a horizontal position. After cooling

and dilute potassium carbonate solution. After drying with sodium sulphate, most of the benzene was distilled off and the residue dissolved in ether. The solution was kept for some hours in a refrigerator and the pure crystals of 3,5-nitrobenzoic *o*-methoxybenzhydryl ester, m. p. 129—130°, that separated were isolated by filtration. (Found: N 6.71. Calc. for $C_{21}H_{16}N_2O_7$: 6.86.)

o-Methoxybenzophenone (10.36 g) was reduced with zinc (9.00 g) in a solution of potassium hydroxide (12.30 g) in water (6.4 ml) and ethanol (85 ml) according to the method previously used for the preparation of di-*o*-tolylcarbinol [4]. After the mixture had been boiled for 4 h, the excess of zinc was filtered off and the filtrate poured into a cold dilute sulphuric acid solution (4 l). The crude product was taken up in ether and the ether solution washed with dilute potassium carbonate solution. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzhydrol, b. p. 181—182°/8 mm, was 82 % (8.50 g). The identity of the substance was established by preparing its 3,5-dinitrobenzoic ester, which was identical with the above *o*-methoxybenzhydryl compound.

o-Methoxybenzhydrol (11.22 g) and a minute quantity of *p*-toluenesulphonic acid (0.02 g) were dissolved in xylene (100 ml). When the solution was heated in an apparatus for the determination of water, an almost quantitative amount of water distilled within 10 min. After cooling, the solution was washed with a dilute sodium hydroxide solution and the solvent was distilled off. The residue consisting of colourless crystals of crude bis-*o*-methoxybenzhydryl ether was treated with ethanol and filtered. After recrystallization from ethanol, the substance (10.14 g) melted at 137—137.5°.

When the experiment was repeated without adding *p*-toluenesulphonic acid, no water distilled within 6 h.

o-Methoxybenzhydrol (13.96 g) was stirred with a cold solution of potassium bichromate (17.7 g) and concentrated sulphuric acid (15 ml) in water (85 ml). When the exothermic reaction was over and the mixture had cooled, it was extracted with ether. The ether solution was washed twice with water, twice with dilute potassium hydroxide, and finally twice with water. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of *o*-methoxybenzophenone, b.p. 177—178°/12 mm, was 73 % (10.12 g). Its 2,4-dinitrophenylhydrazone, prepared according to Wild [5], melted at 173—174° or at the same temperature at which a previously obtained sample melted. By mistake the melting point of this substance was incorrectly reported previously [6].

1. Stoermer, R. and Friderici, E. *Ber.* **41** (1908) 324.
2. Kahil, A. I. M. and Nierenstein, M. *J. Am. Chem. Soc.* **46** (1924) 2556.
3. Martynoff, M. *Ann. Chim. (Paris)* [11] **7** (1937) 424.
4. Holmberg, G. A. *Acta Acad. Aboensis, Math. et Phys.* **16** (1949) No 6.
5. Wild, F. *Characterisation of organic compounds*, Cambridge University Press, Cambridge 1948, p. 114.
6. Holmberg, G. A. *Acta Chem. Scand.* **6** (1952) 1137.

Received February 7, 1959.

The Estimation of Iodine in Urine

GÖSTA WIDSTRÖM and KERSTIN CLAESON

Clinical Laboratories, Centrallasarettet, Danderyd, Sweden

Recently Rodger and Poole [1] have published an investigation on the determination of small amounts of iodine in urine and other biological materials. In order to get acceptable recoveries of iodine, they find it necessary to rely on a distillation method. With a dry ashing technique, slightly modified from that of Barker, Humphrey and Soley [2] for serum, we have obtained a recovery of 96 % of iodine in urine. Our modifications are founded on the experience that the losses of iodine will be smaller when K_2CO_3 is substituted for Na_2CO_3 in the incineration mixture [3], which must have a sufficient content of carboniferous material [4].

Technique: 1 ml 30 % $Zn(Ac)_2$ + 1 ml 0.5 N NaOH are mixed in the tubes used for incineration. The precipitates are allowed to stand about 30 min and centrifuged, the centrifugate is decanted, 1 or 2 ml of the urine and 2 ml of 4 N K_2CO_3 are added and thoroughly mixed with the precipitate.

The tubes are brought in an oven at 90°C in a declined position and their content is dry after 18—20 h. They are then incinerated in a furnace at 600°C for 3 h in a horizontal position. After cooling

the ashes are brought into solution with 5 ml 1 N HCl, cautiously added in small portions with stirring. The tubes are centrifuged. To 2 ml of the clear supernatant are added 2 ml 0.1 N arsenite and the test tubes are held for at least 15 min in a water bath at 25°C for temperature equilibration. 1 ml 0.04 N ceric ammonium sulphate solution of the same temperature is then thoroughly mixed with the contents of the tubes at a noted time and the mixture is kept in the water bath for exactly 30 min when they are read in a (Beckman B) photometer at 440 mμ against distilled water as a blank. The content of iodine is calculated from a calibration curve, plotted from readings of standard solutions.

Urine contains considerably less organic material than serum, but the basic acetate formed when $Zn(Ac)_2$ is precipitated with alkali brings up the total combustible carbon to the same values as those in serum.

Table 1. Apparent iodine content in duplicates from the same urine.

$ZnSO_4$	$Zn(Ac)_2$
3.2	4.2
4.0	4.3
3.9	4.2
4.2	4.2
3.1	

As shown in Table 1 the recovery of iodine in a series of duplicates, of the same urine is improved when $ZnSO_4$ is replaced with $Zn(Ac)_2$.

In a previous publication [3] we have shown, that the substitution of K_2CO_3 for Na_2CO_3 in the incineration mixture will result in considerably smaller losses of iodide in the determination of protein-bound iodine, probably because of the lower vapour pressure of KI.

In Table 2 are given the results of two parallel series where urine samples with added $K^{131}I$ have been incinerated with K_2CO_3 and Na_2CO_3, respectively. The average recovery in the Na_2CO_3 series was 83 % against 96 % in the K_2CO_3 series.

Naturally several types of medication can invalidate the analysis of iodine in urine. Besides iodine compounds, which of course can give very high iodine values, mercurial diuretics can give falsely low values, sulfur-containing drugs, such as sulfonamides, chlorothiazide *etc.* falsely high values, because of their influence on the ceric ammonium sulfate reaction. Urines with high values of calcium must first be treated with ammonium oxalate in order to remove calcium to avoid loss of iodine as CaI_2 during the incineration [3].

Table 2. Recovery of added ^{131}I.

K_2CO_3	Na_2CO_3
Recovery %	Recovery %
87	86
100.5	78
97.4	86
97.6	82
97.0	82
Mean: 96 %	Mean: 83 %

1. Rodgers, K. and Poole, D. B. *J. Biol. Chem.* **70** (1958) 463.
2. Barker, S. B., Humphrey, M. J. and Soley, M. H. *J. Clin. Invest.* **30** (1951) 55.
3. Widström, G. and Björkman, M. *Acta Chem. Scand.* **12** (1958) 1881.
4. Stanbury, J. B. *et al. Endemic Goiter*, Harvard University Press **12** (1954) 24.

Received February 20, 1959.

Sodium Hydroxide in Alcohol as a Base for the Alkylation of Ethyl Acetoacetate

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

In the alkylation of ethyl acetoacetate by means of an alkyl bromide and an alcoholic solution of sodium ethoxide it is usually stated that the quality of the absolute alcohol is important [1]. Most handbooks recommend the use of »superdry» alcohol, *i.e.* alcohol dried with magnesium or by similar methods.

Table 1.

Alkylhalide	*Yield* %	*B. p.*
C_2H_5Br	73	76— 80°/10 mm Hg
n-C_3H_7Br	56	86— 92°/10 » »
n-C_4H_9Br	45	100—105°/10 » »
$CH_2=CH—CH_2Br$	74	90— 95°/10 » »
$C_6H_5CH_2Cl$	59	145—150°/10 » »

In a kinetic investigation of the methylation of ethyl acetoacetate Brändström [2] has recently shown that the hydrolysis of ethyl acetoacetate is slow if the amount of water present in the sodium ethoxide solution is small. This suggests that it is not so important to dry the alcohol which is to be used as a solvent in alkylation of ethyl acetoacetate. In order to test this, some alkylations of ethyl acetoacetate have been undertaken where sodium hydroxide dissolved in 99.5 % alcohol was used as a base. With alkyl halides of an intermediate reactivity such as ethyl bromide the yields obtained were only slightly lower than those obtainable with "superdry" alcohol and sodium.

The use of sodium hydroxide in alcohol is thus strongly recommended as a base in alkylations of ethyl acetoacetate with alkyl halides of an intermediate or high reactivity.

It should be noted that the same base cannot be used in the alkylations of diethyl malonate or other esters which have a much lower acidity than ethyl acetoacetate.

Experimental. 40 g (1 mole) of sodium hydroxide is dissolved in 500 ml of technical absolute alcohol (containing about 0.5 % of water) in a 1 litre three-necked flask, fitted with a stirrer, a reflux condenser and a dropping funnel. 130 g (1 mole) of ethyl acetoacetate is added followed by 1.2 moles of an alkyl halide. The mixture is refluxed for several hours until it has a neutral reaction.

About 300 ml of alcohol is then distilled off. The residue is cooled and 500 ml of water is added. The layers are separated and the aqueous layer is extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate, and then fractionated under reduced pressure. The yields and boiling points obtained are given in Table 1.

The author is indebted to Mr. Ragnvald Lindblom for good help with the manual work.

1. *Organic Syntheses*, Coll. Vol. I (1944) 249.
2. Brändström, A. *Arkiv Kemi* **13** (1958) 51.

Received February 23, 1959.

A Method for the Preparation of *tert*-Alkylacetic Acids

ARNE BRÄNDSTROM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

tert-Alkylacetic acids are very seldom referred to in chemical literature. The reason for this is that they are not readily available by the standard methods for the preparation of aliphatic acids. For instance, the malonic ester method has been used [1] but it gives a very low yield. The halides R-CH_2X where R is a *tert*-alkyl group are not readily available, and they do not react with cyanide ion to the corresponding nitrile. The only general method which has been used is that starting with the corresponding *tert*-alkylcyanoacetic ester which in turn can be prepared from an unsaturated cyanosubstituted ester and a Grignard reagent [2-4]. This method is, however, not very convenient for a large scale synthesis of *tert*-alkylacetic acids.

The following method is however, very useful (see p. 609).

The starting materials RCH_2CH_2Cl where R represents a tertiary alkyl group are readily available from the reaction of a tertiary chloride RCl with ethylene or by other methods [5]. All chlorides used in the following preparations are, *e.g.*, obtained from the reaction of *tert*-butyl chloride with ethylene [6].

The conversion of the chloride into the alcohol is conveniently carried out without isolating the intermediate ester by using polyethylene glycol as a solvent. The overall yield for the preparations of alcohols from the chlorides are 70—80 %.

The oxidation of the alcohols to the corresponding acids are the standard procedures using potassium permanganate or chromic acid.

Experimental. Preparation of 3,3-dimethylbutanol-1. 132 g (2 moles) of potassium hydroxide are dissolved in 600 ml of polyethylene glycole-300 in a 2 litres three-necked flask fitted with a strong stirrer, a reflux condenser, a thermometer and a dropping funnel. 120 g (2 moles) of conc. acetic acid are added with stirring. The water formed in the neutralisation is removed by distillation under reduced pressure. When the mixture starts foaming the distillation is discontinued. 241 g (2 moles)

Table 1.

Alkylhalide	*Yield* %	*B. p.*
C_2H_5Br	73	76— 80°/10 mm Hg
n-C_3H_7Br	56	86— 92°/10 » »
n-C_4H_9Br	45	100—105°/10 » »
$CH_2=CH—CH_2Br$	74	90— 95°/10 » »
$C_6H_5CH_2Cl$	59	145—150°/10 » »

In a kinetic investigation of the methylation of ethyl acetoacetate Brändström [2] has recently shown that the hydrolysis of ethyl acetoacetate is slow if the amount of water present in the sodium ethoxide solution is small. This suggests that it is not so important to dry the alcohol which is to be used as a solvent in alkylation of ethyl acetoacetate. In order to test this, some alkylations of ethyl acetoacetate have been undertaken where sodium hydroxide dissolved in 99.5 % alcohol was used as a base. With alkyl halides of an intermediate reactivity such as ethyl bromide the yields obtained were only slightly lower than those obtainable with "superdry" alcohol and sodium.

The use of sodium hydroxide in alcohol is thus strongly recommended as a base in alkylations of ethyl acetoacetate with alkyl halides of an intermediate or high reactivity.

It should be noted that the same base cannot be used in the alkylations of diethyl malonate or other esters which have a much lower acidity than ethyl acetoacetate.

Experimental. 40 g (1 mole) of sodium hydroxide is dissolved in 500 ml of technical absolute alcohol (containing about 0.5 % of water) in a 1 litre three-necked flask, fitted with a stirrer, a reflux condenser and a dropping funnel. 130 g (1 mole) of ethyl acetoacetate is added followed by 1.2 moles of an alkyl halide. The mixture is refluxed for several hours until it has a neutral reaction.

About 300 ml of alcohol is then distilled off. The residue is cooled and 500 ml of water is added. The layers are separated and the aqueous layer is extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate, and then fractionated under reduced pressure. The yields and boiling points obtained are given in Table 1.

The author is indebted to Mr. Ragnvald Lindblom for good help with the manual work.

1. *Organic Syntheses*, Coll. Vol. I (1944) 249.
2. Brändström, A. *Arkiv Kemi* **13** (1958) 51.

Received February 23, 1959.

A Method for the Preparation of *tert*-Alkylacetic Acids

ARNE BRÄNDSTROM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

tert-Alkylacetic acids are very seldom referred to in chemical literature. The reason for this is that they are not readily available by the standard methods for the preparation of aliphatic acids. For instance, the malonic ester method has been used [1] but it gives a very low yield. The halides R-CH_2X where R is a *tert*-alkyl group are not readily available, and they do not react with cyanide ion to the corresponding nitrile. The only general method which has been used is that starting with the corresponding *tert*-alkylcyanoacetic ester which in turn can be prepared from an unsaturated cyanosubstituted ester and a Grignard reagent [2-4]. This method is, however, not very convenient for a large scale synthesis of *tert*-alkylacetic acids.

The following method is however, very useful (see p. 609).

The starting materials RCH_2CH_2Cl where R represents a tertiary alkyl group are readily available from the reaction of a tertiary chloride RCl with ethylene or by other methods [5]. All chlorides used in the following preparations are, *e.g.*, obtained from the reaction of *tert*-butyl chloride with ethylene [6].

The conversion of the chloride into the alcohol is conveniently carried out without isolating the intermediate ester by using polyethylene glycol as a solvent. The overall yield for the preparations of alcohols from the chlorides are 70—80 %.

The oxidation of the alcohols to the corresponding acids are the standard procedures using potassium permanganate or chromic acid.

Experimental. Preparation of 3,3-dimethylbutanol-1. 132 g (2 moles) of potassium hydroxide are dissolved in 600 ml of polyethylene glycole-300 in a 2 litres three-necked flask fitted with a strong stirrer, a reflux condenser, a thermometer and a dropping funnel. 120 g (2 moles) of conc. acetic acid are added with stirring. The water formed in the neutralisation is removed by distillation under reduced pressure. When the mixture starts foaming the distillation is discontinued. 241 g (2 moles)

$$RCH_2CH_2Cl + CH_3COOK \rightarrow RCH_2CH_2O{-}COCH_3 \xrightarrow{KOH} RCH_2CH_2OH \xrightarrow{Ox.} RCH_2COOH$$

of 1-chloro-3,3-dimethylbutane are added, and the mixture is refluxed for 17 h. The temperature is 118° at the beginning and 132° at the end of the reaction.

197 g (3 moles) of potassium hydroxide are dissolved in 150 ml of water and the solution is cautiously added to the reaction mixture, which is then refluxed for 2 h.

The alcohol is distilled with steam. The steam distillate is separated, and the water layer extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate and distilled. The yield is 165 g (81 %) of 3,3-dimethylbutanol-1, boiling at 136—142°. 3,5-Dinitrobenzoate, m. p. 80.2—80.8°.

Preparation of 3,3-dimethylpentanol-1. The procedure given above is followed with the following modification: When the mixture has been refluxed for 17 h, the temperature is 140°. A water separator [7] is now connected to the flask, and the mixture refluxed for 3 h during which time about 35 ml of water is collected in the separator. The temperature in the flask is then 195°. The solution of potassium hydroxide in water is now added, and the procedure given above is followed.

The yield of 3,3-dimethylpentanol-1 boiling at 76—66°/10 mm is 170 g or 77 %. 3,5-Dinitrobenzoate, m. p. 51.2—51.8°.

Preparation of 3,3,4-trimethylpentanol-1. The procedure given for 3,3-dimethyl-pentanol-1 is followed. The end temperature is 205°. The yield of 3,3,4-trimethylpentanol-1 is 182 g or 69 %. The boiling point is 78—82°/10 mm Hg. The melting point of the 3,4-dinitrobenzoate is 46.6—47.2°.

Preparation of 3,3,6,6-tetramethylheptanol-1. The method given for 3,3-dimethylpentanol is followed with the modification that the water separator is connected to the flask from the beginning. The total time of reflux is 4 h during which time the temperature rises from 150° to 252°. The mixture was then allowed to cool, and the potassium hydroxide solution added. The general procedure was then followed.

The yield of 3,3,6,6-tetramethylheptanol-1 boiling at 103—108°/8 mm Hg is 294 g or 85 %.

Preparation of 3,3-dimethylbutanoic acid. A 3 litres three-necked flask is fitted with a stirrer, a reflux condensor, and a device for intermittent addition of a solid [8]. 210 g of potassium permanganate are added in portions to a refluxing mixture of 102 g (1 mole) of 3,3-dimethylbutanol, 1.5 litres of water, and 20 g of potassium carbonate. The mixture is refluxed for 15 min after the addition, and then filtered still hot. The cake of manganese dioxide is washed with hot water. The alkaline filtrate is acidified with concentrated hydrochloric acid. The layers are separated and the aqueous layer extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate and fractionated under reduced pressure. The yield of 3,3-dimethylbutanoic acid boiling at 76—80°/8 mm is 94 g or 81 %.

Preparation of 3,3-dimethylpentanoic acid The procedure given above is followed using one mole of 3,3-dimethylpentanol. The yield of 3,3-dimethylpentanoic acid is 80.5 g or 62 % The boiling point is 93—97°/8 mm Hg.

Preparation of 3,3,4-trimethylpentanoic acid:
a) *Oxidation with potassium permanganate.* If the procedure given for 3,3-dimethylbutanoic acid is followed, much alcohol remains after the decolourisation of the permanganate solution. The alcohol is therefore removed by distillation with steam before the manganese dioxide is filtered off. 67 g of 3,3,4-trimethylpentanol, b. p. 78—82°/10 mm Hg, are thus recovered. From the alkaline solution obtained after the filtration of the manganese dioxide 39 g of 3,3,4-trimethyl-pentanoic acid are obtained. This is 53 % calculated on the alcohol not recovered. The boiling point is 105—108°/10 mm.

b) *Oxidation with chromic acid.* 118 g of 3,3,4-trimethylpentanol-1 is added to a refluxing solution of 200 g of sodium dichromate, 485 ml of water, and 140 ml of conc. sulphuric acid. The mixture is stirred and refluxed for 2 h. The reaction mixture is then distilled with steam. The distillate is separated, and the water layer is extracted with benzene. The combined organic layers are extracted with a 20 % sodium hydroxide solution. The alkaline extract is acidified with hydrochloric acid, and the acid extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate, and fractionated under reduced pressure. The yield of 3,3,4-trimethylpentanoic acid is 55 g or 38 %.

Preparation of 3,3,6,6-tetramethylheptanoic acid. If 3,3,6,6-tetramethylheptanol-1 is oxidized with chromic acid by the method described above, 3,3,6,6-tetramethylheptanoic acid boil-

ing at 130—133°/10 mm Hg is obtained in a yield of 40 %. The acid is solid at room temperature. The melting point is 44—46°.

The author is indebted to Mr. Holger Blomster for good help with the manual work.

1. Kögl, F. and Salemink, C. A. *Rec. trav. chim.* **74** (1955) 221.
2. Wideqvist, S. *Arkiv Kemi Mineral. Geol.* **B 23** (1946) No. 4.
3. Prout, F. S. *J. Am. Chem. Soc.* **74** (1952) 5915.
4. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1954) 561.
5. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
6. Brändström, A. *Acta Chem. Scand.* **13** (1959) *In press.*
7. Brändström, A. *Arkiv Kemi* **1** (1950) 481.
8. Vogel, A. I. *Practical Organic Chemistry*, Longmans, Green & Co., London 1948, p. 68.

Received February 23, 1959.

A New Method to Prepare Trialkylacetic Acids

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

There are many methods given for the preparation of trialkylacetic acids[1,2] but no one is entirely convenient for a large scale preparation of these acids. They can, however, be prepared by the following satisfactory reactions, where R is a *tert*-alkyl group:

$$\begin{aligned}
&ROH \longrightarrow RCl + H_2O\\
&RCl + CH_2 = CHCl \longrightarrow RCH_2CHCl_2\\
&RCH_2CHCl_2 + KOH \longrightarrow RCH = CHCl + KCl + H_2O\\
&RCH = CHCl + 2\ Na \longrightarrow RCH = CHNa + NaCl\\
&\qquad\qquad \xrightarrow{HCl}\\
&RCH = CHNa + CO_2 \longrightarrow RCH = CHCOOH + NaCl\\
&RCH = CHCOOH + 2\ KOH \longrightarrow RCOOK + CH_3COOK + H_2
\end{aligned}$$

The yields in all these steps are excellent and the procedure very simple and rapid. Thus, great quantities of the acids are easily obtainable. As there are practically no side reactions the acids obtained are easily purified.

The procedure is demonstrated by the synthesis of pivalic acid. Other acids can be prepared by exactly the same method.

Experimental. tert-*Butyl chloride*[3]. 1 kg of *tert*-butanol is shaken with 3.4 litres of conc. hydrochloric acid for 20 min in a 5-litre separatory funnel. The organic layer is separated, washed with 1 litre of water and dried with anhydrous magnesium sulphate at —20° for 10 min. The liquid is then transferred by filtration to a 5-litre three-necked flask. The yield is 1 160 g (93 %) of pure *tert*-butyl chloride.

1,1-Dichloro-3,3-dimethylbutane[4]. The 5-litre three-necked flask containing the *tert*-butyl chloride is chilled to —25° by means of a solid carbon dioxide-acetone bath and fitted with a stirrer, a gas inlet tube ending under the liquid, a thermometer, and a gas escape tube. 25 g of powdered anhydrous aluminium chloride is added, and the gas inlet tube connected to a 1-litre flask containing 860 ml of vinyl chloride. The vinyl chloride is distilled into the reaction mixture at such a rate that the reaction temperature can be kept at —25° to —30°. — *It is very important that the reaction starts before much vinyl chloride has been added.* This can be seen as a rapid rise in temperature if the cooling bath is temporarily removed. — After the reaction has started the vinyl chloride can usually be introduced in one hour. The reaction mixture is then kept at —25° to —30° for 15 min, and is then decanted into 500 ml of water. The mixture is thoroughly shaken, separated and dried with solid potassium hydroxide for 2 h. The yield is 1 870 g (96 %) of a nearly pure 1,1-dichloro-3,3-dimethylbutane.

1-chloro-3,3-dimethylbutene-1. 780 g of potassium hydroxide are dissolved in 3.6 litres of diethylene glycol in a 10-litre three-necked flask fitted with a dropping funnel, a reflux condenser, a thermometer, and a strong stirrer. The solution is heated to 140° and the dichlor ide added. The mixture is refluxed with good stirring for 6 h, whereupon the product is distilled from the flask until the temperature in the liquid is 190°. The product is separated

ing at 130—133°/10 mm Hg is obtained in a yield of 40 %. The acid is solid at room temperature. The melting point is 44—46°.

The author is indebted to Mr. Holger Blomster for good help with the manual work.

1. Kögl, F. and Salemink, C. A. *Rec. trav. chim.* **74** (1955) 221.
2. Wideqvist, S. *Arkiv Kemi Mineral. Geol.* **B 23** (1946) No. 4.
3. Prout, F. S. *J. Am. Chem. Soc.* **74** (1952) 5915.
4. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1954) 561.
5. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
6. Brändström, A. *Acta Chem. Scand.* **13** (1959) *In press.*
7. Brändström, A. *Arkiv Kemi* **1** (1950) 481.
8. Vogel, A. I. *Practical Organic Chemistry*, Longmans, Green & Co., London 1948, p. 68.

Received February 23, 1959.

A New Method to Prepare Trialkylacetic Acids

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

There are many methods given for the preparation of trialkylacetic acids[1,2] but no one is entirely convenient for a large scale preparation of these acids. They can, however, be prepared by the following satisfactory reactions, where R is a *tert*-alkyl group:

$$\begin{aligned}
&ROH \longrightarrow RCl + H_2O\\
&RCl + CH_2 = CHCl \longrightarrow RCH_2CHCl_2\\
&RCH_2CHCl_2 + KOH \longrightarrow RCH = CHCl + KCl + H_2O\\
&RCH = CHCl + 2\ Na \longrightarrow RCH = CHNa + NaCl\\
&\qquad\qquad \xrightarrow{HCl}\\
&RCH = CHNa + CO_2 \longrightarrow RCH = CHCOOH + NaCl\\
&RCH = CHCOOH + 2\ KOH \longrightarrow RCOOK + CH_3COOK + H_2
\end{aligned}$$

The yields in all these steps are excellent and the procedure very simple and rapid. Thus, great quantities of the acids are easily obtainable. As there are practically no side reactions the acids obtained are easily purified.

The procedure is demonstrated by the synthesis of pivalic acid. Other acids can be prepared by exactly the same method.

Experimental. tert-*Butyl chloride*[3]. 1 kg of *tert*-butanol is shaken with 3.4 litres of conc. hydrochloric acid for 20 min in a 5-litre separatory funnel. The organic layer is separated, washed with 1 litre of water and dried with anhydrous magnesium sulphate at —20° for 10 min. The liquid is then transferred by filtration to a 5-litre three-necked flask. The yield is 1 160 g (93 %) of pure *tert*-butyl chloride.

1,1-Dichloro-3,3-dimethylbutane[4]. The 5-litre three-necked flask containing the *tert*-butyl chloride is chilled to —25° by means of a solid carbon dioxide-acetone bath and fitted with a stirrer, a gas inlet tube ending under the liquid, a thermometer, and a gas escape tube. 25 g of powdered anhydrous aluminium chloride is added, and the gas inlet tube connected to a 1-litre flask containing 860 ml of vinyl chloride. The vinyl chloride is distilled into the reaction mixture at such a rate that the reaction temperature can be kept at —25° to —30°. — *It is very important that the reaction starts before much vinyl chloride has been added.* This can be seen as a rapid rise in temperature if the cooling bath is temporarily removed. — After the reaction has started the vinyl chloride can usually be introduced in one hour. The reaction mixture is then kept at —25° to —30° for 15 min, and is then decanted into 500 ml of water. The mixture is thoroughly shaken, separated and dried with solid potassium hydroxide for 2 h. The yield is 1 870 g (96 %) of a nearly pure 1,1-dichloro-3,3-dimethylbutane.

1-chloro-3,3-dimethylbutene-1. 780 g of potassium hydroxide are dissolved in 3.6 litres of diethylene glycol in a 10-litre three-necked flask fitted with a dropping funnel, a reflux condenser, a thermometer, and a strong stirrer. The solution is heated to 140° and the dichlor ide added. The mixture is refluxed with good stirring for 6 h, whereupon the product is distilled from the flask until the temperature in the liquid is 190°. The product is separated

from the water layer in the distillate, dried with anhydrous magnesium sulphate, and then distilled through a Widmer column. The yield is 1 150 g of 1-chloro-3,3-dimethylbutene-1, b. p. 105—115°. 190 g of the dichloride, b. p. 148—153°, is recovered. This corresponds to a yield of 90 % calculated on the dichloride not recovered.

β-tert-Butylacrylic acid. A fine dispersion [5] of 270 g of sodium in 270 ml of pure kerosene, free from sulphurous compounds and aromatics, is added to 4 litres of pure kerosene contained in a 10-litre three-necked flask fitted with a stirrer, a thermometer, a gas inlet tube, a gas escape tube, and a dropping funnel. A slow stream of dry nitrogen is passed through the flask, where the temperature is adjusted to 27°. A solid carbon dioxide acetone bath is kept ready for use and about 100 ml of a solution of 593 g of 1-chloro-3,3-dimethylbutene-1 in 1 litre of kerosene is added. If the reaction starts, which is indicated by a rapid darkning of the contents in the flask and a rapid rise in the temperature, the flask is cooled so that the temperature is kept within 25—30°, and the rest of the solution added as rapidly as possible without exceeding these temperature limits. — *If* the reaction will not begin, a few ml of *n*-butanol is added to help it to start. *Under no conditions more than 200 ml of the chloride solution should be added before the reaction starts or else the reaction may go beyond control.* — When all the chloride solution has been added (about 1 h) the reaction is completed by stirring at 25—30° for 1 h.

The flask is chilled to 0° and carbonated at 0—15° by a rapid stream of dry carbon dioxide, which is bubbled through the black reaction mixture. When the temperature does not rise, even if the cooling bath is removed, carbon dioxide is introduced for 15 min more. Water is then rapidly added through the dropping funnel to destroy the excess of sodium and dissolve the sodium salts. *This can be done without danger if the reaction between the chloride and sodium had started and a stream of carbon dioxide is passed through the flask.*

The layers are separated and the aqueous layer acidified with conc. hydrochloric acid. After cooling the solid acid cake is removed, transferred to a small beaker where it is melted and decanted from the resulting small water layer. The acid thus obtained weighs 550 g (86 %), and is pure enough for the next step.

Pivalic acid [6]. 1 100 g of the acid above is carefully mixed with a solution of 1 150 g of potassium hydroxide (85—87 %) in 1 150 ml of water in a pan made of stainless steel. The pan is provided with a loose cover to avoid splashing and is heated electrically until the temperature reaches 300°. The thermometer is protected from the caustic mixture by means of a copper tube.

The slightly coloured reaction mixture is allowed to cool, dissolved in water, acidified with conc. hydrochloric acid, the organic layer separated and the water layer extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate and distilled through a 30 cm Widmer column. After a small forefraction at 150—162°, 760 g (87 %) of pivalic acid is obtained at 162—165°. The product is completely solidified at room temperature, and melts at 34—35°. The yield is 57 % calculated on the *tert*-butanol.

The author is indebted to Mr. Ragnvald Lindblom for good help with the manual work.

1. Wiberg, K. B. *Diss.* Columbia Univ. 1950; *University microfilms*, Publ. No. 4619.
2. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1953) 351.
3. *Organic Syntheses*, Coll. Vol. **I** (1944) 144.
4. Schmerling, L. *J. Am. Chem. Soc.* **68** (1946) 1650.
5. Foreman, E. L. and McElvain, S. M. *J. Am. Chem. Soc.* **62** (1940) 1438.
6. Varrentrap, F. *Ann.* **35** (1840) 196.

Received February 23, 1959.

A Method for the Preparation of Trialkylacetaldehydes

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

In chemical literature there are given many methods to prepare trialkylacetaldehydes, but the starting materials are usually not readily available, and it is rather difficult to obtain even the most simple aldehyde of this kind (the trimethylacetaldehyde [1]). The following method seems, however, quite satisfactory (p. 612).

From these formulas where R is a tertiary alkyl group it can be seen that there are only readily available starting materials except possibly the sodium periodate but this reagent is easily prepared

from the water layer in the distillate, dried with anhydrous magnesium sulphate, and then distilled through a Widmer column. The yield is 1 150 g of 1-chloro-3,3-dimethylbutene-1, b. p. 105—115°. 190 g of the dichloride, b. p. 148—153°, is recovered. This corresponds to a yield of 90 % calculated on the dichloride not recovered.

β-tert-Butylacrylic acid. A fine dispersion [5] of 270 g of sodium in 270 ml of pure kerosene, free from sulphurous compounds and aromatics, is added to 4 litres of pure kerosene contained in a 10-litre three-necked flask fitted with a stirrer, a thermometer, a gas inlet tube, a gas escape tube, and a dropping funnel. A slow stream of dry nitrogen is passed through the flask, where the temperature is adjusted to 27°. A solid carbon dioxide acetone bath is kept ready for use and about 100 ml of a solution of 593 g of 1-chloro-3,3-dimethylbutene-1 in 1 litre of kerosene is added. If the reaction starts, which is indicated by a rapid darkning of the contents in the flask and a rapid rise in the temperature, the flask is cooled so that the temperature is kept within 25—30°, and the rest of the solution added as rapidly as possible without exceeding these temperature limits. — *If* the reaction will not begin, a few ml of *n*-butanol is added to help it to start. *Under no conditions more than 200 ml of the chloride solution should be added before the reaction starts or else the reaction may go beyond control.* — When all the chloride solution has been added (about 1 h) the reaction is completed by stirring at 25—30° for 1 h.

The flask is chilled to 0° and carbonated at 0—15° by a rapid stream of dry carbon dioxide, which is bubbled through the black reaction mixture. When the temperature does not rise, even if the cooling bath is removed, carbon dioxide is introduced for 15 min more. Water is then rapidly added through the dropping funnel to destroy the excess of sodium and dissolve the sodium salts. *This can be done without danger if the reaction between the chloride and sodium had started and a stream of carbon dioxide is passed through the flask.*

The layers are separated and the aqueous layer acidified with conc. hydrochloric acid. After cooling the solid acid cake is removed, transferred to a small beaker where it is melted and decanted from the resulting small water layer. The acid thus obtained weighs 550 g (86 %), and is pure enough for the next step.

Pivalic acid [6]. 1 100 g of the acid above is carefully mixed with a solution of 1 150 g of potassium hydroxide (85—87 %) in 1 150 ml of water in a pan made of stainless steel. The pan is provided with a loose cover to avoid splashing and is heated electrically until the temperature reaches 300°. The thermometer is protected from the caustic mixture by means of a copper tube.

The slightly coloured reaction mixture is allowed to cool, dissolved in water, acidified with conc. hydrochloric acid, the organic layer separated and the water layer extracted with benzene. The combined organic layers are dried with anhydrous magnesium sulphate and distilled through a 30 cm Widmer column. After a small forefraction at 150—162°, 760 g (87 %) of pivalic acid is obtained at 162—165°. The product is completely solidified at room temperature, and melts at 34—35°. The yield is 57 % calculated on the *tert*-butanol.

The author is indebted to Mr. Ragnvald Lindblom for good help with the manual work.

1. Wiberg, K. B. *Diss.* Columbia Univ. 1950; *University microfilms*, Publ. No. 4619.
2. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1953) 351.
3. *Organic Syntheses*, Coll. Vol. **I** (1944) 144.
4. Schmerling, L. *J. Am. Chem. Soc.* **68** (1946) 1650.
5. Foreman, E. L. and McElvain, S. M. *J. Am. Chem. Soc.* **62** (1940) 1438.
6. Varrentrap, F. *Ann.* **35** (1840) 196.

Received February 23, 1959.

A Method for the Preparation of Trialkylacetaldehydes

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

In chemical literature there are given many methods to prepare trialkylacetaldehydes, but the starting materials are usually not readily available, and it is rather difficult to obtain even the most simple aldehyde of this kind (the trimethylacetaldehyde [1]). The following method seems, however, quite satisfactory (p. 612).

From these formulas where R is a tertiary alkyl group it can be seen that there are only readily available starting materials except possibly the sodium periodate but this reagent is easily prepared

$ROH + HCl$		RCl	93 % yield
$RCl + CH_2=CH_2$	$\xrightarrow{AlCl_3}$	RCH_2CH_2Cl	86 % yield
RCH_2CH_2Cl	$\xrightarrow{KOH}$	$RCH=CH_2$	60 % yield
$RCH=CH_2$	$\xrightarrow{HCOOH+H_2O_2}$	$RCH(OH)CH_2OH$	78 % yield
$RCH(OH)CH_2OH$	$\xrightarrow{Na_3H_2IO_6}$	$RCHO$	79 % yield

from sodium chlorate and iodine [2] and can be recovered.

The method is demonstrated by the synthesis of trimethylacetaldehyde on a large laboratory scale.

Experimental. 1-Chloro-3,3-dimethylbutane[3]. In a 20 litre flask 3 kg of *tert*-butanol are shaken with 10 litres of concentrated hydrochloric acid for 20 min. The layers are separated, the upper layer washed with 1/2 litre of water and dried with 100 g of anhydrous sodium sulphate for 15 min at —20°. The *tert*-butyl chloride which is quite pure is filtered into a 10 litre three-necked flask. The yield of *tert*-butyl chloride is 3.5 kg.

The flask is fitted with a stirrer, a gas inlet tube, a gas escape tube, and a thermometer, and is chilled in a dry ice-acetone bath to —20°. 50 g of powdered anhydrous aluminium chloride are added and pure ethylene introduced as fast as it is consumed. The temperature is kept between —17° and —20° and regulated with the cooling and the rate of stirring (which determines the rate of absorption of ethylene). When the vigorous absorption of ethylene appears to cease (after about 2 h) the liquid is decanted into 200 ml of water, the mixture well shaken, the organic layer separated, dried with solid potassium hydroxide and distilled. The fraction boiling at 115—122° is taken as 1-chloro-3,3-dimethylbutane and weighs 3 900 g.

tert-*Butylethylene.* 2 100 g of potassium hydroxide are dissolved in 9 litres of polyethyleneglycol-300 and the solution is heated to 130° in a 20 litre three-necked flask placed in a heating mantle and fitted with a dropping funnel, a stirrer and a reflux condenser, the top of which is connected to a condenser arranged for distillation into a receiver chilled to —30°. 3 600 g of 1-chloro-3,3-dimethylbutane are added at such a rate that the temperature in the flask is kept at 120—190°, and the temperature at the top of the reflux condenser is kept at 40—60° by regulating the cooling water in the mantle. When necessary, the flask is heated by means of the heating mantle. When no more distillate is obtained, the organic layer is decanted from the ice in the receiver and distilled through a column into a receiver kept at —30°. The fraction, b. p. 40—50°, is taken as *tert*-butylethylene and weighs 1 500 g.

tert-*Butylethylene glycol* [4]. 9 litres of formic acid (98—100 %) are mixed with 2.5 litres of hydrogen peroxide (30 %) and heated to 37° in a 20 litre three-necked flask fitted with a stirrer, a reflux condenser feeded with ice-water, and a dropping funnel. 1.5 g of *tert*-butylethylene are added in 1 h with vigorous stirring and external cooling with ice-water so that the temperature is kept at 37—40°. When all the *tert*-butylethylene has been added, the mixture is stirred at the same temperature for another 1 1/2 h after which it is kept at room temperature over night.

The formic acid is removed under reduced pressure by means of a water-bath kept below 55°. When no more distillate is obtained (about 10 litres), the distillation condenser is replaced by a reflux condenser. 3.2 litres of a 33 % sodium hydroxide solution are added, and the mixture refluxed with rapid stirring for 15 min and then cooled to room temperature. 1.3 litres of benzene are added and the layers separated. The water layer is extracted with 1 litre of benzene and the combined organic layers are distilled under reduced pressure. 1.4 kg of *tert*-butylethylene glycol, b. p. 95—115°/8 mm, are obtained. The residue in the distillation flask is refluxed with the aqueous layer from the separation for 15 min, the mixture extracted with 1 litre of benzene and then distilled. In this way another 250 g of *tert*-butylethylene glycol are obtained. The total yield is thus 1.65 kg. The product is completely solid at room temperature and pure enough for the next step.

Trimethylacetaldehyde. 4.15 kg of $Na_3H_2IO_6$, 1.65 kg of *tert*-butylethylene glycol and 11 litres of water are placed in a 20 litres three-necked flask fitted with a stirrer, a thermometer, a dropping funnel and a condenser

arranged for distillation under reduced pressure. The receiver is kept below —30° and the flask evaporated to about 120 mm Hg. A solution of 770 ml of conc. sulphuric acid in 1.4 litres of water is added at such a rate that the temperature in the flask can be kept at 35—40° by cooling with ice-water. The trimethylacetaldehyde distills during this addition. When the acid has been added, the flask is heated to 45° and the vacuum broken. The aldehyde in the receiver is melted and decanted from the ice. It weighs 1.06 kg and is pure enough for most purposes. If a pure product is desired, it can be obtained by distillation. In this way 950 g of pure trimethylaldehyde, b. p. 71—74°, is obtained.

From the water layer most of the sodium periodate can be recovered by treating the solution containing the sodium iodate with sodium hydroxide and chlorine [2].

Even pure trimethylacetaldehyde is slowly decomposed on standig into gaseous products, and it is advisable to release the pressure developed in the flask at intervals, and to keep it in a dark and cold place.

1. Shirley, D. A. *Preparation of organic intermediates*, New York 1951, p. 290.
2. Booth, H. S. *Inorganic Syntheses*, New York 1939, p. 169.
3. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
4. Criegee, R., Höger, E., Huber, G., Kruck, P., Marktscheffel, R. and Schellenberger, H. *Ann.* **599** (1956) 103.

Received February 23, 1959.

5-*neo*Pentyl-5-Allylbarbituric Acid and Related Compounds

I. On the Preparation of β-*tert*-Alkylpropionic Acids and Derivatives

ARNE BRÄNDSTRÖM

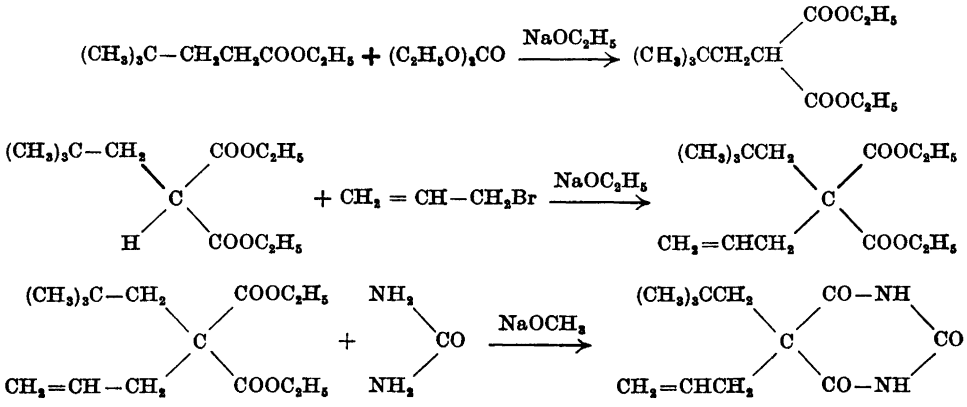
Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

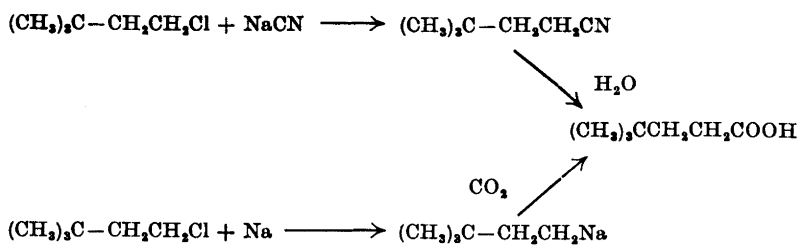
In this laboratory it has been found that *neo*pentylallylbarbituric acid is a very good sedative drug. In order to make this compound available in commercial quantities an investigation of the methods to prepare this barbiturate has been performed and in this connection some intermediates containing a branched chained structure have been prepared. Some of these compounds and methods will be described in this and the two following papers.

For this and similar methods 4,4-dimethylpentanoic acid was needed as a starting material. This compound has been prepared by Moreau and Delange [1] and later by Spindt, Stevens and Bladwin [2]. None of these methods are convenient for a large scale preparation of 4,4-dimethylpentanoic acid or the ethyl ester.

The following methods are, however, very satisfactory. They start with the readily available 1-chloro-3,3-dimethylbutane (obtainable from ethylene and *tert*-butyl chloride).

The method for the conversion of primary alkyl chlorides into cyanides has





recently been studied by Brändström [4] who found that polyethylene glycol-300 is an excellent solvent for the reaction. 3,3-Dimethylbutyl cyanide is obtained in excellent yield by this method and can easily be converted into the corresponding acid or ethyl ester by standard methods.

The preparation of carboxylic acids from alkyl chlorides *via* alkyl sodium compounds has been investigated by Morton *et al.*[5] The yields are usually somewhat low due to the fact that malonic acids are obtained as by-products. When this method is applied to the synthesis of 4,4-dimethylpentanoic acid a very good yield is obtained, probably because the *tert*-butyl group prevents the side reaction to the corresponding malonic acid.

The 4,4-dimethylpentanoic acid can also be prepared from *β-tert*-butylacrylic acid by hydrogenation.

In connection with the preparation of 4,4-dimethylpentanoic acid some homologues have been prepared by the same methods. All alkyl chlorides used in the following preparations are obtained from the reaction of *tert*-butyl chloride with ethylene [6].

Experimental. Preparation of cyanides. The method given by Brändström is followed and for the following cyanides the yields and boiling points are:

3,3-dimethylbutyl cyanide	94 %	57°— 59°/8 mm Hg
3,3-dimethylamyl cyanide	93 »	76°— 80°/8 mm Hg
3,3,4-trimethylamyl cyanide	91 »	88°— 89°/8 mm Hg
3,3,6,6-tetramethylheptyl cyanide	80 »	107°—110°/8 mm Hg

Preparation of acids. A 2 litre three-necked flask is fitted with a high speed stirrer (about 10 000 r.p.m.) a thermometer, a dropping funnel, a gas inlet tube and a gas exit tube. A slow stream of dry nitrogen is passed through the flask. 54 g of clean sodium and 800 ml of dry kerosene (free from aromatics, olefins and sulphurous compounds) are heated to 110°C. The mixture is now stirred at that temperature for 10 min. The stirrer is stopped and the flask chilled to —5° (Danger! The flask must not crack!)

The stirrer is started again. 10 ml of a solution containing 120 g of 1-chloro-3,3-dimethylbutane and 100 ml of pure kerosene are added. In some cases the reaction soon starts, which is indicated by a rapid rise in temperature and a darkning of the contents in the flask. In other cases 2 ml of butanol must be added to start the reaction. When the reaction has started, the flask is chilled in a solid carbon dioxide-acetone bath, and the rest of the chloride solution is added to the *very dark* contents in the flask at such a rate that the temperature is kept between 0° and —10°. The mixture is stirred at that temperature for 15 min after the addition.

Warning! It is very dangerous to add large quantities of the chloride solution to the sodium dispersion before the reaction has started!

The high speed stirrer is changed to an ordinary stirrer and the nitrogen replaced by pure carbon dioxide. The temperature is kept between 0° and —10° by cooling and adjusting the rates of stirring and addition of carbon dioxide. When the temperature begins to decrease, even if the rate of stirring and the stream of carbon dioxide are increased, the cooling bath is removed. If the temperature now only slowly rises, the reaction is finished.

700 ml of water is cautiously added to destroy the excess of sodium and dissolve the salts formed. The layers are separated. The aqueous layer is acidified with conc. hydro-

chloric acid, and the acid, which is liberated, is extracted with benzene. The benzene solution is dried with anhydrous sodium sulphate and then distilled under reduced pressure. The yield is 100 g or 77 % of the theoretical. The boiling point of the 4,4-dimethylpentanoic acid is 104—105°/8 mm Hg.

In exactly the same way the following acids are prepared: 4,4-Dimethylhexanoic acid, b.p. 112—120°/10 mm Hg, yield 46 %; 4,4,5-Trimethylhexanoic acid, b. p. 129—135°/8 mm Hg, yield 50 %.

Hydrolysis of cyanides. The same acids as mentioned above are conveniently prepared from the corresponding cyanides in the following way.

One mole of the cyanide is refluxed with two moles of sodium hydroxide, and 800 ml of water for 24 h or until the evolution of ammonia ceases. The resulting alkaline solution is cooled, extracted with benzene and then treated as above. The yields are almost quantitative.

Hydrogenation of β-tert-butylacrylic acid. *β-tert*-Butylacrylic acid is dissolved in 5 parts of alcohol and hydrogenated with palladium on charcoal as a catalyst at a hydrogen pressure of 50 kg/cm². The hydrogenation is rapid and an almost quantitative yield of 4,4-dimethylpentanoic acid is obtained on distillation of the filtered solution.

Preparation of esters. 475 g of 3,3-dimethylbutyl cyanide are dissolved in 1 080 ml of 99.5 % alcohol in a 5-litre flask fitted with a stirrer, a reflux condenser, and a dropping funnel. 465 ml of conc. sulphuric acid are added during one h. The mixture is then refluxed for 8 h. After cooling the mixture is poured on ice. Water is added to dissolve the salts, and the layers are then separated. The aqueous layer is extracted with benzene, and the combined organic layers are washed with a sodium carbonate solution, dried and distilled. The yield is 580 g of ethyl 4,4-dimethylpentanoate (86 %). The boiling point is 60—62°/8 mm Hg.

In exactly the same way the other cyanides could be converted into the corresponding esters.

The author is indebted to his colleagues and assistants at Pharmacia for good help with this project.

1. Moreau, C. and Delange, R. *Compt. rend.* **136** (1903) 552.
2. Spindt, R. S., Stevens, D. R. and Baldwin. W. E. *J. Am. Chem. Soc.* **73** (1951) 3693.
3. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
4. Brändström, A. *Acta Chem. Scand.* **10** (1956) 1197.
5. Morton, A. A., Richardson, G. M. and Hallowell, A. T. *J. Am. Chem. Soc.* **63** (1941) 367.
6. Brändström, A. *Acta Chem. Scand.* **13** (1959) *In press.*

Received March 9, 1959.

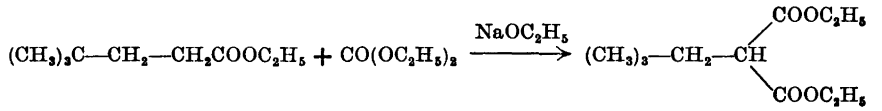
5-*neo*Pentyl-5-Allylbarbituric Acid and Related Compounds

II. On the Preparation of Diethyl *neo*Pentylmalonate and Related Compounds

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

Diethyl *neo*pentylmalonate is an important intermediate in the synthesis of 5-*neo*-pentyl-5-allylbarbituric acid. Due to the very low reactivity of *neo*pentyl halides it cannot be prepared by alkylation of diethyl malonate but other methods must be tried. The most convenient method seems to be the following



This condensation gives an excellent yield of diethyl *neo*pentylmalonate if it is performed under forcing conditions just as described by Brändström [1]. Starting with 3,3-dimethylbutyl cyanide and diethyl carbonate the same method gives ethyl *neo*pentylcyanoacetate.

The *neo*pentyl substituted malonic and cyanoacetic esters can be alkylated just as other monoalkylated malonic and cyanoacetic esters.

Attempts to prepare diethyl *neo*pentylmalonate from ethyl 4,4-dimethylpentanoate and diethyl oxalate by a process

chloric acid, and the acid, which is liberated, is extracted with benzene. The benzene solution is dried with anhydrous sodium sulphate and then distilled under reduced pressure. The yield is 100 g or 77 % of the theoretical. The boiling point of the 4,4-dimethylpentanoic acid is 104—105°/8 mm Hg.

In exactly the same way the following acids are prepared: 4,4-Dimethylhexanoic acid, b.p. 112—120°/10 mm Hg, yield 46 %; 4,4,5-Trimethylhexanoic acid, b. p. 129—135°/8 mm Hg, yield 50 %.

Hydrolysis of cyanides. The same acids as mentioned above are conveniently prepared from the corresponding cyanides in the following way.

One mole of the cyanide is refluxed with two moles of sodium hydroxide, and 800 ml of water for 24 h or until the evolution of ammonia ceases. The resulting alkaline solution is cooled, extracted with benzene and then treated as above. The yields are almost quantitative.

Hydrogenation of β-tert-butylacrylic acid. *β-tert*-Butylacrylic acid is dissolved in 5 parts of alcohol and hydrogenated with palladium on charcoal as a catalyst at a hydrogen pressure of 50 kg/cm². The hydrogenation is rapid and an almost quantitative yield of 4,4-dimethylpentanoic acid is obtained on distillation of the filtered solution.

Preparation of esters. 475 g of 3,3-dimethylbutyl cyanide are dissolved in 1 080 ml of 99.5 % alcohol in a 5-litre flask fitted with a stirrer, a reflux condenser, and a dropping funnel. 465 ml of conc. sulphuric acid are added during one h. The mixture is then refluxed for 8 h. After cooling the mixture is poured on ice. Water is added to dissolve the salts, and the layers are then separated. The aqueous layer is extracted with benzene, and the combined organic layers are washed with a sodium carbonate solution, dried and distilled. The yield is 580 g of ethyl 4,4-dimethylpentanoate (86 %). The boiling point is 60—62°/8 mm Hg.

In exactly the same way the other cyanides could be converted into the corresponding esters.

The author is indebted to his colleagues and assistants at Pharmacia for good help with this project.

1. Moreau, C. and Delange, R. *Compt. rend.* **136** (1903) 552.
2. Spindt, R. S., Stevens, D. R. and Baldwin. W. E. *J. Am. Chem. Soc.* **73** (1951) 3693.
3. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
4. Brändström, A. *Acta Chem. Scand.* **10** (1956) 1197.
5. Morton, A. A., Richardson, G. M. and Hallowell, A. T. *J. Am. Chem. Soc.* **63** (1941) 367.
6. Brändström, A. *Acta Chem. Scand.* **13** (1959) *In press.*

Received March 9, 1959.

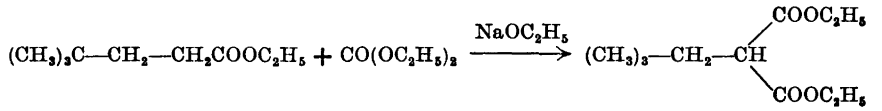
5-*neo*Pentyl-5-Allylbarbituric Acid and Related Compounds

II. On the Preparation of Diethyl *neo*Pentylmalonate and Related Compounds

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

Diethyl *neo*pentylmalonate is an important intermediate in the synthesis of 5-*neo*-pentyl-5-allylbarbituric acid. Due to the very low reactivity of *neo*pentyl halides it cannot be prepared by alkylation of diethyl malonate but other methods must be tried. The most convenient method seems to be the following

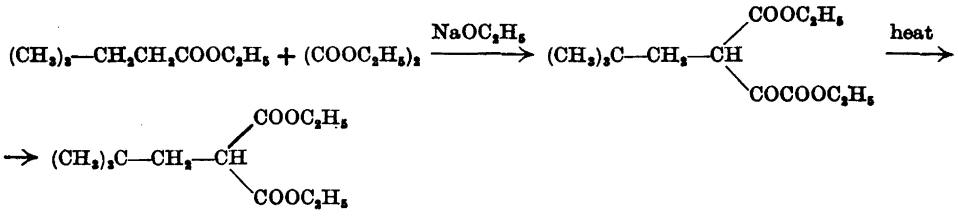


This condensation gives an excellent yield of diethyl *neo*pentylmalonate if it is performed under forcing conditions just as described by Brändström [1]. Starting with 3,3-dimethylbutyl cyanide and diethyl carbonate the same method gives ethyl *neo*pentylcyanoacetate.

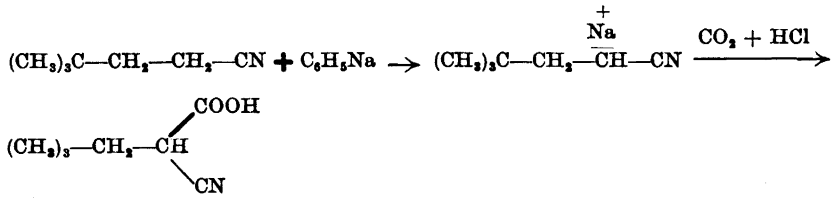
The *neo*pentyl substituted malonic and cyanoacetic esters can be alkylated just as other monoalkylated malonic and cyanoacetic esters.

Attempts to prepare diethyl *neo*pentylmalonate from ethyl 4,4-dimethylpentanoate and diethyl oxalate by a process

similar to that used for the preparation of diethyl phenylmalonate [2], *viz.*

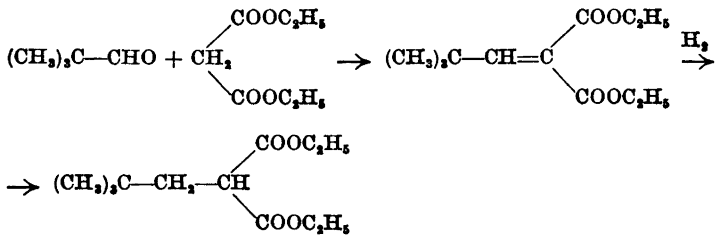


were not particularly successful. A yield of about 25 % could be obtained if the condensation was forced [3]. The following method was tried with success:



The cyano acid can be esterified to ethyl *neo*pentylcyanoacetate or to diethyl *neo*pentylmalonate, and it can be hydrolyzed to give *neo*pentylmalonic acid. The yields obtained are very good and the method is rapid but somewhat dangerous as it involves the manipulation of sodium dispersion.

Diethyl *neo*pentylmalonate can also be prepared by the following method:



This is an excellent method but trimethylacetaldehyde is not a commercially available starting material. A convenient method for a laboratory synthesis of trimethylacetaldehyde has been described by Brändström [4].

If needed in big quantities trimethylacetaldehyde might be prepared from $(CH_3)_3C-CH=CH_2$ or $(CH_3)_3C-CH=CHCl$ by oxidation with ozone [5] but as that method would probably involve many practical problems it was not tried.

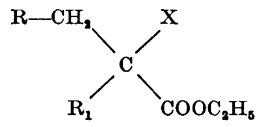
Besides the *neo*pentylderivatives a number of homologues have been prepared, *e.g.* those containing the groups β,β-dimethylbutyl and β,β,γ-trimethylbutyl.

Experimental. 1. Condensations with diethyl carbonate. 2 400 ml of diethyl carbonate are heated to boiling in a 5-litre three-necked flask provided with a stirrer, a reflux condenser and a glass stopper. The heating is then interrupted and 75 g of sodium are added at such a rate that the liquid boils vigorously. — Caution! Carbon monoxide escapes through the condenser! — When the sodium has reacted, 3 moles of the ethyl ester or the cyanide are added.

The reflux condenser is replaced by a Widmer column connected to a condenser arranged for distillation. The alcohol, which is formed in the condensation reaction, is distilled off carefully but rapidly. The temperature in the top of the column is kept below 85°. In this way about 360 ml of distillate can be obtained. The reaction mixture now contains a solution or suspension of the sodium compound of the malonic ester or cyanoester in diethyl carbonate.

The flask is cooled to room temperature and the contents are poured into a mixture of 300 ml of concentrated hydrochloric acid and ice. The mixture is shaken and separated. The ester layer is dried with anhydrous sodium sulphate and the diethyl carbonate distilled off at about 50 mm Hg, whereupon the residue is subjected to a fractional distillation *in vacuo*. The yields and boiling points are given in Table 1.

Table 1. Preparation of esters



R	R_1	X	Yield %	B. p.
$(CH_3)_3C$—	H	$COOC_2H_5$	65	109—113/ 8 mm Hg
»	CH_3-	»	61	131—134/15 » »
»	C_2H_5-	»	59	130—132/ 9 » »
»	CH_2=CH—CH_2-	»	62	136—138/ 8 » »
»	CH_2=CBr—CH_2-	»	43	165—175/ 8 » »
»	C_2H_5O—$COCH_2$-	»	60	170—180/ 8 » »
»	H	CN	65	110—114/ 8 » »
$C_2H_5C(CH_3)_2$	H	$COOC_2H_5$	65	118—128/10 » »
»	C_2H_5-	»	55	144—147/ 7 » »
»	CH_2=CH—CH_2-	»	69	150—155/15 » »
$(CH_3)_2CH$—$C(CH_3)_2$	H	»	65	130—132/ 8 » »

If a second group is to be introduced into the malonic ester or cyanoester it can be done by adding about 3.3 moles of the apropriate halide to the solution or suspension of the sodium compound mentioned above and refluxing the resulting mixture for some h (usually 1—8 h depending on the reactivity of the halide). The mixture is then cooled, poured on ice-water, acidified, separated, dried, and distilled as described above. The yields and boiling points are given in Table 1.

2. Condensations with diethyl oxalate. 3 litres of toluene and 69 g of sodium are heated to boiling in a 5-litre three-necked flask provided with a stirrer, a reflux condenser, and a dropping funnel. The stirrer is started and the heating is interrupted when the sodium begins to melt. To this mixture 180 ml of absolute alcohol are added dropwise in about 1 h. After the addition the vigorously stirred mixture is refluxed for 1 h.

The flask is cooled to 10° and the reflux condenser is replaced by a column connected to a condenser for distillation under reduced pressure. 474 g of ethyl 4,4-dimethylpentanoate and 438 g of diethyl oxalate are added. The flask is evacuated to 120 mm Hg and heated so that alcohol distills off at a temperature below 50° at the top of the column. About 800 ml of distillate are obtained. When no more alcohol distills, the flask is cooled with an ice-bath.

A mixture of 270 ml of conc. hydrochloric acid and an excess of ice is added to the contents of the flask. The mixture is effectively stirred whereupon the toluene layer is separated off, washed with water, and dried with sodium sulphate. The toluene is distilled off at reduced pressure by heating on a boiling water bath. When no more distills even if the pressure is reduced to 1 mm Hg the residue consists of ethyl 2-keto-3-carbethoxy-5,5-dimethylhexanoate, which can be purified by distillation at a pressure of at most 1 mm. B. p. 103—110/1 mm Hg.

If the crude residue is heated to boiling at ordinary pressure a vigorous evolution of carbon monoxide occurs. When this has ceased, the product is distilled under reduced pressure and thereby 175 g of diethyl *neo*pentylmalonate is obtained. B. p. 116—121/8 mm Hg.

If in the condensation above the ethyl 4,4-dimethylpentanoate is replaced by 333 g of 3,3-dimethylbutyl cyanide 210 g of ethyl 2-

keto-3-cyano-5,5-dimethylhexanoate is obtained. B. p. 140—150°/8 mm Hg. This ester does not split off carbon monoxide on heating.

3. Carboxylations of nitriles. In a 2-litre three-necked flask a dispersion of phenyl sodium is prepared from 100 g of sodium, 246 g of chlorobenzene, and 900 ml of toluene by the method given by Nobis and Moormeier [6].

The flask containing the phenyl sodium dispersion is fitted with a Hershberg stirrer [7], a thermometer, a gas inlet tube for dry nitrogen, a gas exit tube and a dropping funnel. The flask is chilled to —5° with a solid carbon dioxide-acetone bath, and a solution of 222 g of 3,3-dimethylbutyl cyanide in 200 ml of dry toluene is added at —5° to 0° during 10 min. The mixture is stirred at that temperature for 15 min and then poured into a 7-litre enamelled pan containing 1 kg of solid carbon dioxide and 100 ml of toluene. The mixture is stirred for some min and then allowed to obtain room temperature.

A few pieces of solid carbon dioxide are added (to function as a fire extinguisher), and then 2 litres of water (Caution! A considerable excess of sodium might be present!) The mixture is stirred and then separated. The aqueous layer contains the sodium salt of *neo*pentylcyanoacetic acid.

A. *Preparation of* neo*pentylmalonic acid.* 100 g of sodium hydroxide are added to the solution containing the sodium salt of *neo*pentylcyanoacetic acid. The mixture is refluxed until the evolution of ammonia ceases. The solution is cooled to room temperature and 400 ml of conc. hydrochloric acid is added while cooling so that the temperature never exceeds 30°. The solution is then extracted with three 200 ml portions of chloroform. The combined chloroform solutions are washed with 40 ml of conc. hydrochloric acid and dried with anhydrous magnesium sulphate. The chloroform is removed by distillation at reduced pressure by warming on a water-bath to 40°. The dry residue is *neo*pentylmalonic acid and weighs about 260 g. It is readily recrystallized from *cyclo*hexane, and has then a melting point of 89.5°—92°. Equiv. wt. found 86.9, calc. 87.1.

In exactly the same way the following malonic acids are prepared: β,β-dimethylbutylmalonic acid, yield of crude acid 242 g, m. p., of recrystallized product 68°—71°, equiv. wt. found 94.3, calc. 94.1; β,β,γ-trimethylbutylmalonic acid, yield of crude product 300 g, m. p. of recrystallized acid 85.5°—86.5°, equiv. wt. found 101.7, calc. 101.1.

B. *Preparation of ethyl* neo*pentylcyanoacetate.* The solution containing the sodium salt of *neo*pentylcyanoacetic acid is acidified with 250 ml of conc. hydrochloric acid at a temperature not exceeding 30°. The layers are separated and the aqueous layer is extracted with two 250 ml portions of benzene. The combined organic layers are washed with 25 ml of conc. hydrochloric acid.

To the benzene solution of *neo*pentylcyanoacetic acid 250 ml of 99.5 % alcohol and 5 ml of conc. sulphuric acid are added. The mixture is refluxed, and the water formed under the esterification removed by means of a water separator [8]. After about 8 h of reflux a second portion of 5 ml of conc. sulphuric acid is added, and the refluxing is continued until no more water is obtained (total time about 15 h). The benzene solution is cooled to room temperature, washed with 500 ml of water then with 200 ml of a 5 % sodium carbonate solution and finally dried with anhydrous magnesium sulphate. The benzene is removed by distillation and the residue is fractionated at reduced pressure. In this way 275 g of ethyl *neo*pentylcyanoacetate is obtained at 105—110°/8 mm. The forefraction contains some ethyl benzoate.

C. *Preparation of diethyl* neo*pentylmalonate.* The benzene solution containing the *neo*pentylcyanoacetic acid is concentrated by warming under reduced pressure in a water bath at a temperature not exceeding 50°. The residue weighs about 260 g. It is dissolved in 800 ml of 99.5 % alcohol, and 190 ml of conc. sulphuric acid is added whereupon the mixture is refluxed overnight with stirring. It is then cooled and poured onto ice. The layers are separated, and the aqueous layer is extracted with benzene. The combined organic layers are washed with water, then with a 5 % sodium carbonate solution, and again with water. The solution is dried with anhydrous magnesium sulphate, the benzene removed by distillation and the residue fractionated under reduced pressure. The yield is 250 g with a boiling point of 110°—115°/8 mm Hg. The forefraction contains some ethyl benzoate.

4. Introduction of a neo*pentyl group in diethyl malonate and ethyl cyanoacetate by means of trimethylacetaldehyde.* A. *Preparation of diethyl trimethylethylidenemalonate.* This ester is readily obtained by condensing trimethylacetaldehyde with diethyl malonate as described by Foreman and McElvain [9].

B. *Preparation of ethyl trimethylethylidenecyanoacetate.* If trimethylacetaldehyde is condensed with ethyl cyanoacetate by the standard method [10] ethyl trimethylethylidenecyanoacetate is obtained in a 89 % yield; b. p. 104°—111°/9 mm Hg.

C. *Preparation of diethyl* neo*pentylmalonate.* Diethyl trimethylethylidenemalonate is readily hydrogenated by means of hydrogen and pal-

ladium on charcoal. The reaction is completed in 1/2 h if alcohol is used as a solvent, and the pressure is 50 atm. The yield is quantitative. B. p. 120°—130°/11 mm Hg.

D. *Preparation of ethyl* neo*pentylcyanoacetate.* Ethyl trimethylethylidenecyanoacetate is hydrogenated as described above to ethyl *neo*pentylcyanoacetate. The yield is quantitative and the boiling point 110°—114°/8 mm Hg.

5. Alkylations of ethyl neo*pentylcyanoacetate.* A. *Preparation of ethyl ethyl*neo*pentylcyanoacetate.* 91.5 g of ethyl *neo*pentylcyanoacetate, 138 g of powdered anhydrous potassium carbonate, 275 ml of dry acetone and 94 g of ethyl iodide are refluxed with good stirring for 3 h. The resulting mixture is cooled, poured into ice-water, separated, extracted with benzene, dried and distilled under reduced pressure. In this way 100 g, 95 %, of ethyl ethyl*neo*pentylcyanoacetate is obtained. B. p. 116°—118°/7 mm Hg. This ester is recovered unchanged after refluxing with 120 ml of alcohol, and 50 ml of conc. sulphuric acid for 16 h.

B. *Preparation of ethyl allyl*neo*pentylcyanoacetate.* 41 g of ethyl *neo*pentylcyanoacetate 62 g of powdered anhydrous potassium carbonate, 125 g of dry acetone and 33 g of allylbromide are refluxed for 5 h. The mixture is then treated as above yielding 49.5 g, 99 %, of ethyl allyl*neo*pentylcyanoacetate boiling at 127°—128°/8 mm Hg.

The author is indebted to this colleagues and assistants at Pharmacia for good help with this project.

1. Brändström, A. *Acta Chem. Scand.* **4** (1950) 1315.
2. *Organic Syntheses*, Coll. vol. **11** (1944) 288.
3. Brändström, A. *Acta Chem. Scand.* **5** (1951) 485.
4. Brändström, A. *Acta Chem. Scand.* **13** (1959) 611.
5. Ecke, G. G., Cook, N. C. and Whitmore F. C. *J. Am. Chem. Soc.* **72** (1950) 1511.
6. Nobis, J. F. and Moormeier, L. F. *Ind. Eng. Chem.* **46** (1954) 539.
7. *Organic Syntheses*, Coll. vol. 11 (1944) 117.
8. Brändström, A. *Arkiv Kemi* **1** (1950) 481.
9. Foreman, E. L. and McElvain, S. M. *J. Am. Chem. Soc.* **62** (1940) 1438.
10. *Organic Syntheses*, Coll. vol. **III** (1955) 399.

Received March 9, 1959.

5-*neo*Pentyl-5-Allylbarbituric Acid and Related Compounds

III. On the Preparation of *neo*Pentyl-substituted Barbituric Acids

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

Since J. von Mering in 1903 discovered the hypnotic activity of a compound he erroneously considered to be 5,5-diethylbarbituric acid [1], 5,5-disubstituted barbituric acids have been extensively studied. In Chemical Abstract Decimal Index 1917—1926 the heading "barbituric acid" covers about 5 pages, in Decimal Index 1937—1945 8 pages and in Subject Index 1956 2 pages. This shows that the interest in substituted barbituric acids is ever increasing.

In spite of the enormous interest devoted to 5,5-disubstituted barbituric acids it appears that no barbituric acid containing a *neo*pentylsubstituent in the 5 position has been described. This is still more remarkable as many very useful hypnotics are barbituric acids with a side chain in the 5 position containing five carbon atoms usually in a branched-chain arrangement.

The introduction of a *neo*pentyl group in a molecule gives often a reactivity very different from that obtained with other aliphatic groups of the same size. This is clearly demonstrated by the well-known extremely low reactivity of *neo*pentyl halides compared with that of other alkyl halides and the tendency of *neo*pentyl compounds to undergo rearrangements instead of simple substitution reactions. Therefore, it is of interest to see if the properties of *neo*pentylsubstituted barbituric acids differs from those substituted with other alkyl groups. Consequently, a number of *neo*pentylsubstituted barbituric acids have been prepared by the methods indicated in paper No. 1 in this series [2].

The chemical properties of the *neo*pentyl-substituted barbituric acids resemble those of other alkyl barbituric acids in all respects. In some cases a slight steric effect of the *neo*pentyl group could be observed resulting in a comparatively low yield in certain syntheses, but this effect is by no means as marked as that produced by a *tert*-butyl group [3].

ladium on charcoal. The reaction is completed in 1/2 h if alcohol is used as a solvent, and the pressure is 50 atm. The yield is quantitative. B. p. 120°—130°/11 mm Hg.

D. *Preparation of ethyl* neo*pentylcyanoacetate.* Ethyl trimethylethylidenecyanoacetate is hydrogenated as described above to ethyl *neo*pentylcyanoacetate. The yield is quantitative and the boiling point 110°—114°/8 mm Hg.

5. Alkylations of ethyl neo*pentylcyanoacetate.* A. *Preparation of ethyl ethyl*neo*pentylcyanoacetate.* 91.5 g of ethyl *neo*pentylcyanoacetate, 138 g of powdered anhydrous potassium carbonate, 275 ml of dry acetone and 94 g of ethyl iodide are refluxed with good stirring for 3 h. The resulting mixture is cooled, poured into ice-water, separated, extracted with benzene, dried and distilled under reduced pressure. In this way 100 g, 95 %, of ethyl ethyl*neo*pentylcyanoacetate is obtained. B. p. 116°—118°/7 mm Hg. This ester is recovered unchanged after refluxing with 120 ml of alcohol, and 50 ml of conc. sulphuric acid for 16 h.

B. *Preparation of ethyl allyl*neo*pentylcyanoacetate.* 41 g of ethyl *neo*pentylcyanoacetate 62 g of powdered anhydrous potassium carbonate, 125 g of dry acetone and 33 g of allylbromide are refluxed for 5 h. The mixture is then treated as above yielding 49.5 g, 99 %, of ethyl allyl*neo*pentylcyanoacetate boiling at 127°—128°/8 mm Hg.

The author is indebted to this colleagues and assistants at Pharmacia for good help with this project.

1. Brändström, A. *Acta Chem. Scand.* **4** (1950) 1315.
2. *Organic Syntheses*, Coll. vol. **11** (1944) 288.
3. Brändström, A. *Acta Chem. Scand.* **5** (1951) 485.
4. Brändström, A. *Acta Chem. Scand.* **13** (1959) 611.
5. Ecke, G. G., Cook, N. C. and Whitmore F. C. *J. Am. Chem. Soc.* **72** (1950) 1511.
6. Nobis, J. F. and Moormeier, L. F. *Ind. Eng. Chem.* **46** (1954) 539.
7. *Organic Syntheses*, Coll. vol. 11 (1944) 117.
8. Brändström, A. *Arkiv Kemi* **1** (1950) 481.
9. Foreman, E. L. and McElvain, S. M. *J. Am. Chem. Soc.* **62** (1940) 1438.
10. *Organic Syntheses*, Coll. vol. **III** (1955) 399.

Received March 9, 1959.

5-*neo*Pentyl-5-Allylbarbituric Acid and Related Compounds

III. On the Preparation of *neo*Pentyl-substituted Barbituric Acids

ARNE BRÄNDSTRÖM

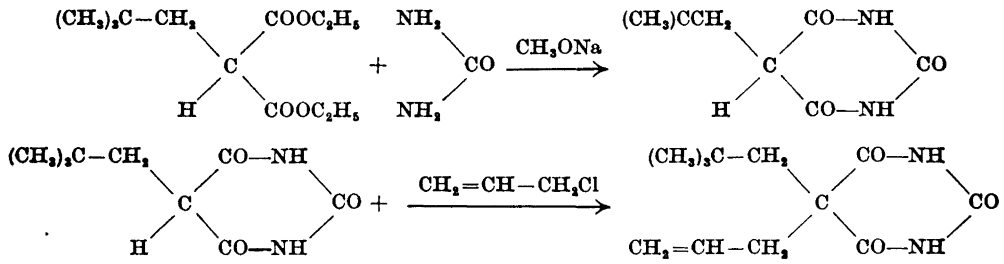
Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

Since J. von Mering in 1903 discovered the hypnotic activity of a compound he erroneously considered to be 5,5-diethylbarbituric acid [1], 5,5-disubstituted barbituric acids have been extensively studied. In Chemical Abstract Decimal Index 1917—1926 the heading "barbituric acid" covers about 5 pages, in Decimal Index 1937—1945 8 pages and in Subject Index 1956 2 pages. This shows that the interest in substituted barbituric acids is ever increasing.

In spite of the enormous interest devoted to 5,5-disubstituted barbituric acids it appears that no barbituric acid containing a *neo*pentylsubstituent in the 5 position has been described. This is still more remarkable as many very useful hypnotics are barbituric acids with a side chain in the 5 position containing five carbon atoms usually in a branched-chain arrangement.

The introduction of a *neo*pentyl group in a molecule gives often a reactivity very different from that obtained with other aliphatic groups of the same size. This is clearly demonstrated by the well-known extremely low reactivity of *neo*pentyl halides compared with that of other alkyl halides and the tendency of *neo*pentyl compounds to undergo rearrangements instead of simple substitution reactions. Therefore, it is of interest to see if the properties of *neo*pentylsubstituted barbituric acids differs from those substituted with other alkyl groups. Consequently, a number of *neo*pentylsubstituted barbituric acids have been prepared by the methods indicated in paper No. 1 in this series [2].

The chemical properties of the *neo*pentyl-substituted barbituric acids resemble those of other alkyl barbituric acids in all respects. In some cases a slight steric effect of the *neo*pentyl group could be observed resulting in a comparatively low yield in certain syntheses, but this effect is by no means as marked as that produced by a *tert*-butyl group [3].

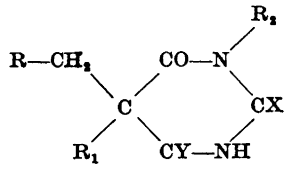


On the other hand the pharmacological properties are very interesting, especially those of 5-*neo*pentyl-5-allylbarbituric acid. This compound has the same depressive effect on the central nervous system as other barbiturates. In contrast to most barbiturates having only alkyl or alkenyl substituents in both 5 positions it has a distinct anticonvulsive effect. Therefore, in this respect it resembles 5-phenyl-5-ethylbarbituric acid. In contrast to this compound 5-*neo*pentyl-5-allylbarbituric acid has a moderate duration of effect just as most barbiturates substituted with branched chained alkyl groups.

The 5-*neo*pentyl-5-allylbarbituric acid has in contrast to, *e.g.*, 5-phenyl-5-ethylbarbituric acid and 5-*iso*amyl-5-ethylbarbituric acid a normalizing effect on rats subjected to a psychical stress.

All these pharmacological results suggest that 5-*neo*pentyl-5-allylbarbituric acid has

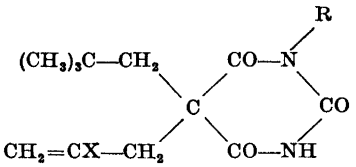
Table 1. Syntheses of barbituric acids



R	R_1	R_2	X	Y	Yield	M. p.	Equiv. wt. Found	Calc.
$(CH_3)_3C$	H	H	O	O	75	—	—	—
	H	H	O	NH	77	—	—	—
	H	H	NH	O	81	—	—	—
	H	H	NH	NH	77	—	—	—
	H	CH_3	O	O	66	144°	214	212.2
	H	C_2H_5	O	O	70	136°	226	226.3
	H	$(CH_3)_2CH$	O	O	57	102°	243	240.2
	CH_3	H	O	O	69	225.5—226.5°	211	212.2
	C_2H_5	H	O	O	62	194.5—195.5°	227	226.3
	C_2H_5	$CH_3CH_2CH_2$	O	O	28	137—139°	269	268.4
	$CH_2=CHCH_2$	H	O	O	56	156—157°	238	238.3
	$CH_2=CHCH_2$	H	O *	O	75	156—157°	238	238.3
	$CH_2=CHCH_2$	CH_3	O	O	53	135—136°	252	252.3
$C_2H_5C(CH_3)_2$	C_2H_5	H	O *	O	63	178.5—179.5°	241	240.2
	$CH_2=CH—CH_2$	H	O	O	36	139.5—140.5°	251	252.3
	$CH_2=CH—CH_2$	H	O *	O	49	139.5—141.5°	251	252.3
	$CH_2=CH—CH_2$	H	S	O	50	143—144.5°	266	268.3

* Guanidine is used instead of urea and the resulting iminobarbituric acid is hydrolyzed by refluxing for 8 h with 1 litre of 20 % hydrochloric acid.

Table 2. Preparation of



X	R	Yield	M. p.	Equiv. wt. Found	Calc.
Br	CH_3—	71	177 —180°	325	331.2
Br	C_2H_5—	68	144.8—145.0°	346	345.2
Br	$(CH_3)_2CH$—	55	177—178°	358	359.3
Br	$CH_3CH_2CH_2$—	64	152—153.5°	358	359.3
Br	$C_2H_5(CH_3)CH$—	51	165—166°	368	373.3
Cl	CH_3—	61	178—181°	289.5	286.8

properties that makes it useful as a sedative drug. Clinical investigations have confirmed this assumption [4].

For the syntheses of *neo*pentylsubstituted barbituric acids all the standard methods have been tried [5]. Most of them can be used with success, but the best method seems to be the condensation of the corresponding malonic ester with urea or guanidine. For the preparation of 5-*neo*pentyl-5-allylbarbituric acid the following reactions seem to be best *(cf.* p. 620).

The condensation of diethyl *neo*pentylallylmalonate with urea seems to give a somewhat low yield of the barbituric acid probably due to steric effects. The disubstituted cyanoacetic esters containing one *neo*pentyl group do not give the corresponding iminobarbituric acid probably due to the very low reactivity of the cyano group.

Experimental. Condensations to barbituric acids. 55 *g* of sodium are dissolved in 1 100 ml of anhydrous methanol in a 3-litre three-necked flask provided with a stirrer, a reflux condenser and a stopper, and 75.5 g of dried urea and 1 mole of the malonic ester are added. The mixture is refluxed for 6 h whereupon the methanol is distilled off as completely as possible by means of an oil-bath, the temperature of which should not exceed 100°. At the end of the distillation the pressure is reduced. The contents of the flask are dissolved in water and the solution extracted with benzene to remove water insoluble products, whereupon the water-layer is acidified with conc. hydrochloric acid while cooling and stirring. The barbituric acid is filtered off, washed with water and dried in a drying oven at 80° to constant weight. The products can usually be recrystallized from dilute methanol. The yields and melting points are given in Table 1.

Allylations of 5-neopentylbarbituric acid. The method given above for the preparation of 5-*neo*pentylbarbituric acid is followed until the methanol has been distilled off. 500 ml of water is now added and the pH of the resulting solution adjusted to about 8 by careful addition of conc. hydrochloric acid (about 128 ml). At this point a slight precipitation may be present which is filtered off. 120 g of allyl bromide is added and the mixture is stirred at 40° in a 2-litre flask fitted with a stirrer, a reflux condensor, and a thermometer. After about 2 h the mixture becomes acidic (pH about 3). The barbituric acid is now filtered off, washed with water and dried. The resulting 5-*neo*pentyl-5-allylbarbituric acid is practically pure, m. p. 154—157°, but may be purified by recrystallisation from dilute methanol. The yield is 63 %, m. p. 156°.

In the method given above the allyl bromide can be replaced by an equivalent amount of allyl chloride together with a solution containing 2 g of cupric sulphate. The yield is the same as above but the product must be dissolved in a dilute sodium hydroxide solution and precipitated with hydrochloric acid in order to obtain a pure product. By rigorous exclusion of all traces of water in the condensation step yields of up to 85 % may be obtained by this method in large scale runs.

Preparation of 5-β-bromoallyl-5-neopentylbarbituric acids. 1 Mole of 1-ethyl-5-*neo*pentylbarbituric acid is dissolved in a solution of 40 g of sodium hydroxide in 1 200 ml of water. 10 g of crystallized cupric sulphate dissolved in a small quantity of water is added, and then 390 ml of 2,3-dibromopropene. The mixture is

stirred at 40° in a 3-litre three-necked flask for 20 h.

The resulting mixture is distilled under reduced pressure (about 60 mm Hg) to remove the excess of 2,3-dibromopropene. About 240 ml are thus recovered. The residue in the flask is filtered and washed with a small quantity of petroleum ether.

The product is dissolved in an ice-cold solution of 65 g of sodium hydroxide in 2 500 ml of water. An insoluble residue of about 10 g is filtered off. The solution is treated with discolouration carbon and then 10 g of ethylenediaminetetraacetic acid are added to remove cupric ions. The filtered solution is carefully added to 150 ml of concentrated hydrochloric acid. The product is filtered off, washed with water and recrystallized from methanol. The yield is 235 g (68 %) of a pure white product with a melting point of 144.8°—145.0°.

In exactly the same way the compounds in Table 2 are prepared.

The author is indebted to his colleagues and assistants at Pharmacia for good help with this project.

1. *Emil Fischer sein Leben und Werk*, Ber. **54** (1921) Sonderheft, S. 241.
2. Brändström, A. *Acta Chem. Scand.* **13** (1959) 613.
3. Bush, M. T. and Beauchamp, W. D. *J. Am. Chem. Soc.* **75** (1953) 2949.
4. Ryde, C. Svenska Läkartidn. **56** (1959) 260.
5. Baggesgaard-Rasmussen, H. and Jespersen, J. C. *De vigtigste barbitursyrederivater og ureider*, Köpenhamn 1938, p. 9.

Received March 9, 1959.

On the Relation between the Reaction Velocity and Dielectric Constant of the Medium

EERO TOMMILA

Institute of Chemistry, University of Helsinki, Finland

According to Laidler and Eyring [1], the effect of the dielectric constant of the solvent on the rate of a bimolecular reaction between two dipole molecules A and B,

$$A + B \rightarrow M^* \rightarrow \text{Products},$$

can be expressed by the equation

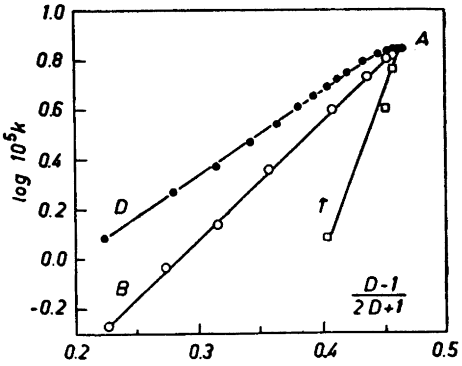


Fig. 1. Plot of log k for the reaction $(C_2H_5)_3N + C_2H_5I \rightarrow (C_2H_5)_4NI$ against $(D-1)/(2D+1)$ at 25°. A = acetone, B = benzene, D = dioxan, T = tetrahydrofuran.

where k and k_0 are the rate constants in a medium of dielectric constant D and in a medium of dielectric constant unity, respectively. The μ's and r's are the dipole moments and radii of the reactants and transition state, respectively. If the non-electrostatic term $\Sigma\Phi/kT$ is small enough to be neglected, and if μ^2_*/r^3_* is independent of the medium, the plot of log k against $(D-1)/(2D+1)$ should be a straight line for the same reaction carried out in different media. Linear plots were obtained for the reactions between pyridine and benzyl bromide and between triethylamine and benzyl bromide in benzene-ethanol mixtures, but not in benzene-nitrobenzene mixtures [1]. In cases where solvation phenomena play an important role, examination over larger areas of solvent mixtures has in general shown departure from linearity.

In Fig. 1 the eqn. (1) has been tested upon the results of some recent investigations concerning quaternary ammonium salt formation, *viz.* the reaction between triethylamine and ethyl iodide in acetone-benzene, acetone-dioxan, and acetone-tetrahydrofuran mixtures. The rate constants for 25° were calculated by the Arrhenius equation from the data of Tommila and Kauranen [2]. The dielectric constants of acetone-benzene mixtures were taken

$$\ln k = \ln k_0 - \frac{1}{kT}\,\frac{D-1}{2D+1}\left(\frac{\mu_A^2}{r_A^3} + \frac{\mu_B^2}{r_B^3} - \frac{\mu_*^2}{r_*^3}\right) + \frac{\Sigma\Phi}{kT} \qquad (1)$$

stirred at 40° in a 3-litre three-necked flask for 20 h.

The resulting mixture is distilled under reduced pressure (about 60 mm Hg) to remove the excess of 2,3-dibromopropene. About 240 ml are thus recovered. The residue in the flask is filtered and washed with a small quantity of petroleum ether.

The product is dissolved in an ice-cold solution of 65 g of sodium hydroxide in 2 500 ml of water. An insoluble residue of about 10 g is filtered off. The solution is treated with discolouration carbon and then 10 g of ethylenediaminetetraacetic acid are added to remove cupric ions. The filtered solution is carefully added to 150 ml of concentrated hydrochloric acid. The product is filtered off, washed with water and recrystallized from methanol. The yield is 235 g (68 %) of a pure white product with a melting point of 144.8°—145.0°.

In exactly the same way the compounds in Table 2 are prepared.

The author is indebted to his colleagues and assistants at Pharmacia for good help with this project.

1. *Emil Fischer sein Leben und Werk*, Ber. **54** (1921) Sonderheft, S. 241.
2. Brändström, A. *Acta Chem. Scand.* **13** (1959) 613.
3. Bush, M. T. and Beauchamp, W. D. *J. Am. Chem. Soc.* **75** (1953) 2949.
4. Ryde, C. Svenska Läkartidn. **56** (1959) 260.
5. Baggesgaard-Rasmussen, H. and Jespersen, J. C. *De vigtigste barbitursyrederivater og ureider*, Köpenhamn 1938, p. 9.

Received March 9, 1959.

On the Relation between the Reaction Velocity and Dielectric Constant of the Medium

EERO TOMMILA

Institute of Chemistry, University of Helsinki, Finland

According to Laidler and Eyring [1], the effect of the dielectric constant of the solvent on the rate of a bimolecular reaction between two dipole molecules A and B,

$$A + B \rightarrow M^* \rightarrow \text{Products},$$

can be expressed by the equation

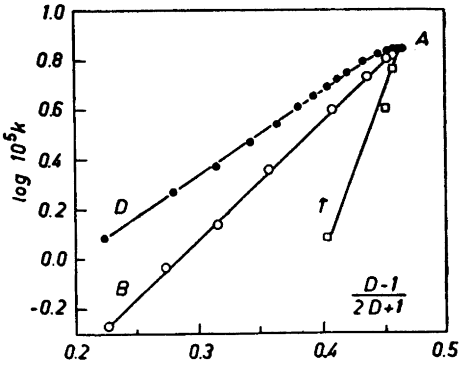


Fig. 1. Plot of log k for the reaction $(C_2H_5)_3N + C_2H_5I \rightarrow (C_2H_5)_4NI$ against $(D-1)/(2D+1)$ at 25°. A = acetone, B = benzene, D = dioxan, T = tetrahydrofuran.

where k and k_0 are the rate constants in a medium of dielectric constant D and in a medium of dielectric constant unity, respectively. The μ's and r's are the dipole moments and radii of the reactants and transition state, respectively. If the non-electrostatic term $\Sigma\Phi/kT$ is small enough to be neglected, and if μ^2_*/r^3_* is independent of the medium, the plot of log k against $(D-1)/(2D+1)$ should be a straight line for the same reaction carried out in different media. Linear plots were obtained for the reactions between pyridine and benzyl bromide and between triethylamine and benzyl bromide in benzene-ethanol mixtures, but not in benzene-nitrobenzene mixtures [1]. In cases where solvation phenomena play an important role, examination over larger areas of solvent mixtures has in general shown departure from linearity.

In Fig. 1 the eqn. (1) has been tested upon the results of some recent investigations concerning quaternary ammonium salt formation, *viz.* the reaction between triethylamine and ethyl iodide in acetone-benzene, acetone-dioxan, and acetone-tetrahydrofuran mixtures. The rate constants for 25° were calculated by the Arrhenius equation from the data of Tommila and Kauranen [2]. The dielectric constants of acetone-benzene mixtures were taken

$$\ln k = \ln k_0 - \frac{1}{kT}\,\frac{D-1}{2D+1}\left(\frac{\mu_A^2}{r_A^3} + \frac{\mu_B^2}{r_B^3} - \frac{\mu_*^2}{r_*^3}\right) + \frac{\Sigma\Phi}{kT} \qquad (1)$$

from the measurements of Drude [3] and those for acetone-dioxan and acetone-tetrahydrofuran mixtures from a recent communication of Lindberg [4]. It is seen that in acetone-benzene mixtures the plots are linear within the limits of experimental error over the whole range of the mixtures. In acetone-dioxan mixtures there is at 25° a weak deviation from the rectilinear course in the range from pure acetone to 12 % dioxan, but from the values of the rate constant one can conclude that this deviation disappears at 40° and higher temperatures. In acetone-tetrahydrofuran mixtures the number of kinetic data is too small for definite conclusions, but it seems probable that in this solvent system the plot is also linear over the whole range of mixtures.

The slope of the plots shows that in eqn. (1) the last term in the brackets is greater than the sum of the first two terms. This is quite natural, since the final products of the reaction are ionized and, accordingly, it is reasonable to assume that the dipole moment of the transition complex is large. The different slopes of the plots show that the term in brackets has different values for the three solvent systems.

1. Laidler, K. J. and Eyring, H. *Ann. New York Acad. Sci.* 39 (1940) 303; Glasstone, S., Laidler, K. J. and Eyring, H. *The Theory of Rate Processes*. McGraw-Hill Book Company New York 1941, p. 419; Laidler, K. J. and Landskroener, P. A. *Trans. Faraday Soc.* 52 (1956) 200.
2. Tommila, E. and Kauranen, P. *Acta Chem. Scand.* 8 (1954) 1152; *Suomen Kemistilehti B* 28 (1955) 170.
3. Drude, P. *Z. physik. Chem.* 23 (1897) 288, 313.
4. Lindberg, J. J. *Suomen Kemistilehti B* 32 (1959) 12.

Received February 20, 1959.

The Structure and Synthesis of *Cyclo*alliin Isolated from *Allium cepa*

ARTTURI I. VIRTANEN and E. J. MATIKKALA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Some years ago we [1] isolated from a 70 % ethanol extract of onion after hydrolysis with 6 N HCl the crystalline hydrochloride of a sulphur-containing amino acid $C_6H_{11}O_3NS \cdot HCl$. Before hydrolysis it was not found in onion. This amino acid could be oxidized by 30 % hydrogen peroxide in glacial acetic acid to the corresponding sulphoxide which was isolated as the crystalline hydrochloride $C_6H_{11}O_4NS \cdot HCl \cdot H_2O$ (Virtanen and Bramesfeld). Because the sulphoxide could be reduced with Raney nickel back to $C_6H_{11}O_3NS$ it was clear that an amino acid sulphoxide and its thioether were in question.

Later on the sulphoxide was found as an original amino acid in onion [2]. The first isolated S-amino acid was obviously formed from the sulphoxide on treatment with strong hydrochloric acid. The amino acid sulphoxide was isolated from a 70 % ethanol extract of onion. The elementary composition of the isolated and recrystallized amino acid hydrochloride corresponded to the formula $C_6H_{11}O_4NS \cdot HCl \cdot H_2O$. (Found: C 31.31 ; H 6.37 ; N 5.90 ; S 13.60 ; Cl 15.20. Calc. C 31.09 ; H 6.09 ; N 6.04 ; S 13.84 ; Cl 15.31.) On spraying with ninhydrin it gives a greenish blue spot, its reduced S-compound a violet one, on the paper chromatogram. The sensitiveness of of the colour reaction of the sulphoxide is about 1/20, and that of the S-compound about 1/10, of that of alanine. R_F-values for the sulphoxide: 0.85 in phenol-NH_3, 0.15 in butanol-acetic acid-water, and for the S-compound: 0.90 in phenol-NH_3, 0.48 in butanol acetic acid-water [2]. Sulphoxide $[\alpha]_D^{20} -17.4°$ (in water).

There is no double bond and also no primary amino group in either of the amino acids. The nitrogen belongs to an imino group. On the basis of these facts and the elementary composition of the substance it should contain a heterocyclic ring.

Detailed investigations of the products formed by heating of the sulphoxide with 6 N HCl at 105°C for 43 h elucidated the structure of this compound. As reduction products the corresponding thioether (II) and as oxidation products 2-aminopropane-1-sulphonic acid (2-methyltaurine) (III) and cysteic acid (IV) were formed. In addition a small spot in the solvent front (phenol), probably an amine, was found on the paper chromatogram (Fig. 1). These results led to structure I for the sulphoxide and structure II for the thioether [3,4].

The elementary composition of compound III corresponded to the formula $C_3H_9O_3NS$. Paper chromatographically it was identical with synthetic 2-methyltauri-

from the measurements of Drude [3] and those for acetone-dioxan and acetone-tetrahydrofuran mixtures from a recent communication of Lindberg [4]. It is seen that in acetone-benzene mixtures the plots are linear within the limits of experimental error over the whole range of the mixtures. In acetone-dioxan mixtures there is at 25° a weak deviation from the rectilinear course in the range from pure acetone to 12 % dioxan, but from the values of the rate constant one can conclude that this deviation disappears at 40° and higher temperatures. In acetone-tetrahydrofuran mixtures the number of kinetic data is too small for definite conclusions, but it seems probable that in this solvent system the plot is also linear over the whole range of mixtures.

The slope of the plots shows that in eqn. (1) the last term in the brackets is greater than the sum of the first two terms. This is quite natural, since the final products of the reaction are ionized and, accordingly, it is reasonable to assume that the dipole moment of the transition complex is large. The different slopes of the plots show that the term in brackets has different values for the three solvent systems.

1. Laidler, K. J. and Eyring, H. *Ann. New York Acad. Sci.* 39 (1940) 303; Glasstone, S., Laidler, K. J. and Eyring, H. *The Theory of Rate Processes*. McGraw-Hill Book Company New York 1941, p. 419; Laidler, K. J. and Landskroener, P. A. *Trans. Faraday Soc.* 52 (1956) 200.
2. Tommila, E. and Kauranen, P. *Acta Chem. Scand.* 8 (1954) 1152; *Suomen Kemistilehti B* 28 (1955) 170.
3. Drude, P. *Z. physik. Chem.* 23 (1897) 288, 313.
4. Lindberg, J. J. *Suomen Kemistilehti B* 32 (1959) 12.

Received February 20, 1959.

The Structure and Synthesis of *Cyclo*alliin Isolated from *Allium cepa*

ARTTURI I. VIRTANEN and E. J. MATIKKALA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

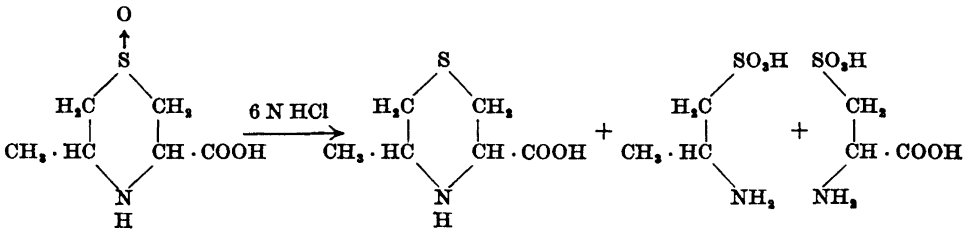
Some years ago we [1] isolated from a 70 % ethanol extract of onion after hydrolysis with 6 N HCl the crystalline hydrochloride of a sulphur-containing amino acid $C_6H_{11}O_3NS \cdot HCl$. Before hydrolysis it was not found in onion. This amino acid could be oxidized by 30 % hydrogen peroxide in glacial acetic acid to the corresponding sulphoxide which was isolated as the crystalline hydrochloride $C_6H_{11}O_4NS \cdot HCl \cdot H_2O$ (Virtanen and Bramesfeld). Because the sulphoxide could be reduced with Raney nickel back to $C_6H_{11}O_3NS$ it was clear that an amino acid sulphoxide and its thioether were in question.

Later on the sulphoxide was found as an original amino acid in onion [2]. The first isolated S-amino acid was obviously formed from the sulphoxide on treatment with strong hydrochloric acid. The amino acid sulphoxide was isolated from a 70 % ethanol extract of onion. The elementary composition of the isolated and recrystallized amino acid hydrochloride corresponded to the formula $C_6H_{11}O_4NS \cdot HCl \cdot H_2O$. (Found: C 31.31 ; H 6.37 ; N 5.90 ; S 13.60 ; Cl 15.20. Calc. C 31.09 ; H 6.09 ; N 6.04 ; S 13.84 ; Cl 15.31.) On spraying with ninhydrin it gives a greenish blue spot, its reduced S-compound a violet one, on the paper chromatogram. The sensitiveness of of the colour reaction of the sulphoxide is about 1/20, and that of the S-compound about 1/10, of that of alanine. R_F-values for the sulphoxide: 0.85 in phenol-NH_3, 0.15 in butanol-acetic acid-water, and for the S-compound: 0.90 in phenol-NH_3, 0.48 in butanol acetic acid-water [2]. Sulphoxide $[\alpha]_D^{20} -17.4°$ (in water).

There is no double bond and also no primary amino group in either of the amino acids. The nitrogen belongs to an imino group. On the basis of these facts and the elementary composition of the substance it should contain a heterocyclic ring.

Detailed investigations of the products formed by heating of the sulphoxide with 6 N HCl at 105°C for 43 h elucidated the structure of this compound. As reduction products the corresponding thioether (II) and as oxidation products 2-aminopropane-1-sulphonic acid (2-methyltaurine) (III) and cysteic acid (IV) were formed. In addition a small spot in the solvent front (phenol), probably an amine, was found on the paper chromatogram (Fig. 1). These results led to structure I for the sulphoxide and structure II for the thioether [3,4].

The elementary composition of compound III corresponded to the formula $C_3H_9O_3NS$. Paper chromatographically it was identical with synthetic 2-methyltauri-



I II III IV

3-Methyl-1,4-thiazane-5-carboxylic acid-1-oxide (*Cyclo*alliin)

3-Methyl-1,4-thiazane-5-carboxylic acid (Thioether of *cyclo*alliin)

2-Aminopropane-1-sulphonic acid (2-Methyltaurine)

Cysteic acid

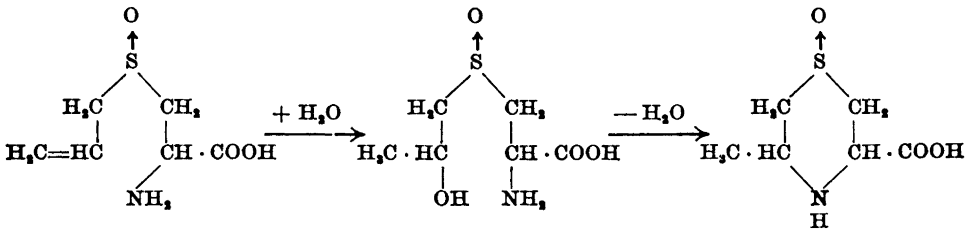
ne which was synthesized from allylamine by the method of Rumpf [4] and separated from salts in an Amberlite IR-120 column. The IR-spectra of the synthetic product and substance III were identical, and hence the formation of 2-methyltaurine as an oxidation product of amino acid I was confirmed.

Cysteic acid was characterized by paper chromatography. Beside these products a red pigment was formed which could be divided into two spots on the paper chromatogram. The chemical nature of these substances is not known yet. Since the oxidation products 2-methyltaurine and cysteic acid do not by far correspond quantitatively to the reduction product, thioether, it is probable that the coloured compounds in question are also oxidation products from *cyclo*alliin.

Because the new amino acid (3-methyl-1,4-thiazane-5-carboxylic acid-1-oxide) which occurs in onion originally has the same elementary composition as alliin, the name *cyclo*alliin is proposed for it [5]. In addition, the biosynthesis of *cyclo*alliin from alliin (V), is possible *e.g.*

The structure of *cyclo*alliin could also be confirmed through the synthesis of 3-methyl-1,4-thiazane-5-carboxylic acid. The way of the synthesis was the following (p. 625).

L-Cystein was used in the synthesis of S-allylcystein. 3 g of S-allylcystein was added to 200 ml of glacial acetic acid and the suspension, saturated with HBr at 0°C, was allowed to stay for 3 1/2 days in a sealed tube. S-Allylcystein then dissolved almost completely. Glacial acetic acid and HBr were distilled off *in vacuo* and pyridine was added to the residue, after which the mixture in a bottle with a reflux condenser was allowed to stay for 4 h in a boiling water bath. Pyridine was removed *in vacuo* and the residue was dissolved into 2.5 N HCl. The amino acids in the solution were then fractionated in a 4.3 × 61 cm column filled with Dowex 50 (200—400 mesh). The resin was in the H-form, in equilibrium with 2.5 N HCl. The dropping rate was about 1 ml/min. Fractions 1 480—2 180 ml contained an unknown amino acid, probably some hydroxyamino acid, which paper-chromatographically differed completely from the thioether of *cyclo*alliin. Fractions 2 530—3 240



V I

$$CH_2{=}CH \cdot CHBr + HS \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow CH_2{=}CH \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH \xrightarrow{HBr}$$

$$CH_2 \cdot CHBr \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH \xrightarrow{\text{pyridine}}$$

$$CH_2 \cdot CH \cdot CH_2 \cdot S \cdot CH_2 \cdot CH \cdot COOH \xrightarrow{H_2O_2} CH_2 \cdot CH \cdot CH_2 \cdot SO \cdot CH_2 \cdot CH \cdot COOH$$
$$\quad\quad\quad

thioether of *cyclo*alliin, was oxidized with hydrogen peroxide into the corresponding sulphoxide this gave the same products both quantitatively and qualitatively as *cyclo*alliin with 6 N HCl at 105°C (Fig. 1). Their IR-spectra resembled each other, but were not identical, however.

As mentioned before, RS I travelled somewhat slower than the thioether of *cyclo*alliin on the paper chromatogram. Both were coloured with ninhydrin in the same way. Oxidized into the sulphoxide RS I gave the same products with 6 N HCl in 105°C as *cyclo*alliin. The IR-spectra differed more from each other than did those of *cyclo*alliin and RS III. Also RS IV travelled slower on the paper chromatogram than RS III, its spot was coloured more strongly with ninhydrin than were those of RS I, RS III, and the thioether of *cyclo*alliin. Also RS III is coloured with ninhydrin somewhat stronger than the natural compound. Although a compound which would have had identical IR-spectra with natural *cyclo*alliin resp. its thioether could not be separated from the synthetical products, all the known facts suggest that the three compounds RS I, RS III and RS IV are stereoisomers of the natural compound. The properties of synthetic RS III and the thioether of *cyclo*alliin are in all respects closest in resemblance.

*Cyclo*alliin represents a new type of natural amino acids. It could been found in all parts of the *Allium* species investigated (*A.cepa*, *A.sativum*, *A. porrum*). The highest content of *cyclo*alliin was found in onion imported from Hungary (3.2 g hydrochloride per kg). From a batch of onions cultivated in Finland 1.4 g per kg was isolated.

The financial support of the *Rockefeller Foundation* is gratefully acknowledged. Thanks are due to Mrs. Anneli Lehtinen for skilful technical assistance.

1. Virtanen, A. I. and Matikkala, E. J. *Suomen Kemistilehti B* **29** (1956) 134.
2. Matikkala, E. J. and Virtanen, A. I. *Suomen Kemistilehti B* **30** (1957) 219.
3. Virtanen, A. I. and Matikkala, E. J. *Suomen Kemistilehti B* **31** (1958) 191.
4. *cf.* Virtanen, A. I. *Schweiz. Z. allgem. Pathol. u. Bakteriol.* **21** (1958) 970.
5. Rumpf, P. *Bull. soc. chim. France* [5] **5** (1938) 871.

Received February 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 627–640

On the Properties of a New Thiol Reagent: 6-Chloromercuri-2-nitrophenol *

STIG ÅKERFELDT

Eastern Pennsylvania Psychiatric Institute, Philadelphia, Pennsylvania, U.S.A.

6-Chloromercuri-2-nitrophenol (CMNP) has been prepared from mercury(II) acetate and *o*-nitrophenol by an improved method.

CMNP was found to react rapidly and quantitatively above pH *ca.* 3.5 with all true and with some potential thiols investigated (giving MNP-thiols). The effect of reaction time, pH and NaCl concentration on this reaction has been studied.

A method is described for quantitative determination of thiols insoluble in toluene or in 1,2-dichloroethane. Concentrations down to the order of 10 μM can thus be determined.

CMNP was found to be a specific reagent for true and potential thiols at pH 4, the only exception found being the cyanide ion.

Mixtures of MNP-thiols have been separated by paper chromatography. R_F-values for some thiols of biochemical interest are given in 4 solvent systems.

A "ghost" spot on the chromatograms was identified as 2,2′-dihydroxy-3,3′-dinitro-diphenylmercury.

Organic mercury compounds, such as the now classical *p*-chloromercuribenzoic acid [1], are among the reagents employed for thiols. As such they have the advantage of being relatively specific and fast reacting.

In this laboratory the study of a few mercury derivatives of nitrophenols was undertaken. These compounds have the advantage of being colored and their reaction with thiols can therefore be conveniently studied colorimetrically.

The substances investigated here were 4-acetoxymercuri-2-nitroresorcinol [2], 4-chloromercuri-2-nitrophenol [2], and 6-chloromercuri-2-nitrophenol [3]. Of these, 6-chloromercuri-2-nitrophenol was found to react rapidly and quantitatively with thiols above pH *ca.* 3.5. This reaction (Fig. 1) was studied in detail.

The results show that at pH 4, 6-chloromercuri-2-nitrophenol is a highly specific reagent for thiols.

---

* Some preliminary investigations for this work were carried out at the Biochemical department of the Nobel Medical Institute, Stockholm, Sweden.

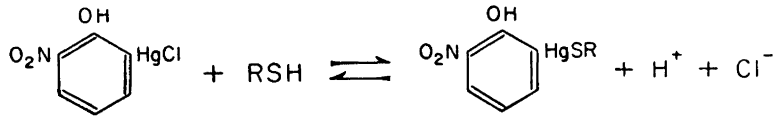


Fig. 1. The reaction between CMNP and thiols.

The reaction products of 6-chloromercuri-2-nitrophenol and various thiols have been separated by paper chromatography. The fact that these thiol derivatives are highly colored renders them especially suitable for chromatographic analysis.

The advantages of 6-chloromercuri-2-nitrophenol over some previously studied colored mercury-containing thiol reagents [4–6] (which all were azo compounds) are: a) the new reagent is easy to prepare in satisfactory yields; b) it can be used as a reagent in chromatography of thiols.

An improved method for the synthesis of 6-chloromercuri-2-nitrophenol is also reported.

MATERIALS AND METHODS

The thiols used in these investigations were from Mann Laboratories, Nutritional Biochemicals Corporation, and Eastman Company. *o*-Nitrophenol (m. p. 44–46°) was obtained from Eastman Company (samples from some other sources were less pure and gave lower yields of 6-chloromercuri-2-nitrophenol). Mercury (II) acetate was of analytical purity obtained from Merck Company. All solvents used in chromatography and elsewhere, as well as other chemicals, were of highest commercially obtainable purity. They were used without further purification.

Buffers used (unless otherwise stated):

pH 1.1– 6.6	0.1 M citrate
7.2– 9.0	0.1 M tris(hydroxymethyl)aminomethane
9.0–12	0.1 M glycine

Unless otherwise stated, all experiments were carried out at room temperature (25°C). All melting points (°C, Kofler method) are corrected. Spectrophotometric measurements were performed in 1 cm cuvettes with a Beckman Model DU spectrophotometer.

Abbreviations used:

CMNP = 6-chloromercuri-2-nitrophenol
MNP-thiol = S-(2-hydroxy-3-nitrophenyl mercuri)-thiol
EDTA = (ethylenedinitrilo) tetraacetic acid
ε_λ = molar extinction coefficient at λ mμ (1 cm cuvette)

Synthesis of 6-chloromercuri-2-nitrophenol.

The mercuration of nitrophenols with mercury (II) acetate generally proceeds easily, but the product obtained practically always seems to consist of a mixture of all theoretically likely monomercury derivatives plus considerable amounts of dimercuri derivatives [2,3,7].

Hodgson [3] synthesized 6-acetoxymercuri-2-nitrophenol by fusing mercury (II) acetate with a large excess of *o*-nitrophenol. However, the yield is poor.

Contrary to Hodgson's statement [3] that 4-acetoxymercuri-2-nitrophenol is preferentially formed by boiling mercury(II) acetate and *o*-nitrophenol in solvent media, we found that when 1-butanol, 1-propanol or methanol were employed as solvents, the 6-acetoxymercuri derivative could be isolated from the reaction product in fairly good yields. However, employing high boiling solvents like 1-butanol caused large amounts of the dimercury derivative to form, and low boiling solvents like methanol gave poor yields. In boiling 1-propanol, however, the reaction proceeded rapidly and the yield of the 6-acetoxymercuri derivative was satisfactory. As has been pointed out by previous investigators [2,3] the 6- resp. 4-acetoxymercuri-2-nitrophenols are difficult to obtain in a pure form, mainly because the dimercuri derivative, which is formed simultaneously, shows somewhat similar solubility properties as the monomercuri derivatives. We found it advantageous to convert the resulting mixture of acetoxymercuri-2-nitrophenols into their chloride forms prior to purification. CMNP could then be preferentially extracted from the crude product with acetone. Another advantage of working with the chloride is that it has a conveniently located melting point (190°) and its purity could thus be checked easily. The acetate does not melt below 300°.

It should be pointed out, however, that there is no substantial difference between the chloride and the acetate in regard to their reactivity toward thiol groups.

Procedure of preparation. To a refluxed solution of 15.0 g (0.108 mole) of *o*-nitrophenol in 50 ml 1-propanol is added 34.5 g (0.108 mole) of mercury (II) acetate in small portions during one hour. The mixture is refluxed for another hour, poured onto 200 g of cracked ice and filtered when ice cold. The yellow precipitate is washed with 50 ml of distilled water and with 25 ml of 95 % ethanol. To convert the substances into their chloride forms they are vigorously stirred for 15 min with 100 ml of a saturated solution of sodium chloride in water. The suspension is then filtered and washed free of chloride ions with distilled water and dried at room temperature in the dark.

The dry, yellow substance obtained (31—34 g) is refluxed for 15 min with 200 ml of acetone and filtered. The undissolved residue is extracted for another 15 min with 100 ml of acetone and filtered. The pooled acetone extracts are evaporated to dryness in a stream of air at room temperature. 18—20 g (yield 45—50 %) of relatively pure CMNP (melting about 185°) is thus obtained. After two recrystallizations from 95 % ethanol it melts at 190°. (Upon storage the melting point tends to drop somewhat). Further recrystallizations do not change the melting point. (Hodgson [3] reports 185°.) The product was found to be fairly soluble in most organic solvents tested, especially in acetone, ethyl acetate and in chlorinated hydrocarbons. CMNP should be stored in a dark, dry and cool place.

Mercury determinations. 0.3 g of CMNP in 50 ml distilled water were mixed with 3 g of potassium iodide and with 1 g of iodine and carefully heated with occasional stirring until a clear solution resulted. Mercury was then determined as HgS in this solution according to Ref.[8] (Found: Hg 53.48, 53.55. Calc. for $C_6H_4ClHgNO_3$ (374.17): Hg 53.61).

Position of the mercury *. 0.15 g of CMNP (0.40 mmole), 0.10 g of iodine (0.40 mmole) and 0.5 g of potassium iodide in 10 ml of distilled water were carefully heated with occasional stirring until a clear solution resulted. The solution was cooled to room temperature, acidified with a few drops of 5 % hydrochloric acid and shaken with 10 ml of ethyl acetate. The ethyl acetate phase was separated and evaporated to dryness and the resulting crystals (0.10 g) were recrystallized once from 50 % ethanol. The golden yellow crystals obtained melted at 109° as reported for 6-iodo-2-nitrophenol [3,9]. No other monoiodo-2-nitrophenol has a melting point close to the one found [10,12].

* A similar technique has been used before [3,7] but no details were given.

It has been presumed that the iodine occupies the same position in the molecule as the mercury it replaces. According to the results of Henry and Sharp [13] this is not always true when halogens (*e. g.* bromine) react with a mercury atom attached to a benzene ring. These authors found, however, that iodine treatment gave the expected derivative in all cases studied.

Furthermore, 4-chloromercuri-2-nitrophenol as prepared by Hodgson [3], has a different melting point (205°) from the one found for CMNP. It seems therefore safe to presume that the mercury in CMNP occupies the 6 position.

The acetone insoluble fraction from the synthesis was treated in a similar manner and after two recrystallizations from 50 % ethanol the product melted at 96°, possibly indicating it to be 4,6-diiodo-2-nitrophenol (m. p. 98°) [11]. However, 5-iodo-2-nitrophenol has a similar melting point (96°) [12]. Mercury determinations performed on the acetone insoluble product were inconsistent, but indicated the presence of two mercury atoms per molecule of *o*-nitrophenol.

The acetone insoluble fraction was then treated with potassium bromide and bromine in an analogous manner to the one described above. After two recrystallizations from concentrated acetic acid the brominated compound melted at 119°, close to that reported for 4,6-dibromo-2-nitrophenol (120—121°) [13].

Thus the acetone insoluble fraction mainly consists of 4,6-di(chloromercuri)- 2-nitrophenol.

Absorption spectra of CMNP. Fig. 2 shows the absorption spectra of CMNP at pH 1.6 and at pH 9.1.

For quantitative measurements the peak at 420 mμ shown only in basic solutions was found convenient to use. From Fig. 3 it is seen that the pH-optimum for absorption at this wavelength is between pH 9—10 ($\varepsilon_{420} = (5.28 \pm 0.02) \times 10^3$ at pH 9.5).

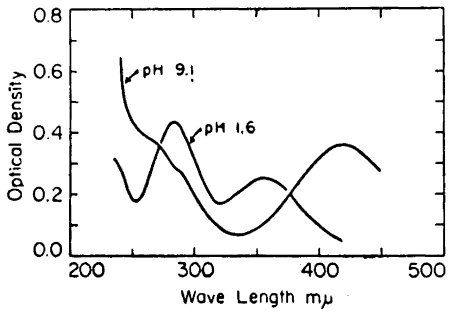


Fig. 2. Absorption spectra of CMNP (66.8 μM) at pH 1.6 and at pH 9.1.

In this pH-range the absorption follows Beer's law.

The absorption spectra of MNP-thiols were found to be almost identical with those presented in Fig. 2, provided the thiol itself does not absorb in this region. MNP-cysteine was found to have the same molar extinction coefficient as CMNP at pH 9.5 (420 mμ). That of MNP-glutathione is practically the same ($\varepsilon_{425} = (5.22 \pm 0.03) \times 10^3$) at this pH, but the absorption maximum was shifted somewhat towards longer wavelengths (425 mμ).

The color at 420 mμ (pH 9.5) was found to be stable within reasonable time limits (Table 1).

Table 1. Color stability of MNP-cysteine (85.8 μM) at pH 9.5.

Time minutes	Optical density at 420 mμ
0	0.452
60	0.453
270	0.452

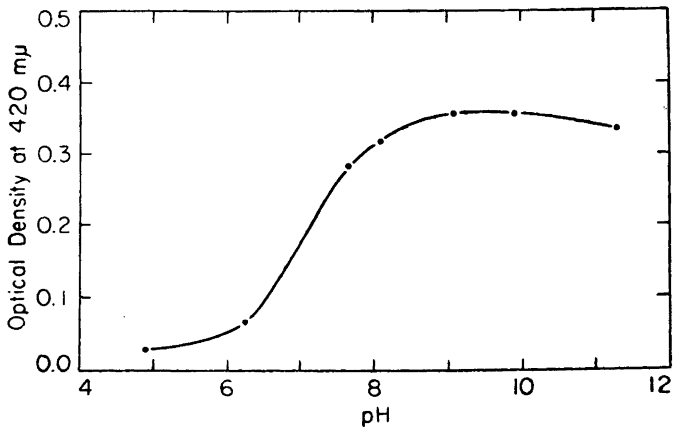


Fig. 3. Effect of pH on absorption peak at 420 mμ. Concentration of CMNP 66.8 μM.

THE REACTION BETWEEN CMNP AND THIOLS

When a thiol dissolved in a buffer of about pH 4 is shaken with a solution of CMNP in an organic solvent, *e.g.* toluene, a yellow color or precipitate generally appears in the water phase. The reaction taking place is described in Fig. 1.

Effect of pH. Cysteine and glutathione dissolved in buffers of varying pH were mechanically shaken with a solution of CMNP in toluene and the decrease in optical density at 355 mμ (the absorption at this wavelength follows Beer's law) in the toluene layer was followed. Simultaneously, blanks containing only the corresponding buffers and reagent in toluene were shaken at the same rate. The difference in optical density between the two toluene layers after completed reaction was taken as a measure of the amount of reacted thiol. From the molar extinction coefficient for CMNP in toluene ($\varepsilon_{355} = (3.91 \pm 0.02) \times 10^3$) it was thus possible to calculate the amount of reacted thiol.

As is seen from Fig. 4 both glutathione and cysteine react quantitatively (97—100 %) under the conditions employed. The difference in structure between glutathione and cysteine apparently does not have a significant influence on the pH dependence of the reaction.

Above pH 5 the reagent becomes very water soluble (Table 2) and the reaction becomes difficult to follow with the described technique. However,

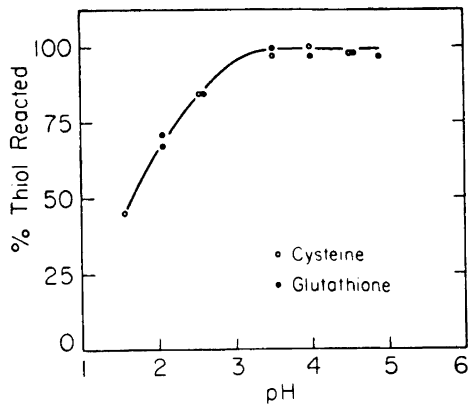


Fig. 4. Effect of pH on the reaction between cysteine, glutathione and CMNP. 6 ml 0.3 mM solutions of thiols in buffers of varying pH (blanks: 6 ml of corresponding buffer) were shaken for 1 minute at the rate of 300 cycles/min with 6 ml 0.03 % CMNP in toluene. For calculation of data see text.

it can be shown by paper chromatography that the thiols investigated also react with the reagent in the pH range 5—12.

Table 2. Distribution of CMNP between buffers and 1,2-dichloroethane. Data were obtained by shaking 0.5 % solutions of CMNP in 1,2-dichloroethane for 10 min at the rate of 300 cycles/minute with an equal volume of buffer of indicated pH. The concentration of CMNP in the water phase was calculated from the optical density at 420 mμ of the water phase after adjustment to pH 9.5. It should be noted that the distribution of CMNP between the phases is much affected by the organic solvent used.

pH	Concentration of CMNP in water phase (μM)
1.56	20
2.59	20
3.52	19
4.00	24
4.90	259

Effect of shaking time. As is shown in Fig. 5 the reaction between glutathione, cysteine and the reagent is completed within one minute under the experimental conditions used. Prolonged shaking increases the optical density in the toluene layer (*i.e.* decreases the difference in readings between blank and sample) thus showing a slight decomposition of the reaction product. This decomposition could be almost completely prevented by the addition of excess cyanide (see Fig. 5), but not by EDTA in similar concentrations.

It thus appears likely that cyanide does not act mainly by binding traces of heavy metals present (which catalyze the autooxidation of thiols) but by forming a cyanide complex with the MNP-thiol. This view is supported by the fact that cyanide, but not EDTA, reacts with CMNP itself under identical

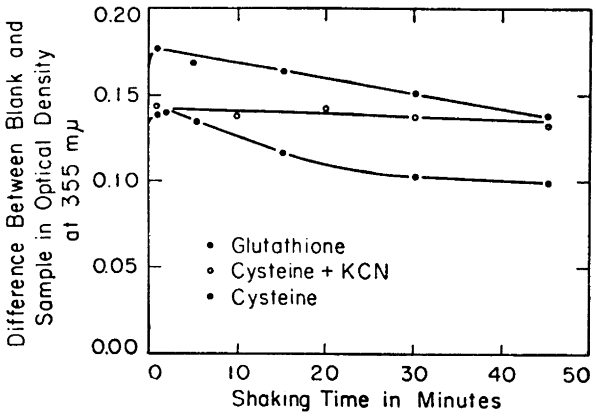


Fig. 5. Effect of shaking time on the reaction between cysteine, glutathione and CMNP. 6 ml thiol of pH 4.0 (blanks: 6 ml buffer of pH 4.0) were shaken at the rate of 300 cycles/min with 6 ml of 0.03 % CMNP in toluene. Optical density of 0.5 ml toluene phase + 3.0 ml of toluene was followed. Concentrations: Cysteine 0.25 mM; glutathione 0.32 mM. KCN in cysteine solution and in corresponding blank: 1.7 mM.

conditions. A possible degradation product is 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury (see later paragraphs).

In basic solutions, however, MNP-thiols seem to be much less subject to decomposition, which can be shown by paper chromatography.

Reversibility of the reaction. By shaking a suspension of a MNP-thiol, such as MNP-glutathione, with an acid of about pH 1 the MNP-thiol is completely decomposed and all the color of the original solution can be taken up in *e.g.*, 1,2-dichloroethane. Analysis of the yellow dissociation product by paper chromatography after treatment of MNP-thiols with 0.1 M hydrochloric acid showed it to consist of CMNP contaminated with a small amount of 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury (see later paragraphs about this compound).

Quantitative measurements. The results of the above experiments suggested that CMNP can be used as a quantitative reagent for thiols. Fig. 6 shows the results of the quantitative determination of cysteine.

The reaction was carried out by shaking cysteine solutions of pH 4.0 with a reagent solution in toluene, and the amount of reacted thiol was determined by measuring the decrease in optical density at 355 mμ in the toluene layer. In each instance 98—100 % reaction was obtained as calculated from ε_{355} for CMNP in toluene. The addition of cyanide to the reaction mixture gave little, if any, improvement when the shaking time was kept short. Furthermore, large excess of cyanide gave low readings.

When very small amounts of thiol were present higher accuracy was achieved by measuring the optical density of the water phase after completed shaking with CMNP. In such cases it is convenient to use a solvent for the reagent which has a density higher than 1 (*e.g.* 1,2-dichloroethane) so that a sample of the water layer can be directly pipetted off after completed reaction.

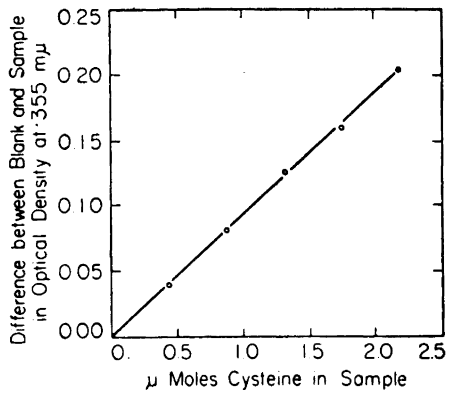


Fig. 6. Determination of cysteine with CMNP. 6 ml of cysteine solution of pH 4.0 (blanks: 6 ml buffer of pH 4.0) were shaken with 6 ml of 0.03 % CMNP in toluene for 1 minute at the rate of 300 cycles/min. The optical density of 0.5 ml toluene phase + 3.0 ml of toluene was determined.

The sample is then adjusted to pH 9.5 by the addition of a buffer of a suitable strength and the optical density at about 420 mμ is determined (the exact wavelength varies somewhat with the thiol used).

This method is generally not applicable when solutions of a high thiol concentration are used, as a precipitate then tends to form in the water-phase.

Care should be taken to avoid very high ionic strengths in the solutions to be measured as this decreases the completeness of the reaction (Fig. 7).

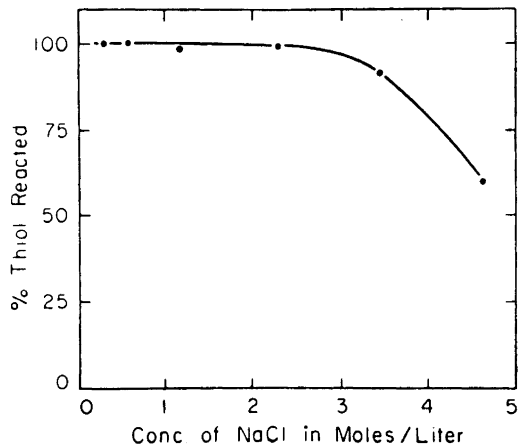


Fig. 7. Effect of NaCl concentration on the reaction between cysteine and CMNP. 0.3 mM solutions of cysteine in buffer containing various amounts of NaCl (final pH adjusted to 4.2—3.8; blanks contained the same buffer — NaCl mixtures) were shaken for 1 min at the rate of 300 cycles/min with equal volumes of 0.03 % CMNP in toluene. The reaction was followed by the decrease in optical density in the toluene phase. The amount of reacted cysteine was calculated as in Fig. 4.

With both methods a thiol concentration of 0.02 mM (3 μg cysteine/ml) gives a change in optical density of the order of 0.1.

Quantitative determination by the above methods is obviously limited to those thiols, which are not soluble in the organic solvents used.

Specificity of the reaction. As is seen from Table 3 CMNP reacts almost exclusively with true or potential thiols at pH 4, the only exception found being the cyanide ion. Thioethers, thiolactones, disulfides, sulfonic acids and sulfinic acids do not seem to react. At higher pH's, however, some substances were found to react with the reagent. *E.g.*, EDTA reacts at pH 7.

Table 3. Reactivity of compounds towards CMNP. Data were obtained by dissolving the compounds to be tested (1 – 2 mg/ml) in 1 M citrate buffer of pH 4.0 and shaking the solutions for one minute with a solution of CMNP (0.2 %) in 1,2-dichloroethane. The appearence of a yellow color or precipitate in the water phase indicated positive reaction. Reactivity values were obtained from quantitative determinations of 0.3 mM solutions of the compounds as described above (1 min shaking time).

Compounds that react with CMNP	Reactivity %	Nonreactive compounds
2-Aminoethanethiol	103	Benzenesulfinic acid
Cysteine	100	DL-(+)-*allo*-Cystathionine
Ergothioneine		L-Cystin
Glutathione	100	6,8-Dithio-*n*-octanoic acid
Homocysteine	100	DL-Homocysteine thiolactone
Hydrogen sulfide		DL-Methionine
Mercaptoacetic acid		Sodium bisulfite
2-Mercaptoethanol *		Taurine
L-2-Mercaptohistidine	100	Adenosine-5′-triphosphate
6-Mercaptopurine		L-Alanine
Mercaptosuccinic acid		L-Arginine
6-Methylthiouracil		DL-Aspartic acid
Potassium cyanide		Betaine
Sodium thiosulfate	100	Choline
2-Thiobarbituric acid	63; 77[a]	Creatinine
2-Thiocytosine	72; 69[a]	Disodium hydrogenphosphate
2-Thiouracilcarboxylic acid		EDTA
Thiourea	100	Glycine
		L-Histidine
		Potassium cyanate
		L-Proline
		DL-Serine
		tris(hydroxymethyl)aminomethane
		DL-Tryptophane
		L-Tyrosine

* = color of organic phase deepens
[a] = 2 minutes shaking time

PAPER CHROMATOGRAPHY OF MNP-THIOLS

Success in applying paper chromatography as a separatory means of MNP-thiols fundamentally depends on the stability of these compounds. As mentioned above MNP-thiols are not quite stable at slightly acid pH's. Fortunately, the MNP-thiols investigated were found to be more stable in alkaline solution. In fact, solutions of most investigated MNP-thiols in 0.1 M sodium hydroxide or in 1 % piperidine did not remarkably change their chromatographic properties after storage for a few days, even if the solutions were kept at room temperature.

Paper chromatography was thus attempted with the main emphasis on basic solvent systems in combination with treated and untreated varieties of paper. Out of some 300 combinations of solvents and varieties of papers tested, the best results were obtained when MNP-thiols were chromatographed on paper, which previously had been impregnated with sodium carbonate. A wide variety of solvent systems were found to be useful in combination with this paper.

"Ghost" spots. When chromatographing MNP-thiols in the manner just described, a small yellow red "ghost" spot was practically always observed accompanying the main MNP-thiol spot. This "ghost" spot had the same R_F-value independent of the MNP-thiol from which it was derived. Its position on the chromatgrams was different from that of CMNP and from MNP-H_2S. It was not identical with *o*-nitrophenol. The "ghost" spot did not contain sulfur, as judged by the cyanide-nitroprussiate reaction. Nor was it ninhydrin positive when derived from amino group containing MNP-thiols.

The size of the "ghost" spot relative to that of the parent MNP-thiol spot varied with the solvent used for the development of the chromatogram. For instance, exceptionally large "ghost" spots were obtained when using solvent systems rich in pyridine. Also, some MNP-thiols, especially MNP-cysteine, gave relatively more "ghost" spot than others. The addition of cyanide to the solvent systems did not significantly decrease the amount of "ghost" spot.

It was found, however, that if the MNP-thiols were prepared at 0° and chromatographed with the solvent systems listed in Table 4, relatively smaller amounts of "ghost" spot was present on the finished chromatograms than when they were prepared at 25°. It is thus clear that much of the compound responsible for the "ghost" spot is formed during the preparation of MNP-thiols, when this takes place at room temperature.

Furthermore, some solvent systems decompose MNP-thiols during chromatography more easily than others.

A substance with the same chromatographic and spectrophotometric properties as the "ghost" spot is formed when MNP-thiols are heated in neutral solutions (*e.g.* when attempts are made to recrystallize MNP-thiols from water or ethanol). A convenient way to obtain this substance in quantity is to boil an ethanol soluble MNP-thiol * (1 g) in 95 % ethanol (50 ml) for 30 min and to recrystallize the crystals appearing upon cooling from the same solvent. The decomposition product thus obtained is chromatographically pure.

Preliminary investigations of the compound, which does not melt below 300°, showed that it contains mercury. Upon treatment with iodine + iodide as described in earlier paragraphs, the compound is slowly converted into 6-iodo-2-nitrophenol and mercury(II) iodide. The substance did not react with thiols when the CMNP procedure was applied, suggesting that the mercury is bound by nonionizable bonds.

* *E.g.*, MNP-2-mercaptoethanol.

Using the molar extinction coefficient for CMNP at pH 9.5 (which seemed justified as an approximation method since all MNP-thiols investigated show very similar molar extinction coefficients) a molecular weight of 257 was obtained. This value, however, does not account for both the mercury and for the nitrophenol residue present. The presence of two nitrophenol residues in the substance must then be presumed. This would approximately double the molar extinction coefficient and give a molecular weight of the compound of the order of 500, which only allows for one mercury atom per molecule of compound.

Elementary analysis of the compound, finally, was in agreement with these findings. (Found: C 29.67; H 1.96; Hg 42.23. Calc. for $C_{12}H_8HgN_2O_6$ (476.81); C 30.23; H 1.69; Hg 42.07)

Thus the isolated compound is identical with 2,2'-dihydroxy-3,3'-dinitrodiphenylmercury, and the decomposition of MNP-thiols by heat *etc.* is most likely represented by the scheme in Fig. 8.

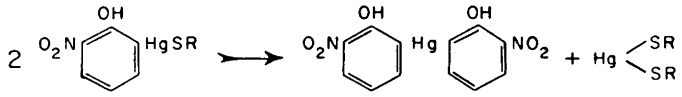


Fig. 8. Probable scheeme for the formation of "ghost" spots.

Preparation of MNP-thiols. Good yields of MNP-thiols were generally achieved by the following procedure carried out at 0°. 0.1 g thiol is dissolved in 5—10 ml of 1 M citrate buffer of pH 4 and an equivalent amount of CMNP in ethylacetate is added under vigorous stirring. A yellow precipitate appears immediately. If changed, pH is adjusted back to 4 with 0.1 M sodium hydroxide. The precipitate is filtered off and washed with 50 ml of water and with 10 ml of ethylacetate (ethylacetate soluble MNP-thiols are preferably washed with a small amount of ethanol). The substances can be further purified by dissolving them in the minimum amount of 0.1 M sodium hydroxide and reprecipitating them at pH 4 by the addition of 10 % acetic acid. After washing with water and with ethylacetate (ethanol) the MNP-thiols are dried at room temperature in the dark.

The MNP-thiols thus prepared are stable when stored in a dark, dry and cool place.

To prepare reference solutions for paper chromatography of thiols not soluble in organic solvents it is generally sufficient to shake the thiol, dissolved in buffer of pH 4 at 0°, with an excess of CMNP in 1,2-dichloroethane at 0°. After centrifugation a sample of the water phase (preferably containing some of the precipitate formed) is pipetted off and 0.1 M sodium hydroxide or 1 % piperidine is added dropwise until the solution turns yellow red (pH 9—10).

Chromatographic procedure. Whatman No. 1 papers were used exclusively. Prior to chromatography the papers were impregnated with a solution of 1 % $Na_2CO_3 \cdot H_2O$ in water by dipping the papers into the solution or by spraying them with the sodium carbonate solution until the papers were completely saturated. The papers were dried at room temperature.

For best results freshly impregnated papers should be used. The dried papers were spotted with MNP-thiols dissolved in 1 % piperidine or in 0.1 M sodium hydroxide.

All chromatograms were developed by the ascending technique and were run for 2—4 h in the dark. Longer runs are preferably carried out in a cold room at 0—4°, in order to decrease the amount of decomposition of MNP-thiols during chromatography. MNP-thiols appear as yellow spots. Amounts down to the order of 5 mμmoles/cm² are easily detected (Fig. 9).

Table 4. Approximate R_F-values of MNP-thiols chromatographed 3—4 h at room temperature on Whatman No. 1 paper impregnated with 1 % $Na_2CO_3 \cdot H_2O$.

Solvents (figures indicate volumes):
1. 95 % ethanol : water, 4:1
2. methanol : N,N-dimethylformamide : water, 3:1:1
3. 95 % ethanol : benzene : water, 4:1:1
4. 95 % ethanol : N,N-dimethylformamide : water, 3:1:1

Substance	Solvent No.			
	1.	2.	3.	4.
CLMNP	0.21	0.12	0.35	0.22
MNP-2-aminoethanethiol	0.55	0.53	0.74	0.63
MNP-cysteine	0.28	0.33	0.45	0.39
MNP-ergothioneine	0.51	0.53	0.75	0.65
MNP-glutathione	0.07	0.13	0.10	0.09
MNP-homocysteine	0.23	0.32	0.35	0.28
MNP-mercaptoacetic acid	0.41	0.47	0.61	0.58
MNP-2-mercaptoethanol	0.85	0.79	0.97	0.90
MNP-2-mercaptohistidine	0.21	0.29	0.41	0.33
2,2′-dihydroxy-3,3′-dinitro-diphenylmercury	0.41	0.51	0.69	0.67

Fig. 9. Paper chromatogram of MNP-thiols (5—10 μg/spot) developed at room temperature (2 h) on Whatman No. 1 paper impregnated with 1 % $Na_2CO_3 \cdot H_2O$. Solvent: 95 % ethanol: N,N-dimethylformamide:water = 3:1:1. The MNP-thiols used were prepared at room temperature to demonstrate the formation of 2,2′-dihydroxy-3,3′-dinitro-diphenylmercury. (The chromatogram was photographed with the aid of a blue filter.)

The solvent systems listed in Table 4 were found to give minimum amounts of "ghost" spots.

Spray reagents. In addition to the R_F-values the following spray reagents were found to be useful aides in the identification of MNP-thiols on the chromatograms. The color reactions described were all obtained on sodium carbonate impregnated paper.

1) All MNP-thiols (and CMNP) give a brown color when sprayed with *p*-nitrobenzenediazonium fluoborate (0.5 % in 50 % ethanol). Thus weak spots can be made to show up more clearly.

2) To confirm the presence of sulfur in the MNP-thiol spots the cyanide-nitroprussiate reagent [14] can be used. Of the MNP-thiols listed in Table 4 all gave an immediate redbrown spot, except MNP-ergothioneine and MNP-2-mercaptohistidine, which do not react.

3) MNP-thiols containing amino groups give brown spots when sprayed with ninhydrine (0.5 % in ethanol). The colors are best developed at room temperature. Depending on the amount of substance present positive response is obtained after 1—12 h.

4) MNP-ergothioneine and MNP-2-mercaptohistidine give grey blue spots immediately when sprayed with N,2,6-trichloro-*p*-benzoquinone imine (0.2 % in 50 % ethanol).

Effect of light on MNP-thiols. Exposure of MNP-thiol spotted chromatograms to ultraviolet radiation prior to chromatography invariably showed a deposit of a brownish colored decomposition product at the starting line on the finished chromatogram (solvent: ethanol:water, 4:1). The blank spots, which had been covered during the UV-exposure, showed no trace of this decomposition product. Even UV lamps with a low wattage (9 watts) when used at a distance of about 10 cm from the paper caused considerable decomposition during one hour.

It was noted on the finished chromatograms that the size of the "ghost" spot relative to the size of the MNP-thiol spot was not significantly changed after UV exposure. Thus the decomposition of MNP-thiols by ultraviolet light is qualitatively different from the decomposition noted by *e.g.* heat, which is represented by Fig. 8.

The author is indebted to Mrs. M. L. Kip for technical assistance.

REFERENCES

1. Chinard, F. P. and Hellerman, L. in Glick, D. *Methods of Biochemical Analysis*, Interscience Publishers Inc., New York 1954, Vol. I p. 12.
2. Raiziss, G. W. and Proskouriakoff, A. *J. Am. Chem. Soc.* **44** (1922) 787.
3. Hodgson, H. H. *J. Am. Chem. Soc.* **49** (1927) 2840.
4. Horowitz, M. G. and Klotz, I. M. *Arch. Biochem. Biophys.* **63** (1956) 77.
5. Bennett, H. S. and Yphantis, D. A. *J. Am. Chem. Soc.* **70** (1948) 3522.
6. Flesch, P. and Kun, E. *Proc. Soc. Exptl. Biol. Med.* **74** (1950) 249.
7. Hantzsch, A. and Auld, S. M. *Ber.* **39** (1906) 1105.
8. Wagner, W., Hull, C. J. and Markle, G. E. *Advanced Analytical Chemistry*, Reinhold Publishing Corporation, New York 1956, p. 140.
9. Hodgson, H. H. and Moore, F. H. *J. Chem. Soc.* **1925** 2260.

10. Roberts, E. *J. Chem. Soc.* **1923** 2707.
11. Hodgson, H. H. *J. Chem. Soc.* **1927** 1141.
12. Hodgson, H. H. and Moore, F. H. *J. Chem. Soc.* **1927** 630.
13. Henry, T. A. and Sharp, T. M. *J. Chem. Soc.* **1924** 1049.
14. Block, R. J., Durrum, E. L. and Zweig, G. *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press Inc. Publishers, New York 1955, p. 94.

Received January 3, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 641–646

A Hemopeptide from a Tryptic Hydrolysate of *Rhodospirillum rubrum* Cytochrome C*

SVEN PALÉUS and HANS TUPPY

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden, and Zweites Chemisches Institut der Universität, Vienna, Austria

Rhodospirillum rubrum cytochrome c purified by chromatography on carboxymethyl cellulose was subjected to hydrolysis with trypsin. A hemopeptide isolated from the digest has been found to consist of thirteen amino-acid residues arranged in a single polypeptide chain, the heme and polypeptide moieties being held together by two thioether bridges. In spite of marked dissimilarities, the sequence of amino-acids in this hemopeptide has certain characteristic features in common with the sequences previously worked out in hemopeptides prepared from other c-type cytochromes.

For the past few years the structure of cytochrome c has been studied with the aim of relating the catalytic activity of this hemoprotein to certain characteristic features of its chemical architecture. Much attention has been given to that portion of the protein moiety which adjoins the prosthetic group and which is supposed to contribute to a catalytically active site. In previous investigations [1,2] the sequence of twelve amino-acid residues including two heme-bound cysteine residues has been elucidated and found to be identical in the cytochromes of three different mammalian species (beef, horse and pig). When, however, the homologous amino-acid sequences were also determined in cytochrome c from fish (salmon)[2], bird (chicken)[2], insect (silk worm)[3] and from a fungus (yeast)[4], they appeared to be dissimilar though resembling each other more or less closely. The present communication will extend this comparative structural investigation to the c-type cytochrome produced by the photosynthetic bacterium *Rhodospirillum rubrum*, first described and subsequently studied in more detail by Kamen *et al.*[5–7]. There is a twofold interest in comparative structural work of this kind. In the first place, one may suppose that only those features which turn up invariably in all various cytochromes c, are likely to be essential to the specific catalytic function, whereas structural differences will indicate points not directly concerned with catalytic

* Presented before the 4th International Congress of Biochemistry, Vienna, September, 1958.

activity. Secondly, the species similarities and dissimilarities as revealed by comparative investigations of amino-acid sequences may give a clue to the genetic relation between the organisms from which the proteins studied have been obtained.

MATERIAL AND METHODS

Preparation of Rhodospirillum rubrum cytochrome c. Lyophilized bacterial cells were extracted with H_2SO_4 at pH 4. From the extract, the cytochrome c was obtained by fractionation with ammonium sulphate [8], dialysis and lyophilization. A sample thus prepared was kindly put at our disposal by Professor M. D. Kamen. It was further purified by chromatography on a column of CM-W cellulose (1.6 × 10 cm) prepared according to Peterson and Sober [9] and buffered with 0.015 M ammonium acetate of pH 6.5. The preparation of cytochrome c (470 mg) was dissolved in 2 ml of this buffer, reduced by the addition of solid $Na_2S_2O_4$ and transferred to the column. A dark brown, fast moving band separated from the red cytochrome band which moved slowly, if at all. When the buffer of pH 6.5 was replaced by 0.05 M ammonium acetate of pH 7.5, the red material started moving down the column and divided into three bands. The main fraction (R = 0.4, E_{270}/E_{551} = 0.96) yielded 71.5 mg of dry, salt-free cytochrome c which was used for all subsequent experiments.

Preparation of the hemopeptide and removal of the heme. After hydrolysis of the bacterial cytochrome c with trypsin, the hemopeptide formed could be precipitated from the digest with ammonium sulphate and purified by adsorption on talc and by column partition chromatography as described previously for other hemopeptides [1,2]. The hemin part of the hemopeptide was split from the peptide moiety using silver sulphate in acetic acid solution according to the method of Paul [10]. Performic acid was used to oxidize the thiol groups of cysteine residues to sulphonic acid residues. The hemin-free oxidized peptide obtained was essentially homogeneous as determined by electrophoresis on paper in a formic acid-acetic acid buffer of pH 2 [11].

Enzymic hydrolyses. The crystalline chymotrypsin used was purchased from the Worthington Biochemical Corporation. The subtilisin was a gift from Ing. M. Ottesen, Carlsberg Laboratory, Copenhagen. Digestions were carried out at pH 7.5 to 7.8 for 20 h at 25°C (with chymotrypsin) or for 24 h at 37°C (with subtilisin), the ratio between enzyme and substrate (w/w) being about 1:15.

End group determinations. For the characterization of N-terminal amino-acid residues the DNP method of Sanger [12,13] was employed. C-Terminal residues were determined using the hydrazinolysis procedure of Akabori *et al.*[14]

RESULTS

In the hydrolysate of the hemin-free oxidized polypeptide (prepared from the hemopeptide as described in the foregoing section) ten different amino-acids were identified by paper chromatography: alanine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, and threonine. The lysine present in the polypeptide may be expected to be the C-terminal residue of the amino-acid sequence, since the polypeptide has been formed by the action of trypsin which is known to split bonds involving the carboxyl groups of lysine and arginine residues. The N-terminal residue was shown to be cysteic acid. After dinitrophenylation of the polypeptide and subsequent hydrolysis, however, the hydrolysate was found to contain, besides DNP-cysteic acid, free cysteic acid as well. This finding is accounted for by the presence in the polypeptide of two cysteic acid residues, one of which is terminal and the other is not.

Table 1. Determination of the amino-acid sequence in a polypeptide obtained from *Rhodospirillum rubrum* cytochrome c by tryptic hydrolysis, removal of the heme moiety, and oxidation with performic acid.

N-terminal residue (DNP method)		$CySO_3H$—. . .
C-terminal residue (specificity of trypsin)		. . .—Lys
Peptides obtained by *chymotryptic* digestion		$CySO_3H$—[Leu, Ala, $CySO_3H$, His] Thr—Phe Asp—Glu—[Gly, Ala, Asp]—Lys
Peptides found after digestion with *subtilisin*	Main split products	$CySO_3H$—Leu—Ala Thr—Phe NH_2 Asp —Lys $CySO_3H$—His [Asp, Glu, Gly, Ala]
	Minor split products	Ala—[$CySO_3H$, His] [Asp, Glu, Gly] [Thr, Phe, Asp, Glu, Gly] $CySO_3H$—[His, Thr] [Gly, Ala]
Sequence		$CySO_3H$—Leu—Ala—$CySO_3H$—His—Thr—Phe—Asp—Glu—Gly—Ala—Asp(NH_2)—Lys

Chymotryptic hydrolysis of the polypeptide (Table 1) gave rise to three main split products which were separated from each other by high-voltage electrophoresis at pH 2 and by paper chromatography in butanol-acetic acid: (I) $CySO_3H$-[Leu, Ala, $CySO_3H$, His], (II) Thr-Phe, and (III) Asp-Glu-[Gly, Ala, Asp]-Lys. Split product (I), with N-terminal cysteic acid, must be derived from the N-terminal side of the polypeptide chain and the lysine-containing peptide (III) from the C-terminal side. Thr-Phe, then, must be located between these two. After dinitrophenylation of peptide III and partial hydrolysis (with conc. HCl at 37°C, for 8 days) two ether-soluble products were separated and characterized, DNP-Asp and DNP-Asp-Glu, which demonstrates that glutamic acid must adjoin the N-terminal aspartic acid residue.

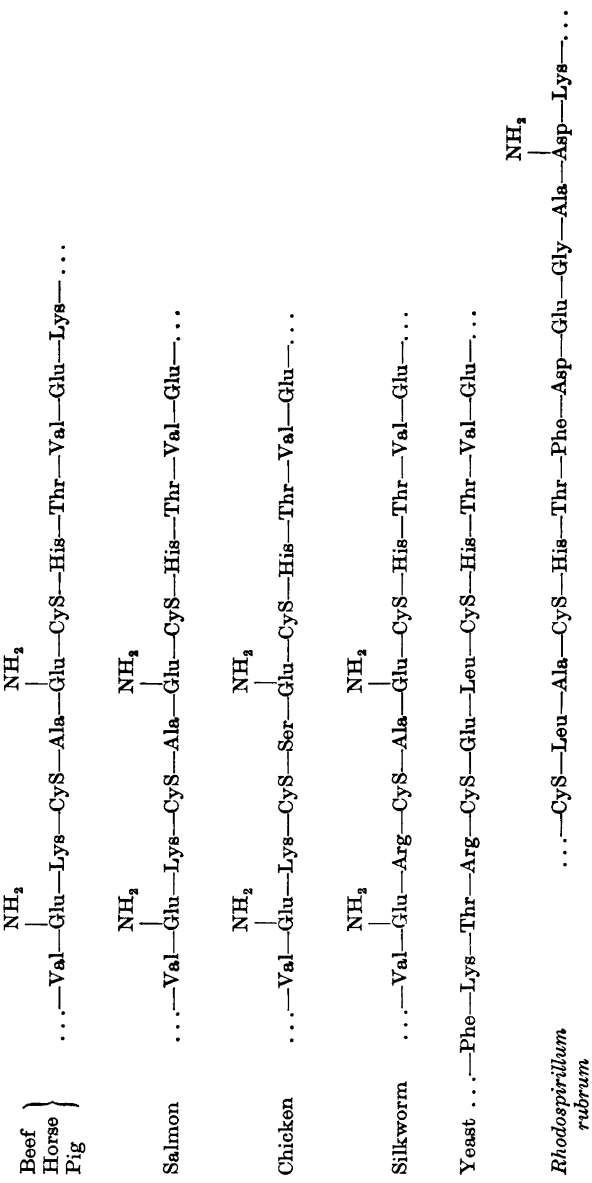
In the subtilisin hydrolysate of the polypeptide (Table 1) five products were present in relatively high concentration: $CySO_3H$-Leu-Ala, $CySO_3H$-His, Thr-Phe, [Asp, Glu, Gly, Ala], and Asp(NH_2)-Lys. Among the split products obtained in small amounts there were two tripeptides containing histidine: Ala-[$CySO_3H$,His] and $CySO_3H$-[His, Thr]. Other minor components of the hydrolysate were shown to be [Gly, Ala], [Asp, Glu, Gly], [Thr, Phe, Asp, Glu, Gly], and free alanine.

From the findings presented above it may be concluded that the amino-acid sequence in the hemin-free oxidized polypeptide is

1 2 3 4 5 6 7 8 9 10 11 12 13

$CySO_3H$-Leu-Ala-$CySO_3H$-His-Thr-Phe-Asp-Glu-Gly-Ala-Asp (NH_2)-Lys.

Table 2. Comparison of the sequences of amino-acids in the vicinity of the prosthetic group in c-type cytochromes of different organisms.



The results obtained leave it undecided whether residues 8 and 9 are free aspartic acid and free glutamic acid or whether these amino-acids are in the amide form, as asparagine and glutamine residues.

The two cysteic acid residues contained in the sequence have arisen from and taken the place of two heme-bound cysteine residues present in the original

Rhodospirillum rubrum cytochrome c and in the hemopeptide prepared from it by tryptic hydrolysis.

The specificity of trypsin already mentioned suggests that the amino-acid residue preceding the above sequence must be either lysine or arginine.

The cleavage by chymotrypsin of a bond between histidine and threonine (residues 5 and 6) may appear unexpected in view of the general belief that chymotrypsin will split bonds involving the carboxyl groups of the aromatic residues phenylalanine and tyrosine. The finding is in agreement with recent observations of Davis [15], however, who has reported that bonds involving the carboxyl groups of histidine residues are somewhat susceptible to chymotryptic hydrolysis.

A comparison of the amino-acid sequences that have been shown to occur in the vicinity of the heme moiety in different cytochromes demonstrates that they have various structural features in common (Table 2). The presence of two cysteine residues linked to side chains 2 and 4 of the porphyrin of the prosthetic group, first indicated by Theorell [16] in 1939 for beef cytochrome c, has been confirmed and found to be characteristic of all c-type cytochromes. The distance between the two cysteine residues along the polypeptide chain is always the same, two other amino-acids being situated in between. Invariably a histidine residue has been found to adjoin one of the heme-bound cysteine residues. Stereochemical considerations of Ehrenberg and Theorell [17] have indicated that the chain of amino-acids in which the two heme-bound cysteine residues and the histidine residue are included is likely to be in the form of an α-helix. This coiling will bring the histidine residue into a steric position favourable for a covalent attachment of an imidazole nitrogen to the iron of the heme disc.

There are still other structural features invariably encountered in all cytochrome c samples examined so far, such, *e.g.*, as the presence of a basic residue (lysine or arginine) close to cysteine and of a threonine residue close to histidine. The significance of these similarities is presently not known.

It is remarkable, anyhow, that in so many structural respects the cytochrome c of *Rhodospirillum rubrum* appears to resemble the c-type cytochromes of other organisms, in spite of an overall amino-acid composition, oxidation-reduction potential, and a specificity of action quite different from those of vertebrate and yeast cytochromes [18].

The authors are most indebted to Professor M. D. Kamen for a gift of *Rhodospirillum rubrum* cytochrome c.

One of the authors (S. P.) wishes in addition to express his thanks to *Statens naturvetenskapliga forskningsråd* and *Magnus Bergvalls stiftelse* for grants provided in support of this investigation.

REFERENCES

1. Tuppy, H. and Bodo, G. *Monatsh.* **85** (1954) 1024, 1182.
2. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
3. Tuppy, H. *Z. Naturforsch.* **12b** (1957) 784.
4. Tuppy, H. and Dus, K. *Monatsh.* **89** (1958) 407.
5. Vernon, L. P. and Kamen, M. D. *J. Biol. Chem.* **211** (1954) 643.
6. Kamen, M. D. and Vernon, L. P. *Biochim. Biophys. Acta* **17** (1955) 10.

7. Bartsch, R. G. and Kamen, M. D. *J. Biol. Chem.* **230** (1958) 41.
8. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc. London* **B122** (1937) 298.
9. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
10. Paul, K. G. *Acta Chem. Scand.* **3** (1949) 1178; **4** (1950) 239.
11. Werner, G. and Westphal, O. *Angew. Chem.* **67** (1955) 251.
12. Sanger, F. *Biochem. J.* **39** (1945) 507.
13. Sanger, F. and Thompson, E. O. P. *Biochem. J.* **53** (1953) 353.
14. Akabori, S., Ohno, K. and Narita, K. *Bull. Chem. Soc. Japan* **25** (1952) 214.
15. Davis, N. C. *J. Biol. Chem.* **223** (1956) 935.
16. Theorell, H. *Enzymologia* **6** (1939) 88.
17. Ehrenberg, A. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1193.
18. Kamen, M. D. and Takeda, Y. *Biochim. Biophys. Acta* **21** (1956) 518.

Received January 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 647–652

The Oxidation of Aminotriazole by Horse Radish Peroxidase (HRP)

K. G. PAUL and E. MARGOLIASH*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The mutual effects of horse radish peroxidase and 3-amino-1,2,4-triazole have been studied spectrophotometrically. With the present technique no evidence was found for the formation of a compound between aminotriazole and peroxidase or any of the peroxidase-peroxide complexes. The complexes I and II reacted with aminotriazole at the rates of 460 and 200 $min^{-1}M^{-1}$ at pH 7.4 and room temperature. Aminotriazole did not inhibit the activity of horse radish peroxidase in the presence of continuously supplied hydrogen peroxide, but rather protected the peroxidase against destruction by hydrogen peroxide.

The observation that plants which had been exposed to 3-amino-1,2,4-triazole (AT) contained less chlorophyll than normal plants led to the discovery that extracts from such plants showed a subnormal catalase activity [1,2]. In livers and kidneys from rats, which have been given AT perorally, intraperitoneally, or intravenously, the catalase activity was decreased to such an extent that only 10 %, occasionally less, remained of the normal value per unit weight or nitrogen. The liver catalase activity dropped remarkably rapidly, maximal reduction being reached already after 3 h. Normal values were found on the seventh day. Blood catalase, haemoglobin, and liver cytochrome c remained normal. In experiments *in vitro* with AT and homogenized livers from normal animals or crystallized catalase considerably higher concentrations of AT were needed to bring about any decrease [2]. The *in vitro* effect could be abolished by dialysis, in contrast to the *in vivo* effect, and was attributed to some unspecific action of AT in high concentration. "Liver peroxidase" was slightly decreased after 3 h but normal again after 24 h. In another investigation [3] the same result with liver homogenate was obtained, but no inhibition of pure catalase occurred unless liver homogenate was added. It was also noticed that molecular oxygen was necessary for the effect. The δ-aminolaevulinic acid dehydrase activity in the AT-rats

* On leave from the Hebrew University—Hadassah Medical School, Jerusalem.

was found to be somewhat reduced although less than the catalase. Minimum activity, 75 % of the normal, was found 8 h after the injection of AT, and the activity had returned to normal after 36—48 h. Quite recently it was demonstrated [5,6] *in vitro* with pure catalase that AT reacts not with catalase itself but with complex I between catalase and hydrogen peroxide, resulting in a complete inhibition of catalatic activity. This reaction was irreversible and one mole of AT was incorporated into the enzyme protein for each mole of catalase haem reacted. These results explain the rapid decrease in catalase activity of liver and kidney observed *in vivo* after the administration of AT [1,2], making it unnecessary to invoke an effect of AT on the formation of the enzyme. The time for restoration of full liver catalase activity agrees well with the life span of liver catalase.

Although several factors can account for the lack of effect of AT on erythrocyte catalase *in vivo* the presence of hydrogen donors in the red cells may be the most important one [6]. Such donors would prevent the irreversible inhibition of catalase by AT by reacting rapidly with catalase-hydrogen peroxide complex I, thus competing with the slow, irreversible reaction of AT with the complex. The possibility that the effects of AT administration could be caused by some metabolite of AT rather than by AT itself was made less likely, although not excluded, by the failure to find any evidence, direct or indirect, for the participation of such a metabolite in the inhibitory reaction [5].

The less pronounced effect on δ-aminolaevulinic acid dehydrase may be caused by inhibition of action or synthesis or both, and may also be quite unspecific.

In the present investigation some of the mutual effects of another hydroperoxidase, horse radish peroxidase, and AT have been studied in the presence of hydrogen peroxide.

METHODS AND MATERIAL

AT from Light & Co Ltd was used without pretreatment. HRP (component C) had been prepared as previously described [7]. Spectra were taken with Beckman spectrophotometers. Dr. R. Ordell, Serafimerlasarettet, Stockholm, kindly put a useful DK 2 instrument at our disposal.

RESULTS AND DISCUSSION

The titration of AT (117 mg in 20 ml of water, room temperature) with molar hydrochloric acid and sodium hydroxide revealed an ionization at pH 4.35. Another group was traced at pH < 2 after correction for the acid, and a third one was seen at pH 10. Although a comparison with pyridine and aniline shows that the three groups are not immediately identifiable the titration made it possible to choose pH-values where only one form of AT is present.

The results in Table 1 show that the peroxide but not AT inactivates HRP under the given conditions and that AT to some extent protects HRP against inactivation by the peroxide. This protection may simply depend upon the continuous consumption of the peroxide passing the cellophane. During the dialysis a yellow colour appeared in the solutions inside and outside the sac.

Table 1. Activity changes in HRP during dialysis against peroxide and AT. 1.5 ml of 10 μM HRP and 20 mM AT in phosphate buffer of pH 7.4 were dialysed at 37° against 100 ml of 20 mM AT and 0.25 mM hydrogen peroxide in the same buffer. Controls were made without AT or peroxide. For the assay of the peroxidatic activity 50 μl of the solution inside the sac were diluted with 1.00 ml of water, 10 μl of the dilution being taken for the assay with 3 ml of guaiacol — buffer (pH 7) mixture. t = the time (in min after the addition of peroxide) during which the absorbancy at 470 mμ increased by 0.050 [8,9].

Hours of dialysis	HRP AT H_2O_2	HRP AT —	HRP — H_2O_2
	Peroxidase activity in min^{-1} $(1/t)$		
0	3.8	4.6	3.9
1	4.0	4.1	3.4
2	3.9	4.4	3.7
3	3.4	4.3	2.4
4	2.9	3.9	1.8
7	3.3	4.3	1.3

It faded only slowly at neutral pH but instantaneously upon acidification or alkalinization. Its formation and disappearance were followed spectrophotometrically at pH 4.9, where it seemed to have its intensity maximum (Fig. 1). As long as any peroxide remained the yellow colour increased in intensity.

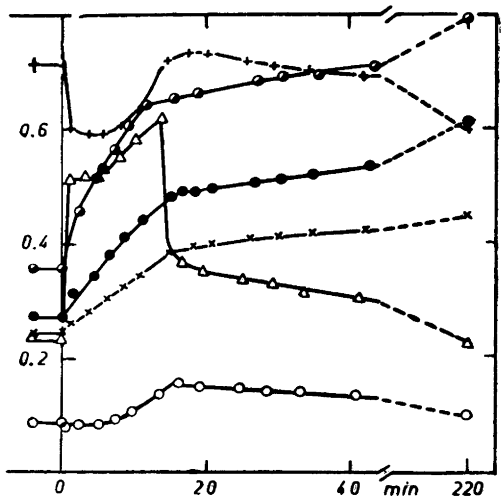


Fig. 1. Spectral changes during the reactions between HRP (10 μM), hydrogen peroxide (10 mM), and AT (5 mM) in acetate buffer of pH 4.9. The peroxide was added to the other reactants at time 0. The HRP-absorption at 460 mμ is independent of the peroxide concentration. Free HRP and complex I are isosbestic at 427 mμ, free HRP and complex II at 412 mμ.

○ 460 mμ + 412 mμ ● 268 mμ
△ 427 mμ × 285 mμ ◒ 250 mμ

When the peroxide had been consumed, as indicated by the sudden changes in the Soret band, the production of the yellow pigment ceased and the colour began to fade. The spectral changes after the termination at about 15 min of the enzymic reactions appeared as first order reactions. The changes must reflect several reactions since the specific rate constant varied from one wavelength to another and since the constant obtained at 427 mμ showed a sudden change at 55 min (Table 2).

Table 2. Specific velocity constants for the non-enzymic reactions of the primary oxidation products occurring during the time range 15—220 min in Fig. 1.

mμ	250	268	285	410	427	460
min^{-1}	1.0	1.3	1.9	−0.9	−1.2 −0.7	− 1.1

The spectral changes during the first minutes were followed by scanning the range 440—380 mμ at suitable time intervals. It was possible and advantageous to work with equimolar concentrations of HRP and peroxide. This

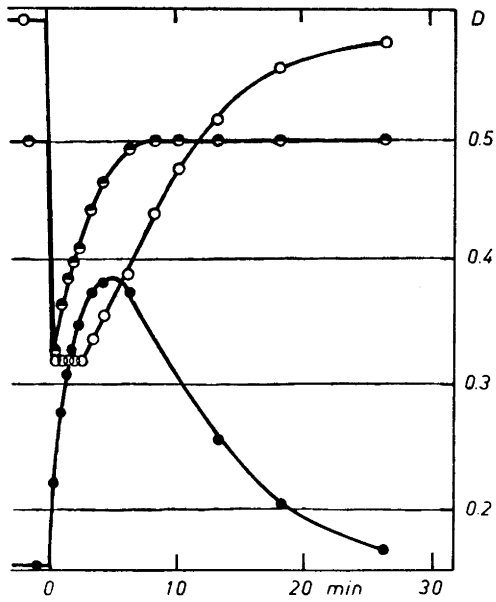


Fig. 2. Spectral changes in the Soret band region during the reactions between HRP (7 μM), hydrogen peroxide (7 μM), and AT (140 μM) in 0.1 M phosphate buffer of pH 7.4. Peroxide and AT were added simultaneously at time 0.

○ 397 mμ (complex I and complex II isosbestic)
× 412 mμ (complex II and free HRP isosbestic)
● 427 mμ (complex I and free HRP isosbestic)

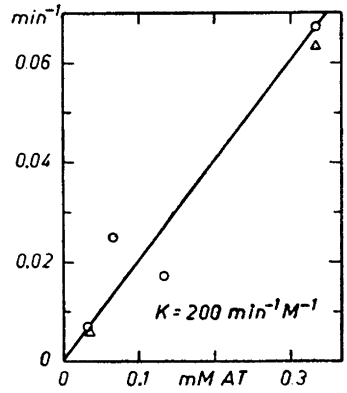


Fig. 3. Velocity constant for the appearance of free HRP (○) and disappearance of complex II (△) as a function of the concentration of AT. HRP, peroxide, and time as in Fig. 2. The values are corrected for the »spontaneous decay» of the complex.

actually converted the enzymatic system to a stoichiometric reaction. The specific rate constant for the spontaneous increase in density at 412 mμ for a solution 7 μM in HRP and 7 μM in hydrogen peroxide in 0.1 M phosphate buffer of pH 7.4 was 0.0035 min^{-1}. An example of the spectral changes during the reactions between HRP-hydrogen peroxide complexes I and II and AT is given in Fig. 2. In these experiments the contribution of the yellow pigment to the absorption in the Soret band region was negligible. With 7—350 μM AT isosbestic points were found at 397 mμ during the initial and at 412 mμ during the terminal part of the reaction. The isosbestic point for complex I/free HRP (427 mμ) could only occasionally be visualized with this technique because of the similarity in shape of the absorption curves of complex II and free HRP.

The specific velocity constant for the appearance of free HRP (Fig. 3) was obtained from the absorbancy changes occurring at 397 mμ after the isosbestic level at 412 mμ had been reached. At a few concentrations of AT it was possible to follow, with this technique, the decrease in absorption at 427 mμ sufficiently accurately to permit the calculation of the velocity constant for the disappearance of complex II (Fig. 3).

The specific velocity constant for the disappearance of complex I was calculated from the changes at 427 mμ during the first minutes when the absorbancy at 397 mμ remained constant (Fig. 4).

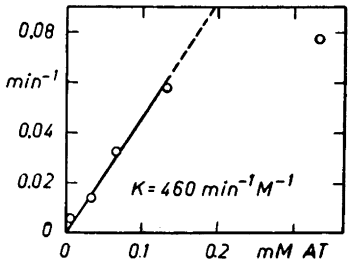


Fig. 4. Velocity constant for the disappearance of complex I. Same conditions as in Fig. 3.

Under the experimental conditions used above (Fig. 2—4) AT gave no spectrophotometrically operable compound with HRP or the HRP-peroxide complexes as long as the concentration of AT was lower than 350 uM. This is obvious from several facts. Two isosbestic points were found, and it is unlikely that both are threefold isosbestic. They were found at the wavelengths which have been reported for the isosbestic points between complex I and II (397 mμ) and complex II and free HRP (412 mμ)[10]. The velocity constants for the appearance of free HRP and the disappearance of complex II agreed fairly well (Fig. 3). At 350 μM AT, however, the disappearance of complex I was markedly hampered (Fig. 4), and the decrease in density at 397 mμ upon the addition of AT and peroxide was not instantaneous. It should be kept in mind, however, that in the case of catalase a compound between AT and the catalase-peroxide complexe was formed only at considerably higher concentrations of AT as well as of the complex. At such a high peroxide concentration as was used in the catalase experiments HRP would have been rapidly inactivated.

At the concentrations of AT used in the present investigation nothing has been found that would put AT in a class of its own as regards the reactions with HRP. The two HRP-peroxide complexes seem to react with AT at the rates of 460 and 200 min^{-1} M^{-1}, thus quite slowly. This fact, and the purity of the HRP preparation, makes the system a suitable one for studies of the peroxidative reactions.

This investigation was financially supported by *Statens Naturvetenskapliga Forskningsråd*. One of us (E.M.) is grateful for a fellowship from the *Dazian Foundation for Medical Research*.

REFERENCES

1. Heim, W. G., Appleman, D. and Pyfrom, H. T. *Science* **122** (1955) 693.
2. Heim, W. G., Appleman, D. and Pyfrom, H. T. *Am. J. Physiol.* **186** (1956) 19.
3. Sugimura, T. *Gann.* **47** (1956) 159.
4. Tschudy, D. P. and Collins, A. *Science* **126** (1957) 168.
5. Margoliash, E. and Novogrodsky, A. *Biochem. J.* **68** (1958) 468.
6. Margoliash, E., Nowogrodsky, A. and Schejter, A. *Unpublished.*
7. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1312.
8. George, P. *J. Biol. Chem.* **201** (1953) 413.
9. Maehly, A. C. *Methods in Biochemical Analysis* **I** (1954) 357.
10. Chance, B. *Arch. Biochem.* **21** (1949) 416.

Received November 3, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 653–658

Studies on Humic Acids

VII.* The Structure of the Dihydroxy-dioxidoterphenyl Obtained by the Action of Sulphuric Acid on *p*-Benzoquinone

HOLGER ERDTMAN and NILS E. STJERNSTRÖM

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Acid polymerisation of *p*-benzoquinone gives, among other products, a dioxidoterphenyl for which structure V has been suggested. This has now been shown to be incorrect; the structure is very probably X. Arguments in favour of this view are presented.

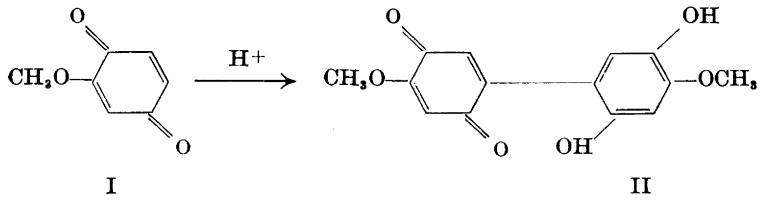
Quinones sometimes exhibit a considerable reactivity in the presence of acids undergoing intermolecular coupling reactions with the formation of di- or terphenyl derivatives and amorphous high molecular weight quinonoid products of unknown constitution which are of obvious interest in connection with the chemistry of humic acids and melanins.

Thus, even with very dilute sulphuric acid, the highly reactive methoxyquinone I is rapidly and almost quantitatively transformed into the insoluble diphenyl derivative II (or the intermolecular quinhydrone alternative) which is relatively stable to acids.[1] The far less reactive toluquinone undergoes a similar change in the presence of strong sulphuric acid with the formation of toluhydroquinone, a diphenyl derivative analogous to II (CH_3 instead of OCH_3), and about 10 % of a terphenyl derivative to which the structure III has been assigned. The main reaction product is an amorphous, quinonoid "polymer".

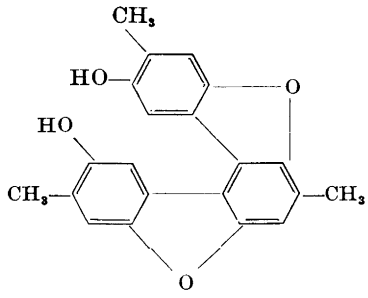
In the same way *p*-benzoquinone yields about 50 % hydroquinone, a large amount of polymeric material and a small amount of a terphenyl derivative for which structure V has been suggested [2].

It appears probable that these quinones react in a similar way with acids and alkali since hydroquinones and quinonoid polymers are formed in both cases. Intermediate low molecular weight polyphenols have been less frequently isolated from the products of alkaline decomposition of quinones but the formation of a very small amount of dihydroquinone has been demonstrated in the case of *p*-benzoquinone [3].

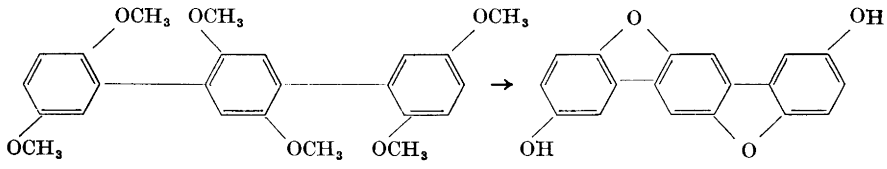
* Part VI. *Acta Chem. Scand.* 8 (1954) 1442.



I II



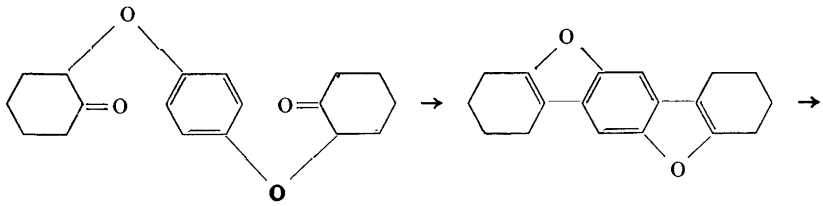
III (III a: C_2H_5 instead of CH_3)
(The positions of the alkyl groups in III and III a uncertain)



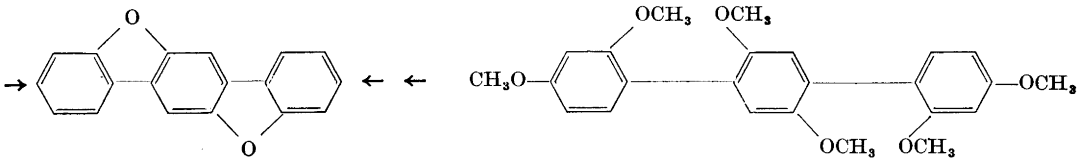
IV V

The termolecular products obtained with acids are probably intermediates in the acid polymerisation of benzoquinone and toluquinone which have been stabilised by dehydration and removed from further reactions. Methoxyquinone is exceptional in its preferential formation of a dimer.

Some years ago Schimmelschmidt [4] described the dioxidoterphenyl VIII obtained by alkali fusion of 2,2″-dichloro-2′,5′-dihydroxy-*p*-terphenyl. It melts at 265—266°. We have synthesised the same compound in a different way starting with 2-bromo*cyclo*hexanone [5] and the disodium salt of hydroquinone. These compounds reacted to give 1,4-di-(2-keto*cyclo*hexyloxy)-benzene (VI) which was treated with a polyphosphoric acid mixture to give what must be 3,4,5,6,3″,4″,5″,6″-octahydro-2,2′,5′,2″-dioxido-*p*-terphenyl (VII) as a result of intramolecular condensation. This substance was dehydrogenated with palladium to give a compound identical with Schimmelschmidt's compound VIII. The same compound has also been obtained in the following way. 4-Iodo-resorcinol dimethyl ether and 2,5-diiodo-hydroquinone dimethyl ether were condensed by the Ullmann procedure. The resulting hexamethoxyterphenyl (IX) when heated with hydrobromic acid furnished a dihydroxy-



VI VII



VIII IX

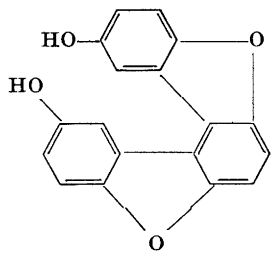
dioxido-*p*-terphenyl which on distillation with zinc dust gave compound VIII (Stjernström, forthcoming publication). Schimmelschmidt's substance must have the structure he assigned to it.

In order to relate the dihydroxy-dioxidoterphenyl from *p*-benzoquinone with Schimmelschmidt's compound it was subjected to distillation with zinc dust. The neutral product obtained was, however, isomeric, not identical with VIII. Moreover, a compound which must have the structure V was obtained by prolonged boiling of the hexamethoxyterphenyl IV [6] with hydrobromic acid. It was isomeric with the dihydroxy-dioxidoterphenyl obtained from *p*-benzoquinone and the suggested structure (V) for the compound from *p*-benzoquinone must therefore be incorrect.

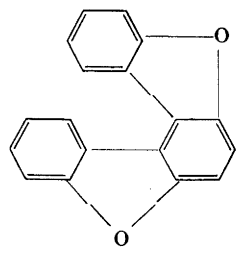
Structure V was advanced on the likely assumption that *p*-benzoquinone would polymerise stepwise and, for sterical reasons, preferentially in the 2,5-positions. In the case of toluquinone this is not possible because of the obstructing methyl groups and condensation with other molecules of toluquinone can only take place (the slight possibility of methyl group migration being ignored) in positions 5 and 6 in order to provide a tri-toluhydroquinone able to furnish a dioxidoterphenyl by dehydration.

The exact mechanism of the quinone polymerisation is not known and it is, of course, possible that the hypothesis that trihydroquinones are formed first is an oversimplification. At present, the only likely alternative to structure V for the termolecular compound from *p*-benzoquinone appears to be X and attempts were therefore made to convert compound III into X by elimination of the methyl groups.

The dimethyl ether of III was oxidised in the hope that a tricarboxylic acid might be formed which on decarboxylation should give the dimethyl ether of X. All attempts to effect this oxidation with permanganate or chromic acid invariably resulted in acid mixtures, from which no pure tricarboxylic acid could be obtained.



X



XI

On decarboxylation neutral products were obtained which were very similar to the dimethyl ether of the dihydroxy-dioxidoterphenyl from *p*-benzoquinone but they were obviously not pure compounds. As the oxidation of the methyl ether of the toluquinone product appeared to offer insurmountable difficulties ethylquinone was subjected to treatment with sulphuric acid and a termolecular product, obviously the ethyl analogue of III (III a) was obtained. The dimethyl ether of III a was oxidised with potassium permanganate in pyridine and an acid product was obtained which on decarboxylation furnished a neutral compound identical with the dimethyl ether of the termolecular product from *p*-benzoquinone.

It follows that the dihydroxy-dioxidoterphenyl from *p*-benzoquinone will almost certainly have the structure X.

EXPERIMENTAL

All melting points are uncorrected

1,4-Di-(2-ketocyclohexyloxy)-benzene (VI). 2-Bromo-*cyclo*hexanone was prepared according to Trippett [5]. Sodium (2.3 g) was dissolved in absolute ethanol (50 ml) and a solution of hydroquinone (5.5 g) in absolute ethanol (50 ml) was added. The alcohol was evaporated under reduced pressure care being taken to avoid oxidation. The dry hydroquinone sodium salt, 2-bromo*cyclo*hexanone (17.6 g) and dry benzene (200 ml) were stirred for three days in a coal gas atmosphere. After filtration and evaporation of the solvent a dark brown oil was obtained that soon crystallised partly on cooling. The crystals were collected and recrystallised from *n*-butanol. Yield 1 g. M. p. 190.5—193°. (Found: C 71.4; H 7.2. Calc. for $C_{18}H_{22}O_4$: C 71.5; H 7.3.)

3,4,5,6,3'',4'',5'',6''-Octahydro-2,2',5',2''-dioxido-p-*terphenyl (VII)*. 1,4-Di-(2-keto*cyclo*hexyloxy)-benzene (VI) (720 mg) was added to a solution of phosphorus pentoxide (10 g), in phosphoric acid (*d* 1.75, 20 ml). The mixture was stirred at 100° for 3 h and then diluted with water. The precipitate was collected, washed and dried. The gray, crude product (520 mg) was recrystallised from *n*-butanol, m. p. 201—202°. (Found: C 80.5; H 6.7. Calc. for $C_{18}H_{18}O_2$: C 81.2; H 6.8.)

2,2',5',2''-Dioxido-p-*terphenyl (VIII)*. Compound VII (150 mg) was mixed with a palladium-charcoal catalyst (10 % Pd, 100 mg) and the mixture was heated to 230° for 5 h in an atmosphere of nitrogen. The reaction product was extracted with boiling bromobenzene from which the substance crystallised on cooling. Yield 60 mg, m. p. 265.5—

266.5°. Mixed melting point with Schimmelschmidt's dioxidoterphenyl, generously placed at our disposal by Farbwerke Höchst, showed no depression (m. p. 265—266°).

2,6′,3′,2″-Dioxido-o-terphenyl (XI). 5,5″-Dihydroxy-2,6′,3′,2″-dioxido-*o*-terphenyl (X) was prepared by acid polymerisation of *p*-benzoquinone according to Erdtman [2]. This phenol (1 g) was thoroughly mixed with zinc dust (10 g) and the mixture was introduced into a hard melting glass tube (1 × 50 cm) followed by zinc dust (20 g) and zinc dust impregnated pumice. Carbon dioxide was passed through the tube to remove oxygen. The pumice and the zinc dust layer were then heated until red hot followed by similar heating of the phenol-zinc dust layer. After about twenty minutes some 300 mg of reduced material could be collected from the cool zone of the tube. The substance was recrystallised from acetic acid and sublimed *in vacuo*, m. p. 143—144°. (Found: C 83.4; H 4.0. Calc. for $C_{18}H_{10}O_2$: C 83.7; H 3.9.)

*5,5″-Dihydroxy-2,2′,5′,2″-dioxido-*p*-terphenyl (V) and its dimethyl ether.* 2,5, 2′,5′,2″, 5″-Hexamethoxy-*p*-terphenyl [6] (IV) (440 mg) was heated under reflux with hydrobromic acid (48 %, 25 ml) in an atmosphere of carbon dioxide for three days. The reaction mixture was diluted with water (100 ml) and filtered. The collected phenol (300 mg) was dark blue due to superficial oxidation. M. p. *ca* 330° (decomp.). The phenol was suspended in methanol and quantitatively converted to the corresponding dimethyl ether by methylation with an excess of dimethyl sulphate and sodium hydroxide (40 %). The dimethyl ether was recrystallised from dioxane and sublimed *in vacuo*, m. p. 255—257°. (Found: C 75.1; H 4.3. Calc. for $C_{20}H_{14}O_4$: C 75.5; H 4.4.) Neither the phenol nor its dimethyl ether were identical with the corresponding compounds from the *p*-benzoquinone polymerisation.

Phenol III a and the corresponding diacetate and dimethyl ether. Ethylquinone was prepared from ethylhydroquinone by the method described by Gulland [7] using ferric chloride as oxidising agent. Ethylquinone (28 g) was shaken for three days with a mixture of acetic acid (50 ml) and dilute sulphuric acid (33 %, 100 ml). The thick blue black precipitate was collected, washed thoroughly with water and dried in air. The dry, very finely divided material (19 g) was shaken vigorously with excess zinc dust, acetic anhydride (45 ml) and pyridine (25 ml). The reaction mixture became very hot and after boiling for some minutes it was filtered. Crystals of the diacetate of III a soon began to separate from the filtrate and after one day they were collected, washed with acetic acid and recrystallised from acetic anhydride. Yield 3.5 g, m. p. 226.5—227°. (Found: C 73.4; H 5.9. Calc. for $C_{28}H_{26}O_6$: C 73.4; H 5.7.)

The acetate was refluxed for one hour in an atmosphere of nitrogen with a mixture of conc. sulphuric acid and acetic acid (1:50, 100 ml). The reaction mixture was poured into a large volume of boiling water. The precipitated phenol was a slightly bluish powder that very rapidly darkened in the air due to superficial oxidation and melted about 230°. Yield almost quantitative.

The freshly prepared phenol suspended in methanol was methylated with an excess of dimethyl sulphate and sodium hydroxide (40 %). The reaction mixture was poured into water and the product was washed with water, dried and recrystallised from ligroin. (Yield from 2 g acetate 1.7 g.) The *methyl ether* melted at 161—162.5° after sublimation *in vacuo*. (Found: C 78.1; H 6.8. Calc. for $C_{26}H_{26}O_4$: C 77.6; H 6.5.)

Oxidation of the dimethyl ether of phenol III a and decarboxylation of the resulting acid. The dimethyl ether of phenol III a (540 mg) was dissolved in a mixture of pyridine (25 ml) and potassium hydroxide solution (5 M, 4 ml). The solution was refluxed and very finely divided potassium permanganate (4 g) was added in small portions during 1.5 h. The reaction mixture was cooled, acidified with dilute hydrochloric acid and decolourised with sulphur dioxide. The yellowish green jelly was collected by centrifuging. The jelly was treated with dilute potassium hydroxide, the neutral material was removed by filtration and the clear filtrate was acidified with hydrochloric acid. The acid amorphous precipitate was collected, washed thoroughly with water and dried in air. It did not crystallise. The dry product (114 mg) was heated for 3 h at 200° in an atmosphere of nitrogen together with a copper chromite catalyst (30 mg) and quinoline (3 ml). The filtered reaction mixture was acidified with dilute sulphuric acid and the brown precipitate collected and washed with large amounts of water. After drying and sublimation *in vacuo* 11 mg of neutral material was obtained, m. p. 173—220°. This material was fractionally sublimed *in vacuo*, a fraction, m. p. 185—200°, was isolated and recrystallised from a pyridine water mixture and sublimed *in vacuo*, m. p. 211—214°. The dimethyl

ether of phenol X from the polymerisation of *p*-benzoquinone melts at 216—218° and the mixed m. p. was 215—218°. The infrared and ultraviolet spectra of the two samples were identical.

We are grateful to Dr. B. Thomas for checking the manuscript.

REFERENCES

1. Erdtman, H. *Proc. Roy. Soc. London* **A 143** (1933) 191.
2. Erdtman, H. *Proc. Roy. Soc. London* **A 143** (1933) 228.
3. Erdtman, H. and Granath, M. *Acta Chem. Scand.* **8** (1954) 811.
4. Schimmelschmidt, K. *Ann.* **566** (1950) 184.
5. Trippett, S. *J. Chem. Soc.* **1957** 421.
6. Erdtman, H., Granath, M. and Schultz, G. *Acta Chem. Scand.* **8** (1954) 1442.
7. Gulland, J. M. *Biochem. J.* (London) **26** (1932) 32.

Received January 16, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 659–664

On the Kinetics of the Decomposition of Cyanic Acid

II. The Carbonate Catalysis

MOGENS BALLUND JENSEN

The Royal Danish School of Pharmacy, Copenhagen, Denmark

The uncatalyzed decomposition of cyanate in dilute aqueous solution takes place according to the reactions: $OCNH + H_3O^+ \rightarrow NH_4^+ + CO_2$ (I), $OCNH + H_2O \rightarrow NH_3 + CO_2$ (II) and $OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^-$ (III). Reaction III has been shown to be catalyzed by bicarbonate ion: $OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^-$ (catalyzed by HCO_3^-) (IV). The results can also be interpreted in that way that reaction II is catalyzed by carbonate ion: $OCNH + H_2O \rightarrow NH_3 + CO_2$ (catalyzed by CO_3^{--}) (V). The rate expression for the decomposition of cyanate in the presence of carbonate (HCO_3^- or CO_3^{--}) is: $dc_{OCN^-}/dt = -(k_{OCNH} \cdot a_{H^+} \cdot f / K_{OCNH} + k_{OCN^-} + k' \cdot c_{HCO_3^-}) \cdot c_{OCN^-} = -(k_{OCNH} + k_{OCN^-} \cdot K_{OCNH} / a_{H^+} \cdot f + k'' \cdot c_{CO_3^{--}}) \cdot c_{OCNH}$. In this expression k' and k'' are the velocity constants for reactions IV and V, respectively. k'' is equal to $k' \cdot K_{OCNH} / K_{HCO_3^-}$, where K_{OCNH} is the acidic dissociation constant for cyanic acid and $K_{HCO_3^-}$ is the acidic dissociation constant for the bicarbonate ion. k_{OCNH} (0.047 (min^{-1}) at 18°C) and k_{OCN^-} ($10^{-6.48}$ (min^{-1}) at 18°C) are the previously determined velocity constants for reactions II and III, respectively. f is an activity coefficient.

The uncatalyzed decomposition will take place mainly according to reactions II and III in solutions where the carbonate catalysis is appreciable. The decomposition after I has therefore been disregarded in the above stated expression. (The velocity constant for reaction I is 3.61 (l $mole^{-1}$ min^{-1}) at 18°C).

k' (l $mole^{-1}$ min^{-1}) was found to be 4.76×10^{-4} (18°C), 9.21×10^{-4} (25°C) and 76.2×10^{-4} (50°C) yielding an energy of activation of 16.2 kcal $mole^{-1}$. The corresponding value of k'' is $10^{3.54}$ (l $mole^{-1}$ min^{-1}) at 18°C.

In a previously published paper [1] the decomposition of cyanic acid and cyanate in dilute aqueous solution was found to take place according to three simultaneous reactions:

$$OCNH + H_3O^+ \rightarrow NH_4^+ + CO_2 \quad \text{(I)}$$
$$OCNH + H_2O \rightarrow NH_3 + CO_2 \quad \text{(II)}$$
$$OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^- \quad \text{(III)}$$

In the experiments carried out in carbon dioxide-bicarbonate buffers at pH = approx. 6.7 it was observed that the decomposition was catalyzed by the bicarbonate ion. In this pH-range the uncatalyzed decomposition will take place mainly according to reaction II. The rate in the presence of bicarbonate was found to be

$$dc_{OCN^-}/dt = -(k_{OCNH} \cdot a_{H^+} \cdot f/K_{OCNH} + k' \cdot c_{HCO_3^-}) \cdot c_{OCN^-} \quad (1)$$

Eqn. (1) can also be written

$$dc_{OCN^-}/dt = -(k_{OCNH} + k'' \cdot c_{CO_3^{--}}) \cdot c_{OCNH} \quad (2)$$

$$\text{where } k'' = k' \cdot K_{OCNH}/K_{HCO_3^-}.$$

k_{OCNH} is the velocity constant for reaction II. f is an activity coefficient. K_{OCNH} and $K_{HCO_3^-}$ are the acidic dissociation constants for cyanic acid and for the bicarbonate ion, respectively. k' is the velocity constant for the reaction

$$OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^- \text{ (catalyzed by } HCO_3^-). \quad (IV)$$

Reaction IV can also be written

$$OCNH + H_2O \rightarrow NH_3 + CO_2 \text{ (catalyzed by } CO_3^{--}) \quad (V)$$

k'' is the velocity constant for reaction V. The experiments do not give any information about the actual reaction being IV or V. The value of k' was previously found to be approx. 0.0006 at 18°C.

The present investigation deals with the decomposition of cyanate in the presence of bicarbonate or carbonate, or both, under more varied conditions.

The method of analysis was the same as has been used previously, *viz.* an argentometric titration of the cyanate.

The velocity constants refer to molar concentration, the unit of time being the minute. The solutions were prepared at room temperature.

Sodium Cyanate Pure (Hopkin and Williams Ltd.) was used for the experiments. The salt was found to contain only a slight trace of carbonate.

RESULTS

The experiments on the decomposition of cyanate in solutions containing bicarbonate (pH = approx. 8.4) are listed in Table 1. The solution contained both carbonate and bicarbonate (pH = approx. 10) in runs 4 and 9.

The uncatalyzed decomposition will take place mainly according to reactions II and III in this pH-range. The rate expression may be written

$$dc_{OCN^-}/dt = -(k_{OCNH} \cdot a_{H^+} \cdot f/K_{OCNH} + k_{OCN^-} + k' \cdot c_{HCO_3^-}) \cdot c_{OCN^-} \quad (3)$$

identical with

$$dc_{OCN^-}/dt = -(k_{OCNH} + k_{OCN^-} \cdot K_{OCNH}/a_{H^+} \cdot f + k'' \cdot c_{CO_3^{--}}) \cdot c_{OCNH} \quad (4)$$

Table 1. The velocity constant, k', for the reaction: $OCN^- + 2H_2O \rightarrow NH_3 + HCO_3^-$ (catalyzed by HCO_3^-).

Run	°C	Initial solution			t min.	% OCN^- left	k	$k' = \frac{k}{c_{HCO_3^-}}$
		$c_{HCO_3^-}$	$c_{CO_3^{--}}$	c_{OCN^-}				
1	18	0.108		0.021	1 430 7 499 14 246 18 391	92.5 67.2 47.1 38.0	0.0000540 0.0000532 0.0000529 0.0000526 mean: 0.0000532	0.000482
2	18	0.179		0.020	1 313 3 076 7 467 14 169 18 349	89.7 76.7 53.0 29.8 20.0	0.0000828 0.0000861 0.0000850 0.0000855 0.0000878 mean: 0.0000854	0.000470
3	25	0.188		0.020	1 185 2 690 4 365 5 647 10 035 37 400	81.9 62.9 46.5 36.8 17.5 0	0.000169 0.000172 0.000176 0.000177 0.000174 mean: 0.000174	0.000911
4	25	0.100	0.050	0.019	1 299 4 051 7 046 11 211	88.2 67.6 50.1 32.4	0.0000961 0.0000966 0.0000979 0.0001006 mean: 0.0000978	0.000931
9	50	0.100	0.050	0.020	162 292 405 1 508	87.8 79.7 72.2 26.2	0.000799 0.000780 0.000805 0.000890 mean: 0.000818	0.00762

k' (mean): 0.000476 (18°C) 0.000921 (25°C) 0.00762 (50°C).

k_{OCN^-} is the velocity constant for reaction III. The velocity of the uncatalyzed decomposition, however, is negligible in comparison with that of the catalyzed in the experiments listed in Table 1. Consequently eqn. (3) will be reduced to

$$dc_{OCN^-}/dt = -k' \cdot c_{HCO_3^-} \cdot c_{OCN^-} = -k \cdot c_{OCN^-}.$$

The concentration of bicarbonate is approximately constant during each experiment and k was obtained from the expression for a unimolecular reaction: $k \cdot t = \ln a/a-x$. k and k' are listed in Table 1 ($k' = k/c_{HCO_3^-}$). k'

is 0.000476 (18°C), 0.000921 (25°C) and 0.00762 (50°C). The energy of activation is 16.2 kcal mole^{-1} calculated from the data at 18°C and 25°C, and 16.1 kcal mole^{-1} calculated from the data at 25°C and 50°C. The corresponding value of k'' is $10^{3.54}$ at 18°C. The values $10^{-3.54}$ (Ref.[1]) and $10^{-10.40}$ (Ref.[2]) for K_{OCNH} and $K_{HCO_3^-}$, respectively, have been used. $k' = 0.000476$ at 18°C is in a relatively good agreement with 0.0006 found previously in the above mentioned experiments in more acid solution.

It should be noted that ammonium ions are formed during the process, *i.e.* the gross reaction: $NH_4^+ + OCN^- \rightarrow NH_2 \cdot CO \cdot NH_2$ will also occur in solutions containing initially only cyanate. From the velocity constant for this reaction (0.000577 at 18°C [3]) can be estimated that only a small amount of urea is formed in the experiments listed in Table 1 (the concentration of NH_4^+ does not exceed 0.02).

The experiments on the decomposition of cyanate in strongly alkaline solutions of sodium carbonate and sodium hydroxide are listed in Table 2.

We get

$$dc_{OCN^-}/dt = -(k_{OCNH} \cdot a_{H^+} \cdot f/K_{OCNH} + k_{OCN^-} + k' \cdot c_{HCO_3^-}) \cdot c_{OCN^-} \simeq$$
$$-(k_{OCN^-} + k' \cdot c_{HCO_3^-}) \cdot c_{OCN^-} =$$
$$-(k_{OCN^-} + (k' \cdot K_{H_2O}/K_{HCO_3^-}) \cdot c_{CO_3^{--}}/c_{OH^-}) \cdot c_{OCN^-}$$

if we assume that the decomposition still takes place in accordance with eqn. (3). (The term $k_{OCNH} \cdot a_{H^+} \cdot f/K_{OCNH}$ is without significance in strongly alkaline solution).

Substituting $p = k' \cdot K_{H_2O}/K_{HCO_3^-}$ we get

$$dc_{OCN^-}/dt = -(k_{OCN^-} + p \cdot c_{CO_3^{--}}/c_{OH^-}) \cdot c_{OCN^-} \qquad (5)$$

or

$$dc_{OCN^-}/dt = -k \cdot c_{OCN^-}$$

where $k = k_{OCN^-} + p \cdot c_{CO_3^{--}}/c_{OH^-}$.

The concentration of carbonate and hydroxyl ion is approximately constant during each experiment. The values of k listed in Table 2 were hence calculated from the expression for a unimolecular reaction.

The experimental results in Table 2 show clearly that k depends not only upon the concentration of carbonate ion but also upon the concentration of hydroxyl ion as required from eqn. (5). The values of p calculated from $p = (k - k_{OCN^-}) \cdot c_{OH^-}/c_{CO_3^{--}}$ are also listed in Table 2 (k, c_{OH^-} and $c_{CO_3^{--}}$ are mean values). The previously determined values 0.00170 at 94°C and 0.000170 at 70°C for k_{OCN^-} have been used. Although the difference between k and k_{OCN^-} is rather small a fairly constant value for p is obtained in runs 5, 6, and 11 yielding the mean value 0.00031 for p at 94°C. The value found in run 7 has been disregarded. The difference between k and k_{OCN^-} is very small in this run ($c_{OH^-} = 0.2$). At 70°C the value 0.000032 was found for p.

Run 8 shows that the addition of sulfate instead of carbonate has very little influence on the velocity. Run 12 shows that the velocity in strongly alkaline medium is not affected by the addition of ammonia (0.1 M).

Table 2. The decomposition of OCN^- in $NaOH + Na_2CO_3$.

Run	°C	Initial solution					t min.	% OCN^- left	k	$p = (k - k_{OCN^-}) \times \frac{c_{OH^-}}{c_{CO_3^{--}}}$ *
		c_{OH^-}	$c_{CO_3^{--}}$	c_{NH_3}	$c_{SO_4^{--}}$	c_{OCN^-}				
11	94	0.040	0.070			0.018	83 220 306 403	82.5 57.9 46.9 35.4	0.00231 0.00248 0.00247 0.00258 mean: 0.00246	0.00034
5	94	0.100	0.070			0.020	121 265 368 525	79.1 59.4 49.5 36.7	0.00194 0.00197 0.00191 0.00191 mean: 0.00194	0.00030
6	94	0.100	0.150			0.019	120 280 392 430	78.2 54.2 41.0 37.5	0.00205 0.00219 0.00227 0.00228 mean: 0.00220	0.00030
7	94	0.200	0.070			0.019	174 329 392 493	73.9 55.8 50.4 41.5	0.00174 0.00177 0.00175 0.00178 mean: 0.00176	(0.00016)
8	94	0.100			0.150	0.020	158 252 336 1 139	77.2 65.7 57.7 14.2	0.00164 0.00167 0.00164 0.00171 mean: 0.00167	
12	94	0.180		0.10		0.018	76 141 207 362 458	88.1 77.9 70.2 55.6 44.8	0.00167 0.00178 0.00171 0.00162 0.00175 mean: 0.00171	
10	70	0.100	0.149			0.019	1 189 1 534 2 625 4 339	76.7 71.0 56.2 37.8 **	0.000223 0.000222 0.000219 0.000225 mean: 0.000222	0.000032

* k_{OCN^-} (0.00170 at 94°C 0.000170 at 70°C) is the velocity constant for the uncatalyzed reaction.

** In this run the ammonia was distilled from the filtrate from the silver cyanate and titrated. The following percentages of ammonia were found: 22.0 29.1 44.0 62.7.

An extrapolation from the data at 94°C and 70°C gives $p = 3.8 \times 10^{-6}$ at 50°C. k' calculated from this value of p by means of $k' = p \cdot K_{HCO_3^-}/K_{H_2O}$ is 0.0047 (for $K_{HCO_3^-} = 6.73 \times 10^{-11}$ (Ref.[2])) or 0.0062 (for $K_{HCO_3^-} = 8.98 \times 10^{-11}$ *); *cf.* the value 0.00762 found in run 9 (Table 1). In view of the small differences on which this calculation is based the agreement between k' calculated from p and k' found in run 9 is regarded as satisfactory. This means that eqn. (3) (or eqn. (4)) accounts for the catalyzed decomposition as well in moderately acid or basic medium as in strongly basic medium.

DISCUSSION

The catalytic effect of carbonate on the decomposition of cyanate was first observed by Masson and Masson [6] who found the formation of carbonate from sodium cyanate to be autocatalytic. More recently Lister [7] and Kemp and Kohnstam [8] have examined the carbonate catalysis.

Lister finds that the decomposition of cyanate conforms to the equation

$$dc_{OCN^-}/dt = -(k_1' \cdot c_{OCN^-} + k_2' \cdot c_{CO_3^{--}})$$

in solutions containing sodium carbonate and approximately equal moles of sodium hydroxide and sodium cyanate. This near-equality will hold throughout the reaction (the gross reaction is: $OCN^- + OH^- + H_2O \rightarrow NH_3 + CO_3^{--}$). Eqn. (5) in this case takes the form

$$dc_{OCN^-}/dt = -(k_{OCN^-} \cdot c_{OCN^-} + p \cdot c_{CO_3^{--}})$$

i.e. eqn. (5) is under the stated experimental conditions consistent with the expression of Lister. Lister has $k_2' (= p) = 6.2 \times 10^{-4}$ at 100°C. An extrapolation to 100°C from the data listed in Table 2 gives $p = 5.2 \times 10^{-4}$.

The expression proposed by Kemp and Kohnstam is in agreement with eqn. (3). At 60.1°C Kemp and Kohnstam have $k' = 0.0175$, which agrees well with $k' = 0.0164$ obtained by extrapolation from the data listed in Table 1.

The author whishes to thank Professor, Dr.phil. Carl Faurholt for his very kind interest in this investigation.

REFERENCES

1. Jensen, M. B. *Acta Chem. Scand.* **12** (1958) 1657.
2. Harned, H. S. and Scholes Jr., S. R. *J. Am. Chem. Soc.* **63** (1941) 1706.
3. Jensen, M. B. *Acta Chem. Scand.* **13** (1959) 289.
4. Kauko, Y. and Mantere, V. *Z. physik. Chem.* **A 176** (1936) 187.
5. Harned, H. S. and Hamer, W. J. *J. Am. Chem. Soc.* **55** (1933) 2194.
6. Masson, O. and Masson, I. *Z. physik. Chem.* **70** (1910) 290.
7. Lister, M. W. *Can. J. Chem.* **33** (1955) 426.
8. Kemp, I. A. and Kohnstam, G. *J. Chem. Soc.* **1956** 900.

Received December 22, 1958.

* $K_{HCO_3^-} = 8.98 \times 10^{-11}$ at 50°C was obtained by extrapolation from 5.70×10^{-11} at 25°C and 7.15×10^{-11} at 37°C[4]. $K_{H_2O} = 5.47 \times 10^{-14}$ has been used at 50°C [5].

ACTA CHEMICA SCANDINAVICA 13 (1959) 665–670

Kinetics of Solvolysis of Acetic Anhydride in Methanol-Water Mixtures

JOUKO KOSKIKALLIO

Department of Chemistry, University of Helsinki, Helsinki, Finland

The rates of simultaneous hydrolysis and methanolysis of acetic anhydride in various methanol-water mixtures were measured at three different temperatures. The great variation in the second-order rate constants of the solvolysis reactions when the composition of the solvent mixture is altered is discussed and it is concluded that both methanolysis and hydrolysis are catalysed by the weak base water. The changes observed in the ratio methanolysis/hydrolysis are discussed in terms of hydrogen bonding in the methanol-water mixtures.

In addition to the spontaneous hydrolysis and alcoholysis of acetic anhydride, these reactions are catalysed by acids and bases [1-2]. The spontaneous solvolyses of acetic anhydride in alcohol-water mixtures could be described as reactions catalysed by the weak base water. In order to study this problem the rates of hydrolysis and methanolysis of acetic anhydride were measured in different methanol-water mixtures.

EXPERIMENTAL

The water used was freshly distilled. The methanol (Merck, analytical grade) was dried with aluminium methylate [3] and distilled. Finally it was distilled over anhydrous copper sulphate. The amount of water remaining after this treatment, usually about 0.005 mole/litre, was determined by the Karl Fischer method. The aniline was freshly distilled. The acetic anhydride used was a product of Merck, analytical grade.

The solvent mixtures were made up by volume in a 100 ml volumetric flask and the components were also weighed. The solvent mixture and anhydride were separately allowed to obtain the temperature of the thermostat during about one hour. At 0°C a stirred 10 l Dewar flask thermostat, half filled with ice, was used and for other temperatures the thermostats were electrically controlled to within $\pm 0.01°$. The reaction was started by adding about one milliliter of acetic anhydride to 100 ml of the solvent which yielded an approximately 0.1 M anhydride solution. The reaction was followed by taking 5 ml samples at suitable time intervals in order to cover the range from about 10 % to 90 % change. One sample was mixed with 15 ml of saturated aniline water (about 0.3 M aniline) and titrated with 0.02 M baryta. Another sample was taken immediately after the first (about 1 min) and mixed with 20 ml of 0.6 M ammonia. The mixture

was made sligtly acid by adding 1—3 ml of 0.1 M hydrochloric acid and then titrated with 0.6 M ammonia potentiometrically (Radiometer PHM 22h). These results were interpolated to correspond to the same reaction time. The difference between these two titrations gives the amount of anhydride present in the sample.

During the reaction of acetic anhydride with methanol in dry methanol the acetic acid produced in the reaction was esterified to some extent (2—3 %). This did not interfere with the calculation of the rate of methanolysis of acetic anhydride when the method of two different titrations was used. In methanol-water mixtures the consecutive reactions of esterification of acetic acid or hydrolysis of methyl acetate would have complicated the calculations of the ratio of the rates of simultaneous hydrolysis and methanolysis of acetic anhydride. Fortunately these consecutive reactions were so slow, when compared with the solvolysis of acetic anhydride, that they could be neglected. This was verified by taking samples from mixtures in which all the anhydride had reacted.

The rate constants were calculated from the usual first-order equation, which in the case of two simultaneous first-order reactions gives the sum of the two rate constants $k_1^h + k_1^m$. The ratio of these rate constants was obtained from the mean of the ratios of the changes in the values of the aniline and ammonia titration. Thus the first-order rate constants of hydrolysis k_1^h and methanolysis k_1^m could be calculated in each solvent mixture from a single run. The experimental error of the rate constants is of the order of magnitude of about 1—2 % in cases where the rates of hydrolysis and methanolysis do no much differ from each other and increases as the proportion of the rate of reaction decreases. The results are given in Table 1.

RESULTS AND DISCUSSION

From Table 1 it is seen that the first-order rate constant of hydrolysis of acetic anhydride k_1^h decreases continuously when methanol is added to water, but the first-order rate constant of methanolysis k_1^m shows a maximum. This contrast in the behaviour of hydrolysis and methanolysis disappears in the second-order rate constants k_2^h and k_2^m. Over the whole range of solvent mixtures there is nevertheless a considerable change of the second-order rate constant, of about 80-fold in the case of hydrolysis and 320-fold in methanolysis. There are different ways of explaining these great changes in rates. If the transition state in the hydrolysis and methanolysis of acetic anhydride is much more polar than the reactants, a solvent of lower solvating power such as methanol would retard the rate. Still, methanol is a solvent of fairly high dielectric constant ($D = 35.0$ at 0°) and good solvating power. Therefore it would be necessary to postulate an highly polar transition state. But it would then be difficult to account for the increase in the second-order rate constant of methanolysis of acetic anhydride when a solvent of low dielectric constant and poor solvating power such as benzene ($D = 2.29$ at 18°) is added to methanol [4].

An other possible explanation is based on changes in the acid-base properties in the methanol-water mixtures. Water is 137 times as strong a proton acceptor as methanol [5] at 25° and about one-eighth as strong a proton donator as methanol [6] at 25°. When water is replaced by methanol there is a considerable decrease in the basicity of the solvent. The solvolyses of acetic anhydride are known to be accelerated by various bases such as hydroxyl ion [4], acetate ion [2], pyridine [1,2] and various tertiary amines [1]. The great sensitivity of acetic anhydride towards bases makes it possible to assume that the spontaneous

Table 1. The first-order k_1, second-order k_2 and catalytic k_k rate constants of hydrolysis and methanolysis of acetic anhydride in methanol-water mixtures.

	wt. %	mole · l^{-1}		sec^{-1}		$l \cdot mole^{-1} \cdot sec^{-1}$		$l^2 \cdot mole^{-1} \cdot sec^{-1}$		
$t°$	MeOH	$[H_2O]$	[MeOH]	$10^5 \cdot k_1^h$	$10^5 \cdot k_1^m$	$10^7 \cdot k_2^h$	$10^7 \cdot k_2^m$	$10^7 \cdot k_k^h$	$10^7 \cdot k_k^m$	k_2^m / k_2^h
0	100	0.007	25.2		0.258	1.0 *	1.02			
	98.9	0.507	25.0	0.009	0.483	1.8	1.93	1.6	1.8	1
	97.8	1.01	24.8	0.019	0.728	1.9	2.94	0.9	1.9	1.5
	95.0	2.28	24.5	0.060	1.41	2.6	5.75	0.7	2.08	2.2
	93.4	3.03	24.1	0.11	1.88	3.6	7.80	0.85	2.24	2.2
	89.0	5.06	23.3	0.25	2.96	5.0	12.7	0.8	2.12	2.5
	83.6	7.57	22.3	0.56	5.07	7.4	22.8	0.85	2.88	3.1
	73.2	12.9	20.1	1.63	9.44	12.7	47	0.90	3.57	3.7
	42.5	29.6	12.5	8.21	17.4	27.8	139	0.90	4.67	5.0
	16.2	45.5	4.96	24.1	13.1	52.4	246	1.13	5.78	4.7
	8.03	50.4	2.48	31.9	7.14	63.4	290	1.23	5.7	4.6
	5.00	52.5	1.49	36.8	4.80	70.0	320	1.31	6.1	4.6
	2.50	54.0	0.743	40.3	2.85	74.7	320	1.36	5.9	4.3
	0	55.5	0	46.5		83.8		1.46		
25	100	0.001	24.6		2.15	10 *	8.75			
	99.87	0.057	24.6		2.26		9.20			
	99.74	0.113	24.6		2.36		9.60			
	99.46	0.224	24.5		2.63		10.7			
	97.5	1.10	24.2	0.16	4.04	15	16.7	4.5	7.2	1.1
	95.1	2.20	23.8	0.41	6.52	19	27.4	4.1	8.5	1.4
	87.8	5.50	22.4	1.86	16.2	34	72.3	4.3	11.6	2.1
	76.6	11.0	20.3	6.41	32.7	58	161	4.4	13.8	2.8
	0	55.5	0	255		460		8.1		
50	100	0	23.9		20.9	60 *	87.5			
	95.1	2.14	23.1	2.0	32.7	95	141	16	25	1.5
	87.8	5.36	21.8	8.3	57.6	155	264	18	33	1.7
	76.6	10.7	19.8	24	96.7	230	489	16	37.6	2.1
	0	54.9	0	972		1 770		31		

* extrapolated values.

solvolysis of acetic anhydride in methanol-water mixtures is catalysed by the weak base water. The catalytic rate constant of methanolysis k_k^m is obtained when the second-order rate constant of methanolysis in pure methanol k_2^m is subtracted from the rate constants in methanol-water mixtures and divided by the concentration of water. The catalytic rate constant k_k^m decreases much less than k_2^m when methanol is added to water (Table 1 and Fig. 1). The catalytic rate constant of hydrolysis k_k^h is obtained in the same way when an extrapolated value of k_2^h in absolute methanol is used. Such an extrapolation may not be quite justified, but the choice of the extrapolated value only affects the value of k_k^h at low water concentrations and has a negligible effect upon their

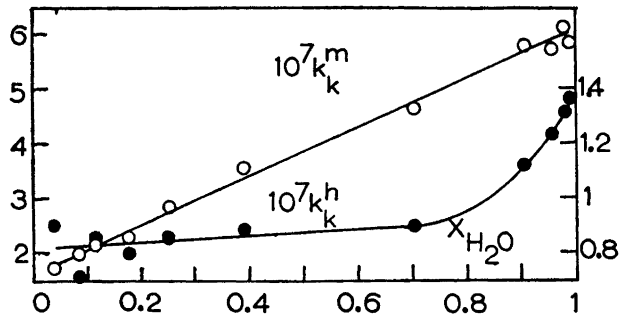


Fig. 1. The variation of the water-catalysed rates of hydrolysis and methanolysis of acetic anhydride in methanol-water mixtures.

values at water concentrations higher than 50 %. Furthermore, these rate constants change much less than the corresponding second-order rate constants k_2^h. The variation of the catalytic rate constants when the composition of the solvent is altered at the temperatures 0°, 25° and 50° is essentially the same. The remaining small changes in k_k-values with changing composition of the solvent mixture may be ascribed to solvent effects, for example hydrogen bonding.

Further insight into these "second-order" effects can be obtained from a comparison of the ratios of the rates of hydrolysis and methanolysis, because this treatment excludes the effect of water as a catalyst. From Table 1 and Fig. 2 it is seen that considerable changes in these ratios occur when temperature or solvent composition is altered. For comparison the writer's [4] previous values in ethanol-water mixtures are included, and likewise the values of Caundri [7]. In both cases the ratio of the rate constants changes in the same way when the composition of alcohol-water mixtures are altered, but the reactivities of the alcohols and water are in an unexpected order of magnitude. The rates of hydrolysis and ethanolysis are not very different from each other but the rate of methanolysis is considerably greater than that of hydrolysis, especially in mixtures with a high water concentration. This high reactivity of methanol in methanol-water mixtures may be attributed to hydrogen bonding in solvent mixtures. When methanol and water are mixed, considerable amounts of heat are evolved [8]. The strong interaction between methanol and water molecules probably occurs *via* hydrogen bonding. This can take place in two different ways, but because of the dominating acidic properties of methanol and the basic properties of water, it is likely to occur in the way: $MeOH...OH_2$. Higher hydrogen-bonded associations also occur, but their occurrence does not invalidate the following argument. The hydrogen bonding between methanol and water molecules would then make the oxygen of methanol more and the oxygen of water less nucleophilic or reactive to the anhydride molecule. The same argument also applies to ethanol-water mixtures. When the temperature is increased, the hydrogen bonds are partially broken and the ratios of the rates of alcoholysis and hydrolysis decrease. At

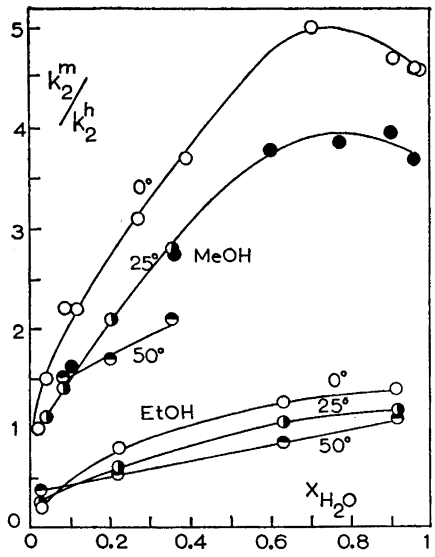


Fig. 2. The variation in the ratio of the second-order rate constants of hydrolysis and alcoholysis of acetic anhydride in methanol-water mixtures. In addition to the present values the writers previous [4] data and those of Caundri [7] (black points) are also included.

high enough temperatures the ratios could well be less than one in all mixtures of water and alcohol.

In mixtures containing less water than alcohol the ratio is decreased as the amount of water is decreased. In mixtures containing little water, all the water is hydrogen-bonded, mainly in the form MeOH...OH_2, and in addition the excess of methanol molecules are associated between themselves MeOH...OHMe. Therefore there is no change in the species reacting in the hydrolysis but an increasingly greater proportion of methanolysis occurs between the anhydride and various polymers of methanol as the amount of water in the solvent mixture decreases. On steric grounds the rate of methanolysis will decrease as compared with that of hydrolysis and the ratio of the rates will decrease as the proportion of water decreases. The reverse effect of temperature at very low water concentrations may likewise be explained on the same lines (Fig. 2).

In mixtures containing small amounts of methanol the ratio also decreases as the amount of methanol decreases. In these mixtures the methanol is mainly bonded as MeOH...OH_2 but the excess of water forms associations with itself HOH...OH_2 and these may be slightly more reactive in the hydrolysis than the associations with methanol.

The low reactivity of acetic anhydride with associations of alcohol is clearly seen from the increase in the rate when the associations are depolymerised by addition of an inert solvent such as benzene [4] and change of temperature which yields high apparent activation energies [4] in alcohols (about 18 kcal) compared with those in benzene (14 kcal) or water (10 kcal).

The author wishes to record his indebtedness for a research grant from the *Alf. Kordelin Foundation.*

REFERENCES

1. Gold, V. and Jefferson, E. G. *J. Chem. Soc.* **1953** 1909; Bafna, S. L. and Gold, V. *Ibid.* **1953** 1406.
2. Koskikallio, J. *Acta Chem. Scand* **13** (1959) 671.
3. Walden, P. *Z. physik. Chem.* **114** (1925) 181.
4. Koskikallio, J. *Ann. Acad. Sci. Fennicae* **A II 57** (1954).
5. Koskikallio, J. *Suomen Kemistilehti* **B 30** (1957) 43.
6. Koskikallio, J. *Suomen Kemistilehti* **B 30** (1957) 111.
7. Caundri, J. F. M. *Rec. trav. chim.* **49** (1930) 1.
8. Bose, E. *Z. physik. Chem.* **58** (1907) 585.

Received January 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 671–676

Kinetics of the Acid-Catalysed Solvolysis of Acetic Anhydride in Methanol-Water Mixtures

JOUKO KOSKIKALLIO

Department of Chemistry, University of Helsinki, Helsinki, Finland

The rate constants of the acid-catalysed hydrolysis and methanolysis of acetic anhydride were measured in various methanol-water mixtures. In solvent mixtures containing more than 27 % of water, the third-order rate constants are almost unaffected by the change of solvent composition. In solvent mixtures containing less than about 27 % of water, the increase of the rate constant is due to a shift in the proton transfer equilibria between the methoxonium ion, water and anhydride. From these values of the rate constants the equilibrium constant of the proton transfer reaction between methoxonium ion and water was calculated. The values obtained are $K = 209$ at 0° and 118 at 25° and they agree well with the previous investigations. With the aid of these values it was concluded that the acid-catalysed solvolysis of acetic anhydride involves a proton transfer pre-equilibrium instead of a bimolecular reaction between hydroxonium or methoxonium ions and acetic anhydride.

The hydrolysis of acetic anhydride in water and the alcoholysis in alcohols are catalysed by strong acids [1,2]. In organic solvents or in solvent mixtures with water, the rate of spontaneous hydrolysis is much less than in water, and acetic anhydride becomes more sensitive towards acids. In the present study the rates of the acid-catalysed methanolysis and hydrolysis of acetic anhydride in methanol-water mixtures were measured. From these measurements it is possible to calculate the equilibrium constant for the proton transfer reaction (1) between methoxonium ion and water and compare these values with those previously obtained by the author [3] from e.m.f. measurements.

$$MeOH_2^+ + H_2O = MeOH + H_3O^+ \qquad (1)$$

EXPERIMENTAL

The materials used in the experiments were the same as in the previous work [4]. Gaseous hydrogen chloride obtained from sodium chloride and concentrated sulphuric acid was absorbed in methanol and a sample of the solution obtained was titrated with baryta before use. The amounts of water remaining in the dried methanol were titrated by the

Karl Fischer method. The rate measurements and the calculations of the rate constants were made as before [3]. In dry methanol and also in mixtures containing less than 6 % of water the acetic acid formed in the reaction between anhydride and methanol (2)

$$AcOAc + MeOH = AcOH + AcOMe \quad (2)$$

$$AcOH + MeOH = AcOMe + H_2O \quad (3)$$

was slowly esterified with methanol (3) during the experiments. Because the method of taking two samples simultaneously [2], mixing with aniline and ammonia, and titrating, allows the calculation of the amount of anhydride in the mixture, the sum of the first-order rate constants of hydrolysis and methanolysis in methanol-water mixtures could be exactly calculated. The consecutive reaction (3) interfered only with the calculations of the ratio of the rate constants of hydrolysis and methanolysis. Fortunately, the rate of hydrolysis of acetic anhydride was small compared with that of methanolysis in the mixtures containing 6 % or less of water and hence it could be neglected. In the 5.4 % water mixture the hydrolysis was estimated at about 5 % of the methanolysis and in mixtures containing less water it was a proportionally smaller fraction of the total rate. The esterification of acetic acid (3) also produced water and since the rate of methanolysis in absolute methanol was especially sensitive to small amounts of water, the rate constants in each run decreased as the reaction proceeded. Between 0 and 50 % change the decrease was about 10 %. These rate constants were extrapolated to zero reaction time. In methanol-water mixtures the change of rate constants was much less, and the mean of the rate constants and the mean of the water concentrations were used. In mixtures containing 27 % or more water, the esterification of acetic acid or the hydrolysis of methyl acetate were negligible. Thus it was possible to calculate separately the rates of simultaneous hydrolysis and methanolysis of acetic anhydride catalysed by hydrochloric acid.

The estimated error in the rate constants of methanolysis is about ± 2 % except in the two mixtures containing more water, in which the error is about ± 5 %. In view of the unfavourable conditions for measuring the acid-catalysed hydrolysis of acetic anhydride owing to the comparatively high proportion of the spontaneous reaction and the simultaneous, faster methanolysis, these rate constants are subject to an error of about ± 20 % and are included only for a rough comparison. The results are given in Table 1, which contains the first-order rate constants of methanolysis k_1^m and hydrolysis k_1^h, from which the rate constants of the respective spontaneous solvolyses [4] have been subtracted, the catalytic rate constants k_k^m and k_k^h obtained from the first-order rate constants by dividing by the concentration of hydrochloric acid, the third-order rate constants k_s^m and k_s^h obtained by dividing the catalytic constants by the concentrations of water and methanol, respectively, and in the last columns the equilibrium constants calculated for the proton transfer equilibria 1.

RESULTS AND DISCUSSION

The equilibrium constant of the proton transfer reaction 1 is known from previous measurements (see Table 2). This equilibrium constant can also be calculated from the present kinetic measurements. The methanolysis of acetic anhydride catalysed by hydrogen chloride is especially suitable for this purposes, because the rate of the reaction is very little affected by the change in the composition of the methanol-water mixtures used as solvents. From Table 1 it is seen that between 27 % and pure water the rates of acid-catalysed methanolysis and hydrolysis change very little; the small changes observed are probably due to experimental error. At low water concentrations the rate increases as the proportion of water decreases and the change is about 30-fold between 27 and 0 % water. This change can be ascribed to changes in the distribution of protons between methanol, water and anhydride.

Table 1 shows the difference between the first-order experimental rate constants of methanolysis k_1^m and hydrolysis k_1^h in a solvent mixture containing hydrochloric acid and the rate constant of the spontaneous reaction k_o, the respective catalytic rate constants $k_k = (k_1 - k^o)/c_{HCl}$, the third-order rate constants $k_3^m = k_k^m / c_{MeOH}$ and $k_3^h = k_k^h / c_{H_2O}$ and the equilibrium constant of the proton transfer reaction 1.

$t°$	wt. % H_2O	mole · l^{-1}			sec^{-1}		l · mole^{-1} · sec^{-1}		l^2 · mole^{-2} · sec^{-1}		l · mole^{-1}
		c_{H_2O}	c_{MeOH}	c_{HCl}	10^5 $k_1^m - k_o$	10^5 $k_1^h - k_o$	10^4 k_k^m	10^4 k_k^h	10^5 k_3^m	10^5 k_3^h	$K = \frac{[H_3O^+]}{[MeOH_2^+] \cdot [H_2O]}$
0	0	0	25.2	0.00250	10.05		422		168		
	0	0.001	25.2	0.00250	10.00		400		159		
	0.55	0.250	25.2	0.00245	3.28		138		54.8		7.6
	1.09	0.495	25.1	0.00243	1.96		81		32.2		7.9
	0	0	25.2	0.00525	21.1		402		160		
	0	0.007	25.2	0.00525	20.4		388		81		
	0	0	25.2	0.0130	55.0		424		168		
	0	0.007	25.2	0.0130	52.3		402		160		
	0.20	0.103	25.2	0.0129	29.1		226		90		8.8
	0.40	0.204	25.2	0.0128	20.8		163		64.7		8.1
	1.11	0.504	25.1	0.0127	10.6		83		33.1		8.2
	2.17	0.983	25.0	0.0126	6.54		52		20.8		7.2
	5.40	2.47	24.4	0.0121	3.16		26.1		10.7		6.0
	27.0	13.0	19.8	0.099	9.9	0.9	10.0	0.9	5.2	0.8	
	27.0	13.0	19.8	0.198	21.0	2.5	10.6	1.2			
	57.5	29.6	12.3	0.099	7.6	4.0	7.6	4.0	5.6	1.3	
	57.5	29.6	12.3	0.198	12.3	7.4	6.2	3.7			
	100	55.5	0	0.099		5.3		5.3		0.89	
	100	55.5	0	0.198		9.1		4.6			
10	100	55.5	0	0.198		31.7		16.0		2.88	
20	100	55.5	0	0.099		41		41.4		7.45	
	100	55.5	0	0.198		86		43.4		7.80	
25	0	0	24.6	0.00237	54.5		2 300		935		
	0	0.003	24.6	0.00237	54.0		2 280		925		
	0.23	0.103	24.6	0.00236	37.4		1 585		644		4.7
	0.45	0.201	24.5	0.00235	28.4		1 210		494		4.7
	0.91	0.398	24.4	0.00235	18.4		783		321		5.0
	2.31	1.01	24.3	0.00234	9.5		416		171		4.7
30	100	55.5	0	0.198		225		113		20.4	

This problem was first discussed by Goldschmidt [5], but he did not take into account the decrease in the concentrations of water when hydroxonium ions are formed in reaction 1. In absolute methanol there is a proton transfer equilibrium between methoxonium ions and anhydride

$$MeOH_2^+ + A = AH^+ + MeOH \qquad (4)$$

The rate of the acid-catalysed methanolysis of acetic anhydride is proportional to the concentration, or rather to the activity of the protonated anhydride. In the following calculations concentrations are used instead of activities because both the rate constants and the equilibrium constants involve only

ratios of similarly charged species and at the low electrolyte concentrations used in the experiments the changes of the ratios of the activity coefficients were small compared with the experimental error involved in determining the rate constants. The equilibrium constants obtained from rate measurements at two different acid concentrations do not differ more from each other than the experimental error (Table 1, last column).

When a base such as water is added to methanol, the concentration of methoxyl ions decreases according to reaction 1. Since only a small fraction of the protons were combined with anhydride, the concentration of the protonated anhydride is decreased in proportion to the concentration of methoxonium ions. Therefore the ratio of the rate constants of the acid-catalysed methanolysis of acetic anhydride in absolute methanol and in a methanol-water mixture is equal to the ratio of the concentrations of protonated anhydride and also the methoxonium ions in the two solvents. The difference between the total acid concentration and the methoxonium ion concentration is equal to the concentration of hydroxonium ions. Finally the concentration of water is obtained by subtracting the concentration of hydroxonium ions. All the necessary equilibrium concentrations for calculation of the equilibrium constant of reaction 1 are now known. As a first approximation, a K-value was obtained which was used to calculate the rate constant in absolute methanol from the rate constants at the lowest water concentrations. The correction was only about 1—2 %, as is seen from Table 1. The mean of these rate constants was then used to calculate the K-values shown in Table 1 in the last column. The error in K is estimated as about $\pm$ 3 %. The K-values decrease as the concentration of water increases and are probably slightly affected by the change in the concentration of hydrochloric acid, too. An extrapolation to zero water concentration yields $K = 8.3$ at 0° and $K = 4.8$ at 25°. When these values are multiplied by the concentration of methanol, values of 209 and 118 are obtained. These values may be compared with the previous values obtained by various methods.

Table 2. Values of the equilibrium constant $K = [ROH] \cdot [H_3O^+] / [H_2O] \cdot [MeOH_2^+]$.

	Method				
$t°$	kinetically *	e.m.v. [5]	indicator [6]	conductivity [7]	kinetically [8]
0°	209				
25°	118	137	111	107	109

* present work

The heat of the proton transfer reaction 1 is $\Delta H = -3.7$ kcal mole^{-1} and the entropy $\Delta S = -2.7$ cal degree^{-1} mole^{-1} as calculated from the values of K of the present work. The great difference in the basicity between methanol and water is probably due to the strong hydrogen bonding occurring in methanol. In acetic acid [9] water is only about eight times as strong a base as methanol at 25°. The heat of depolymerisation of hydrogen-bonded polymers of alcohols is of the same order of magnitude as the heat of the proton transfer reaction 1.

The activation energies and entropies calculated from the rate constants of hydrolysis in water and methanolysis in methanol given in Table 1 are

	E kcal · mole^{-1}	A l · mole^{-2} · sec^{-1}	ΔS^* at 25° cal · degree^{-1} · mole^{-1}
Hydrolysis	17.13	$3.95 \cdot 10^9$	−17.06
Methanolysis	10.97	$1.4 \cdot 10^6$	−33.2

The values of the activation energy is not far from the respective value obtained for the acid-catalysed hydrolysis of simple esters, *e. g.* ethyl acetate [10] $E = 16.76$ kcal mole^{-1}, $A = 3.75 \times 10^6$ l · mole^{-2} · sec^{-2} and $\Delta S^* = -30.5$ cal · degree^{-1} · mole^{-1}. Although the entropies of activation differ considerably the mechanism of the two reactions is probably the same, involving a fast proton transfer equilibrium 4 and a subsequent bimolecular reaction between the protonated anhydride and a water molecule to form either an intermediate addition product or a transition state. A unimolecular mechanism was proposed by Gold [1] for the acid-catalysed hydrolysis of acetic anhydride in water at 0° on the grounds that the rate was proportional to the Hammett acidity function h_0 and not to the concentration of the catalyst at high acid concentrations. The validity of this method has recently been criticized in a work of Koskikallio and Whalley [11] concerning the volume of activations of acid-catalysed hydrolyses.

The experimental activation energies given above are actually the sums of the activation energies of the slow step of the reaction and the proton transfer equilibria 4 and 5 in methanol and water, respectively.

$$H_2O^+ + A = AH^+ + H_2O \qquad (5)$$

The ratio of the equilibrium constants of the two proton transfer reactions 4 and 5 in the same solvent, *e. g.* a methanol-water mixture, is equal to the equilibrium constant of the proton transfer reaction 1 in the same solvent. Because the solvent effects are small for these reactions the above statement is approximately valid for values obtained in different solvents, *e. g.* pure methanol and water. Thus the greatest part of the difference between activation energies in water and in methanol, $\Delta E = -7.2$ kcal · mole^{-1}, is due to the heat of the proton transfer reaction 1, $\Delta H = -3.7$ kcal · mole^{-1}, and the rest of the difference is due the difference between the activation energies of the two reactions, hydrolysis and methanolysis, and their solvent effects.

Similarly, differences in the entropies of activation can be corrected for the changes in the proton transfer equilibria 4 and 5. If the concentrations of the protonated anhydride in water and in methanol containing hydrochloric acid were known, the entropies of activation of the slow step of the solvolysis of acetic anhydride could be calculated. Assuming the value of the equilibrium constant of the reaction 1 to be unaffected by the change in the composition of the solvent mixture, $K = 209$ at 0°, the ratio of the concentrations of the protonated anhydride in methanol and in water can be calculated to be $25.2 \cdot 209/55.5 = 94.2$. Because the rate is proportional to the concentrations of the protonated anhydride, this would also be the ratio of the rate constants.

It would cause a difference of about 9.0 units in the experimental activation entropies. The observed difference in the entropies is 16.1 units. Thus the differences in the rates, activation energies and entropies of the acid-catalysed solvolysis of acetic anhydride in methanol-water mixtures are partly explained by the changes in the proton transfer equilibria when the composition of the solvent mixture is altered and the remaining small differences indicate that other solvent effects are only of minor importance.

That there are fast proton transfer pre-equilibria (4 and 5) in the acid-catalysed solvolyses of acetic anhydride is obvious from the obsevration of Gold [1] that the rate of the hydrolysis is much more closely proportional to the Hammett acidity function h_0 than to the concentration of the catalyst at high acid concentrations. The same result is obtained from the present experiments. One of the possible mechanisms of the acid-catalysed solvolysis of acetic anhydride in addition to that involving a fast proton transfer pre-equilibria is a bimolecular reaction between methoxonium or hydroxonium ions and the uncharged anhydride. Because all the species are thermodynamically in equilibrium, a choice between these two mechanisms is impossible. For the following reasons the mechanism which includes the proton transfer equilibrium is more probable. If the value of $K = 209$ is used to calculate the ratio of hydroxonium and methoxonium ions in the mixture containing 27 % water, a value of $[H_3O^+]/[ROH_2^+] = 137$ is obtained. The ratio of the first-order rate constants of the acid-catalysed hydrolysis and methanolysis of acetic anhydride is $k_1^h/k_1^m = 0.132$ in the same solvent mixture. Thus the bimolecular reaction between anhydride and hydroxonium ion would be about 10^3 times slower than the corresponding reaction between anhydride and methoxonium ion. Such a large difference between these two similar reactions is unexpected. Thus the mechanism is that involving a proton transfer pre-equilibrium. According to this mechanism the ratio of the acid-catalysed methanolysis and hydrolysis (about 4 to 6) is almost identical with the value for the ratio of the spontaneous reactions in the same solvent mixtures [4]. Also this observation is in accordance with the mechanism involving the proton transfer pre-equilibrium.

The author wishes to record his indebtedness for a research grant from the *Alf. Kordelin Foundation.*

REFERENCES

1. Gold, V. and Hilton, J. *J. Chem. Soc.* **1955** 838.
2. Koskikallio, J. *Ann. Acad. Sci. Fennicae* **A II 57** (1954).
3. Koskikallio, J. *Suomen Kemistilehti* **B 30** (1957) 43.
4. Koskikallio, J. *Acta Chem. Scand.* **13** (1959) 665.
5. Goldschmidt, H. *Z. physik. Chem.* **89** (1914) 129; *ibid.* **81** (1912) 30.
6. Salomaa, P. *Acta Chem. Scand.* **11** (1957) 191.
7. Thomas, L. and Marum, E. *Z. physik. Chem.* **143** (1929) 191.
8. Schmidt, H. A. and Reichardt, C. H. *J. Am. Chem. Soc.* **63** (1941) 605.
9. Kolthoff, J. M. and Bruckenstein, S. *J. Am. Chem. Soc.* **78** (1956) 1.
10. Tommila, E. and Hella, A. *Ann. Acad. Sci. Fennicae.* **A II 53** (1954).
11. Koskikallio, J. and Whalley, E. *Canad. J. of Chem.* **37** (1959) 788.

Received January 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 677–678

Studies on Pyrazolones

XII. Alkaline Decomposition of Furlone Yellow

GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

Alkaline hydrolysis of furlone yellow [1] (I) at room temperature gives a salt of the base II, which on acidification splits off carbon dioxide to form the compound III.

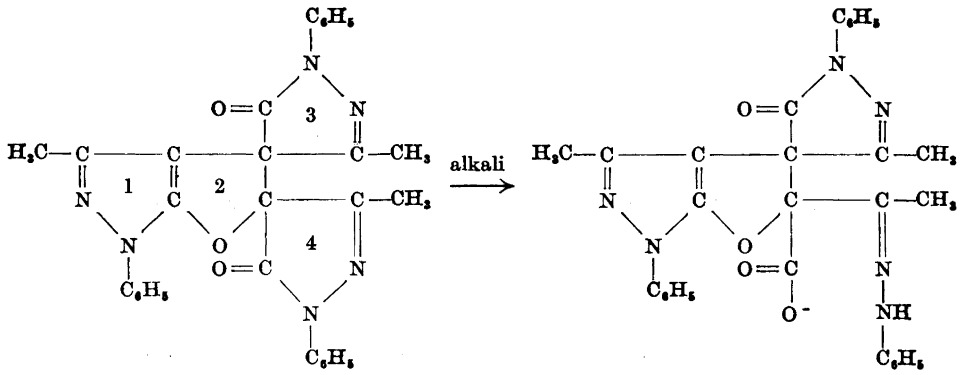
The pyrazolone ring of 1-phenyl-3-methyl-2-pyrazolin-5-ones is stable towards alkali, unless both the 4-hydrogen atoms are substituted, at least one of them by an electron-attracting group. Cleavages of pyrazolone rings by alkali have been described, *e.g.* for rubazonic acid [2] and for 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione [3]. In the present paper one of the alkaline cleavage products of furlone yellow [1] is studied.

From the acidified, alkaline hydrolysate of furlone yellow (I) a colourless compound can be isolated, the elemental analyses of which are in accordance with the structure III. The product is insoluble in aqueous alkali. In solution it decomposes in a couple of days with formation of 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione [2] (IV). This degradation, which is catalysed by acids and bases, excludes a cleavage of the pyrazolone ring 3 of I during the hydrolysis. As ring 1 has a stable pyrazole structure the ring 4 must have been split up in accordance with formula II. The intermediate II has not been isolated. On acidification it splits off carbon dioxide.

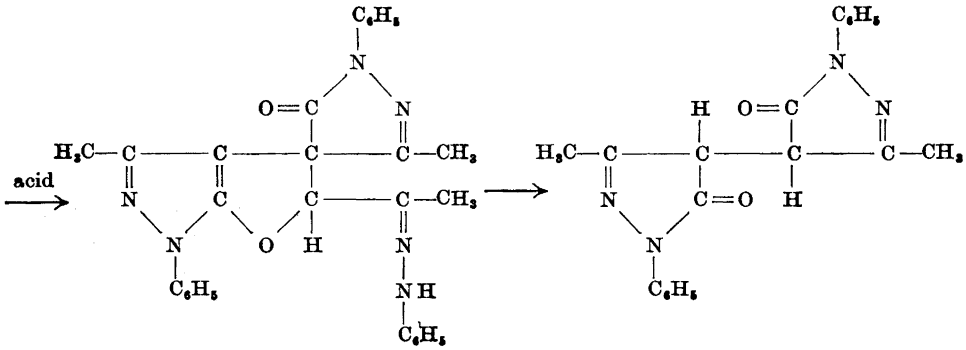
In addition coloured products of hydrolysis are formed from I. They have not been investigated and may involve cleavage of ring 3 of I.

EXPERIMENTAL

Preparation of III. Furlone yellow [1] (5.27 g) was dissolved in 300 ml of boiling ethanol, the solution was cooled to room temperature, and 2.5 N sodium hydroxide (75 ml) was added. After 2 h the solution was acidified with 2.5 N hydrochloric acid (75 ml) and immediately precipitated with water to prevent decomposition of the product III formed. The precipitate was collected by filtration, washed with water and air-dried (5.3 g). The product obtained was dissolved in ethyl acetate (150 ml, room temperature). Insoluble bispyrazolone (IV) was sometimes present and had to be removed by filtration. After evaporation of the excess of ethyl acetate at room temperature, colourless crystals of



I II



III IV

III separated on scratching. They were filtered and washed with ethyl acetate (yield 3.35 g). For further purification the treatment with ethyl acetate may be repeated. M. p. about *155°* (decomp.). (Found: C 70.9; H 5.3; N 17.1; O 6.6. Calc. for $C_{29}H_{26}N_6O_2$: C 71.0; H 5.3; N 17.1; O 6.5.)

Degradation of III with formation of IV. A solution of III (0.25 g) in chloroform (4 ml) was kept at room temperature for 24 h. The colourless precipitate formed (0.15 g) was filtered and washed with chloroform. It was almost insoluble in boiling ethanol, melted >300° (decomp.) and was oxidized by nitrous acid to form pyrazole blue. Accordingly it must be 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione [2] (IV). — When hydrogen chloride was passed through a benzene solution of III, the degradation to the bispyrazolone IV was rapid. Analogously the addition of alkali to an ethanol solution of III accelerated the formation of IV. (Found: C 69.1; H 5.2; N 16.4. Calc. for $C_{20}H_{18}N_4O_2$: C 69.3; H 5.2; N 16.2.)

The financial support of *Statens naturvetenskapliga forskningsråd* is gratefully acknowledged.

REFERENCES

1. Westöö, G. *Acta Chem. Scand.* 7 (1953) 360.
2. Knorr, L. *Ann.* 238 (1887) 137.
3. Westöö, G. *Acta Chem. Scand.* 9 (1955) 797.

Received January 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 679–682

Studies on Pyrazolones

XIII. The Reactions of 1-Phenyl-3-methyl-2-pyrazolin-5-one with Some Diketones

GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

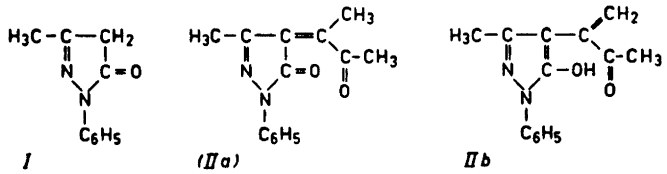
1-Phenyl-3-methyl-2-pyrazolin-5-one (I) reacts with 2,3-butanedione to form the acid 1-phenyl-3-methyl-4-(1-acetylvinyl)-5-hydroxypyrazole (II b). With 2,4-pentanedione I gives a neutral, tetracyclic product (III d) and with 2,5-hexanedione the bispyrazolone, V, which is stable in solid form but in solution is easily oxidized to VI even by the air at room temperature.

2,3-Butanedione and 1-phenyl-3-methyl-2-pyrazolin-5-one (I) react at room temperature with formation of a colourless acid, II b, a tautomer of the expected, coloured, neutral reaction product, II a. A CH_2=C-test on the acid according to Bricker and Roberts [1] was positive. The IR spectrum contains only one C=O stretching frequency (1 670 cm^{-1}) and a broad OH band around 2 500 cm^{-1} (solid state).

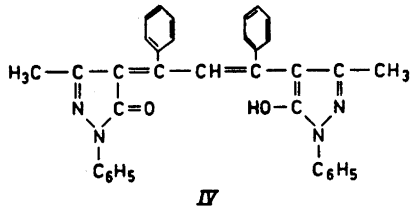
When I is heated with 2,4-pentanedione in methanol, a neutral, colourless compound separates. Piperidine catalyses the reaction. The analyses of the compound formed are in accordance with the structure III a, but since the lack of colour excludes this formula, the reaction sequence, Scheme I, leading to the structure III d, is proposed. The UV-curve of the product in methylene chloride solution (λ_{max} = 254 mμ, log ε = 4.26) is similar to the corresponding curve of 1-phenyl-3-methyl-5-methoxypyrazole in hexane [2]. The IR spectrum of the solid product also confirms the structure III d, having neither C=O, nor OH absorption bands. A very strong band at 1 090 cm^{-1} is probably associated with the ether linkages.

Only neutral solvents like ligroin, methylene chloride and chloroform dissolve the product in the cyclic form (III d). Alcoholic solutions are violet-red, indicating that III d is here in equilibrium with the open forms (ionized III b has a λ_{max} at 545 mμ). III d can be recovered from the red solutions.

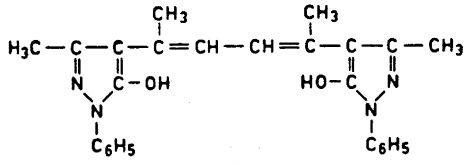
When dry hydrogen chloride is introduced into a chloroform or methylene chloride solution of III d, the ether linkages are split up, for the IR spectrum of the colourless compound which separates shows OH and NH bands. The



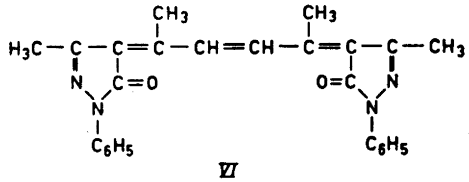
I (*IIa*) *IIb*



IV



V

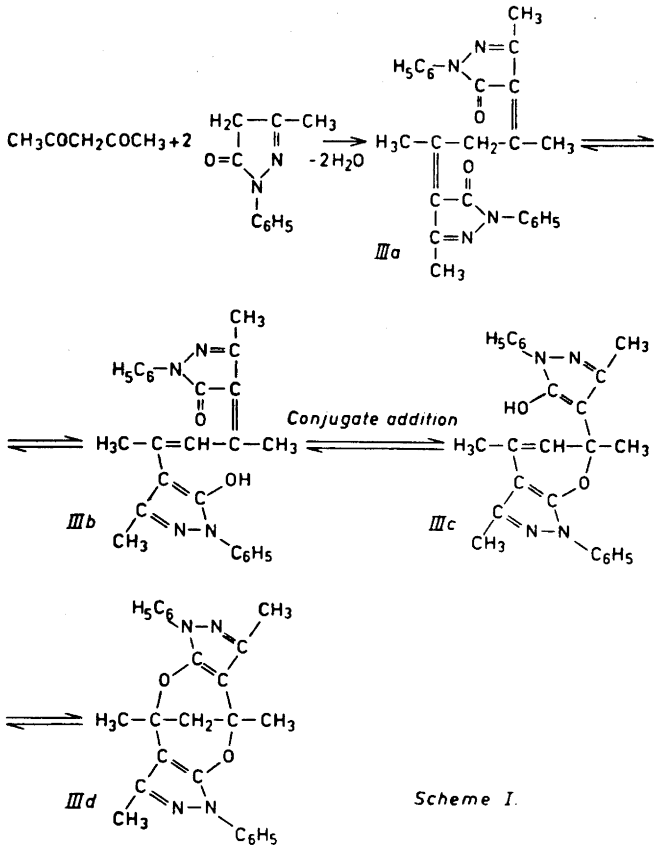


VI

structure of the reaction product is difficult to settle on account of its great instability. Both in solution and in the solid state the compound splits off hydrogen chloride with reformation of III d.

When the methyl groups in 2,4-pentanedione are exchanged for phenyl groups in the above reaction, the open form IV is strongly stabilized by resonance and no cyclization takes place. As is to be expected on account of the acidic hydroxyl group and the resonance possibilities of the corresponding ion, the colour of IV is pH-dependent, being blue in alkaline solution, red in the solid form and in acid solution.

Heating of I in 2,5-hexanedione gives rise to a colourless product, the properties of which are in accordance with the structure V. The IR curve of the compound in KBr has no carbonyl band but a broad hydroxyl band at about 2 600 cm^{-1}. V is stable in the solid state, but in solution it is rapidly oxidized by the air to form the deep red compound VI.



Scheme I.

EXPERIMENTAL

Preparation of 1-phenyl-3-methyl-4-(1-acetylvinyl)-5-hydroxypyrazole (II b). 1-Phenyl-3-methyl-2-pyrazolin-5-one (I) (10 g) was dissolved in 2,3-butanedione (30 ml), and the solution was kept at room temperature for 18 h. After cooling the crystals formed were collected by filtration and washed with cold ethanol. Yield: 11.7 g. After recrystallization from ethanol the product melted at 136° (decomp.). (Found: C 69.4; H 5.9; N 11.7; O 13.3; equiv. wt 242. Calc. for $C_{14}H_{14}N_2O_2$: C 69.4; H 5.8; N 11.6; O 13.2; equiv. wt 242.)

Preparation of III d. 2,4-Pentanedione (5.0 ml), I (5.0 g), methanol (20 ml) and one drop of piperidine were refluxed for 48 h. The piperidine was neutralized by addition of 2 drops of 5 N hydrochloric acid. The boiling was continued for 10 min. After cooling in ice for one hour, the colourless crystals, which had separated, were filtered and washed with methanol and water. Yield: 5.0 g, m. p. 171° (decomp.). The product can be recrystallized from benzene. (Found: C 72.7; H 5.9; N 13.6; O 7.7; mol. wt * 417. Calc. for $C_{25}H_{24}N_4O_2$: C 72.8; H 5.9; N 13.6; O 7.8; mol. wt 412.)

Preparation of IV. Dibenzoylmethane (2.2 g), I (3.5 g) and *n*-butanol (10 ml) were refluxed overnight. After addition of ethanol, the solution was poured into water and

* Cryoscopic determination in glacial acetic acid.

acidified with hydrochloric acid. The precipitate was filtered and washed with water. Yield of crude IV: 3.7 g. The product was recrystallized from ethanol repeatedly (0.8 g of deep red product remained). M. p. 211°. It was dried at 100° and 0.5 mm Hg. (Found: C 77.9; H 5.5; N 10.1. Calc. for $C_{35}H_{28}N_4O_2$: C 78.3; H 5.3; N 10.4.)

Preparation of V. 2,5-Hexanedione (5 ml), I (5.0 g) and ethanol (30 ml) were heated in a boiling water bath in N_2-atmosphere for 24 h. Colourless crystals of V separated. After cooling they were filtered, washed with ethanol and chloroform and dried in N_2-atmosphere. Yield: 4.45 g; m. p. about 220° (decomp.). (Found: C 73.0; H 6.2; N 13.2; O 7.6. Calc. for $C_{26}H_{26}N_4O_2$: C 73.2; H 6.15; N 13.1; O 7.5.)

V is only slightly soluble in most solvents. In solution it is easily oxidized by *e. g.*, perchloric acid or 1-phenyl-3-methyl-4,4-dibromo-2-pyrazolin-5-one and even by the air, the red compound VI being formed. In *e. g.*, methylenechloride in N_2-atmosphere, V is soluble without change of colour. When air is admitted the solution rapidly turns red. Besides by the colour change the formation of VI is proved by the IR spectrum, which, contrary to the spectrum of V, has a C=O band but no OH band. M. p. of VI: 217° (decomp.). (Found: C 73.5; H 5.9; N 13.2; O 7.7. Calc. for $C_{26}H_{24}N_4O_2$: C 73.6; H 5.7; N 13.2; O 7.5.)

The analyses were performed by Mikroanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany. Dr. Andreas Rosenberg, University of Uppsala, carried out the IR measurements.

The author wishes to thank *Statens naturvetenskapliga forskningsråd* for financial support.

REFERENCES

1. Bricker, C. E. and Roberts, K. H. *Anal. Chem.* **21** (1949) 1331.
2. Valyashko, N. A. and Bliznjukov, V. I. *J. Gen. Chem.* (U.S.S.R.) **11** (1941) 23.

Received January 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 683–688

Studies on *Cyclo*propanes

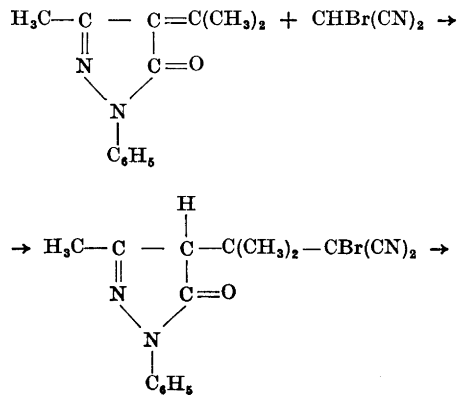
III. On the Isomerization of Certain Hexasubstituted *Cyclo*propanes

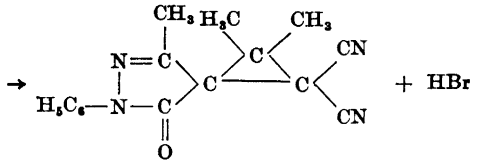
GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

The rearrangement of certain hexasubstituted *cyclo*propanes, involving cleavage of the *cyclo*propane ring with migration of a hydrogen atom from a methyl group attached to the ring, which was described in a previous paper [1], has been further studied. I a and b isomerize at elevated temperatures, IV a, b being formed. Boiling with acids or bases transforms IV a, b into V. II rearranges easily with formation of VI and VII, but no rearrangement was observed with the compounds III a–e.

The hexasubstituted, neutral *cyclo*propanes (I—III), the isomerizations of which are studied in the present investigation, all have four electrophilic groups (1,1- and 2,2-positions) and — except for III e — two alkyl groups, one of which is a methyl, attached to the *cyclo*propane ring. With the exception of III a, b and e, they have all been prepared by a Michael addition of a bromo compound to an α,β-unsaturated carbonyl compound, followed by ring closure with expulsion of hydrogen bromide, *e. g.*





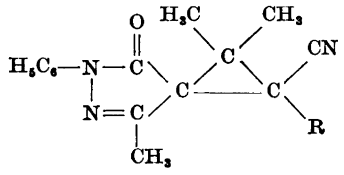
Alkali has been used to catalyse the reactions.

III a has been prepared by Ramberg and Wideqvist [2] and III b by Wideqvist [3], and their method of preparation could be used also for III e.

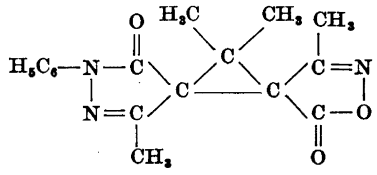
I a and I b are both stable at room temperature even in alcoholic solution and much more resistant towards heat than were the *cyclo*propanes, described in Studies on *Cyclo*propanes I [1]. However, heating in *n*-butanol on a boiling-water bath for several hours causes rearrangement to monobasic acids, which must have the structures IV a and IV b, on account of the fact that heating with concentrated hydrochloric acid or dilute alkali solution transforms them into a monobasic acid of the structure V. A qualitative test of end unsaturation according to Bricker and Roberts [4] was distinctly positive for IV but doubtable for V. This indicates a rearrangement of V from the β,γ-unsaturated nitrile structure to the α,β-form. The IR-spectrum of V presents a nitrile stretching band at 2 200 cm^{-1}, showing that the CN group is in strong resonance [5] (α,β-form). V exhibits a remarkable stability towards hydrolysis, a fact which also supports the α,β-unsaturated structure.

The compound II is less stable than I, and rearranges on boiling in ethanol solution for half an hour. The two possible isomerization products, the acids VI and VII, are both formed. They can be separated by recrystallization from ethanol. The isomer that is least soluble in ethanol is similar to IV in being rather soluble in concentrated hydrochloric acid, indicating that it has a pyrazolonyl group in the end position (VI), enabling the formation of NH tautomer. The other isomer (VII) is insoluble in concentrated hydrochloric acid. The UV spectra support these structures. In VI as well as in VII the main absorbing group in the UV range is the pyrazolonyl group. This group can form salts with acids in VI but not in VII. In accordance with this fact, the UV curve of VII is only slightly affected by strong acids, whereas the absorption of VI is appreciably depressed at wavelengths >250 mμ.

The compounds III a—d are all stable and do not isomerize even on prolonged boiling in ethanol or *n*-butanol. III e decomposes when boiled in *n*-butanol. No isomerization products could be isolated.



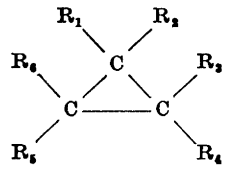
I



II

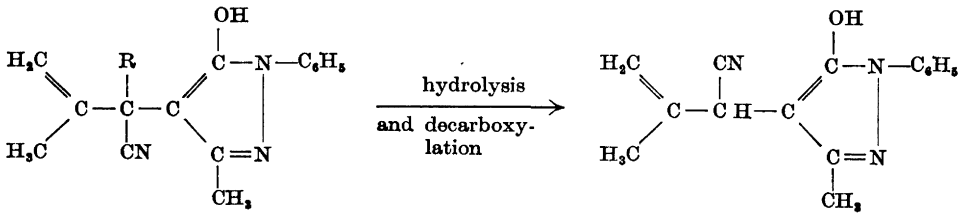
a. R=CN

b. R=$CONH_2$



III

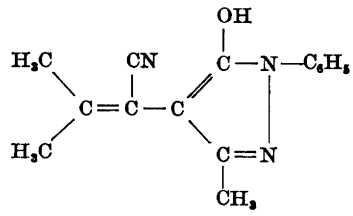
a. $R_1=R_2=CH_3$; $R_3=R_4=R_5=R_6=CN$
b. $R_1=CH_3$; $R_2=C_2H_5$; $R_3=R_4=R_5=R_6=CN$
c. $R_1=R_2=CH_3$; $R_3=R_4=R_5=CN$; $R_6=COOC_2H_5$
d. $R_1=CH_3$; $R_2=C_2H_5$; $R_3=R_4=R_5=CN$; $R_6=COOC_2H_5$
e. $R_1=CH_3$; $R_2=CH_3COCH_2CH_2$; $R_3=R_4=R_5=R_6=CN$



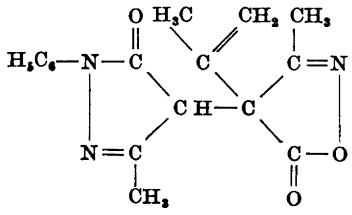
IV

a. R=CN
b. R=CONH$_2$

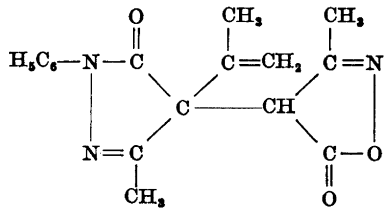
⇅



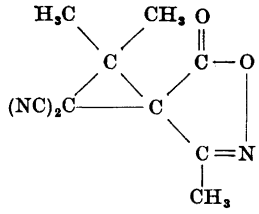
V



VI



VII



VIII

Unsuccessful attempts have been made also to prepare and study *cyclo*propanes with the substituents $R_1 = R_2 = CH_3$, $R_3 = CN$, $R_4 = CN$ or $COOC_2H_5$ and $R_5 = R_6 = COCH_3$. Acetone, sodium bromoacetylacetonate and malonitrile or ethyl cyanoacetate were used as starting material, but the reaction products isolated did not contain any component emanating from the acetone and were not *cyclo*propanes. From the nitrile and the bromoacetylacetone a furan derivative was obtained and from ethyl cyanoacetate and the bromoacetylacetone a substituted pyran. These products are described in a separate paper [6].

EXPERIMENTAL

Preparation of I a. 1-Phenyl-3-methyl-4-*iso*propylidene-2-pyrazolin-5-one (4.3 g) was dissolved in 250 ml of hot ethanol, the solution was immediately cooled to room temperature and bromomalonitrile (2.90 g) was added. As soon as it had dissolved, 2.39 N sodium hydroxide solution (8.4 ml) was added dropwise with stirring. The colourless I a soon started separating, and after 4 h it was collected by filtration and washed with 50 % ethanol. Yield: 4.5 g. The product was recrystallized from ethanol. M. p. 163° (decomp.). (Found: C 69.2; H 5.0; N 20.0. Calc. for $C_{16}H_{14}N_4O$: C 69.05; H 5.1; N 20.1.)

Preparation of I b. 1-Phenyl-3-methyl-4-*iso*propylidene-2-pyrazolin-5-one (4.3 g) and bromocyanoacetamide (3.3 g) were dissolved in 250 ml of ethanol. Sodium hydroxide (8.4 ml of 2.39 N solution) was added dropwise with stirring to the cold solution. The next day the colourless crystals which had separated were collected by filtration and washed with 50 % ethanol. Yield: 3.7 g; m. p. about 160° (very unsharp, decomp.). (Found: C 64.8; H 5.6; N 18.8; O 10.9. Calc. for $C_{16}H_{16}N_4O_2$: C 64.8; H 5.4; N 18.9; O 10.8.)

Preparation of II. 1-Phenyl-3-methyl-4-bromo-2-pyrazolin-5-one (5.07 g) was dissolved in ethanolic sodium hydroxide solution (10 ml of ethanol + 8.27 ml of 2.42 N aqueous sodium hydroxide solution) and immediately poured into a solution of 3-methyl-4-*iso*propylidene-2-isoxazolin-5-one [7] (2.78 g) in ethanol (75 ml). II separated at once. It was filtered and washed with water and ethanol. Yield of almost colourless product: 5.90 g, m. p. 160° (decomp.). The product was recrystallized by dissolving 1 g of it in 100 ml of boiling ethanol and immediate cooling of the solution in ice. (Found: C 65.4; H 5.4; N 13.5. Calc. for $C_{17}H_{17}N_3O_3$: C 65.6; H 5.5; N 13.5.)

The UV curve of II is closely related to the corresponding curves of the *cyclo*propane derivatives described in Studies on *Cyclo*propanes I [1]. The product is neutral towards thymolphthalein but is rapidly hydrolysed by alkali.

Preparation of III c. Ethyl *iso*propylidenecyanoacetate (3.06 g) and bromomalonitrile (2.90 g) were dissolved in ethanol (10 ml), and 8.55 ml of 2.34 N sodium hydroxide solution were added with stirring. III c started separating immediately, and after 3 h it was filtered and washed with 50 % ethanol. Yield: 3.3 g of colourless product; m. p. about 135° (decomp.). The product was purified by recrystallization from ethanol. (Found: C 60.8; H 5.2; N 19.35; O 14.7. Calc. for $C_{11}H_{11}N_3O_2$: C 60.8; H 5.1; N 19.35; O 14.7.)

Preparation of III d. Ethyl *sec.*-butylidenecyanoacetate (5.0 g of crude product) and bromomalonitrile (4.35 g) were dissolved in ethanol (20 ml). Sodium hydroxide (12.6 ml of 2.34 N solution) was added. III d separated in colourless crystals, and after 4 h it was filtered and washed with 50 % ethanol. Yield: 4.5 g; m. p. 89°. The product was purified by recrystallization from ethanol. M. p. 89.3°. (Found: C 62.3; H 5.7; N 18.1; O 13.7. Calc. for $C_{12}H_{13}N_3O_2$: C 62.3; H 5.7; N 18.2; O 13.8.)

Preparation of III e. 2,5-Hexanedione (1.14 g) and bromomalonitrile (2.90 g) were dissolved in ethanol (10 ml), and a solution of potassium iodide (7.0 g) in water (10 ml) was added. The following day the precipitate formed (III e) was filtered and washed with 50 % ethanol. Yield: 1.15 g. The compound was purified by recrystallization from ethanol, when a colourless, neutral product was obtained. M. p. 165—166° (decomp.). (Found: C 63.7; H 4.5; N 24.8; O 7.1. Calc. for $C_{12}H_{10}N_4O$: C 63.7; H 4.5; N 24.8; O 7.1.)

Isomerization of I a to form IV a. I a (3.0 g) was heated in 1-butanol (120 ml) on a boiling-water bath for 6—7 h. The butanol was evaporated under reduced pressure, and the crude IV a left was recrystallized repeatedly from ethanol. M. p. 163—164° (decomp.). (Found: C 69.2; H 5.1; N 20.0; O 5.6; equiv. wt 278. Calc. for $C_{16}H_{14}N_4O$: C 69.05; H 5.1; N 20.1; O 5.75; equiv. wt 278.) A CH_2=C determination according to Bricker and Roberts [4] was positive.

Boiling of IV a with a) concentrated hydrochloric acid or b) dilute sodium hydroxide solution to form V. a) IV a (1.35 g) was heated with concentrated hydrochloric acid (20 ml) on a water-bath for 3 h. The hydrochloric acid was removed under reduced pressure, and the solid residue was boiled with water. On cooling colourless crystals separated. They were filtered and washed with water. Yield: 1.03 g. After recrystallization from ethanol twice the product melted at 173° on slow heating. When the melting tube was inserted in the heating-block at ~130°, the product melted, but solidified again and then melted at ~173°. (Found: C 70.9; H 6.1; N 16.4; O 6.4; equiv. wt 252. Calc. for $C_{15}H_{15}N_3O$: C 71.1; H 6.0; N 16.6; O 6.3; equiv. wt 253.) A qualitative test on unsaturation according to Bricker and Roberts gave only weak colour. The product is not hydrolysed by concentrated hydrochloric acid, nor by 2 N sodium hydroxide solution at 100°.

b) A solution of IV a (0.2 g) in 2.4 N sodium hydroxide solution (2.5 ml) was heated on a water-bath for 8 h. On acidification V separated. It was collected by filtration and washed with water. From the solution more V could be extracted with ether. Mixed melting point with an authentic sample of V was 172°.

Isomerization of I b to form IV b. I b (2.0 g) and 1-butanol (60 ml) were heated in a boiling-water bath for 6 h. The butanol was evaporated under reduced pressure and the solid residue was recrystallized from ethanol. Yield: 1.6 g of colourless IV b; m. p. about 215° (decomp.). (Found: C 64.8; H 5.5; N 18.9; O 10.9; equiv. wt 296. Calc. for $C_{16}H_{16}N_4O_2$: C 64.85; H 5.4; N 18.9; O 10.8; equiv. wt 296.) A test on end unsaturation according to Bricker and Roberts was positive.

IV b, analogously to IV a, could be transformed into V by heating with concentrated hydrochloric acid on a boiling-water bath.

Isomerization of II to form VI and VII. A solution of II (5.05 g) in ethanol (500 ml) was boiled vigorously for half an hour. At that time so much solvent had evaporated that crystals of VI started separating. After cooling the product was collected by filtration and washed with cold ethanol. Yield of VI: 2.35 g. The compound can be recrystallized from ethanol. M. p. 187° (decomp.). (Found: C 65.6; H 5.5; N 13.5; O 15.2; equiv. wt 308. Calc. for $C_{17}H_{17}N_3O_3$: C 65.6; H 5.5; N 13.5; O 15.4; equiv. wt 311.) A qualitative test on CH_2=C according to Bricker and Roberts [4] was positive. The product is soluble in concentrated hydrochloric acid.

The mother liquor and washings from VI were evaporated to dryness in vacuum. The product obtained was recrystallized from ethanol, treated with concentrated hydrochloric acid to remove VI, filtered, washed with hydrochloric acid and water and once more recrystallized from ethanol. Yield of VII: 1.25 g, m. p. 179° (decomp.). (Found: C 65.5; H 5.7; N 13.5; O 15.5; equiv. wt 309. Calc. for $C_{17}H_{17}N_3O_3$: C 65.6; H 5.5; N 13.5; O 15.4; equiv. wt 311.) A qualitative test on end unsaturation was positive.

The UV curves of VI and VII are distinctly different (λ_{max_1} = 246 mμ, log ε_1 = 4.09 and λ_{max_2} = 275 mμ, log ε_2 = 3.98 for VI, λ_{max} = 248 mμ, log ε = 4.34 for VII).

Preparation of VIII. When bromomalonitrile (4.35 g) was dissolved in a solution of 3-methyl-4-*iso*propylidene-2-isoxazolin-5-one (4.17 g) in ethanol (150 ml), VIII started separating immediately. After 4 h it was filtered and washed with 50 % ethanol. Yield:

4.95 g of colourless product; m. p. ~173° (decomp.). The compound was recrystallized from ethanol. (Found: C 59.3; H 4.5; N 20.7. Calc. for $C_{10}H_9N_3O_3$: C 59.1; H 4.5; N 20.7.) The product is rapidly hydrolysed by alkali. A solution of the compound in concentrated acetic acid does not react with bromine at room temperature. Accordingly it does not contain the isomeric acid. The IR spectrum shows absorption at 1 025 cm^{-1}, which is within the range characteristic of *cyclo*propane rings [8,9].

Attempts to isomerize III a—e. Heating of ethanol or 1-butanol solutions of III a, b, c, d or e on a boiling-water bath did not result in rearrangements with ring cleavage. Except with III e most of the *cyclo*propane derivative could be recovered unchanged.

The analyses were performed by Mikroanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany. Dr Andreas Rosenberg, University of Uppsala, carried out the IR measurements.

The author wishes to thank *Statens naturvetenskapliga forskningsråd* for financial support.

REFERENCES

1. Westöö, G. *Acta Chem. Scand.* **11** (1957) 1290.
2. Ramberg, L. and Wideqvist, S. *Arkiv Kemi, Mineral., Geol.* **14 B** (1941) No. 37.
3. Wideqvist, S. *Arkiv Kemi, Mineral., Geol.* **20 B** (1945) No. 4.
4. Bricker, C. E. and Roberts, K. H. *Anal. Chem.* **21** (1949) 1331.
5. Kitson, R. E. and Griffith, N. E. *Anal. Chem.* **24** (1952) 334.
6. Westöö, G. *Acta Chem. Scand.* **13** (1959) 692.
7. Khromov, N. V. and Porai-Koshits, A. E. *J. Gen. Chem.* (U.S.S.R.) **17** (1947) 1816; *C. A.* **42** (1948) 4169i.
8. Bartleson, J. D., Burk, R. E. and Lankelma, H. P. *J. Am. Chem. Soc.* **68** (1946) 2513.
9. Derfer, J. M., Pickett, E. E. and Boord, C. E. *J. Am. Chem. Soc.* **71** (1949) 2482.

Received January 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 689–691

Studies on *Cyclo*propanes

IV. On the Isomerization of 1,1-Dimethyl-2,3-dicyano-2-ethoxycarbonyl-3-*cyclo*propanecarboxamide

GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

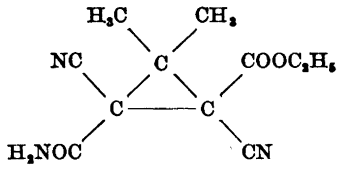
1,1-Dimethyl-2,3-dicyano-2-ethoxycarbonyl-3-*cyclo*propanecarboxamide (I) is easily rearranged to form the iminoimide II.

1,1-Dimethyl-2,3-dicyano-2-ethoxycarbonyl-3-*cyclo*propanecarboxamide (I) can be prepared from ethyl *iso*propylidenecyanoacetate and bromocyanoacetamide in alkaline solution in a way analogous to the preparations of *cyclo*propane derivatives described in Studies on *Cyclo*propanes 1—3 [1-3].

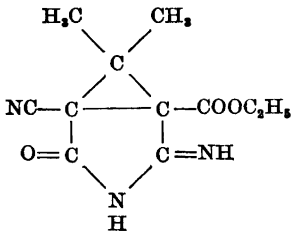
When kept in ethanol solution at room temperature for 1—2 days, the neutral I rearranges to form an amphoteric isomer, which is very weak both as an acid and as a base but can be sharply titrated in anhydrous medium with strong bases or acids. The rearrangement is strongly catalysed by bases, retarded by acids. The properties of the isomer are in accordance with the structure II. The structure III is excluded, a qualitative test on end unsaturation being distinctly negative. Thus the isomerization of I is not related to the rearrangements of the *cyclo*propanes described earlier [1,3].

The IR spectra of I and II both have an absorption band within the range characteristic of *cyclo*propane rings [4,5], I at 1 025 cm^{-1} and II at 1 005 cm^{-1}. As is to be expected the IR curve of I (in potassium bromide) shows two NH_2 stretching bands (3 150 cm^{-1} and 3 310 cm^{-1}), the spectrum of II (in potassium bromide) only one NH stretching band (3 330 cm^{-1}).

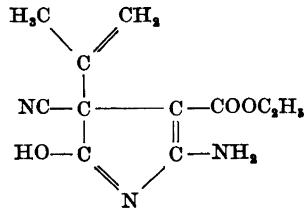
The structure II may seem strange, but ring closure with formation of iminoimides has been described earlier by, *e. g.*, Thorpe and collaborators [6-9]. Furthermore, 3,5-bridged diketopiperidines, which possess structures related to II, are stable and easily prepared as shown by Guareshi and Grande [10], Ghiglieno [11], and Wideqvist [12].



I



II



(III)

EXPERIMENTAL

Preparation of I. Sodium hydroxide (21.6 ml of 2.34 N solution) was added dropwise with stirring to a solution of ethyl *iso*propylidenecyanoacetate (7.7 g) and bromocyanoacetamide (8.2 g) in ethanol (45 ml). The precipitate formed was filtered after 5 h, washed with 50 % ethanol and air-dried. Yield of colourless product containing <50 % of I: 2.7 g. For removal of II the crude product was mixed with 5 ml of ethanol, the suspension was poured into 50 ml of 5 N hydrochloric acid, and the mixture was stirred for some minutes, filtered and washed with water and a small amount of cold ethanol. M. p. 168° (decomp.). (Found: C 56.1; H 5.6; N 17.9; O 20.3. Calc. for $C_{11}H_{13}N_3O_3$: C 56.2; H 5.6; N 17.9; O 20.4.)

The isomerization caused by bases of the neutral I to form II is so rapid that the product can be titrated as an acid in dimethylformamide solution using sodium methoxide in benzene-methanol solution as the titrant and azoviolet as the indicator. Acids retard the isomerization. Hence the compound I is stable in glacial acetic acid-chlorobenzene solution and does not consume any perchloric acid on titration using methyl violet as indicator.

Rearrangement of I to form II 1) in ethanol solution 2) in ethanol solution catalysed by alkali. 1) When a saturated ethanol solution of I was left at room temperature for a few days, colourless crystals of an isomeric, amphoteric product separated (II). In a more dilute solution the isomerization, which is almost quantitative, could be observed by UV measurements, log ε at 245 mμ increasing from 1.95 (measured in 0.05 N hydrochloric acid ethanol) to 4.06 (for a solution of recrystallized II log ε at 245 mμ was found to be 4.07). Addition of one drop of 2.5 N sodium hydroxide solution to this dilute solution of I made the isomerization complete in a few seconds. Boiling of an ethanol solution of I for ten minutes also effected isomerization. M. p. 190° (decomp.). (Found: C 56.2; H 5.8; N 17.8; O 20.4; equiv. wt on titration with perchloric acid in glacial acetic acid (solvent: chlorobenzene; indicator: methyl violet) 237; equiv. wt on titration with sodium methoxide in benzene-methanol solution (solvent: dimethylformamide; indicator: azoviolet) 236. Calc. for $C_{11}H_{13}N_3O_3$: C 56.2; H 5.6; N 17.9; O 20.4; equiv. wt 235.) II is soluble in dilute hydrochloric acid. A test on end unsaturation according to Bricker and Roberts [13] was negative.

2) A 0.83 N sodium ethoxide solution (5.38 ml) was added with stirring to the crude product from the preparation of I (1.05 g). When all crystals had dissolved (about 4 min), the solution was poured into 25 ml of 0.076 N hydrochloric acid. The colourless precipi-

tate formed was filtered and washed with water. Yield: 0.80 g. M. p. 190° (decomp.). (Found: C 56.2; H 5.6; N 17.8; O 20.5. Calc. for $C_{11}H_{13}N_3O_3$: C 56.2; H 5.6; N 17.9; O 20.4.) UV and IR curves of this product were identical with the curves of the product obtained in the preceding isomerizations.

The analyses were performed by Mikroanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany.

The author wishes to thank *Statens naturvetenskapliga forskningsråd* for financial support.

REFERENCES

1. Westöö, G. *Acta Chem. Scand.* **11** (1957) 1290.
2. Westöö, G. *Acta Chem. Scand.* **11** (1957) 1359.
3. Westöö, G. *Acta Chem. Scand.* **13** (1959) 683.
4. Bartleson, J. D., Burk, R. E. and Lankelma, H. P. *J. Am. Chem. Soc.* **68** (1946) 2513.
5. Derfer, J. M., Pickett, E. E. and Boord, C. E. *J. Am. Chem. Soc.* **71** (1949) 2482.
6. Thole, F. B. and Thorpe, J. F. *J. Chem. Soc.* **99** (1911) 422.
7. Thorpe, J. F. and Wood, A. S. *J. Chem. Soc.* **103** (1913) 1586.
8. Kon, G. A. R. and Thorpe, J. F. *J. Chem. Soc.* **115** (1919) 686.
9. Day, J. N. E. and Thorpe, J. F. *J. Chem. Soc.* **117** (1920) 1465.
10. Guareschi, I. and Grande, E. *Chem. Zentr.* (1899) II, 439.
11. Ghiglieno, M. *Chem. Zentr.* (1910) II 806.
12. Wideqvist, S. *Arkiv Kemi, Mineral., Geol.* **20 B** (1945) No. 4.
13. Bricker, C.E. and Roberts, K.H. *Anal Chem* **21** (1949) 1331.

Received January 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 692–694

The Reactions of 3-Chloro- and 3-Bromo-2,4-pentanedione with Malonitrile and with Ethyl Cyanoacetate

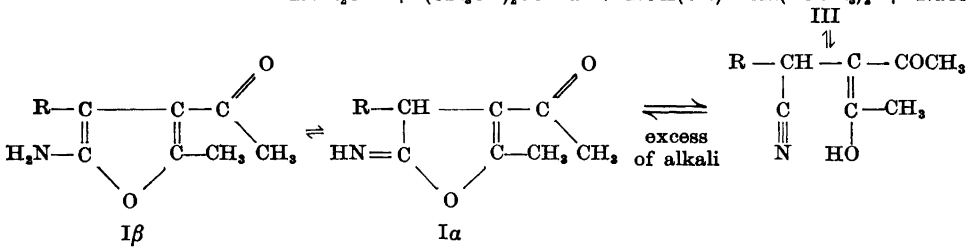
GUNNEL WESTÖÖ

Institute of Organic Chemistry, University of Lund, Lund, Sweden

2-Amino-3-cyano-4-acetyl-5-methylfuran (I a), 2-amino-3-ethoxycarbonyl-4-acetyl-5-methylfuran (I b) and 2-amino-3-ethoxycarbonyl-4,4,5-triacetyl-6-methylpyran (II) have been prepared.

When the sodium salt of 3-chloro-2,4-pentanedione reacts with malonitrile or ethyl cyanoacetate in ethanol-water solution, cyclization of the primarily formed III a, b occurs with formation of the neutral compounds, 2-amino-3-cyano-4-acetyl-5-methylfuran (I a) or 2-amino-3-ethoxycarbonyl-4-acetyl-5-methylfuran (I b).

$$RCH_2CN + (CH_3CO)_2CClNa \rightarrow RCH(CN)-CH(COCH_3)_2 + NaCl$$

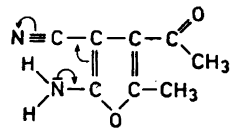


Iβ Iα

a. R=CN

b. R=$COOC_2H_5$

I a and b are stable (*cf.* unsubstituted 2-aminofuran which is very unstable), probably on account of the resonance possibilities offered by the 3-cyano- or the 3-ethoxycarbonyl groups, *e.g.*

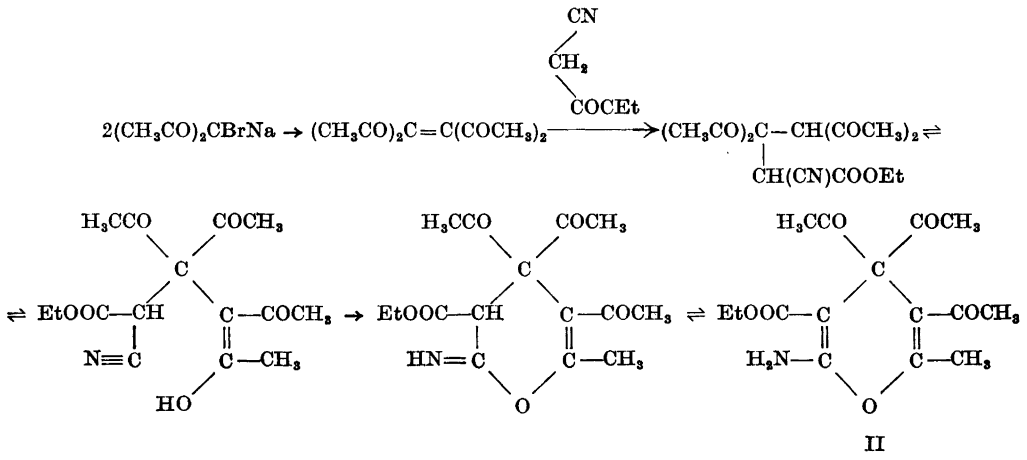


The IR absorption curve of I a (in KBr and in nujol) has one single nitrile band at 2 175 cm^{-1}. This means a displacement of about 75 cm^{-1} from the unconjugated nitrile band at 2 250 $\pm$ 10 cm^{-1}, indicating a strong resonance effect on the nitrile group [1]. A nujol suspension of the product shows absorption at 3 100 and 3 250 cm^{-1}, which is within the region characteristic of associated amino groups. After equilibration with D_2O, ND_2-bands at 2 330 and 2 500 cm^{-1} were obtained. Thus I a β is probably the main structure of the product formed. With *o*-nitrobenzaldehyde a Schiff's base can be prepared from I a, and with 2,4-dinitrophenylhydrazine a 2,4-dinitrophenylhydrazone.

The IR spectrum of I b in potassium bromide has no nitrile band and shows the expected NH_2-bands at 3 200 and 3 390 cm^{-1}.

The basic character of the amino group of I a, b is so weak that the products cannot be titrated with perchloric acid in glacial acetic acid. Excess of alkali dissolves I a and b with opening of their furan rings. On acidification ring closure with reformation of I a and I b takes place, but only in low yields because of alkaline hydrolysis of the nitrile and carbethoxy groups.

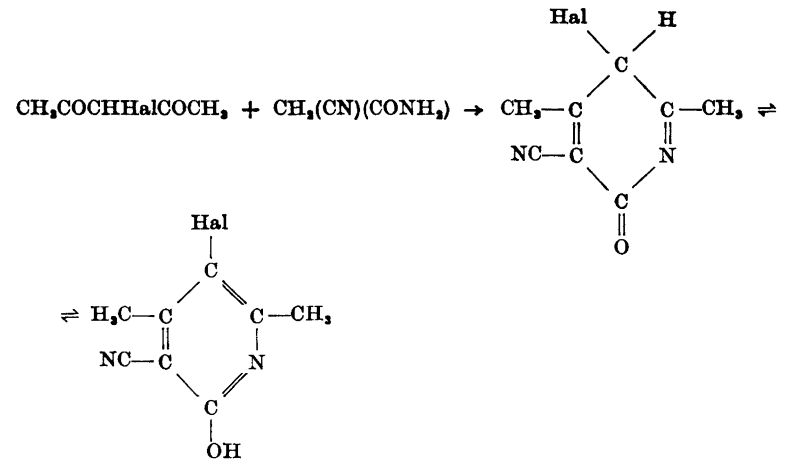
The reaction of the sodium salt of 3-bromo-2,4-pentanedione with malonitrile is analogous to the reaction described above of the corresponding chloro compound, I a being formed though in lower yield. The reactions of ethyl cyanoacetate with 3-bromo-2,4-pentanedione and with 3-chloro-2,4-pentanedione, however, are essentially different. With the bromo compound a pyran derivative, II, is formed evidently according to the equations:



II

The IR absorption curve of the product (in KBr) has no nitrile band but NH_2 stretching bands at 3 225 and 3 325 cm^{-1}. The amino group is basic enough to be titrated with perchloric acid in glacial acetic acid, though not very sharply.

It should be mentioned that the reaction of 3-chloro (or bromo)-2,4-pentanedione with cyanoacetamide in alkaline solution [2] follows other pathways than the reactions described above, pyridine derivatives being formed according to the equations



EXPERIMENTAL

Preparation of 2-amino-3-cyano-4-acetyl-5-methylfuran (I a). 3-Chloro-2,4-pentanedione (2.69 g) and malonitrile (1.32 g) were dissolved in ethanol (10 ml), and sodium hydroxide (8.26 ml of 2.42 N solution) was added. Colourless crystals of I a started separating immediately. The next day they were collected by filtration and washed with 50 % ethanol. Yield: 3.05 g, m. p. about 240° (decomp.). The product can be recrystallized from ethanol. (Found: C 58.5; H 4.85; N 17.1; O 19.5. Calc. for $C_8H_8N_2O_2$: C 58.5; H 4.9; N 17.1; O 19.5.)

2,4-Dinitrophenylhydrazone: M.p. 166—168° (decomp.) (Found: C 48.9; H 3.7; N 24.3; O 23.4. Calc. for $C_{14}H_{12}N_6O_5$: C 48.8; H 3.5; N 24.4; O 23.2.) Schiff's base with *o*-nitrobenzaldehyde (Found: C 60.6; H 4.0; N 14.0. Calc. for $C_{15}H_{11}N_3O_4$: C 60.6; H 3.7; N 14.1).

Preparation of 2-amino-3-ethoxycarbonyl-4-acetyl-5-methylfuran (I b). 3-Chloro-2,4-pentanedione (2.69 g) and ethyl cyanoacetate (2.26 g) were dissolved in ethanol (7 ml). When sodium hydroxide (8.26 ml of 2.42 N solution) was added, colourless crystals of I b soon started separating. The following day the product was filtered and washed with 50 % ethanol. Yield: 3.30 g; m. p. after recrystallization from ethanol: 136°. (Found: C 57.0; H 6.2; N 6.7; O 30.1. Calc. for $C_{10}H_{13}NO_4$: C 56.9; H 6.2; N 6.6; O 30.3.)

Preparation of 2-amino-3-ethoxycarbonyl-4,4,5-triacetyl-6-methylpyran (II). Bromine (3.2 g) was added with stirring to a cooled (12°) solution of 2,4-pentanedione (2.0 g) in 8.26 ml of 2.42 N sodium hydroxide solution and 15 ml of ethanol. After cooling the solution to 8°, sodium hydroxide (4.1 ml of 2.42 N solution) was added and — one minute later — ethyl cyanoacetate (1.38 g) and another 5.0 ml of sodium hydroxide solution. After 5 h the precipitate formed was filtered and washed with 50 % ethanol. Yield: 0.56 g; m. p. 166° (decomp.). (Found: C 58.2; H 6.2; N 4.7; O 30.9; mol. wt 320. Calc. for $C_{15}H_{19}NO_6$: C 58.2; H 6.2; N 4.5; O 31.0; mol. wt 309.)

The analyses were performed by Mikroanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mülheim, Germany. Dr. Andreas Rosenberg, University of Uppsala, carried out the IR measurements.

The author wishes to thank *Statens naturvetenskapliga forskningsråd* for financial support.

REFERENCES

1. Kitson, R. E. and Griffith, N. E. *Anal. Chem.* **24** (1952) 334.
2. Coover, H. W. and Bowman, N. J. U.S. Patent 2.481.573; Sept. 13, 1949.

Received January 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 695–704

Oxidation of Metals

The General Oxidation Equation

TOR HURLEN

Central Institute for Industrial Research, Blindern-Oslo, Norway

A detailed expression of a general rate equation for metal oxidation processes governed by the transport of material in the oxide film, is derived on the basis of Eyring's theory of absolute reaction rates and on the assumption that the transport is accomplished by defect species, *i. e.* interstitial ions and ion vacancies.

Following previous treatments by Evans and Hoar, the conditions at which this equation may be reduced to the more classical parabolic (Wagner) and inverse logarithmic (Mott-Cabrera) equations are discussed. It is further shown that the general equation under certain conditions may be satisfactorily replaced also by a cubic equation.

All the deductions have been applied to the special case of titanium being oxidized to rutile.

1.0 INTRODUCTION

On the basis of the theory of absolute reaction rates (Eyring [1]), it is possible to derive a general oxidation equation for cases of control by rate of transport of material in the oxide film. In two limiting cases, the general equation may be replaced by simpler forms known as the parabolic and inverse logarithmic equations. Under certain conditions, it may satisfactorily be replaced also by a cubic type of equation.

The general equation and its importance to metal oxidation studies seem first to have been pointed out by Evans [2], who derived a simplified, formal expression for this equation and also showed how it could be reduced to the more conventional oxidation equations. On this basis, Hoar [3] later calculated the limiting conditions of temperature and oxide film thickness under which the general equation for some metals (Fe, Zn, Cu) may be satisfactorily represented either by the inverse logarithmic equation or by the parabolic equation.

Gulbransen [4] seems to have been the first to apply the theory of absolute reaction rates to metal oxidation. He thereby derived an absolute expression for the parabolic rate constant.

To our knowledge, nobody has so far applied the absolute rate theory in deriving the general oxidation equation.

2.0 DERIVATION OF THE GENERAL EQUATION

From the absolute rate theory, the following expression for the net rate of transport (v) of a species in an ideal solution under the action of chemical and electrical potential fields, may easily be derived (see Ref.[1], pp. 552—555):

$$v = \frac{kT}{h}\lambda C \exp\left(-\frac{\Delta G_o^{\ddagger}}{RT}\right)\left[\exp\left(-\frac{\alpha\lambda zF}{RT}\frac{d\emptyset}{dx}\right) - \left(1+\lambda\frac{d\ln C}{dx}\right)\exp\left(\frac{(1-\alpha)\lambda zF}{RT}\frac{d\emptyset}{dx}\right)\right] \quad (1)$$

R is the gas constant, k the Boltzmann constant, h the Planck constant, T the Kelvin temperature, F the Faraday, $\Delta G_o^{\ddagger}$ the standard chemical energy of activation, C the concentration of the species, $\emptyset$ the inner potential, x a distance in direction of the transport, z the charge carried by a single migrating entity, λ the distance between equilibrium positions for these entities, and α the so called symmetry factor.

According to the theories of Frenkel [5] and Schottky [6], ionic crystals usually contain reversible defects (ions in interstitial positions and ion vacancies in connection with an equivalent number of quasi-free electrons and electron holes). It seems further well recognized that the transport of material in ionic crystals may be described as a transport of such defects (see *e.g.* Ref.[7]). As a normal crystal with good approximation also may be considered an ideal solution of reversible defects in a perfect crystal, the rate of transport of any one type of such defects in the crystal should be represented by eqn. (1) above.

The thickening of an oxide film on a metal certainly requires transport of material across the film. The defects, which may take part in this transport, generally are:

$O_v^{\cdot\cdot}$ (oxide ion vacancies)
$O_i^{''}$ (oxide ion interstitials)
$M_v^{z\prime}$ (metal ion vacancies)
M_i^{z+} (metal ion interstitials)

where the symbols $\cdot$ and $\prime$ represent a positive and a negative surplus charge, respectively.

During oxidation, oxide ion vacancies and metal ion interstitials will move *outwards* and oxide ion interstitials and metal ion vacancies *inwards* through the film. If we now choose the outward direction (from the metal to the gas) as our single reference direction or x-direction (instead of having to consider two transport directions), the total net rate of transport of defects through a plane at a distance x from the metal/oxide interface is given by:

$$v_x(\text{total}) = \sum_i \pm v_{i,x} \quad (2)$$

$v_{i,x}$ means here the rate of transport of the defect species i in the x direction and is given by (1) when $(d\emptyset/dx)$ and (dC/dx) are considered gradients also in this direction (not in the actual transport directions). The signs + and

— apply to positively and negatively charged species, respectively. In this way, all the rate terms in (2) become positive.

When + and — are put together as in the above equation, it will in this paper always mean that the upper sign applies to positive species and the lower to negative ones.

As both the concentration and the concentration gradient of a defect species as well as the electric potential gradient at any given time may vary from point to point within the film, it is obvious that v_x(total) also may vary. It is a question, therefore, how the momentary rate of oxidation is connected to the various momentary rates of transport in the oxide film.

What is actually measured however by gravimetric and volumetric methods of investigation of oxidation of metals, is the rate of entrance of oxygen from the gas phase into the oxide phase. The other rate processes in the system (such as the transfer of metal ions from the metal phase into the oxide phase and the migration of ions in the oxide phase) are not directly susceptible to measurements of this kind, and information on these processes must be obtained either by theoretical deductions from the oxidation measurements or by other types of measurements.

The measured oxidation rate can be assumed to be equal to the rate of entrance of oxygen into the oxide, provided the concentration of chemisorbed oxygen on the oxide surface does not change during oxidation. On this basis, the oxidation rate, v(ox), may be represented by the following formula:

$$v(\mathrm{ox}) = \lim_{x \to y} v_x(\mathrm{total}) \tag{3}$$

where y is the oxide film thickness, and where the various rates must be expressed in equivalent units (number of equivalents per unit area per unite time).

Eqn. (3) combined with (1) and (2) may thus be regarded as a general oxidation equation for cases of control by rate of transport of material in the oxide film. In practice, one also has to add the requirement that the area undergoing oxidation must be constant with time as experimental oxidation rates usually are determined on the basis of the apparent surface area of the specimens at the start of the experiment.

The general equation will be somewhat easier to handle if we introduce the following approximations:

$$v(\mathrm{ox}) = \frac{1}{V}\,(\mathrm{d}y/\mathrm{d}t) \tag{4a}$$

$$\mathrm{d}\emptyset/\mathrm{d}x = \Delta\emptyset/y \tag{4b}$$

$$\mathrm{d}C/\mathrm{d}x = \Delta C/y \tag{4c}$$

$$F\Delta\emptyset = \Delta G \tag{4d}$$

$$\alpha = \tfrac{1}{2} \tag{4e}$$

and the following substitute:

$$V\lambda(kT/h)\,\exp(-\Delta G_o^{\ddagger}/RT) = K \tag{5}$$

V is the equivalent volume of the oxide, $\Delta\emptyset$ the total potential increase in the film from metal to gas, ΔC the total concentration increase (for species

i) in the film from metal to gas, and ΔG the energy of formation of one gram-equivalent oxide at the actual oxidation conditions.

On this basis (eqns (1) to (5) above), the general oxidation equation may be written:

$$\frac{dy}{dt} = \sum_i \pm K_i C_{i,y} \left[\exp\left(-\frac{\lambda_i z_i \Delta G}{2 RT y}\right) - \left(1 + \frac{\lambda_i \Delta C_i}{y C_{i,y}}\right) \exp\left(\frac{\lambda_i z_i \Delta G}{2 RT y}\right)\right] \quad (6)$$

where $C_{i,y}$ means the limiting value of C_i for $x = y$ (*i.e.* when approaching the oxide/gas interface).

3.0 DEDUCTIONS FROM THE GENERAL EQUATION

The general oxidation equation (6) is rather clumsy. Under special conditions, however, it may be replaced by equations of simpler forms. This greatly increases its applicability and facilitates the testing of its validity.

Following previous treatments by Evans [2] and Hoar [3], we shall in the following discuss how the more classical parabolic (Wagner [8]) and inverse logarithmic (Mott-Cabrera [9]) equations may be regarded as limiting cases of the general oxidation equation. It shall also be shown how the general equation under certain conditions may be approximately, though satisfactorily, replaced even by a cubic type of equation.

The parabolic equation. At sufficiently high temperatures and large film thicknesses, the general equation (6) may be reduced to the simpler form:

$$\frac{dy}{dt} = \frac{1}{y} \sum_i \mp K_i \lambda_i C_{i,y} \left(\frac{z_i \Delta G}{RT} + \frac{\Delta C_i}{C_{i,y}} \right) \quad (7)$$

which is the parabolic oxidation equation ($y^2 = k_p t$) in its differential form.

This simplification is easily performed by extending the exponential functions in (6) and omitting the higher powers of the exponents. This is very nearly the same as omitting p^2 in the expression $6 + p^2$, where p in our case is given by:

$$p = \mp \lambda z \Delta G / 2RTy \quad (8)$$

The parabolic equation should thus be expected to represent the oxidation rate within about 10 % accuracy when $p^2 < 6/10$, which means:

$$y > \mp 0.65 \lambda z \Delta G / RT \quad (9)$$

A similar requirement has previously been pointed out by Hoar [3].

The inverse logarithmic equation. At sufficiently low temperatures and small film thicknesses, one of the exponential terms in (6) vanishes as compared to the other one, and it is easily seen that the general equation under these conditions may be reduced to:

$$\frac{dy}{dt} = \sum_i K_i C_{i,y} \exp\left(\mp \frac{\lambda_i z_i \Delta G}{2RTy}\right) \quad (10)$$

Through integration and some simplification we obtain:

$$\frac{1}{y} = \sum_i \pm 2.303 \frac{2RT}{\lambda_i z_i \Delta G} \left[\log\left(\frac{K_i C_{i,y}}{y}\right) + \log\ t\right] \tag{11}$$

assuming $y = 0$ when $t = 0$.

For small values of y, log $1/y$ may be considered a constant as compared to $1/y$, whereby (11) represents a so called inverse logarithmic oxidation equation ($1/y = \mathrm{A} - \mathrm{B} \log t$). It also gives an interpretation of the constants (A and B) in this equation.

In integrating (10) to obtain (11), one has to make the requirement that exp $(\pm\ \lambda z \Delta G/2RTy) \ll 1$, and in reducing (6) to obtain (10), the necessary requirement is that exp $(\pm\ \lambda z \Delta G/2RTy) \ll$ exp $(\mp\ \lambda z \Delta G/2RTy)$. Of these requirements, the former seems to be strongest. As this is the most difficult one to evaluate, however, we shall here assume that the other requirement is sufficient.

The inverse logarithmic equation should thus represent the general equation within about 10 % accuracy when

$$y < \mp\ \lambda z \Delta G/2.32\ RT \tag{12}$$

Also this requirement has previously been pointed out by Hoar [3].

The cubic equation. Under conditions at which exp(p) — exp$(-p)$ with sufficient accuracy might be replaced by ap^2, where a is a constant and p as given by eqn. (8), the general equation reduces to:

$$\frac{\mathrm{d}y}{\mathrm{d}t} = \frac{a}{y^2} \sum_i K_i C_{i,y} \left(\frac{\lambda_i z_i \Delta G}{2RT}\right)^2 \tag{13}$$

when $\Delta C/C_y$ is negligible compared to 1.

This is the cubic oxidation equation ($y^3 = k_c t +$ const.) in its differential form, and it gives an interpretation of the cubic rate constant (k_c).

In Fig. 1, a graphical test is made on the validity of the approximation on which the reduction of the general equation to the cubic type of equation is based. As may be seen from this test, the approximation may be considered valid with reasonable accuracy through at least a part of the film-thickness region between those covered by the inverse logarithmic equation and the parabolic equation, respectively.

In accordance with the requirements suggested there (Fig. 1), the following film-thickness limits may be given for the validity of the cubic approximation:

$$\mp\ \lambda z \Delta G/1.72\ RT > y > \mp\ \lambda z \Delta G/2.32\ RT \tag{14}$$

To our knowledge, this is the first explanation of the cubic oxidation equation not implying any assumptions as to the semiconductor properties of the oxide. The cubic equation derived by Cabrera and Mott [9] and the one derived by Engell, Hauffe, and Ilschner [10] are only valid for p-type conducting oxides. Rutile, for instance, which we are going to discuss below, is an n-conductor [11–13].

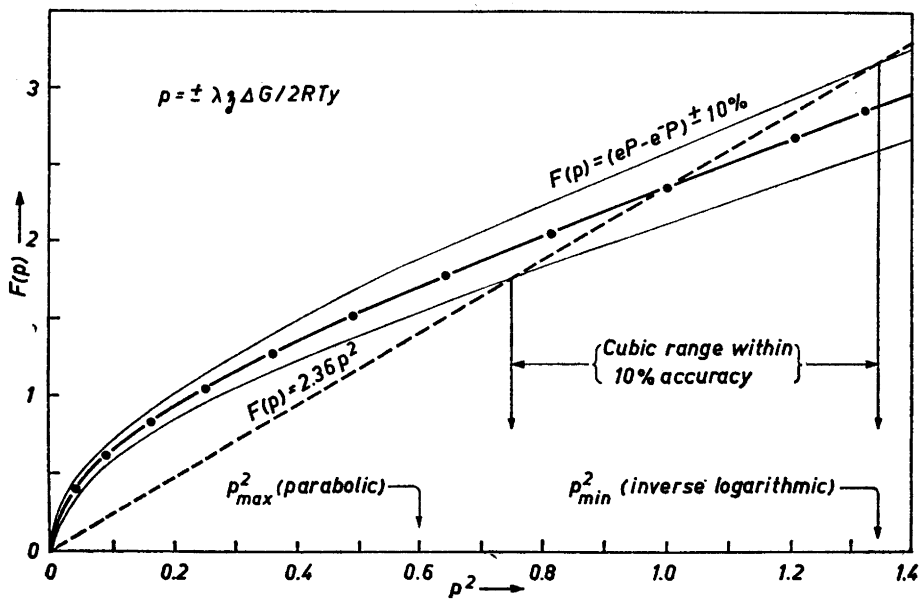


Fig. 1. Test on the validity of the "cubic" approximation (see eqn. 13).

By comparing the expressions for the parabolic (7) and the cubic (13) equations it is seen that one should expect to find the same Arrhenius energy of activation for the cubic as for the parabolic oxidation of a metal, provided the variation with temperature of the pre-exponential factors might be neglected. This could possibly be used as a basis for testing if an observed cubic and parabolic oxidation of a metal should have a common root in the general equation, or if the cubic oxidation should have another origin.

4.0 APPLICATION OF THE DEDUCTIONS

As an example, we shall here apply the above deductions to the special case of titanium being oxidized to rutile.

To simplify the discussion, we shall at first assume that it is mainly a titanium transport in rutile ($z = \pm 4$), that $\lambda = 3$ Å (which is close to the shortest Ti—Ti distance in rutile [15,16]), and that the oxidation takes place at an oxygen pressure of one atmosphere. On this basis, and using the ΔG-values given by Mah, Kelley, Gellert, King, and O'Brien [22] for rutile (see Table 1), one may from (9), (12), and (14) calculate the limiting film-thickness values for the validity of the parabolic (7), the cubic (13), and the inverse logarithmic (11) equations.

Table 1. Energy of formation of one gram-equivalent rutile at an oxygen pressure of one atmosphere (Ref.[22])

T (°K)	600	700	800	900	1 000
$-\Delta G$ (Kcal)	49.75	48.75	47.75	46.75	45.50

The results of some calculations of this kind are graphically presented in Fig. 2, where the various validity regions and their temperature dependence are clearly shown. The weight increase (w) connected to the film thickening (y), is also indicated. The following formula have been used in relating w to y:

$$w = \frac{32}{47.9 + 32} 4.2\ y$$

The shortest distance between interstitial positions for titanium ions in rutile has been shown [16] to be the half only of the shortest Ti—Ti distance in an ideal rutile lattice. If this should be determining for the elementary diffusion step, one should have to lower the film-thickness values in Fig. 2 by a factor of two in case the interstitial transport mechanism predominates over the vacancy mechanism. This is easily seen from (9), (12), and (14).

If it should be mainly an oxygen transport in rutile ($z = \pm 2$), and if a λ-value of 3 Å should apply also to this transport, the limiting film-thickness values should also in this case be the half only of those calculated on the assumption of a titanium vacancy transport (Fig. 2).

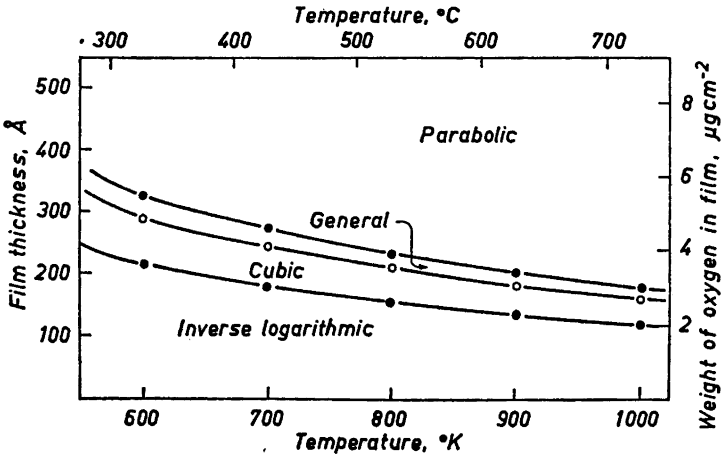


Fig. 2. Calculated regions for the validity of the varius rate equations in the case of titanium being oxidized to rutile (see text for underlying assumptions).

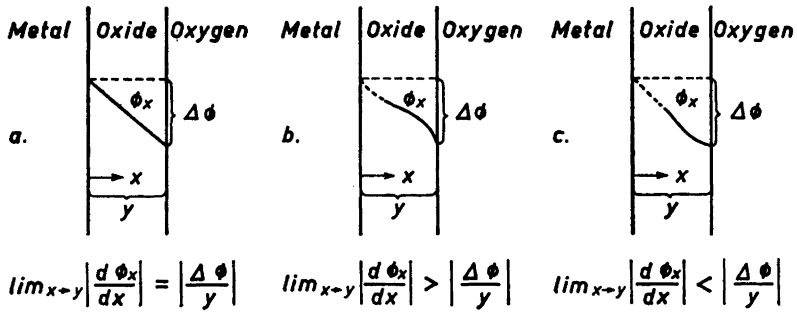


Fig. 3. Hypothetical variations of electric potential within oxide films.

If the oxidation should be governed by grain boundary diffusion instead of volume diffusion, the calculations should mainly be affected by a possible difference in the λ-values for these two types of diffusion. It seems likely, however, that this difference should be rather small, whereby a possible grain boundary diffusion should not be expected to alter the above picture very much. Another point is that a grain boundary diffusion — because of a lower energy of activation — may increase the oxidation rate and thereby decrease *the duration* of the inverse logarithmic and cubic types of oxidation at a given temperature. It is here important clearly to distinguish between time periods and film-thickness regions.

The correctness of calculations of this kind depends on the validity of the approximations (4). Of these, assumption (4b), which implies

$$\lim_{x \to y} (\mathrm{d}\Phi/\mathrm{d}x) = \Delta\Phi/y$$

probably is the most questionable one. The physical significance of this assumption is somewhat illustrated by the sketches in Fig. 3. From the general knowledge of space charge phenomena in phase boundary layers [7,17], the situation sketched in Fig. 3b seems the most probable one. This means that the limiting film-thickness values calculated from (9), (12), and (14) would be too low. It seems very difficult at present to estimate the actual electric potential gradient at the oxide/oxygen interface, to which gradient the limiting film-thicknesses should be proportional.

5.0 SUMMARY AND CONCLUSION

It has been shown how a general oxidation equation under certain conditions may be derived from the theory of absolute reaction rates, chapter 2.0, and how this equation may be represented by an inverse logarithmic, a cubic, and a parabolic type of equation at low, medium, and high values, respectively, of the oxide film thickness (chapter 3.0). These derivations do not involve any assumptions as to the semi-conductor properties of the oxide, and should apply just as well to metals forming *p*-type oxides as to those forming *n*-type oxides.

The general equation — and all the deductions made from this equation — are only valid for cases in which the oxidation is controlled by the rate of transport of material (not electrons) in the oxide film, and in which the area undergoing oxidation is constant with time. When these conditions are sufficiently satisfied, one should thus expect to observe oxidation/time curves running smoothly from an initial period of inverse logarithmic oxidation, through a medium period of cubic oxidation and general oxidation, into a final period of parabolic oxidation.

The "transitions" between the oxidation equations ought not occur at too low values of the film thickness if these values are to be measured with reasonable accuracy. From (9), (12), and (14) it is seen that metals with a high free energy of oxidation are to be expected the most suitable in this respect. Most of these unnoble metals, however, either do not fulfill the condtions on which the theory is based, or they have unsuitably high oxidation rates. All considerations of this kind taken into account, it seems that titanium might be one of the most suitable metals for testing the above theory.

Some approximate calculations have been made, therefore, of the film-thickness regions in which one should expect the inverse logarithmic, the cubic, and the parabolic equations to be satisfactory substitutes for the genral equation in the case of titanium being oxidized to rutile (chapter 4.0).

From previous work on the oxidation of titanium [17,18] is known, however, that titanium at low oxide film-thickness and low temperatures usually is not oxidized according to an inverse logarithmic equation, but rather according to a direct logarithmic one, and that this logarithmic film growth probably is best explained by the theory of Evans [19] based on the assumption of the formation of cavities at the metal/oxide interface. This assumption implies that the effective area undergoing oxidation is not constant, whereby one of the conditions for the validity of the general equation is not satisfied.

As generally discussed by Birchenall [20] and quite recently shown by Engell and Wever [21] in the case of iron, there seems to be an inherent tendency of the oxide film to try to follow the retreating metal interface by a plastic flow mechanism, thereby counteracting the cavity formation. It seems quite reasonable to assume that this effect also is present in the case of titanium, and probably also that it increases in importance both with increasing film thickness and with increasing temperature, whereby the basic conditions for the validity of the general oxidation equation subsequently may be reached.

A further discussion of this matter will be given in a separate paper on the oxidation mechanism of titanium.

Acknowledgement. This work forms part of a research project sponsored by the *Air Research and Development Command*, US Air Force, through its European Office under Contract AF 61(514)-892.

The author is moreover much indebted to Cand. real. J. Markali, Siv.ing. H. Kjøllesdal, Siv.ing. J. Lothe and other members of the staff at the Central Institute for Industrial Research, Blindern, Oslo, Norway, for helpful discussions.

REFERENCES

1. Glasstone, S., Laidler, K. J. and Eyring, H. *Theory of Rate Processes*, Mc Graw-Hill, New York 1941.
2. Evans, U.R. *Revs. Pure and Appl. Chem. Australia* **5** (1955) 1.
3. Hoar, T. P. *J. Chim. Phys.* **53** (1956) 826.
4. Gulbransen, E. A. *Ann. N. Y. Acad. Sci.* **58** (1954) 830.
5. Frenkel, J. *Z. Physik.* **35** (1926) 652.
6. Schottky, W. *Z. physik. Chem.* **(B) 11** (1930) 163.
7. Hauffe, K. *Reaktionen in und an festen Stoffen*, Springer Verlag, Berlin 1955.
8. Wagner, C. *Z. physik. Chem.* **(B) 21** (1933) 25.
9. Cabrera, N. and Mott, N. F. *Repts. Progr. in Phys.* **12** (1949) 163.
10. Engell, H. J., Hauffe, K. and Ilschner, B. *Z. Elektrochem.* **58** (1954) 478.
11. Breckenridge, R. G. and Hosler, W. R. *Phys. Rev.* **83** (1951) 227.
12. Hauffe, K., Grunewald, H. and Tränckler-Greese, R. *Z. Elektrochem.* **56** (1952) 937.
13. Cronemeyer, D. C. *Electrical and Optical Properties of Rutile Single Crystals.* Technical Report 46, Laboratory for Insulation Research, M.I.T., August 1951.
14. Kofstad, P., Hauffe, K. and Kjøllesdal, H. *Acta Chem. Scand.* **12** (1958) 239.
15. Anderson, S., Collén, B., Kuylenstierna, U. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1641.
16. Hurlen, T. *Acta Chem. Scand.* **13** (1959) 365.
17. Hauffe, K. *Oxydation von Metallen und Metallegierungen*, Springer Verlag, Berlin 1956.
18. Kofstad, P. and Hauffe, K. *Werkstoffe u. Korrosion* **7** (1956) 642.
19. See Ref.[2], pp. 5–6.
20. Birchenall, C. E. *J. Electrochem. Soc.* **103** (1956) 619.
21. Engell, H. J. and Wever, F. *Acta Met.* **5** (1957) 695.
22. Mah, A. D., Kelley, K. H., Gellert, N. L., King, E. G. and O'Brien, C. J. *Thermodynamic Properties of Titanium-Oxygen Solutions and Compounds*, Bureau of Mines, U.S.A., Report of Investigations 5316, March 1957.

Received December 17, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 705–710

Studies of Mono-C-Methylquinalizarins in Relation to a Phenolic Metabolite of *Penicillium islandicum*

STEN GATENBECK

Institute of Biochemistry, University of Lund, Lund, Sweden

A polyhydroxyanthraquinone obtained from a metabolite of *Penicillium islandicum* is shown to be a methylquinalizarin. Three isomeric methylquinalizarins have been synthesized. The isolated polyhydroxyanthraquinone has been shown to be identical with one of them, 1,4,7,8-tetrahydroxy-2-methylanthraquinone.

In an earlier paper [1] a new phenolic metabolite, called compound A, was isolated from the culture medium of *Penicillium islandicum*. As this compound is easily transformed to a polyhydroxyanthraquinone by treatment with concentrated sulfuric acid, the structure of compound A may have an interest in the view of the biogenesis of polyhydroxyanthraquinones.

Since compound A is available only in very small amounts, it has not been possible to make any derivatives or degradation experiments on it, other than the transformation to the hydroxyanthraquinone. A structural analysis of the polyhydroxyanthraquinone is apt to give some information about the unknown structure of compound A. The investigation of the structure of the anthraquinone obtained from compound A will be the subject of this paper.

The elementary analysis of the unknown anthraquinone indicates a tetrahydroxymethylanthraquinone (Found: C 61.8; H 3.9. $C_{15}H_{10}O_6$ requires: C 62.9; H 3.5).

The anthraquinone shows a weak fluorescence when dissolved in acetic acid. According to Raistrick *et al.*[2] this is a characteristic property of polyhydroxyanthraquinones with two hydroxyl groups in the *para* position to each other. The occurrence of hydroxyl groups in the *para* position is further established by the strong absorption in the region 490—520 mμ which the anthraquinone shows in ethanolic solution.

When zirconium nitrate in diluted hydrochloric acid is added to an acetone solution of the anthraquinone, a blue colouration is obtained which is the usual behaviour of a polyhydroxyanthraquinone with two hydroxyl groups in the *ortho* position [3].

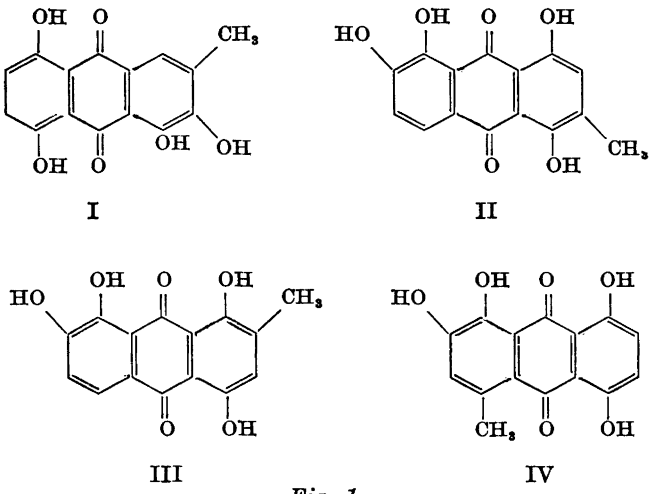


Fig. 1.

It is well known that a polyhydroxyanthraquinone with one hydroxyl group in β-position is soluble in 1 N Na_2CO_3 and there are several examples that polyhydroxyanthraquinones with more than one β-hydroxyl group is soluble in 0.5 N $NaHCO_3$. As reported in an earlier paper [1] the unknown anthraquinone is soluble in 1 N Na_2CO_3 but insoluble in 0.5 N $NaHCO_3$ which would then suggest one β-hydroxyl group in the molecule.

If the conclusions from the reactions above are true the unknown anthraquinone has to contain two hydroxyl groups in the *para* position and two hydroxyl groups in the *ortho* position. Of the latter one occupies an α-position and the other a β-position. These positions of the hydroxyl groups leave two possible alternatives. The pairs of *ortho* and *para* hydroxyl groups are either located in different rings (quinalizarin type) or the arrangement may be that of one hydroxyl group in one ring and three in the other (purpurin type). That the former alternative is the most probable is shown by two further properties of the unknown anthraquinone.

Polyhydroxyanthraquinones with hydroxyl groups in *para* position are oxidized by lead tetraacetate in glacial acetic acid to coloured diquinoid compounds. All polyhydroxyanthraquinones with *para* hydroxyl groups tested form a rather stable colouration with this reagent except for purpurin whose colour disappears rapidly since it is very easily oxidized. The unknown anthraquinone gives a stable colouration indistinguishable in appearence from that of quinalizarin. The relation of the unknown anthraquinone to quinalizarin rather than to purpurin is further indicated by the similarity of the absorption spectra of the former two compounds (Fig. 4).

As a working hypothesis the unknown anthraquinone is supposed to be a C-methylquinalizarin. Its structure is further proven by synthesis as described below.

There are theoretically four different isomers of methylquinalizarin (Fig. 1).

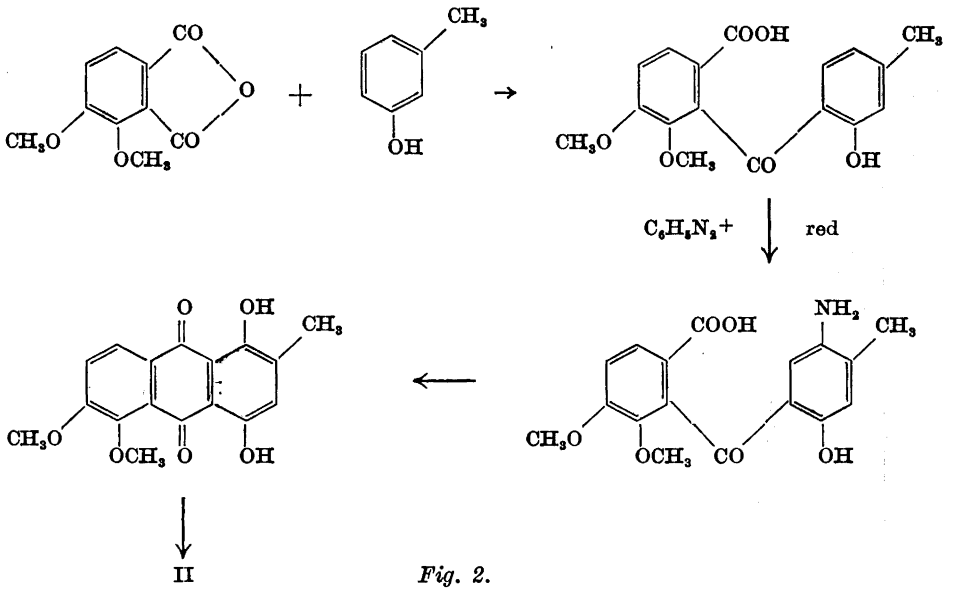


Fig. 2.

Of these four possibilities, structure IV is considered as less probable since a hydroxyanthraquinone with a C-substituent in an α-position has never been found among the mold pigments. The other three methylquinalizarins have been synthesized and their properties compared with those of the unknown anthraquinone.

I was synthesized by condensing 3,6-dihydroxyphthalic anhydride and 3-methylpyrocatechol with $AlCl_3$. I has m. p. 280—282°C and forms wool-like crystals from toluene.

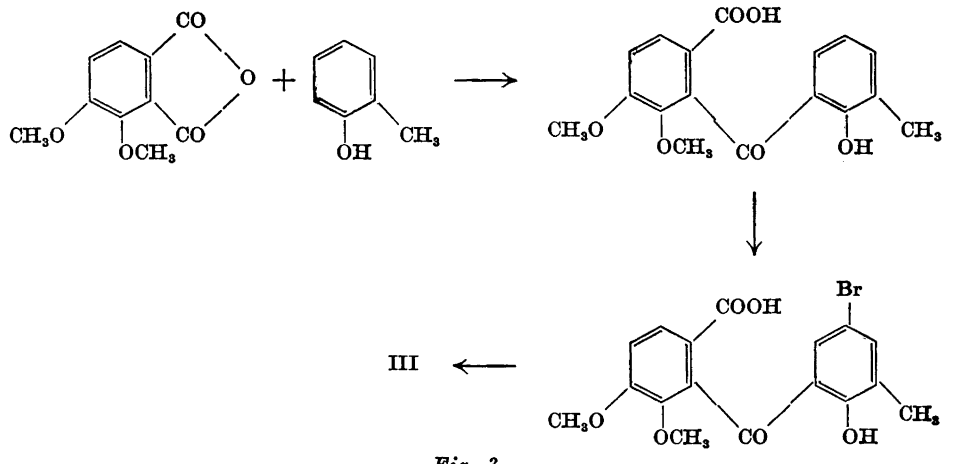


Fig. 3.

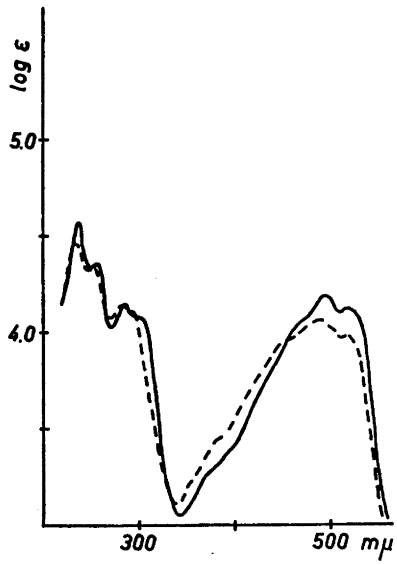


Fig. 4. Molecular extinction curves of quinalizarin — — — — — and II, III, the unknown anthraquinone ————.

II was prepared according to the reaction series in Fig. 2. It has m. p. 230°C and crystallizes in small plates from toluene.

III has been synthesized as indicated in Fig. 3 in very poor yield. It was easily obtained by a direct condensation of hemipinic anhydride and toluhydroquinone with $AlCl_3$, followed by demethylation with hydrobromic acid. III has m. p. 255°C and gives red needles when crystallized from toluene.

The unknown anthraquinone melts at 255°C and forms red needles when crystallized from toluene. The melting point of the unknown anthraquinone was not depressed on admixture with III.

II, III and the unknown anthraquinone have the same R_F value (0.75) on paper chromatogram developed in benzene-formic acid (2 %) (10:1). They have identical color reactions with methanolic magnesium acetate (blue), ethanolic ferric chloride (blue violet) and zirconium nitrate in diluted hydrochloric acid (light blue). They have practically identical absorption in UV and visible light (Fig. 4). The colors produced by I in the reactions mentioned had a tinge of red compared with those produced by the other compounds. The absorption curve of I shows small deviations from the others.

The data presented indicate the structure of the unknown anthraquinone to be identical with III.

The difference in structure of the identified anthraquinone and compound A should apparently be one molecule of water. Addition of one molecule of water to the anthraquinone gives theoretically rise to several possibilities of the structure of compound A, *e. g.* benzoylbenzoic acids, hydratized anthraquinones and naphthoquinone derivatives. These possibilities will be further investigated.

EXPERIMENTAL

Preparation of the unknown anthraquinone. Compound A was treated with concentrated H_2SO_4 (*d*, 1.84) for half an hour at room temperature. The violet sulfuric acid solution was poured onto crushed ice. The anthraquinone formed a red precipitate. After extraction with ether and evaporating the ether extract to dryness the anthraquinone was sublimed in vacuum at 150—160°C (bath temperature). The sublimate was recrystallized from acetic acid and toluene until a constant melting point (255°C) was obtained.

Synthesis of I. An intimate mixture of 0.6 g of 3,6-di-hydroxyphthalic anhydride and 0.5 g of 3-methylpyrocatechol was added in portions to 10 g of anhydrous $AlCl_3$ and 3 g of NaCl and fused together at 160°C. The temperature was kept at 160°C for 10 min after the last portion had been added. After cooling, the melt was boiled with diluted HCl for 15 min and the anthraquinone extracted with ether. The residue obtained after evaporation of the ether extract was sublimed in vacuum at 170—180°C (bath temperature). The anthraquinone was recrystallized from acetic acid and toluene (m. p. 280—282°C). (Found C 62.0; H 3.9. Calc. C 62.9; H 3.5.) At the condensation described no isomer anthraquinone was formed which was tested by paper chromatography.

The tetraacetyl derivative was prepared by boiling the anthraquinone for 2 min with acetic anhydride and a few drops of concentrated H_2SO_4. The acidic solution was poured onto crushed ice and the tetraacetylanthraquinone isolated by filtration and recrystallization from acetic acid, m. p. 208°C.

Synthesis of II. 3,4-dimethoxy-2(2-hydroxy-4-methylbenzoyl)-benzoic acid was synthesized as described by Simonsen [4].

Keeping the temperature below +5°C, a diazonium solution, prepared in the usual way from 0.852 g of aniline, was added with stirring to 2 888 g of the benzoylbenzoic acid dissolved in 50 ml of 1 N NaOH. The temperature and stirring were maintained for one hour, after which the solution was acidified with concentrated HCl. The precipitated yellow brown azocompound was filtered off, washed with water and dried at 100°C. It was recrystallized from benzene. Yield 2.4 g, m. p. 239°C.

To 2.4 g of the azocompound dissolved in 100 ml of ethanol, 8 g of solid sodium hydrosulphite was added. After heating the suspension to boiling, 300 ml of water was slowly added. The colour of the red solution faded rapidly and a lemon yellow solution was obtained. The amine precipitated in light yellow crystals on cooling the solution. (Found: N 3.9. Calc. N 4.2.) Yield 1.7 g, m. p. 148—250°C.

In a mixture of 4 ml of concentrated H_2SO_4 and 0.5 ml of water, 0.632 g of the amine was dissolved at 0°C. An equivalent amount of solid $NaNO_2$ was added in portions to the sulfuric acid solution. The reaction mixture was kept cold for half an hour at which time the solution became almost solid. Further 15 ml of concentrated H_2SO_4 were added and the temperature was raised to 150°C during half an hour and kept constant until the nitrogen evolution had ceased. After cooling to room temperature, the solution was poured onto crushed ice. The anthraquinone was filtered off and washed with water. The yield was almost quantitative. The air-dried anthraquinone was demethylated by refluxing for 12 h with a mixture of 20 ml of aqueous HBr (48 %) and 60 ml of acetic acid. The acetic acid was driven off in vacuum and the anthraquinone filtered off and washed with water. After repeated recrystallization from acetic acid and toluene it had m. p. 230°C. (Found: C 63.1; H 3.7. Calc. C 62.9; H 3.5.)

The tetraacetyl derivative was prepared in the same way as described for I, m.p. 210—212°C.

Synthesis of III. III was synthesized in two ways.

3,4-Dimethoxy-2(2-hydroxy-3-methylbenzoyl)-benzoic acid was prepared according to Jacobson and Adams [5].

In 15 ml of acetic acid, 0.709 g of the benzoylbenzoic acid (m.p. 251°C) was dissolved and then 0.115 ml of Br_2 in 5 ml of acetic acid added in small portions at 60°C. The reaction mixture was kept at 60°C for two hours and then at room temperature over night. The acetic acid was distilled off in vacuum and the residue recrystallized twice from methanol-water, m.p. 238°C.

Treatment of the bromocompound with a mixture of boric acid and concentrated H_2SO_4 at 150°C for 3 h resulted in a brown solution. III could be isolated, in very poor yield, by pouring the sulfuric acid solution on ice and extracting the water solution with ether. The ether extract was developed on a thick paper chromatogram (Whatman 3

MM) in benzene-formic acid. The anthraquinone was extracted from the paper with acetone. The acetone solution was evaporated to dryness and the anthraquinone recrystallized from acetic acid, m. p. 255°C. The amount of pure anthraquinone obtained in this way was too small for characterization.

A more convenient method for the preparation of III is to condense 0.5 g of hemipinic anhydride and 0.5 g of toluhydroquinone using the same procedure as described for the preparation of I. The demethylated product was recrystallized several times from acetic acid and toluene. Yield 0.09 g, m. p. 255°C. (Found: C 62.8; H 3.7. Calc. C 62.9; H 3.5.)

The tetraacetyl derivative was synthesized as described above, m. p. 208–210°C.

Thanks are due to Professor G. Ehrensvärd for valuable discussions.

REFERENCES

1. Gatenbeck, S. *Acta Chem. Scand.* **12** (1958) 1985.
2. Raistrick, H., Robinson, R. and Todd, A. R. *Biochem. J.* **28** (1934) 559.
3. Feigl, F. *Spot Tests in Organic Analysis*, 5th Ed. Elsevier, Amsterdam 1956, p. 193.
4. Simonsen, J. L. *J. Chem. Soc.* **125** (1924) 721.
5. Jacobson, R. A. and Adams, R. *J. Am. Chem. Soc.* **47** (1925) 288.

Received January 24, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 711–716

The Occurrence of 7β-Hydroxylated Bile Acids in Human Bile

Bile Acids and Steroids 76

JAN SJÖVALL*

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Ursodeoxycholic acid has been crystallized from human duodenal contents and identified by means of chromatographic properties, melting point, sulphuric acid spectra and infrared spectra. Column and paper chromatographic analyses and sulphuric acid spectra have indicated the presence of 3α,7β,12α-trihydroxycholanic acid in some human bile samples.

During a study of the quantitative relationships between bile acids in human bile, it was found that, besides the glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acids, some bile samples contained other compounds which behaved like bile acids on paper chromatograms [1]. The present paper gives some results regarding the nature of these compounds.

EXPERIMENTAL

The bile acid analyses were done either with gallbladder bile (collected during abdominal operations) or with duodenal contents (collected through a polyvinyl tubing introduced through the nose).

Paper chromatography of bile, duodenal contents and purified bile acids was carried out according to Sjövall [2].

Reversed-phase partition chromatography according to Sjövall [3] and Norman [4] was used for the purification of the bile acids present in the biological material.

The following phase systems were used:

Phase system	Moving phase (ml)	Stationary phase (ml)
F 1	Methanol/water 165/135	Chloroform/heptane 45/5
C 1	Methanol/water 150/150	Chloroform/*iso*octanol 15/15
C 2	Methanol/water 144/156	Chloroform/*iso*octanol 15/15

* Present address: Statens Rättskemiska Laboratorium, Stockholm 60.

When the conjugated bile acids were to be separated, about twenty volumes of ethanol were added to the bile or duodenal contents, respectively, and after filtration, the clear ethanol solution was evaporated to dryness *in vacuo*. The residue was dissolved in 1 N sodium hydroxide and, after acidification, the bile acids were extracted with butanol [5]. When the free bile acids were to be analyzed, the bile or the duodenal contents was hydrolyzed at 120°C for 6 h in 2 N sodium hydroxide. After acidification, the bile acids were extracted with ether.

Sulphuric acid spectra were recorded with a self-recording spectrophotometer (Perkin-Elmer, Spectracord 4 000) after dissolving the bile acid samples in 65 % sulphuric acid (20—40 μg per ml) and heating them for 15 min at 60°C.

RESULTS

A. 3α,7β-Dihydroxycholanic acid (ursodeoxycholic acid). Descending paper chromatography of bile and duodenal contents, with ethylene chloride/heptane 50/50 as moving phase and 70 % acetic acid as stationary phase, reveals the presence of glycocholic (GC), glycochenodeoxycholic (GCD) and glycodeoxycholic (GD) acids in human bile. In several samples, a fourth spot which is weaker than the other ones can be detected between the spots of GC and GCD. In order to purify the compound causing this spot, the conjugated bile acids from about 10 ml of gallbladder bile (patient N.A.) were subjected to reversed-phase partition chromatography with phase system C 1. The effluent from the column was collected by means of a fraction collector and titration of the fractions with 0.02 N sodium hydroxide gave titration peaks at the places of taurine conjugated bile acids, GC and GCD. The fractions containing the latter two acids were combined and paper chromatography revealed only spots of GC and GCD, respectively. The fractions between these two acids from the column chromatogram were combined and evaporated to dryness. After butanol extraction, rechromatography with phase system C 1, on a column 1/5 as large as the first column, showed a titration peak corresponding to about 3 mg of bile acid. Paper chromatography of this material produced one spot at the place of the unknown compound. The residue was then hydrolyzed and after acidification extracted with ether.

Descending paper chromatography of the hydrolyzate with ethylene chloride/heptane 20/80 as moving phase and 70 % acetic acid as stationary phase gave one spot which appeared between the spots of cholic and chenodeoxycholic acids, run simultaneously as reference compounds. The position of the spot suggested that the compound (if a bile acid) might be a dihydroxy bile acid with two equatorial hydroxyl groups which caused the relatively slow rate of movement. Therefore, ursodeoxycholic acid (3α, 7β-dihydroxycholanic acid) and hyodeoxycholic acid (3α, 6α-dihydroxycholanic acid) were used as reference compounds and the unknown substance was found to move like the acid mentioned first. The sulphuric acid spectrum of the compound, eluted from the paper chromatograms [2], was identical with that of ursodeoxycholic acid.

The taurine conjugated bile acids from the bile of patient N.A. were hydrolyzed and subjected to chromatography with phase system F 1. Titration peaks were found at places of cholic (C), ursodeoxycholic (UD) and chenodeoxycholic (CD) acids (Fig. 1). Hyodeoxycholic acid is known to be eluted at approxim-

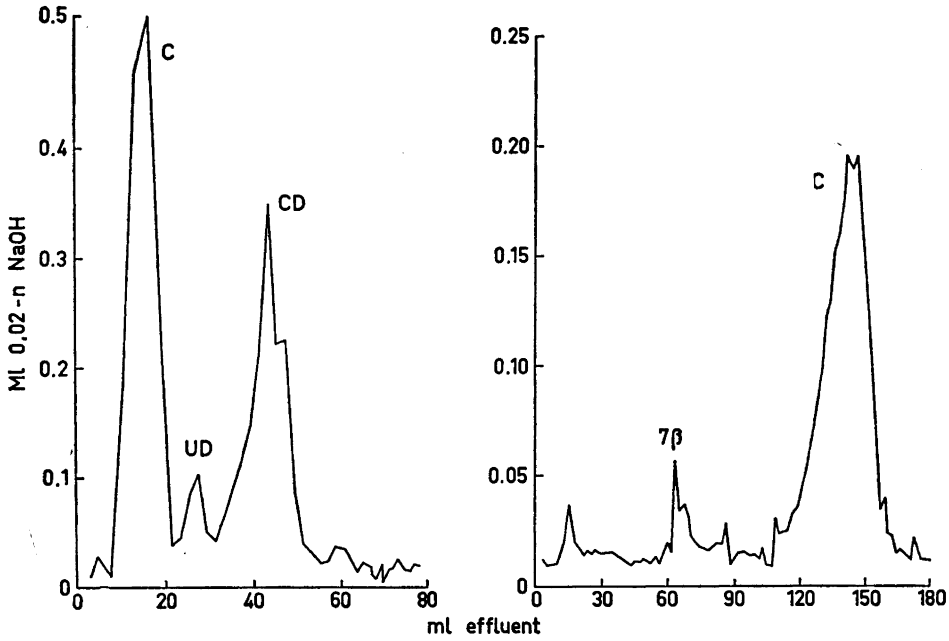


Fig. 1. Chromatography of hydrolyzed taurine conjugated bile acids from patient N.A. The front band from the chromatography with phase system F 1 (C, left curve) was rerun with phase system C 2 (right curve).

ately the same place as ursodeoxycholic acid, but paper chromatography of the peak at this place showed that the substance moved as UD.

The two samples of the compound, moving as ursodeoxycholic acid, isolated from the bile of patient N.A., were combined and weighed about 5 mg. A slow crystallization from ethyl acetate light petroleum took place, but, due to the small amount of material, only one recrystallization could be carried out resulting in crystals which had a melting point of 195—197°. The sulphuric acid spectrum was identical with that of UD.

The largest amount of the compound causing the spot between GC and GCD on the paper chromatograms was found in the duodenal contents of a one-year-old child (patient R.A.). About 10 ml of duodenal contents were hydrolyzed and the free bile acids separated with phase system F 1. Titration peaks were found at the places of C, UD and CD. The material in the fractions corresponding to UD was collected and extracted with ether from an acidified water solution. The residue, weighing about 10 mg, was dissolved in ethyl acetate and light petroleum was added. Crystals slowly appeared, were recrystallized twice, and gave about three mg of crystals which had a melting point of 201—202° not depressed by authentic ursodeoxycholic acid. Paper chromatographic behavior and sulphuric acid spectrum were identical with those of UD as was also the IR spectrum shown in Fig. 2.

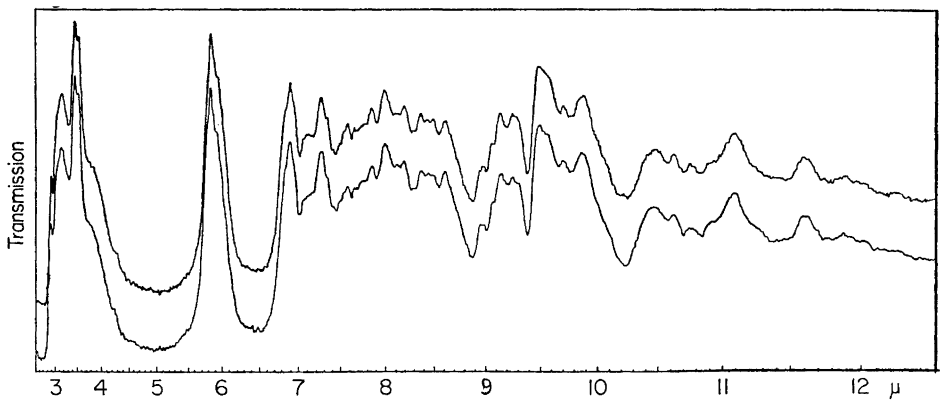


Fig. 2. Infrared spectra of authentic ursodeoxycholic acid (upper curve) and ursodeoxycholic acid isolated from the duodenal contents of patient R.A. (lower curve).

B. 3α,7β,12α-Trihydroxycholanic acid. This acid has been prepared recently by Samuelsson [6]; its presence in rat bile and feces has been demonstrated [7]. In chromatograms with phase system F 1, the acid appears together with cholic acid. In order to separate these acids, the "cholic acid bands"

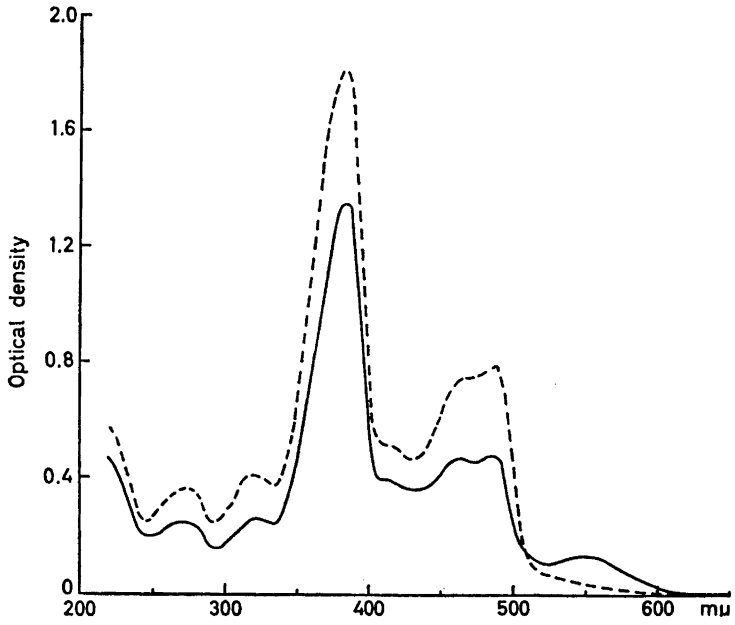


Fig. 3. Absorption spectra of 3α,7β,12α-trihydroxycholanic acid (– – – –) and the compound with identical chromatographic behaviour as this acid, isolated from the bile of patient N.A. (———). The samples were heated for 15 min at 60°C in 65 % sulphuric acid.

from patient N.A. (C, Fig. 1, left curve) and patient R.A. were subjected to chromatography with phase system C 2. The chromatogram of the band from patient N.A. is shown in Fig. 1 (right curve). A large titration peak, corresponding to cholic acid, is present (C), and there is also a minor peak at the place where 3α,7β,12α-trihydroxycholanic acid is known to be eluted [6]. Ascending paper chromatography of the material contained in these fractions, with ethylene chloride/heptane 50/50 as moving phase and 70 % acetic acid as stationary phase, showed the presence of a compound moving with the same rate as 3α,7β,12α-trihydroxycholanic acid. The sulphuric acid spectrum of this compound eluted from the chromatogram was very similar to that of 3α,7β,12α-trihydroxycholanic acid, kindly supplied by Dr. Samuelsson (Fig. 3). The low absorption maximum at 555 mμ of the compound isolated from bile, is due to the presence of small amounts of bromothymol blue used as indicator in the titration of the fractions from the column chromatography. Due to the small amounts of acid obtained from the bile (about 0.8 mg), further purification and identification could not be carried out.

The chromatogram of the "cholic acid band" from patient R.A. showed no titration peak at the place of 3α,7β,12α-trihydroxycholanic acid. However, paper chromatograms of the fractions where this acid could be expected to be eluted clearly showed a spot that had moved with the same rate as the reference acid 3α,7β,12α-trihydroxycholanic acid. If this compound was identical with the reference acid, it was present in amounts less than 1 % of the total bile acids of patient R.A.

DISCUSSION

The non-ketonic bile acids in human bile hitherto isolated are cholic, chenodeoxycholic, deoxycholic and lithocholic acids. These acids all contain hydroxyl groups in one or several of the positions 3α, 7α and 12α. Bile acids with a 7β hydroxyl group have been isolated, *e. g.* from the bile of a certain bear species and from the coypu and the rat [7-9]. The present investigation has shown the presence of ursodeoxycholic, 3α,7β-dihydroxycholanic acid, in samples of human bile and suggests the presence of 3α,7β,12α-trihydroxycholanic acid. Except in the duodenal contents of a one year old child, the ursodeoxycholic acid accounted only for a few per cent of the total bile acids as judged from the spots on the paper chromatograms. In most samples, no spot could be detected at all at the place of ursodeoxycholic acid when the amount of bile used for routine analysis of bile acids was run (a total bile acid amount of about 50 μg).

The chromatographic analysis of bile from patient N.A. showed that ursodeoxycholic acid occurred among the taurine as well as the glycine conjugated bile acids. It is therefore reasonable to assume that it was conjugated with taurine and glycine. The ratio between these conjugates was roughly equal to that of the other bile acids.

Further work must be done for the final identification of the compound behaving like 3α,7β,12α-trihydroxycholanic acid. In the rat, this acid has been shown to be formed from cholic acid, probably through the action of microorganisms in cecum [7]. Several strains of *E. coli* have been shown to oxidize

the hydroxyl group of cholic and chenodeoxycholic acids at C—7, giving a 7-keto group [10]. The 3α-hydroxy-7-ketocholanic acid, that might thus be formed from chenodeoxycholic acid in the rat cecum, is known to be converted to ursodeoxycholic acid in the rat liver [9–11]. Bile acids are known to be absorbed from the cecum [7] and ursodeoxycholic acid could thus be formed from chenodeoxycholic acid (see also Ref.[9]). Whether the 7β-hydroxylated bile acids discussed in this paper are formed in the same way, remains to be investigated. The general importance of the intestinal flora for the composition of the biliary bile acids is, however, clearly seen in the case of deoxycholic acid, which has been shown to be formed by microbial action on cholic acid in man as well as in animals [12–13].

The technical assistance of Mrs. Ann-Mari Andersson is gratefully acknowledged. This work is part of investigations supported by *Statens Medicinska Forskningsråd.*

REFERENCES

1. Sjövall, J. *Clin. Chim. Acta* **4** (1959). *In press.*
2. Sjövall, J. *Acta Chem. Scand.* **10** (1956) 1051.
3. Sjövall, J. *Acta Physiol. Scand.* **29** (1953) 232.
4. Norman, A. *Acta Chem. Scand.* **7** (1953) 1413.
5. Norman, A. *Acta Physiol. Scand.* **32** (1954) 1.
6. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) *In press.*
7. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
8. Haslewood, G. A. D. *Physiol. Rev.* **35** (1955) 178.
9. Mahowald, T. A., Yin, M. W., Matschiner, J. T., Hsia, S. L., Doisy, E. A. Jr., Elliott, W. H. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 581.
10. Bergman, S. and Norman, A. *To be published.*
11. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 236.
12. Lindstedt, S. *Arkiv Kemi* **11** (1957) 145.
13. Ekdahl, P.-H. and Sjövall, J. *Acta Chir. Scand.* **114** (1957) 439.

Received January 31, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 717–721

Physical Properties of Isotopic Forms of Azeotropic Systems Formed from Acetic Acid and Some Tertiary Amines

K. E. HOLMBERG

Section for Nuclear Chemistry, Chemical Research and Production Div., AB Atomenergi, Stockholm, Sweden

Azeotropes of CH_3COOH and CH_3COOD with pyridine, trimethylamine and triethylamine have been prepared. The boiling point differences between the H and D forms of the azeotropes are larger than those between the isotopic forms of pure acetic acid. The composition and some physical properties of the azeotropes have been determined. Upon distillation of the azeotropes, there is some separation of the nitrogen isotopes and, possibly, also of the carbon and oxygen isotopes of the carboxyl group in the acetic acid.

Acetic acid gives, with suitable tertiary amines, maximum boiling point azeotropes. In these azeotropes, the strength of the chemical bond or hydrogen bond between the components will depend on the isotopic constitution of the atoms forming the bond. Different isotopic forms of the azeotropes may therefore have different compositions (*i. e.* ratios amine:acid) and different physical properties.

The azeotropes with acetic acid or acetic acid deuterated in the carboxyl group as one component and pyridine, trimethylamine or triethylamine as the other component have been prepared and investigated with respect to composition and physical properties. After Rayleigh distillation, the isotopic composition has been determined for other elements which may be involved in the compound formation, *viz.* the nitrogen of the amine and the carbon and oxygen of the carboxyl group.

The results are collected in Tables 1 and 2. For convenience, the following abbreviations are used for the azeotropes: PyHAc, PyDAc, Me_3NHAc, Me_3NDAc, Et_3NHAc, Et_3NDAc, in spite of the fact that they are not composed of equimolecular amounts of acid and amine.

The H and D forms of the azeotropes differ rather little in composition. Except for the case with pyridine, the azeotropes seem to contain a compound consisting of one mole amine and four moles acetic acid. Measurements, not

Table 1. Composition and physical properties of the H and D forms of azeotropes of acetic acid and tertiary amines.

	PyHAc	PyDAc	Me_3NHAc	Me_3NDAc	Et_3NHAc	Et_3NDAc
Weight% of HAc or DAc	52.2	51.6	81.2	81.8	69.9	70.7
Mole% of HAc or DAc	59.0	58.0	80.95	81.3	79.7	80.0
Formula	C_5H_5N. 1.44 CH_3COOH	C_5H_5N. 1.38 CH_3COOD	$(CH_3)_3N$. 4.25 CH_3COOH	$(CH_3)_3N$. 4.35 CH_3COOD	$(C_2H_5)_3N$. 3.92 CH_3COOH	$(C_2H_5)_3N$. 4.00 CH_3COOD
Boiling point at 760 mm Hg, °C	138.00	136.66	152.9	151.8	161.85	161.01
Boiling point difference, °C	1.34		1.1		0.84	
$(dp/dt)_{760}$, mm Hg/°C	21.3		24.1		27.0	
Melting point, °C	—51		glass		glass	
d_4^{25}, g/ml	1.03678	1.04114	1.03887	1.05365	1.01638	1.02834
η_{25}, centipoise	2.21	2.06	11.35	11.47	14.47	14.59
n_D^{25}	1.4520	1.4510	1.4087	1.4080	1.4218	1.4212

Table 2. Ratio of the vapour pressures of H and D forms of azeotropes and separation factors for other isotopes upon distillation. The errors given refer to the mass spectrometric measurements.

	PyHAc at 138°C	Me_3NHAc at 153°C	Et_3NHAc at 162°C
$p(D)/p(H)$	1.038	1.032	1.030
$\frac{(^{14}N/^{15}N)_{vap}}{(^{14}N/^{15}N)_{liq}}$	1.0034 ± 0.0012	1.0097 ± 0.0012	1.0075 ± 0.0012
$\frac{(^{18}O/^{16}O)_{vap}}{(^{18}O/^{16}O)_{liq}}$	1.0007 ± 0.0009	1.0010 ± 0.0009	1.0018 ± 0.0009
$\frac{(^{13}C/^{12}C)_{vap}}{(^{13}C/^{12}C)_{liq}}$	1.0017 ± 0.0006	1.0005 ± 0.0006	1.0012 ± 0.0006

reproduced here, of the density and viscosity of mixtures of Py and HAc have shown a maximum deviation from linear relationship at about the composition Py.4HAc. It is therefore possible that the liquid pyridine-acetic acid azeotrope is a mixture of a compound Py.4HAc together with free, or less strongly bound, pyridine.

The ratio of the vapour pressures of the H and D forms of an azeotrope at the boiling point (b.p.) of the H form is larger than the corresponding ratio for acetic acid at its normal b.p. Rabinowitsch *et al.*[1] have investigated the vapour pressures of CH_3COOH and CH_3COOD at different temperatures. From their results, $p(CH_3COOD)/p(CH_3COOH)$ is 1.017 at 118°C and passes through a maximum of 1.032 at about 50°C. They give a qualitative explanation of their results.

Similar points of view may perhaps apply for the azeotropes but it is felt that a discussion is of no avail so long as we do not know the composition and amounts of the various possible associates occurring in the liquid and the vapour. An investigation to clarify this situation was beyond the scope of the present work.

The ratio of the vapour pressures of the H and D forms of an azeotrope is not to be confused with the separation factor α for H/D on distillation of the azeotrope, $\alpha = (H/D)_{vap}/(H/D)_{liq}$. The two quantities may differ since the composition of the H and D forms of the azeotrope differ, since part of the acetic acid distills as double molecules and also since the vapour phase contains a certain amount of amine.

The separation factors for the N isotopes are quite large considering the rather high distillation temperatures. The low value for the PyHAc azeotrope may be explained by the assumption above that only part of the pyridine is strongly bound by the acetic acid as Py.4HAc. If all of the Py had been bound in this way, the separation factor would be expected to be

$$1 + 0.0034\ (4/1.44) = 1.0094$$

in fair agreement with the values obtained for the other azeotropes. Of course the different structure of Py compared with the other tertiary amines may offer another explanation.

The separation factors for ^{13}C and ^{18}O are very small — of the same order as the errors of the mass spectrometer measurements. However, since they occur in the same direction in all cases, they may have some real meaning. These factors indicate that the C and O atoms of the carboxyl group are very little influenced by the formation of a compound between HAc and an amine.

EXPERIMENTAL

Preparations

Deuterated acetic acid (CH_3COOD) was prepared from acetic anhydride and D_2O (99.7 %). The acetic anhydride was purified by distillation through a 1 meter column with glass helices and reacted with the calculated amount of D_2O. The product was distilled using the 1 meter column to give a product with a 0.1°C boiling point range. The boiling point was compared with that of ordinary CH_3COOH prepared in the same way. HAc b.p.$_{760}$ = 117.70°C, DAc b.p.$_{760}$ = 116.80°C. In another experiment, however, a b.p. difference of only 0.63°C was found.

The azeotropes. Pyridine was mixed with an equal weight of HAc or DAc and the mixture was then fractionated to give a product with a 0.1—0.2°C b.p. range. Et_3NHAc and Et_3NDAc were prepared in a similar way.

HAc was added to 25 % aqueous Me_3N, water was distilled off and the remaining Me_3NHAc fractionated to a narrow b.p. range.

Me_3N was expelled by heating from its 25 % aqueous solution. The product was dried with solid NaOH and absorbed in anhydrous DAc. The resulting Me_3NDAc was fractionated to a narrow b.p. range.

Analyses

A weighed amount of azeotrope dissolved in water was washed through a cation exchanger in the H^+ form. The HAc in the eluate was titrated with standard alkali.

Physical properties

Boiling points were determined in a special all-glass apparatus with thermometer pocket and reflux cooler. Mercury thermometers were used and the pockets were filled with mercury. The H and D forms of the azeotrope were measured simultaneously. Stem and pressure corrections were applied. There may have been some uncertainty in these corrections and thus the absolute values of the boiling points may not be very exact. The b.p. differences, however, are probably correct to ±0.02°C. The rate of change of b.p. with pressure has been determined by a direct comparison with the b.p. of water at three pressures near atmospheric, generally p, $p + 20$, and $p + 40$ mm Hg.

The melting point of PyHAc was determined on a small sample in a test tube and the value is thus rather rough. It was taken as the temperature at which the greater part of the sample had passed into the liquid state. Me_3NHAc and Et_3NHAc could not be made to crystallize. They formed glasses which began to soften at about −100°C.

Densities were determined with a 10 ml pycnometer at 25.00°C.

Viscosities were determined with a probable error of less than 1 % using an Ostwald viscometer.

Rayleigh distillations

A 100—200 ml sample was distilled from a 250 ml flask surrounded by a vapour thermostat at a few (1—4) degrees above the b.p. of the azeotrope. The bottom of the flask rested on an asbestos plate with a hole through which the flask was heated with a small flame. The heating was such that the distillation occurred without the formation of bubbles. The vapour thermostat prevented refluxing from the walls of the flask and from the tube leading to the condenser. 5—10 % of the total amount were taken as a first distillate and about an equal amount was left behind as residue. The first distillate and the residue were analysed for the isotope abundance of ^{15}N, and of ^{13}C and ^{18}O in the carboxyl group of the acetic acid.

The precautions described above were such as to minimize all possibilities for introducing errors giving too high values for the separation factor. Several tests with H_2O—HDO mixtures gave separation factors of 1.0222—1.0235 instead of the accepted value of 1.026 *i. e.* the separation obtained was only 85—90 % of the expected value. Similar errors may occur in the case of the azeotropic systems. No corrections have been introduced, however, since it is not certain that the errors will be of the same magnitude as those in the case of water.

Preparations for isotopic analyses

The N of the amine was transformed by Kjeldahl digestion to NH_3 and then to H_4NCl. Pyridine was digested for several days in the presence of mercury sulphate to insure complete decomposition.

Calculated amounts of $Ba(OH)_2$ solution and sample were evaporated and dried to form anhydrous Ba acetate. This was pyrolyzed in a slow stream of nitrogen to give $BaCO_3$ which was reacted in an evacuated tube with concentrated (96 %) sulphuric acid. The CO_2 formed was purified by freezing out with liquid air and analyzed for ^{13}C and ^{18}O.

Acknowledgement. Thanks are due to Mr. R. Cedenholt for assistance with the experimental work and to AB Atomenergi for permission to publish the results. The isotope abundance measurements were performed by the Mass Spectrometer Laboratory, Karolinska Institutet, Stockholm.

REFERENCE

1. Rabinovitsch, I. B., Sokolov, N. N. and Artjuchin, P. I. *Doklady Akad. Nauk SSSR* **105** (1955) 762.

Received January 31, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 722–736

Equilibrium Studies of Polyanions

III. Silicate Ions in $NaClO_4$ Medium

GÖSTA LAGERSTRÖM*

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Equilibria in silicate solutions have been studied in 0.5 m (molal) $Na(ClO_4)$ medium at 25°C and 50°C, and in 3 m $Na(ClO_4)$ medium at 25°C, using emf's with a hydrogen electrode, at a series of total Si concentrations, B. The data are given in the form of curves $Z(\log h)_B$, or $Z(\log [OH^-])_B$, where Z is the average number of OH^- bound per $Si(OH)_4$ in the solution, thus the average negative charge per Si. The data for 0.5 m medium, $Z = 0.70$ to 1.0, could be explained in terms of the species $Si(OH)_4$, $SiO(OH)_3^-$, $SiO_2(OH)_2^{2-}$, and $Si_4O_6(OH)_2^{2-}$. The data in 3 m medium, $Z = 0.8$ to 1.6, could be explained assuming equilibria between the three mononuclear species mentioned, and in addition $Si_2O_3(OH)_4^{2-}$ and $Si_4O_8(OH)_4^{4-}$. The equilibrium constants arrived at are given in Table 5; they are not considered as final. Equilibria are rapidly obtained, and the solutions are clear, as long as no part of the solution is allowed to pass, even momentarily, into the "instability range", an area in the (B, log h) field, where a colloid precipitates. This may have been one source of error in older work.

Although silicate solutions are being used extensively for various purposes, very little is known with certainty about the formulas of the silicon-containing species. This is borne out by the survey of previous work in the following which, however, is by no means a complete historical review; for such, the reader is referred to monographs by Iler [1] and Vail [2].

The aim of the present work has been to study equilibria in silicate solutions by experimental and mathematical methods worked out in this laboratory, thus to measure data over as wide a range of concentrations as possible, and to deduce the formulas of the species present and the equilibrium constants involved.

As in the previous work on borate equilibria (Part II [3]), no electrode could be found which responds to the free anion concentration. On the other hand, the concentrations of H^+ (and OH^-) could be measured accurately using a hydrogen electrode. As previously, they were recalculated to the form $Z(\log h)_B$, and these curves (Figs 2, 3, and 4) were the basis of the calculations.

* Present address: Columbia-Southern Chemical Corporation, Barberton, Ohio, USA.

An initial difficulty was the existence of an "instability range" of the solutions, which was eventually found out and will be discussed below.

The present work, which was, like the following Part IV, generously supported by Columbia-Southern Chemical Corporation, was already completed in 1955, though the publication has been delayed for external reasons.

SYMBOLS

The symbols are in general the same as in Part II[3]. Thus, B is the total concentration of Si in the solution, b the free concentration, $[Si(OH)_4]$, h the concentration of free H^+, H the analytical excess of H^+ over H_2O and $Si(OH)_4$ (H was negative in most of our experiments), and Z is the average number of OH^- bound per $Si(OH)_4$, thus the average charge per Si.

The silicate species, or complexes, will (in analogy with other work from this laboratory [3,4]) be written as A_pB_q where A stands for a proton deficiency, ($-H^+$), or OH^-, and B for $Si(OH)_4$. Equilibrium measurements like the present ones cannot distinguish between species with various contents of water, and only indicate the charge, $p-$, and the number of Si atoms, q. For instance, A_2B_4 stands for a complex with 4 Si atoms and the charge -2. It might have been written as $Si_4O_9^{2-}$, but this is certainly not correct. In the final formulas of Table 5, it is written as $Si_4O_6(OH)_6^{2-}$ which seems plausible from what is known of the structural chemistry of silicates. However, it is only the charge and the number of Si which can be concluded from the equilibrium data.

To conform with the notations in the IUPAC Tables of Stability constants [5] the formation constants for the complexes will be denoted as β_{pq} when they are formed from $Si(OH)_4$ and OH^-, and with $^*\beta_{pq}$, when they are written with $Si(OH)_4$ and H^+ as reactants (see Table 5).

The composition of silicate solutions has often been expressed by r, the "ratio" SiO_2/Na_2O, in moles (or grams, which is practically the same). To transform r to the quantity Z, which is more practical for equilibrium calculations, we may use the relations

$$BZ = h - H - [OH^-] = h - H - K_w h^{-1} \qquad (1)$$
$$H = -2B/r$$

In solutions with silicate anions, h is always negligible in comparison with BZ, and so is $[OH^-]$, at least for $Z \leq 1$. Then we have simply $Z = 2/r$.

EXPERIMENTAL

Reagents

Sodium perchlorate, perchloric acid, and *silver perchlorate* were prepared and analysed as described in Part II[3].

From p.a. *sodium hydroxide,* a 50 % solution was prepared and filtered through a G4 Jena glass filter. Dilute solutions were prepared from this stock solution and deaerated water. The solutions were kept in polythene bottles which had previously been soaked with warm dilute alkali.

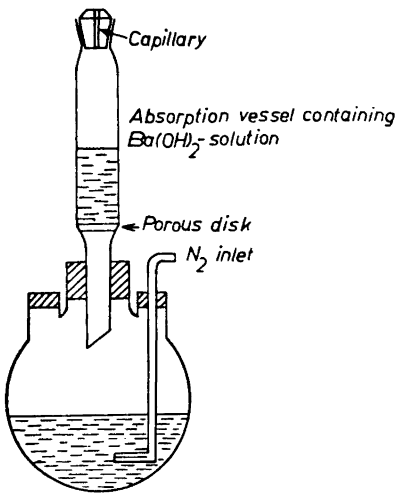


Fig. 1. Apparatus for determining carbonate ions in alkaline solution.

To analyse the 50 % stock solution, a measured quantity of it was transferred to a flask (Fig. 1), filled with nitrogen gas free from CO_2. The absorption vessel was filled with 2/3 of its volume of a $Ba(OH)_2$ solution. The NaOH solution was rapidly acidified with dilute sulfuric acid, and the CO_2 was driven off by heating and passing a stream of nitrogen. The precipitate of $BaCO_3$ was sucked off, washed with 50 % ethanol and dissolved in dilute HCl. $Ba(IO_3)_2$ was precipitated, separated, and determined by titration with $Na_2S_2O_3$ after acidification.

The dilute NaOH solutions were analysed as described in Part II[3]. The ratio CO_3^{2-}/OH^- was always less than 0.001.

A silicic acid sol was prepared by recrystallizing "sodium metasilicate", $Na_2H_2SiO_4(H_2O)_8$ twice from 5 % NaOH, dissolving it in water, and passing the solution through a H^+-saturated Dowex-50 ion-exchanger. A weighed sample of the sol was evaporated in a Pt crucible, and the residue ignited for 2 h at 950°C. The crucible was weighed, treated with HF in the usual way, and weighed again. The difference gave the concentration of SiO_2 in the solution. The sol proved to be extremely pure, with no components other than SiO_2 and water in measurable amounts.

Sodium silicate solutions were not prepared from solid sodium silicate since this product after storage will be contaminated with carbonate, which cannot then be removed. Instead, silicate solutions were prepared from silicic acid sol, NaOH solution or perchloric acid, deaerated water, and $NaClO_4$ solution.

In the analysis of the stock solutions, the concentrations were obtained in M (mole/liter), since all additions were made from burets. To recalculate the concentrations to the unit m (mole/kg H_2O), the water content of each solution was calculated from its concentration in M, and its density. All density determinations and mixing operations were carried out in a room thermostated at 25°C.

Apparatus

Since it was feared that glassware would be attacked by the alkaline solutions, a considerable effort was made to use polythene vessels. It was not possible to obtain commercially a polythene vessel of the desired size and shape and so attempts were made to coat a suitable glass vessel with polythene. However, the coating was easily scratched, and, once this was done, the alkaline solution crept in between the glass and the coating, which was a nuisance.

Next, attempts were made with all-polythene apparatus. It proved a difficult task to fit an ordinary polythene bottle with extra air-tight necks. Moreover, polythene is water-repellent; a great number of droplets splashed from the rinsing gas stream etc, and kept sitting on the polythene surface. This caused an error in the composition of the solution, which could be overcome by occasional shaking but this was objectionable for other reasons. An additional disadvantage was that polythene is opaque so that the formation of a colloid or a precipitate in the titration vessel could not be observed.

Hence, the use of glass vessels was reconsidered. The dissolution of Si from Jena-Geräte glass was investigated in a series of experiments, in which the conditions (time, volume, stirring, composition of solutions) during the emf titrations was reproduced as carefully as possible. If the Jena-Geräte glass, before the experiment, had been treated first with acid and then with alkali, the maximum concentration of dissolved Si was 0.03 mm (3×10^{-5} mole/kg H_2O). When the glass had first been treated only with alkali, no more than one tenth of this concentration was obtained. For 50°C, the figures are approximately doubled. Considering that the total concentration, B, of Si added in the experiments is 2.5—80 mm, little objection can be raised to using glass vessels and burets in the study. In order to depress the error in the experiments at 50°C, the buret containing a strongly alkaline solution was kept at 25°C and was only inserted in the vessel very rapidly each time an addition was made.

The *titration vessel* was made of Jena-Geräte glass, which proved more resistant to alkali than Pyrex. The vessel was of the same type as described in other papers from this institute, and the salt bridge was of the "Wilhelm" type [6]. The hydrogen was purified, the hydrogen and Ag, AgCl electrodes were prepared, and the emf measured as described in Part II[3].

TITRATION PROCEDURES

The cell, including the titration vessel, was kept in an oil thermostat at 25.0 ± 0.1°C, and the whole equipment kept in a room thermostated at 25°C. For the experiments at 50°C, an air thermostat of 50.0 ± 0.5°C was used.

In the various solutions, h, or (OH^-) which is equivalent, was determined by means of the cell

—Pd, H_2(1 atm)/solution studied//SE+

where the reference silver electrode is

I m $NaClO_4$//(I — 0.01)m $NaClO_4$, 0.01 m $AgClO_4$/AgCl, Ag = SE

Assuming the activity factors to be constant, the E may be represented by the equations

$$E = E_{0a} + 59.15 \log [OH^-] + E_j = E_{0a} + 59.15 \log K_w - 59.15 \log h + E_j$$

As in Part II[3], the constant E_{0a} was determined using solutions of known [OH^-]; such measurements also gave the diffusion potential $E_j = 60[OH^-]$ for $I = 0.5$, and $E_j = 10[OH^-]$ for $I = 3.0$. The values obtained for K_w are given in Table 5.

As ionic medium, 0.5 or 3.0 m $Na(ClO_4)$ was used ($I = [Na^+] = 0.5$ or 3.0.) For each titration, two solutions were prepared, S for the titration vessel, and T for the buret, both with the same total silicate concentration, B. To vary Z and log h, two different procedures were used, both extensively.

Procedure 1:

S_1: I m Na^+, B m $Si(OH)_4$, $H = -C_1(C_1$ m $OH^-)$, $(I - C_1)$ m ClO_4^-

T_1: I m Na^+, B m $Si(OH)_4$, $H = C_2(C_2$ m $H^+)$, $(I + C_2)$ m ClO_4^-

Procedure 2:

S_2: I m Na^+, B m $Si(OH)_4$, $H = -C_3(C_3$ m $OH^-)$, $(I - C_3)$ m ClO_4^-

T_2: I m Na^+, B m $Si(OH)_4$, $H = -C_4(C_4$ m $OH^-)$, $(I - C_4)$ m ClO_4^-

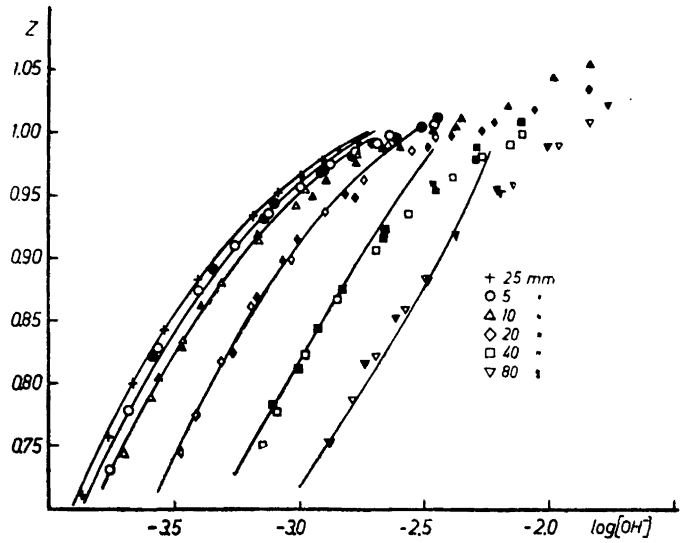


Fig. 2. Silicate equilibria, $Z(\log [OH^-])_B$, 0.5 m $Na(ClO_4)$, 25°C. Procedure 1, open symbols; procedure 2, filled symbols. Curves calculated with equilibrium constants in Table 3.

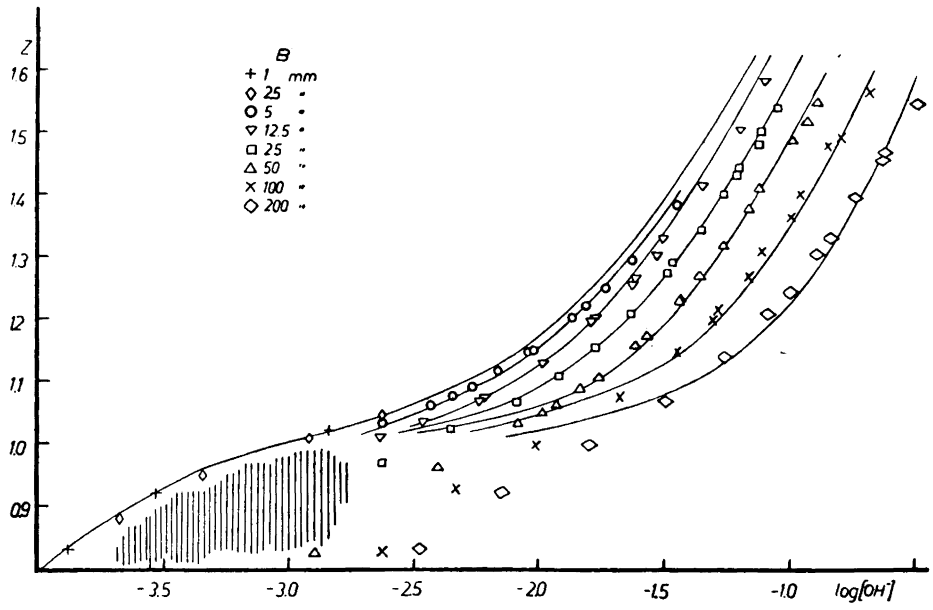


Fig. 3. Silicate equilibria, $Z(\log [OH^-])_B$ for 3 m $Na(ClO_4)$, 25°C. The instability range is shaded. Curves calculated with equilibrium constants in Table 5.

Table 1. Typical titration according to procedure 1.

S: 0.500 m Na^+, 0.020 m $Si(OH)_4$, 0.02595 m OH^-, 0.474 m ClO_4^-. Total water content = 59.67 g
T (v ml): 0.500 m Na^+, 0.020 m $Si(OH)_4$, 0.01939 m H^+, 0.500 m ClO_4^-. 1 ml contains 0.976 g H_2O

v ml	*E* mV	log[OH^-]	*Z*
0.00	1 357.00	2.238	1.008
2.72	1 347.90	2.392	0.998
5.15	1 337.87	2.5619	0.983
8.11	1 323.40	2.810	0.952
10.43	1 311.63	3.009	0.915
12.76	1 301.61	3.179	0.869
15.14	1 293.73	3.312	0.818
17.54	not stable		

At first, an extensive series of experiments was carried out using procedure 1. They proved to be very time-consuming, since equilibrium was obtained very slowly indeed. At first we thought — like so many previous investigators — that the formation of polysilicate anions is a slow process. It was learned by experience, however, that slow equilibria are only observed when the solution or part of it has been through a region denoted as the "instability range" (shaded in Fig. 3). With procedure 1, for instance, local excess of acid caused a precipitate of colloid, which dissolved only slowly.

With procedure 2, the composition of S_2 is chosen outside of the "instability range", but as close to it as practicable. On addition of an alkaline solution, neither precipitate nor colloid is formed, and equilibrium is established very rapidly. This procedure was preferred in later work. However, with both procedures, true equilibrium states are obtained: in Fig. 2, experiments by procedure 2 (open symbols) and procedure 1 (filled symbols) are seen to give points on the same curves in the $Z(\log [OH^-])_B$ diagram, within the experimental error.

Table 1 gives a representative part of a titration with procedure 1. After addition of 15.14 ml of T_1, the H_2 stream was replaced by N_2, and the apparatus left over night. Next morning, H_2 was again switched on, and the titration continued. After each addition of acid, E (and thus [OH^-]) decreased, first rapidly, then slowly, and finally reached a constant value. The final E-value was obtained more rapidly at the beginning of the titration than in the later part. The direction of the change is expected, if the local excess of acid first forms a precipitate, $Si(OH)_4$ (with $Z = 0$), or at least one with Z less than in the solution, and this then dissolves slowly, consuming OH^-. Before each new addition, the solution was observed to be clear and free from precipitate.

After addition of 17.54 ml, a new addition of acid caused an initial decrease of E (and OH^-), but then E increased again, which would be expected if an increasing amount of precipitate is slowly formed. A visible precipitate remained, and no constant E could be obtained.

The titrations at 50°C were very tedious, mainly because of difficulties with thermostating. Only a few series of measurements were made; for a more extensive study, an improved technique would be desirable.

The available range in B and Z was limited by the instability range, and also by some considerations of experimental accuracy. When the values of B or [OH^-] cease to be negligible in comparison with I, variations in the

activity factors may be feared. A closer limit on $[OH^-]$ was set by the difficulty of determining Z accurately at high $[OH^-]$, when BZ is obtained as the difference between two numbers of comparable magnitude.

The instability range is more pronounced in 3 m $NaClO_4$, which on the other hand permits higher Z values; thus, the two ionic media allow measurements to be made in different ranges of Z(*cf.* Fig. 2—4).

Some observations in the "instability range". In Fig. 3, for 3 m $Na(ClO_4)$ medium, the "instability range" is shaded. If a solution corresponding to a point outside this range (say, $Z = 0.8$, $B = 0.2$) was diluted with 3 m $NaClO_4$, Z remained practically constant, whereas B was lowered, and the solution eventually entered the "instability range". The solution might appear absolutely clear for some time but the E drifted, indicating slow reactions, and no equilibrium could be obtained. On longer standing the solution became turbid, and finally a precipitate formed. The precipitate might appear after a few minutes, or several hours, depending on B and Z. The maximum rate seemed to be obtained around $B = 0.020$ m. If the precipitate was separated from the apparently clear solution, and the latter heated, further precipitation occurred.

The precipitate formed at room temperature was very fluffy and voluminous; on heating it rapidly contracted to a dense, sticky substance, which finally became brittle on boiling.

SURVEY OF DATA. CALCULATION OF EQUILIBRIA

The experimental data are given in Tables 2—4, and Figs. 2—4. It is seen that the different ionic media allow different Z-ranges to be covered. In all cases, the curves $Z(\log [OH^-])_B$ are seen to differ for different B values,

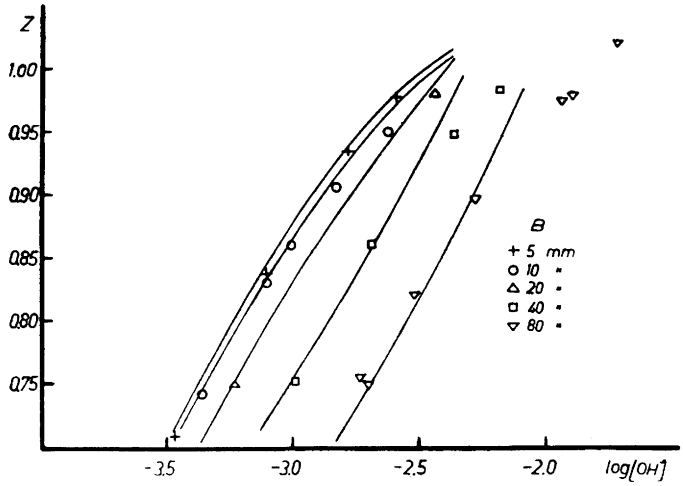


Fig. 4. Silicate equilibria, $Z(\log [OH^-])_B$, 0.5 m $Na(ClO_4)$, 50°C. Curves calculated with equilibrium constants in Table 5.

Table 2. Experimental data for 25°C and 0.5 m $Na(ClO_4)$. For each experimental point, Z is given first, and then $-\log [OH^-]$.

$B = 0.0025$ *m:* Z, -log $[OH^-]$; 0.711 3.8719; 0.757 3.7640; 0.800 3.6611; 0.843 3.5406; 0.883 3.4089; 0.910 3.2958; 0.934 3.1832; 0.952 3.0833; 0.966 2.9948; 0.978 2.9074; 0.986 2.8377; 0.992 2.7620 (proc. 2).

$B = 0.005$ *m:* Z, -log $[OH^-]$; 0.822 3.5896; 0.892 3.3470; 0.944 3.1024; 0.968 2.9123; 0.982 2.7764; 0.996 2.6109; 1.006 2.4572 (proc. 1). 0.932 3.1433; 0.970 2.9037; 0.992 2.7061; 1.004 2.5186; 1.012 2.4417 (proc. 1). 0.731 3.7961; 0.779 3.6827; 0.829 3.5632; 0.875 3. 4023; 0.910 3.2583; 0.936 3.1246; 0.957 2.9970; 0.973 2.8751; 0.985 2.7733; 0.991 2.6937; 0.998 2.6372 (proc. 2).

$B = 0.010$ *m:* Z, -log $[OH^-]$; 0.743 3.7011; 0.788 3.5952; 0.835 3.4657; 0.881 3.3113; 0.916 3.1646; 0.943 3.0154; 0.962 2.8925; 0.977 2.7733; 0.989 2.6619 (proc. 2). 0.830 3.4735; 0.950 2.9484; 0.988 2.5942; 1.004 2.3700; 1.021 2.1687; 1.044 1.9856; 1.055 1.8420. 0.805 3.5610; 0.863 3.3993; 0.919 3.1748; 0.955 2.9723; 0.982 2.7721; 0.991 2.6425; 1.005 2.4605; 1.011 2.3587 (proc. 1).

$B = 0.020$ *m:* Z, -log $[OH^-]$; 0.746 3.4748; 0.775 3.4173; 0.818 3.3156; 0.862 3.1905; 0.898 3.0668; 0.937 2.8955; 0.962 2.7394; 0.986 2.5460; 0.996 2.4517 (proc. 2). 0.818 3.3124; 0.869 3.1788; 0.915 3.0091; 0.952 2.8095; 0.983 2.5619; 0.998 2.3923; 1.008 2.2375; 0.825 3.2679; 0.899 3.0360; 0.948 2.7691; 0.988 2.4845; 1.001 2.2666; 1.018 2.0517; 1.034 1.8472 (proc. 1).

$B = 0.040$ *m:* Z, -log $[OH^-]$; 0.751 3.1545; 0.777 3.0967; 0.823 2.9784; 0.867 2.8482; 0.906 2.6999; 0.935 2.5671; 0.964 2.3874; 0.980 2.2677; 0.990 2.1537; 0.998 2.1109 (proc. 2). 0.922 2.6608; 0.960 2.4632; 0.988 2.2828; 0.782 3.1115; 0.812 3.0123; 0.843 2.9273; 0.875 2.8274; 0.916 2.6653; 0.956 2.4545; 0.978 2.2909; 1.008 2.1195 (proc. 1).

$B = 0.080$ *m:* Z, -log $[OH^-]$; 0.752 2.8785; 0.787 2.7884; 0.821 2.6919; 0.859 2.5761; 0.883 2.4905; 0.915 2.3666; 0.951 2.1880; 0.958 2.1391; 0.988 1.9630; 1.007 1.8367 (proc. 2). 0.815 2.7361; 0.852 2.6168; 0.887 2.4975; 0.918 2.3705; 0.954 2.2088; 0.988 2.0022; 1.021 1.7667 (proc. 1).

Table 3. Experimental data for 25°C and 3.0 m $Na(ClO_4)$. For each experimental point, Z is given first, and then $-\log [OH^-]$. Procedure 2.

$B = 0.005$ *m:* Z, -log $[OH^-]$; 1.061 2.4317; 1.116 2.1550; 1.149 2.0130; 1.220 1.8070; 1.292 1.6200; 1.089 2.2650; 1.145 2.0356; 1.201 1.8690; 1.247 1.7255; 1.380 1.4433.

$B = 0.0125$ *m:* Z, -log $[OH^-]$; 1.073 2.2150; 1.198 1.7814; 1.253 1.6190; 1.329 1.4980; 1.413 1.3367; 1.502 1.1900; 1.578 1.0920; 1.010 2.6307; 1.035 2.4630; 1.072 2.2410; 1.201 1.7703; 1.265 1.6098; 1.306 1.5200.

$B = 0.025$ *m:* Z, -log $[OH^-]$; 1.066 2.0886; 1.107 1.9187; 1.153 1.7720; 1.207 1.6253; 1.290 1.4613; 1.349 1.3408; 1.398 1.2595; 1.440 1.1988; 1.499 1.1075; 1.537 1.0421; 0.968 2.6300; 1.022 2.3517; 1.107 1.9236; 1.274 1.4813; 1.429 1.2020; 1.479 1.1166.

$B = 0.050$ *m:* Z, -log $[OH^-]$; 0.823 2.9007; 0.960 2.4010; 1.060 1.9293; 1.105 1.7551; 1.171 1.5607; 1.225 1.4348; 1.267 1.3518; 1.374 1.1579; 1.532 0.9265; 1.033 2.0803; 1.050 1.9837; 1.088 1.8313; 1.156 1.6085; 1.229 1.4390; 1.317 1.2577; 1.406 1.1155; 1.480 0.9902; 1.546 0.8937.

$B = 0.100$ *m:* Z, -log $[OH^-]$; 0.827 2.6728; 0.927 2.3262; 0.996 2.0107; 1.072 1.6738; 1.144 1.4473; 1.213 1.2823; 1.305 1.1063; 1.397 0.9584; 1.475 0.8502; 1.197 1.3007; 1.265 1.1567; 1.360 0.9907; 1.486 0.7951; 1.560 0.6808.

$B = 0.200$ *m:* Z, -log $[OH^-]$; 1.240 0.9951; 1.328 0.8328; 1.454 0.6273; 1.542 0.4856; 0.832 2.4841; 0.923 2.1481; 0.999 1.7907; 1.068 1.4865; 1.138 1.2546; 1.206 1.0832; 1.303 0.8925; 1.393 0.7384; 1.466 0.6168.

Point interpolated from titrations at constant ratio $r = 2$, 1.82, 1.33, or 1.0:

$B = 0.001$ *m:* 0.831 3.8827; 0.921 3.5307; 1.020 2.8400. $B = 0.0025$ *m:* 0.882 3.6813; 0.954 3.3491; 1.008 2.9240; 1.045 2.6262. $B = 0.005$ *m:* 1.032 2.6310; 1.075 2.3401. $B = 0.0125$ *m:* 1.067 2.2370; 1.129 1.9782.

Table 4. Experimental data for 50°C and 0.5 m $Na(ClO_4)$. For each experimental point, Z is given first, and then $-\log [OH^-]$. Titrations always with procedure 2.

B = 0.005 m: *Z*, $-\log [OH^-]$; 0.708 3.4713; 0.837 3.1042; 0.934 2.7731; 0.978 2.5819; 1.004 2.4007. *B = 0.010 m:* 0.746 3.3613; 0.830 3.1029; 0.860 3.0027; 0.906 2.8216; 0.950 2.6193. *B = 0.020 m:* 0.750 3.2330; 0.980 2.3811. *B = 0.040 m:* 0.752 2.9905; 0.860 2.6875; 0.948 2.3590; 0.982 2.1726. *B = 0.080 m:* 0.750 2.7201; 0.756 2.7236; 0.820 2.5183; 0.896 2.2731; 0.974 1.9298; 0.978 1.8875; 1.020 1.7030.

proving that the solutions must contain polynuclear complexes. For low B, they are seen to approach a curve, with an inflexion point at $Z = 1.0$. Since the shape of this limiting curve agrees very well with that expected for equilibria between three mononuclear species with $Z = 0$, 1, and 2, there is every reason to conclude that at low B the predominant species are $Si(OH)_4$, $SiO(OH)_3^-$, and $SiO_2(OH)_2^{2-}$. *The mononuclear curve* was taken either as the one for the lowest concentration, or from a short extrapolation. The curve was fitted to a normalized curve for two-step equilibria [7]. The results are given in the final Table 5.

For the *polynuclear* complexes, it would have been desirable to start with an approach free from any assumptions (except possibly the constants for the mononuclear equilibria), as was done with the borate equilibria (Part II[3]). For the borates, the data for each value B could be extended to a range with $Z \sim 0$ ($B(OH)_3$ predominates) and $Z = 1$ ($B(OH)_4^-$ predominates) which made a double check possible. For the silicates, the curves for the higher B's have no experimental point where the main species of Si is known from the onset, and so the integration method [3] is impossible.

With a limited range of experimental data available, the only approach that could be invented was to start with the simplest possible hypothesis, namely that, in addition to the mononuclear complexes B, AB and A_2B, a single predominating polynuclear complex A_pB_q is formed. Using procedures outlined by Sillén [8] and applied by Biedermann and Sillén [9] and in Part II[3], sets of normalized curves $\log B$ ($\log \alpha)_Z$ were constructed for combinations of

Table 5. Survey of equilibrium constants obtained in the present work. Unit: m (mole/kg H_2O).

Reaction		I=0.5, 50°C	I=0.5, 25°C	I=3, 25°C
$H_2O \rightleftharpoons H^+ + OH^-$	$\log K_w =$	—12.97	—13.73	—14.03
$A + B \rightleftharpoons AB$; $OH^- + Si(OH)_4 \rightleftharpoons SiO(OH)_3^- + H_2O$	$\log K_1 =$	3.84	4.27	4.60
$A+AB \rightleftharpoons A_2B$; $OH^- + SiO(OH)_3^- \rightleftharpoons SiO_2(OH)_2^{2-} + H_2O$	$\log K_2 =$	1.06	1.17	1.32
$2A+B \rightleftharpoons A_2B$; $2OH^- + Si(OH)_4 \rightleftharpoons SiO_2(OH)_2^{2-} + 2H_2O$	$\log \beta_2 =$	4.90	5.44	5.92
$2A+4B \rightleftharpoons A_2B_4$; $2OH^- + 4Si(OH)_4 \rightleftharpoons Si_4O_6(OH)_6^{2-} + 6H_2O$	$\log \beta_{24} =$	13.38	14.89	—
$4A+4B \rightleftharpoons A_4B_4$; $4OH^- + 4Si(OH)_4 \rightleftharpoons Si_4O_8(OH)_4^{4-} + 8H_2O$	$\log \beta_{44} =$	—	—	23.64
$2A+2B \rightleftharpoons A_2B_2$; $2OH^- + 2Si(OH)_4 \rightleftharpoons Si_2O_3(OH)_4^{2-} + 3H_2O$	$\log \beta_{22} =$	—	—	9.94
$A + B \rightleftharpoons AB$; $Si(OH)_4 \rightleftharpoons SiO(OH)_3^- + H^+$	$\log {}^*K_1 =$	— 9.13	— 9.46	— 9.43
$A + AB \rightleftharpoons A_2B$; $SiO(OH)_3^- \rightleftharpoons SiO_2(OH)_2^{2-} + H^+$	$\log {}^*K_2 =$	—11.91	—12.56	—12.71
$2A + B \rightleftharpoons A_2B$; $Si(OH)_4 \rightleftharpoons SiO_2(OH)_2^{2-} + 2H^+$	$\log {}^*\beta_2 =$	—21.04	—22.02	—22.14
$2A+4B \rightleftharpoons A_2B_4$; $4Si(OH)_4 \rightleftharpoons Si_4O_6(OH)_6^{2-} + 4H_2O + 2H^+$	$\log {}^*\beta_{24} =$	—12.56	—12.57	—
$4A+4B \rightleftharpoons A_4B_4$; $4Si(OH)_4 \rightleftharpoons Si_4O_8(OH)_4^{4-} + 4H_2O + 4H^+$	$\log {}^*\beta_{44} =$	—	—	—32.48
$2A + 2B \rightleftharpoons A_2B_2$; $2Si(OH)_4 \rightleftharpoons Si_2O_3(OH)_4^{2-} + H_2O + 2H^+$	$\log {}^*\beta_{22} =$	—	—	—18.12

$p = 2$, 3, and 4 with $q = 2$, 3, and 4. These normalized "projection maps" were then compared with the experimental data log $B(\log [OH^-])_Z$.

For the data in the range attainable with $I = 0.5$ m, no acceptable fit could be obtained with any complex other than A_2B_4. The agreement obtained assuming this complex and the equilibrium constants given in Table 5 is shown in Figs. 2 and 4. It seems quite satisfactory, except for $Z = 0.9$ at the highest B. One might probably obtain complete agreement, within experimental error, on assuming that A_3B_4 is also present, and assigning to it a suitable equilibrium constant.

In the experiments at $I = 3$ m, and higher Z-values (Fig. 3), fair agreement could be obtained assuming either A_3B_4 or A_4B_4 as the single polynuclear complex; with the other single complexes tested, no acceptable fit could be obtained.

Slight deviations at low values of B indicate the presence of a complex with q between 1 and 4. Fig. 3 shows the best agreement obtained assuming the species AB, A_2B, A_2B_2, and A_4B_4 and the constants in Table 5. The agreement around $Z = 1.1$ might be improved by introducing the species B, A_3B_4, and A_2B_4, which might well be expected to exist in this region.

Deviations at low B might also be explained assuming complexes with 3 B instead of A_2B_2. However, one would then have to assume such a high percentage of B_3-complexes that it is remarkable that they have not showed up in the Z-range given in Fig. 2.

One might certainly "improve" the agreement between experimental data and calculated values by increasing the number of complexes assumed. However, it is felt that such a procedure would be arbitrary, if there was no other evidence for the formulas of the complexes. As a rule, more than two independent equilibrium constants for the formation of polynuclear ions may be handled simultaneously with confidence only in experimentally very favorable cases — and silicate equilibria is not one of them.

One could certainly set to the task of refining the equilibrium constants for the A_pB_4 species more whole-heartedly, if there were other data available that would confirm the predominance of tetranuclear species, and could indicate which other q would be important.

To sum up, the data may be explained with reasonable accuracy assuming, apart from the mononuclear species B, AB, and A_2B, only tetranuclear species; although only A_2B_4 and A_4B_4 have been assumed in the calculations, it seems reasonable that at least A_3B_4 should also exist.

SURVEY OF PREVIOUS SOLUTION DATA

Contrary to common practice, we prefer to give a short survey of previous pertinent work after, rather than before the description of our work. For one thing, the concept of an "instability range" makes it easier to understand many observations by previous workers. Those who want a complete historical review, giving due credit for each step in the development of aqueous silicate chemistry, are referred to the monographs by Iler [1] and Vail [2]. Iler also summarizes the abundant literature on the precipitation of silica gel

and various metal silicates from silicate solutions, a subject which has been left out here completely.

Reversibility of silicate equilibria. There is a wide-spread belief — shared, in fact, by ourselves at the beginning of this work — that equilibria in silicate solutions are reached rather slowly. This is only partly true; on the contrary, equilibria are obtained very rapidly so long as clear solutions, free from colloid or precipitate, are studied. When the B and Z of the solution are in the "instability range", where a clear solution no longer corresponds to equilibrium, the solution changes its properties over a long period.

In the light of our present knowledge, it is for instance easy to explain the observations of Kohlrausch [10]. When a sodium silicate solution of ratio $SiO_2/Na_2O = r = 3.4$, thus $Z = 0.59$, was diluted, and then NaOH added after a certain time, the conductance of the resulting solution changed over a period of many hours. The sooner NaOH was added after the dilution, the more rapid was the approach to the final value. This is easy to understand: the concentrated solution is close on the border-line of the "instability range". On dilution, a precipitate is formed (polysilicic acid, perhaps with some adsorbed Na^+), and this then redissolves very slowly, especially if it has had time to grow. With the initial $r < 2$ ($Z > 1.0$), Kohlrausch found no such timeeffect.

Even if the average composition of a solution is kept within the "clear" area, precipitation of colloid may occur because of local excess of acid. Thus, in commercial silicate solutions, the carbon dioxide of the air has often precipitated a colloid. Titration with acid of a sodium silicate solution (as in procedure 1 above) produces local precipitates, which are not easily redissolved. Such phenomena may explain the divergence of some previous data and the time-effects observed, *e.g.*, by Treadwell and Wieland [11].

On the other hand, research workers who have taken great trouble to avoid acid impurities, such as Hägg [12], and Nauman and Debye [13], seem to agree that in clear solutions equilibria are obtained instantaneously so that the properties of a clear silicate solution depend only on its composition and not on its history.

Freezing-point data may be used to calculate the average number of silicate complexes (charged or uncharged) per Si, which will be denoted by R as in Part II. The inverted value, R^{-1}, is the average number of Si-atoms per complex. Souchay [14] titrated 0.66 M "Na_2SiO_3" with H_2SO_4 in a $Na_2SO_4(H_2O)_{10}$ melt, measuring the freezing-point. He concluded that the "Na_2SiO_3" ($Z = 2$) gives mononuclear ions ($R = 1$), whereas at the point corresponding to "$NaHSiO_3$" ($Z = 1$), he found $R^{-1} = 4$; for the predominant species Souchay gave the formula $Si_4O_{10}^{4-}$.

Thilo and Miedreich [15,16] also studied the transition point of $Na_2SO_4(H_2O)_{10}$, the melt incidentally providing constant ionic medium. In dilute solutions of $Z = 1$, they found $R = 1$ and concluded that $H_3SiO_4^-$ predominates. With increasing B, R decreases from 1.0 to ≈ 0.25, which might be interpreted by the formation of a tetrasilicate ion; however, Thilo and Miedreich do not commit themselves to giving any formula for it. They also studied solutions of the salts previously denoted as $Na_2SiO_3(H_2O)_9$ and $Na_6Si_2O_7(H_2O)_{11}$, and concluded that they are really $Na_2H_2SiO_4(H_2O)_8$ and $Na_3HSiO_4(H_2O)_5$. Since $[OH^-]$ was high it was not possible to calculate R^{-1} accurately.

Miedreich [16] has also recalculated Hägg's [12] and Harman's [17] freezing-point data for ice in sodium silicate solutions. These data are somewhat less accurate, since a correction must be made for $[Na^+]$; the activity factor correction has been neglected. Nevertheless, R varies from around 1.0 at low B to around 0.25 at high B, as before.

Alexander [18] prepared a solution of $Si(OH)_4$, from crystalline $Na_2H_2SiO_4(H_2O)_8$ and a slurry of H^+-saturated ion exchanger at 0°C. By freezing-point measurements he proved it to be mononuclear ($R = 1$). Iler [19,20] measured the freezing-point of a polysilicic acid sol, obtained from a sodium silicate solution with $Z = 0.61$, adding acid [19], or H^+ ion exchanger [20]. In the former case he found $R^{-1} = 3.25$, in the latter $R^{-1} = 4.7$; subsequent polymerization was corrected for by extrapolation to zero time.

All this might seem to support the conclusions from the present emf work that the predominant complexes are mononuclear and tetranuclear. However, it is fair to point out that freezing-point measurements for other systems have repeatedly been interpreted in ways that differ from the conclusions obtained by the emf method, which we consider far more accurate, if applied properly.

For instance, freezing-point data have been interpreted [14,21] as a proof that the main hydrolysis product of Be^{2+} is $(BeOH)_4^{4+}$, whereas the emf data of Kakihana and Sillén [22] in this laboratory give a good fit over a wide concentration range assuming $(BeOH)_3^{3+}$, and give no evidence for $(BeOH)_4^{4+}$.

Optical data. Nauman and Debye [13] studied the light-scattering of carefully filtered sodium silicate solutions. With $Z \sim 2$ and higher ($r = 1$ and lower), the calculated molecular weights were around 70, corresponding to monomers, whereas with increasing r (up to 3.75, $Z = 0.53$) they rose to a few hundreds. There may thus be polynuclear ions with a small number of Si, but certainly no "colloidal" particles in the usual sense. The viscosity of highly concentrated solutions can also be well explained without assuming large particles: the large rise in viscosity occurs, just as with sucrose, only when the molecules come very close together in the solution.

For $Z = 0.53$, Nauman and Debye observed an increase in turbidity (and thus molecular weight) with time for $B = 0.26$ M, whereas the turbidity remained constant with $B = 0.65$ M. This is another example of the instability of solutions of low Z (high r) on dilution.

Brady, Brown and Huff [23] have found and studied an increase in turbidity with time for potassium silicate solutions of $r = 3.8$, which are thus in the "instability range".

Diffusion data. Jander and Heukeshoven [24] found that the diffusion coefficient of silicate ions is the same for $B = 0.167$, 0.10, and 0.05 M in 3 M OH^-. They concluded that a single mononuclear species, SiO_3^{2-}, was present. With decreasing pH, the diffusion coefficient at first decreased and then, between pH = 13.5 and 10.9, it kept at a constant value, which was different for different B's. In this range they assumed $Si_2O_5^{2-}$ to be the predominant species.

Emf (pH) data. Several investigators have measured the pH of silicate solutions, obtained for instance during titrations of silicate with acid, or of silicic acid with alkali, or by mixing or diluting solutions of pure salts like NaH_3SiO_4. The data have in general been interpreted in terms of the dissocia-

tion constants of the acid H_4SiO_4 (in older work often called H_2SiO_3, by analogy with H_2CO_3). Some of these workers have noticed deviations which they have ascribed to the formation of polysilicate ions; with the exceptions to be mentioned below, no attempt was made to calculate the constants for their formation.

For the mononuclear constants, the following values have been given: (the figures are pK_{a1} etc, thus $-\log {}^*K_1$, ($-\log {}^*K_2$, $-\log {}^*K_3$, $-\log {}^*K_4$), in our notation) Hägg [12] (20°C) 9.13; Harman [25] (25°C) 9.4 16.3; Hahn and Klockmann [26] (20°C) 10.64; Treadwell and Wieland [11] (20°C) 9.7 12; Flint and Wells [27] 9.66 11.7 12 12; Oka, Kawagaki and Kadoya [28] (25°C) 9.51 11.77; Miedreich [16] (20°C) 9.4 11.4 13.7. Miedreich calculated the constants from his own data, and also from recalculated data of Bogue [29] (30°C), Hägg [12], and Harman [30]. Alexander, Heston and Iler [31], from the increase of solubility of "amorphous silica" with increasing pH concluded $pK_{a1} \sim 9.8$.

When comparing these data and those of the present work (Table 5) we should notice that the ionic media have been different in different papers. The differences in the constants given may be explained partly by the activity factor correction and partly by the neglect of polynuclear silicate ions by most authors.

Roller and Ervin [32] studied the solubilities of calcium silicates in solutions of various pH and $[Ca^{2+}]$. They postulated that pK_a for H_2SiO_3 and $H_3Si_2O_6^-$ is 9.8, and for $H_2Si_2O_6^{2-}$ 12.8. On this assumption they calculated for $pK_{a2} = 12.16 \pm 0.02$, and moreover $\log K\ (2H_3SiO_4^- \rightleftharpoons H_2Si_2O_6^{2-} + 2H_2O) = 3.34 \pm 0.03$.

In a previous communication by the author [33] it was shown that data for a restricted range (B,Z) could be explained assuming complexes AB_n^-, thus with charge -1. Since this mechanism cannot explain the data for the wider range now available, it will not be upheld.

From emf and freezing point data, Thilo and Krüger [34] deduced the formation of a series of complexes, $(AB)_n^{n-}$, in the brief notation, with the equilibrium constant, $\log K(nAB^- \rightleftharpoons (AB)_n^{n-}) = -1.23(n-1)$.

Conductivity titrations have been reported by Constable and Tegul [35]. From breaks in the conductivity curves they inferred the existence in the solution of "ions from $Na_4Si_3O_8$, $Na_2Si_2O_5$ and $NaHSiO_3$" at 15°C, and "$Na_2O.2SiO_2$ and $Na_2O.4SiO_2$" at 100°C. In general, the conductivity method does not seem to be well suited for studying complicated equilibria.

SOLUBILITY OF $Si(OH)_4$, AND INSTABILITY RANGE

According to several authors (Alexander, Heston and Iler [31]; Goto, Okura and Kayama [36]; and Gardner [37]) the solubility of "amorphous silica" in acidic and neutral solutions is around 2 mM $= 10^{-2.7}$ M, probably in the form of $Si(OH)_4$. Solutions of a higher concentration may be prepared, but they are unstable and a gel will eventually precipitate.

It is a natural suggestion, that this solubility should determine at least one limit to the range in which stable solutions can be obtained. To test this suggestion, the concentration $b = [Si(OH)_4]$ has been calculated as a function of $\log B$ for the constant values $Z = 0.75$ and 0.80, and $I = 0.5$, 25°C, and 50°C, using the equilibrium constants K_1, β_2, and β_{24} from Table 5. The results are given in Fig. 5.

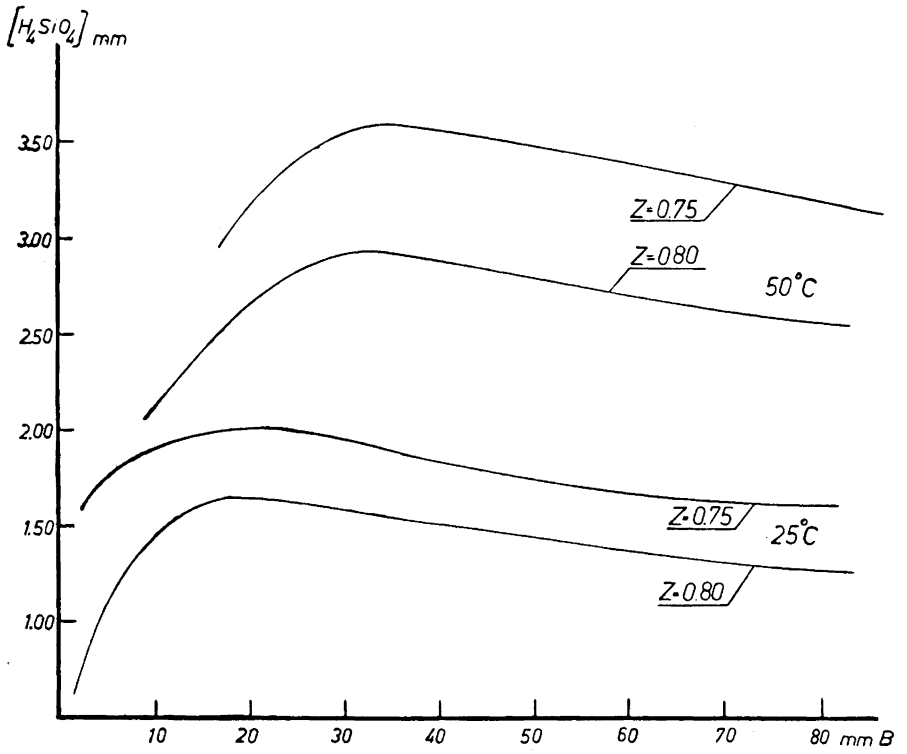


Fig. 5. Equilibrium concentration, b, of $Si(OH)_4$ calculated as a function of B, for constant $Z = 0.75$ or 0.80; 0.5 m $Na(ClO_4)$, 25°C. Equilibrium constants from Table 5.

Since h and $[OH^-]$ are negligible in comparison with B in (1), the curves will show the behavior of the solution when it is diluted with ionic medium, which keeps Z constant with good approximation.

It is seen that on dilution, b passes through a maximum, for $Z = 0.75$, and 25°C, of $b_{max} \sim 2$ mm ($B = 20$ mm) and for 50°C of $b_{max} \sim 3.6$ mm (at $B = 40$ mm). The figures 2 mm and 3.6 mm approximately correspond to the solubility of $Si(OH)_4$ at these temperatures, and it may be inferred from Fig. 5 that, at lower values for Z, the solubility will be exceeded.

This agrees with experience: stable solutions have not been obtained for $Z < 0.75$ except in the very dilute range, $B < 5$ mm, where the limiting Z-value decreases with B, and roughly seems to correspond to a saturated solution of $Si(OH)_4$.

For $I = 3.0$, the limiting value for Z was in the range 0.80—0.95, depending on B. Here, as in the dilute medium, the distinction between stable and unstable solutions was sharp.

The data have not been considered accurate enough to justify a similar calculation for the lower limit at $I = 3.0$, nor for the intermediate "instability

range" for $I=3.0$. It seems likely that a similar intermediate range should exist for $I=0.5$ although it has not been met with in the range of B and Z studied.

Although the explanation given here is reasonable and agrees fairly well both with present data and with the results of earlier work, it is not considered that the present constants are a final answer to the question. Attempts will be made to study silicate ions in other media, and with other experimental methods, and also to study some related anions.

Acknowledgements. This work was made possible by a generous grant from Columbia-Southern Chemical Corporation whose permission to publish is very much appreciated. I wish to thank Professor Lars Gunnar Sillén for introducing me to this interesting type of equilibrium studies and for his invaluable help and encouragement throughout the work. I also wish to thank Dr. Franklin Strain and Mr. Björn Holmström for their stimulating interest and Mr. Frans Ericson for his valuable help with experimental equipment.

REFERENCES

1. Iler, R. K. *The Colloid Chemistry of Silica and Silicates*, Cornell University Press, Ithaca, N.Y. 1955.
2. Vail, J. G. *Soluble silicates*, Reinhold Publishing Company, New York, 1952.
3. Ingri, N., Lagerström, G., Frydman, M. and Sillén, L. G. *Acta Chem. Scand.* **11** (1957) 1034.
4. Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 1607.
5. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability constants, Part II, Inorganic ligands, The Chemical Society Special Publication* **No. 7** (1958).
6. Forsling, W., Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **6** (1952) 901.
7. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 186.
8. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 803.
9. Biedermann, G. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 1011.
10. Kohlrausch, F. *Z. physik. Chem.* **12** (1893) 773.
11. Treadwell, W. D. and Wieland, W. *Helv. Chim. Acta* **13** (1930) 842.
12. Hägg, G. *Z. anorg. Chem.* **155** (1926) 21.
13. Nauman, R. V. and Debye, P. *J. Phys. Chem.* **55** (1951) 1.
14. Souchay, P. *Bull. soc. chim. France* **1948** 143.
15. Thilo, E. and Miedreich, W. *Z. anorg. Chem.* **267** (1951) 76.
16. Miedreich, W. *Thesis*, Frankfurt am Main 1954.
17. Harman, R. W. *J. Phys. Chem.* **31** (1927) 355.
18. Alexander, G. B. *J. Am. Chem. Soc.* **75** (1953) 2887.
19. Iler, R. K. *J. Phys. Chem.* **57** (1953) 604.
20. Iler, R. K. US Pat. 2588389, quoted in Ref[1], p. 27.
21. Faucherre, J. *Bull. soc. chim. France* **1953** 1117.
22. Kakihana, H. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 985.
23. Brady, A. P., Brown, A. G. and Huff, H. *J. Colloid. Sci.* **8** (1953) 252.
24. Jander, G. and Heukeshoven, W. *Z. anorg. Chem.* **201** (1931) 301.
25. Harman, R. W. *J. Phys. Chem.* **31** (1927) 616.
26. Hahn, F. L. and Klockmann, R. *Z. physik. Chem.* **146** (1930) 373.
27. Flint, E. P. and Wells, L. S. *J. Research Natl. Bur. Standards* **12** (1934) 751.
28. Oka, Y., Kawagaki, K. and Kadoya, R. *J. Chem. Soc. Japan* **64** (1943) 718.
29. Bogue, R. H. *J. Am. Chem. Soc.* **42** (1920) 2575.
30. Harman, R. W. *J. Phys. Chem.* **30** (1926) 1100.
31. Alexander, G. B., Heston, W. M. and Iler, R. K. *J. Phys. Chem.* **58** (1954) 453.
32. Roller Jr, P. S. and Ervin Jr, G. *J. Am. Chem. Soc.* **62** (1940) 461.
33. Lagerström, G. *Rec. trav. chim.* **75** (1956) 721.
34. Thilo, E. and Krüger, G. *Z. Elektrochem.* **61** (1957) 24.
35. Constable, F. N. and Tegul, S. *Nature* **160** (1947) 55.
36. Goto, K., Okura, T. and Kayama, I. *Kagaku Tokyo* **23** (1953) 426.
37. Gardner, L. U. *Am. Inst. Mining. Met. Engrs. Techn. Publ.* **No. 929** (1938) p. 7.

Received January 8, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 737–749

Studies on the Vitamin B_{12}, Cyanocobalamin-Binding Capacity, Desoxyribosides, and Methionine in Some Commercial Milk Products and Cheese

DANLEY A. CALLIERI

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

1. Part of the vitamin B_{12} in cow milk is destroyed during commercial pasteurization (74°C for 16 sec).

2. There is no "free" vitamin B_{12} (estimated as *E. coli* activity) in unpasteurized cow milk. On the other hand, this activity is considerable in pasteurized milk.

3. The cyanocobalamin-binding capacity of cow milk amounted on a average to 0.29 mμg/ml. Different results were obtained with different samples of milk, the range being 0.15–0.45.

4. Commercially pasteurized cow milk (74°C, 16 sec) has no cyanocobalamin-binding capacity.

5. The cyanocobalamin-binding capacity of cow milk, determined as shown in Table 4, is destroyed by heating to a temperature between 50–60°C.

6. The substance responsible for the cyanocobalamin-binding capacity of cow milk loses this property upon heating, at the same time as the cyanocobalamin is released from the complex.

7. Freezing has no influence on the cyanocobalamin-binding capacity of cow milk.

8. The "total" vitamin B_{12} activity of sour milk and yoghurt is lower than that of the milk from which they have been prepared. This indicates that the vitamin is used by the bacteria during the lactic fermentation of the milk. The total activity in yoghurt is due mainly to methionine and desoxyribosides, both of which are not at all present, or only in very small amounts, in the ultrafiltrate of cow milk.

9. Sour milk has no, or only a very low, cyanocobalamin-binding capacity. This property is on the other hand very high in yoghurt, being in the range 150–190 mμg/ml, *i. e.* about 500–600 times higher than in fresh unpasteurized milk.

10. Preliminary results with saline suspensions of *Lactobacillus bulgaricus* and of *L. thermophilus*, used in the preparation of yoghurt, indicate that these organisms are able to bind cyanocobalamin.

11. Cheddar and Roquefort had on average about the same B_{12} activity (28 and 27 mμg/g fresh basis, respectively), and so had Camembert and Svecia but on a lower level (11.5 and 11.3 mμg/g fresh basis).

The high contents of desoxyribosides made impossible the assay of the "free" vitamin B_{12} in Camembert and Roquefort by the micro-

biological method used here. The results obtained with Cheddar and Svecia show that the vitamin occurs in these cheeses mainly in a bound form.

13. Camembert contained about 400 μg methionine/g fresh basis (*Leuconostoc mesenteroides* activity) while the other varieties had amounts which were 3 to 7 times greater. The *L. delbrückii* activity of Cheddar and Svecia was of the same order of magnitude, 0.75—1.9 μg/g fresh basis, calculated as thymine desoxyriboside. The activity of Camembert and Roquefort was 20—35 times greater.

Numerous articles have been published in different countries on the determination of the content of cyanocobalamin in cow milk and the seasonal variations of this content as well as the influence of the animal diet composition etc. In the present work, determinations of the total vitamin B_{12} activity of cow milk were only made in order to see if the vitamin is destroyed during commercial pasteurization of the milk, and if so, to what extent. The total vitamin B_{12} activity in the whey was also determined as well as the "free vitamin B_{12}" activity in pasteurized and untreated cow milk. The results obtained are summarized in points 1—3 above.

In 1955 Gregory and Holdsworth [2] demonstrated that milk from different animal species possessed the property of binding added cyanocobalamin. The amount of cyanocobalamin which could be bound was different for different species, being greatest for sow milk (240 mμg Cy/ml) and smallest for cow milk (0.5 mμg Cy/ml). It has been found previously in this laboratory by Neujahr *et al.*[3] that dry skim milk powder has no cyanocobalamin-binding capacity and, even if not fully investigated, it was pointed out that also commercially pasteurized milk might have lost the ability to bind cyanocobalamin.

In the present work, the cyanocobalamin-binding capacity of pasteurized and untreated cow milk was further investigated. As it was found that the pasteurized product does not bind cyanocobalamin, an attempt was made to determine at which temperature this property is destroyed by heating. The results are summarized in points 3—7 above.

There seems to be no published data about the vitamin B_{12} activity and cyanocobalamin-binding capacity of yoghurt and sour milk. In the present work, the vitamin B_{12} content of both products was determined and compared with that of the milk used in their preparation. The ability to bind cyanocobalamin was also investigated. The results are summarized in points 8—10 above.

The vitamin B_{12} activity, total as well as "free", and the cyanocobalamin-binding capacity of four different kinds of cheese were determined. The cheese extracts were also analyzed for methionine and desoxyribosides. The results are summarized in points 11—13 above.

PART I. THE VITAMIN B_{12} ACTIVITY, TOTAL AND "FREE", IN PASTEURIZED AND UNPASTEURIZED COW MILK

Experimental

Determination of the vitamin B_{12} content. A 50 ml sample of milk, containing 100 μg/ml of KCN, was autoclaved for 10 min at 120°C. After cooling, the liquid was ultrafiltered and the ultrafiltrate analyzed for vitamin B_{12} using the tube method and *E. coli* 113—3 as test-organism.

The ultrafiltration was carried out using a piece of Visking cellulose tubing (The Visking Corp., Chicago), diameter 1/4 inch, as described by Gregory [1] but modified in the following way: Two small pieces of plastic or rubber tubing (c) were pushed on to the stem of a funnel (a) (Fig. 1) and a short piece of glass tubing (b). A 20—25 cm length of cellulose tubing was moistened with distilled water at both ends and then slipped over the sleeves (c). Cotton thread was tied tightly around the cellulose tubing in order to make both joints secure. Hence, in contrast to the original method, the cellulose tubing did not have a cotton-tied end with which the liquid to be ultrafiltered came into contact. To facilitate the fastening, the tube (b) can first be loosened from the rubber stopper and can then, after the joints have been made secure, be pushed back into its place. The cellulose tube must be carefully moistened in order to prevent it from cracking during the ultrafiltration. This was performed by keeping it overnight, after it had been fastened to the funnel and the glass tube, in a desiccator, the lower compartment of which was half-filled with distilled water. The desiccator was placed in a refrigerator in order to prevent any possible microbiological attack on the cellulose. To perform the ultrafiltration, the cellulose tubing *etc.* was put into a tube (d) which could be evacuated through (e). It was possible to connect several ultrafilters to the same suction device when there were numerous samples to be treated. During the present work, up to 30 simultaneous ultrafiltrations have been run successfully. After autoclaving, a 10 ml sample was poured

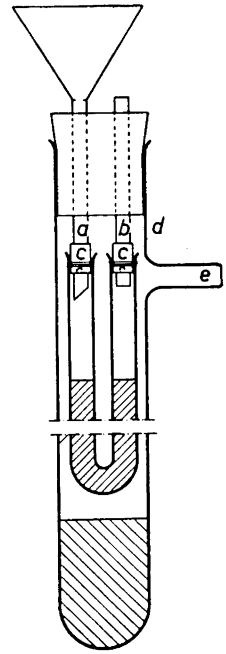


Fig. 1. Ultrafilter.

into the funnel. Suction was applied and then removed when about 1—1.5 ml ultrafiltrate had been collected. This took approximately one hour.

Working with this proportion between the sample and the ultrafiltrate, the amount of free vitamin B_{12} per ml in both of them should be the same [2]. The ultrafiltrate was diluted appropriately and its vitamin B_{12} content determined using the tube method and *E. coli* 113—3 as test organism. To get the standard curve, crystalline cyanocobalamin was added to six sets of tubes in quadruplicate in the following quantities: 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mμg per tube. In several cases, the pH of the milk was adjusted to 5 before autoclacing. The whey, thus produced, was ultrafiltered and its vitamin B_{12} content determined. The values were fundamentally the same as those obtained by ultrafiltration of the whole milk, after the above treatment. This is in agreement, with the findings of Neujahr et al [3].

To determine the amount of "free" vitamin B_{12} (as *E. coli* activity), samples of milk were directly ultrafiltered and the ultrafiltrate analyzed.

Bioautography. Spots of the samples were put on sheets of Whatman No. 1 paper and developed in two different solvent systems according to Neujahr [6], the compositions of which are given below, for 24 or 48 h. The spots were made by releasing drops from a capillary. After development, the paper sheets were dried and placed on agar plates seeded with the appropiate test microorganism.

Solvent system I		Solvent system II	
Sec. butanol	75 %	*Sec.* butanol	75 %
Acetic acid (conc.)	1 %	NH_3 (conc.)	1 %
H_2O	24 %	H_2O	24 %
KCN	0.01 %	KCN	0.01 %

Results and discussion

The samples were provided by Mjölkcentralen, Stockholm.

The values found for the total vitamin B_{12}, given as *E. coli* activity, were 3—6 mμg/ml for untreated milk and 2.5—5.3 mμg/ml for pasteurized milk. These results show that a part of the vitamin B_{12} is destroyed during the commercial pasteurization of the milk (16 sec at 74°C). In untreated milk, the "free" B_{12} content is zero, or very low, while in pasteurized milk it is 0.6—0.14 mμg/ml. During pasteurization the milk loses its cyanocobalamin-binding capacity and also a part of the naturally bound vitamin is released. (See Part II).

The results of two typical experiments are shown in Table 1.

Table 1. Total and "free" vitamin B_{12} in pasteurized and unpasteurized cow milk.

Unpasteurized cow milk		Pasteurized cow milk	
Total vitamin B_{12} activity mμg Cy/ml	"Free" vitamin B_{12} activity mμg Cy/ml	Total vitamin B_{12} activity mμg Cy/ml	"Free" vitamin B_{12} activity mμg Cy/ml
6	0.025	5.3	0.56
5.1	0	4.7	0.60

The samples were analyzed also by the bioautographic technique with solvent systems I and II [6] (see Experimental), using *E. coli* 113—3 and *L. leichmannii* 313. In this way, it was shown that the vitamin B_{12} activity was due only to cyanocobalamin.

PART II. CYANOCOBALAMIN-BINDING CAPACITY OF COW MILK. TEMPERATURE AT WHICH THIS PROPERTY IS DESTROYED. EFFECT OF LOW TEMPERATURES ON THE BINDING CAPACITY

Experimental

Determination of cyanocobalamin-binding capacity of pasteurized and unpasteurized cow milk. To four samples of milk from the same batch, 0.25, 1.0, 2.0, and 4.0 mμg cyanocobalamin/ml of milk was added. The samples were kept at 4—7°C for 2 h stirring for 5 min every 30 min. The samples were then ultrafiltered in the usual way, each sample being divided equally between two ultrafilters. In this way, two ultrafiltrates were obtained from each sample and their cyanocobalamin contents were determined separately, the average being taken as the cyanocobalamin content of the sample in question. The cyanocobalamin determination was made using the tube-method with *E. coli* 113—3 and/or *L. leichmannii* 313 A.T.C.C. 7830 as test-organisms. The quantity of cyanocobalamin bound additionally is obtained as the difference between the amount of cyanocobalamin added and that found in the ultrafiltrate. Samples of milk without any added cyanocobalamin were also ultrafiltered to determine the activity of the ultrafiltrate for the test-organism.

Influence of temperature on the cyanocobalamin-binding capacity of cow milk. A 2 l quantity of milk was heated under continuous stirring in a water bath. When the milk reached the desired temperature, a sample was taken. After having kept the milk at the same temperature for a certain time, another sample was taken (Table 4). The milk was then heated until the next temperature was reached and a fresh sample was taken, and so on. The samples were then kept at 4—7°C to permit them to reach a uniform temperature. The binding capacity of the different samples was then determined as above, after adding 2 mμg cyanocobalamin/ml. In the last four samples of assay No 3 taken at 70—85°C, some of the cyanocobalamin naturally present in the milk had been released (contents over the 2 mμg cyanocobalamin added). The remaining part of each sample was kept at —20°C for several days, and then ultrafiltered and analyzed. The results were the same.

Results and discussion

The cyanocobalamin-binding capacity of pasteurized and unpasteurized cow milk was investigated using the ultrafiltration technique (see Experimental). The results show that the pasteurized milk (Table 3) has no binding capacity while the unpasteurized milk (Table 2) does bind cyanocobalamin.

Table 2. Cyanocobalamin-binding capacity of unpasteurized cow milk. The results are given as "coli activity".

Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml	Bound Cy mμg/ml	Average
1	0.76	0.24	
2	1.61	0.39	0.29
4	3.77	0.23	

Table 3. Cyanocobalamin-binding capacity of pasteurized cow milk. The results are given as "coli-activity".

Assay No.	Activity of the milk before adding Cy mμg/ml	Cy added mμg/ml	Activity of the ultrafiltrate mμg/ml
1	0.20	0.25	0.5
		1.0	1.11
		2.0	2.10
		4.0	4.10
2	0.28	1.0	1.1
		2.0	2.3
		4.0	4.3
3	0.14	0.25	0.45
		1.0	1.08
		2.0	2.0
		4.0	4.2

This is in agreement with the results of previous investigations in this laboratory performed by Neujahr *et al.*[3] The table shows the average of a large number of determinations using *E. coli* and *L. leichmannii* as test-organisms in the tube method.

The cyanocobalamin-binding capacities of different samples of unpasteurized milk were different, the range being 0.15—0.45 mμg cyanocobalamin/ml. Since the amount of vitamin is small, this variation can be due to the method employed *.

Since pasteurized milk has no binding capacity, the temperature at which this property is destroyed was determined. It was found (Table 4) that, for the conditions under which this work was done (see Experimental), the critical temperature is between 50 and 60°C. Above this temperature the "free" vitamin B_{12} content increases considerably with increasing temperature. This indicates that part of the bound vitamin B_{12} is released by heating over 50—60°C and is thereafter unable to rebind. Hence, this seems to indicate that the substance responsible for the binding of cyanocobalamin in milk is altered by the heating at the moment when the vitamin is released from the complex. To make this conclusion more certain, samples of milk, heated to different temperatures above 50—60°C, were ultrafiltered after they had reached room temperature and also after they had been kept for several days at either 4 or 7°C. It was found that the quantities of vitamin B_{12} in the ultrafiltrates were the same. No recombination of the vitamin took place.

* It it also possible that milk samples from different cows, and even from the same cow on different occasions, have not the same cyanocobalamin-binding capacity. Such possibilities were not especially studied it the present work.

In several assays, the values of which were discarded, a considerable increase in the binding capacity was observed when the amount of cyanocobalamin added was increased. This observation has been made by many authors before, and has been attributed by Gregory [1] to the method of determination of the vitamin.

Table 4. Influence of heating on the cyanocobalamin-binding capacity of cow milk. The results are given as "coli-activity".

Assay No.	Temp °C	Time used to pass from a given temp. to the next. Min	Time kept at given temp. Min	Cy added mμg/ml	Cy found in the ultra- mμg/ml (a)	(b)
	15			2	1.8	
	30	8	5	2	1.8	1.85
	40	6	5	2	(1.35)	(1.32)
1	50	6	5	2	*1.92*	*2.15*
	60	6	5	2	2.32	2.7
	70	7	5	2	2.32	2.52
	80	6	5	2	2.50	2.8
	90	6	5	2	3.2	3.5
	17					
	30	7	5			
	40	6	5	2	1.15	(1.92)
2	50	6	5	2	1.20	1.27
	60	6	5	2	*2.45*	*2.45*
	70	7 1/2	5	2	2.20	2.07
	80	6	5	2	2.37	2.80
	90	7		2	3.90	
	18					
	50	11	0	2	1.77	
	55	1	0	2	*2.15*	
	60	1	0	2	broken	
3	65	1	0	2	2.10	
	70	1	0	2	2.73	
	75	1	0	2	2.45	
	80	1	0	2	(1.97)	
	85	1	0	2	3.90	

(a) Samples were taken at the moment at which the milk reached the given temperature.
(b) Samples were taken after keeping for 5 min at the given temperature.

The effect of a low temperature on the cyanocobalamin-binding capacity of cow milk was also studied. Three assays were made with the same sample: before freezing and after keeping at —20°C for 3 days and 10 days. No differences were found.

PART III. VITAMIN B_{12} ACTIVITY AND CYANOCOBALAMIN-BINDING CAPACITY OF SOUR MILK AND YOGHURT. PRELIMINARY STUDY ON BINDING CAPACITY OF *LACTOBACILLUS BULGARICUS* AND *L. TERMOPHILUS*.

Experimental

1. *Determination of "total" vitamin B_{12} activity.* The samples of both sour milk (pH 4.2—4.4) and yoghurt (pH 4.0—4.05) were diluted with an equal amount of distilled water. The pH was adjusted to 5.5 and 100 mμg KCN/ml of diluted sample was added

before autoclaving for 10 min at 120°C. After cooling to room temperature, the solutions were ultrafiltered as usual and the ultrafiltrates analyzed for vitamin B_{12} by the tube method using *E. coli* 113—3 and/or *L. leichmannii* 313 A.T.C.C. 7830 as test-organisms.

2. *Determination of "free" vitamin B_{12} activity.* The samples were diluted with an equal amount of distilled water, ultrafiltered and the ultrafiltrates were assayed as above.

3. *Determination of cyanocobalamin-binding capacity.* This assay was performed as described for milk (see Part II), the appropriate amount of cyanocobalamin being added to the samples after they had been diluted with an equal amount of distilled water.

4. *Chromatography in order to identify methionine* was done on sheets of Whatman No. 1 paper, using a solvent system composed of methylethylketone (55 %), acetone (25 %) and NH_3 conc. (10 %), for 5—8 h. The papers were, after drying, developed by spraying with 0.5 % ninhydrine in acetone [5].

Results and discussion

Yoghurt and sour milk are obtained by the lactic fermentation of milk. Industrially, the milk is first pasteurized and then inoculated with the appropriate microorganism. The vitamin B_{12} activity of these products and also that of the pasteurized milk from which they were prepared was investigated in the present work. As shown above (see Part II), pasteurized milk does not bind cyanocobalamin. The cow milk used for the production of yoghurt and sour milk is treated more rigorously (85°C for 16 sec) than the commercially pasteurized milk (74°C for 16 sec). Any cyanocobalamin-binding capacity in the soured products must therefore be due to changes occurring during the fermentation. The results are shown in Table 5 and can be summarized as follows:

Sour milk. The total B_{12} activity was 30—40 % lower than that of the milk from which the sour milk was prepared. Thus a large part of the vitamin is used by the *Lactobacillus* during the fermentation. Bioautographs were performed using solvent systems I and II (see Part I) and *E. coli*. Only one spot corresponding to cyanocobalamin was obtained. Furthermore, the agreement between the results obtained in assay No 4 with two different test-organisms also indicates that the activity is due to only one factor.

In all assays, the "free" vitamin B_{12} content was found to be greater than that in the milk from which the sour milk was prepared. Bioautographs were, in addition, performed with the ultrafiltrates in which the "free" vitamin B_{12} had been determined. Also here, only one spot, corresponding to cyanocobalamin, was obtained. As before, the results with two different test-organisms (assay No 4) were the same.

The presence of "free" B_{12} would indicate that sour milk has no binding capacity for this vitamin. This seems to be confirmed, in general, by the assays made to study this property. As shown in Table 5, there seems to exist, in some cases and especially in assay No 2, a certain cyanocobalamin-binding capacity since it should be remembered that the solution already contained a certain amount of "free" vitamin B_{12}. In conclusion, it can be said that sour milk has only a very small, or no, cyanocobalamin-binding capacity.

Yoghurt. As can be seen in Table 5, practically all the vitamin B_{12} of the milk was used during the lactic fermentation. Bioautographs were made, in solvent systems I and II (see Experimental Part I) and using *E. coli* 113—3

Table 5. Cyanocobalamin-binding capacity of sour milk and yoghurt, vitamin B_{12} activity of pasteurized cow milk, sour milk, and yoghurt.

Assay No.	Pasteurized cow milk				Sour milk								Yoghurt									
	"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml		Bound Cy mμg/ml	"Total vitamin B_{12}" mμg/ml		"Free vitamin B_{12}" mμg/ml		Added Cy mμg/ml	Cy found in the ultrafiltrate mμg/ml			Bound Cy mμg/ml	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)		(a)	(b)	*	(a)	(b)	(a)	(b)		(a)	(b)	Average		Average
1	6.5		0.95		4.7		1.23		2 4 8	3.1 6.7 11.0			1.6		0.52		2 4 8	0.56 0.52 0.52			>2 >4 >8	
2	5.5		0.6		3.25		0.92		2 4 8	2.6 4.0 6.8												
3	4.7		0.56		3.5		1.14		2 4 8	2.46 6.30 9.00			1.25		0.42		160 200 300 400	16 56 140 206			144 144 160 194	160
4	3.6	3.41	0.025	0.067	2.44	2.22	0.6	0.51	2 4 8	2.34 4.16 7.40	2.50 4.54 7.20		0.49	0.10	0.55	0	80 160 200 300	0 3.4 4.9 64.0	0 2.8 2.7 62.0	0 3.1 3.8 63.0	>80 157 196 237	197

(a) The results are given as *E. coli-activity.*
(b) » » » » » *L. leichmanii-activity.*
* See page 744.

as test-organism, with the ultrafiltrates corresponding to the assays of "total" and "free" vitamin B_{12}. Several diffuse spots were obtained in this way, none of them being cyanocobalamin. The different results obtained in the determination of vitamin B_{12} using *E. coli* and *L. leichmannii* as test-organisms in the tube method (assay No 4) also indicate that the activity is due to more than one factor. Hence, a qualitative assay of the ultrafiltrates was made by putting them simultaneously on four agar plates seeded with *E. coli* 113—3, *L. leichmannii* 313 A.T.C.C. 7830, *L. delbrückii* A.T.C.C. 9649, and *Leuconostoc mesenteroides* P. 60 A.T.C.C. 8042 (the first microorganism is sensitive to cyanocobalamin and methionine, the second to cyanocobalamin and desoxyribosides, the third to desoxyribosides and the last one to methionine). This assay showed that the active factors in the ultrafiltrates were mainly methionine and desoxyribosides. Methionine was later also identified by chromatography (see Experimental).

The cyanocobalamin-binding capacity of yoghurt is surprisingly high (Table 5), *viz.* 150—190 mμg/ml.

As mentioned before, yoghurt is prepared from pasteurized cow milk by inoculating with *Lactobacillus bulgaricus* and *L. thermophilus* and fermenting at 45°C. Obviously, the cyanocobalamin-binding capacity of yoghurt appears during this fermentative process and can be due to transformations of the natural constituents of milk and/or can be caused by the substances belonging to the metabolism of the above-named lactobacilli. Hence, preliminary assays were made with suspensions of *L. bulgarius* and of *L. thermophilus* in saline solution. It was found that they are able to bind cyanocobalamin. A further investigation will be carried out on this subject.

PART IV. VITAMIN B_{12} ACTIVITY AND CYANOCOBALAMIN-BINDING CAPACITY OF FOUR DIFFERENT KINDS OF CHEESE. DETERMINATION OF METHIONINE AND DEOXYRIBOSIDES IN THE EXTRACTS USED TO TEST VITAMIN B_{12} CONTENT

Experimental

1. *Determination of total vitamin B_{12} activity.* 200—300 g of cheese was cut into small pieces and mixed, in order to obtain a random sample. Three different methods for the extraction of the vitamin B_{12} were compared:

a) 20 g of cheese was emulsified in a blender for 5 min with 70—75 ml of fresh aqueous emulsifying solution (see below) at 50°C. The volume was adjusted to 100 ml with the same solution. The emulsion was ultrafiltered as usual (see Part I) and the ultrafiltrates diluted and assayed for vitamin B_{12} activity with *Lactobacillus leichmannii* 313 A.T.C.C. 7830 in the tube method. The emulsifying solution had the following composition [4]:

KH_2PO_4	1.36 g
$Na_4P_2O_7$	0.75 g
KCN	0.005 g
H_2O	to 100 ml

b) 20 g of cheese was emulsified as in (a). Before adjusting the volume to 100 ml, KCN was added in order to get a final concentration of 100 μg KCN/ml of emulsion. The emulsion was then autoclaved for 10 min at 120°C, the volume readjusted to 100 ml and the solution ultrafiltered and assayed as before.

c) 20 g of cheese was emulsified as in (a) using, however, glassdistilled water containing 100 μg KCN/ml instead of the emulsifying solution. The volume was adjusted to 100 ml and the pH to 5.5. The emulsion was then autoclaved for 10 min at 120°C, the volume readjusted before ultrafiltering and the ultrafiltrates assayed.

As mentioned before, no appreciable differences in the vitamin B_{12} activity were found when using the methods described. The aqueous extraction (c) was thus adopted.

2. *Determination of "free" vitamin B_{12}.* 20 g of cheese was emulsified in a blender for 5 min with 70—75 ml glass-distilled water at 40—45°C. The emulsion was made up to 100 ml, ultrafiltered and the ultrafiltrate assayed.

3. *Determination of cyanocobalamin-binding capacity.* Emulsions of the cheeses, obtained as described above, were assayed for cyanocobalamin-binding capacity using the ultrafiltration method (see Part II). The activity of the emulsions without addition of cyanocobalamin was also determined after ultrafiltering.

Results and discussion

Several methods for the extraction of vitamin B_{12} from cheese were tested (see Experimental) but no significant difference was found. Consequently, the aqueous extraction method was adopted because of its simplicity.

The determination of the vitamin B_{12} activity was performed by taking the whole of the cheese. The variations in vitamin B_{12} content which may exist in different parts of the same cheese were not tested.

The samples of cheese were extracted (see Experimental) and ultrafiltered. The ultrafiltrates were assayed, using first of all the cup method and *E. coli* 113—3 as test-organism in order to get an approximate idea of the activity. All the four cheeses tested (see below) gave double zones of growth, the inner ring being sharper. This indicates that there was present in the extracts more than one substance which stimulated the growth of *E. coli*. It was thought that the smaller and sharper ring could be due to vitamin B_{12}, and the larger and more diffuse to methionine.

A simultaneous qualitative test was then made using four agar plates, each seeded with a different microorganism (see Part III). The results were as follows:

	Vitamin B_{12}	Methionine	Desoxyribosides
Camembert	+	++	+++
Roquefort	+	++++	+++
Svecia	+	++++	+
Cheddar	+	++++	+

The presence of methionine was confirmed by chromatography and bioautography (see Part III).

The presence of considerable amounts of methionine and desoxyribosides in the extracts made uncertain the use of *E. coli* or *L. leichmannii* for the determination of vitamin B_{12}. It was first necessary to determine these substances quantitatively in order to calculate the appropriate degree of dilution of the extracts required to prevent their interference in the vitamin B_{12} assay. Methionine and desoxyribosides were determined by the tube method using *Leuconostoc mesenteroides* and *Lactobacillus delbrückii*, respectively, as test-organisms. As can be seen in Table 6, all four cheeses have a considerable methionine content, high enough to make the determination of vitamin B_{12}

impossible using *E. coli* as test-organism in the tube method without any special treatment of the extracts.

The amount of desoxyribosides in Cheddar and Svecia is 0.6 — 1.5 μg/g fresh basis. In Camembert and Roquefort the content is 20—40 times higher (*L. delbrückii* activity, calculated as thymine desoxyriboside). Because of the ratio between the quantities of cheese used and the final volume of the extract (see Experimental), the amount of desoxyribosides per ml of extract enabled a direct determination of vitamin B_{12}, using the tube method and *L. leichmannii* as test-organism, provided that the vitamin B_{12} content was not too low, in which case the necessary dilution of the extracts would be impossible.

Preliminary assays were made to determine the approximate vitamin B_{12} activity of the extracts. It was found that, at the dilution necessary in order to prevent the influence of deoxyribosides on the *L. leichmannii* growth, there was sufficient of the vitamin to permit an accurate assay. The results obtained using this microorganism in the tube method are shown in Table 6.

Table 6. Cyanocobalamin, methionine, and desoxyribosides contents of aqueous extracts from four kinds of cheese. The results are given as *L. leichmanii, Leuconostoc mesenteroides* and *L. delbrückii activity*, respectively, each being the average of three different assays.

	Extract from "total vitamin B_{12}" determination			Extract from "free vitamin B_{12}" determination		
	Methionine μg/g fresh basis	Desoxyribosides μg/g fresh basis	Cy mμg/g fresh basis	Methionine μg/g fresh basis	Desoxyribosides μg/g fresh basis	Cy mμg/g fresh basis
Cheddar	1 478	0.75	28	1 635	1.2	1.7
Camembert	390	70	11.5	565	67	*
Roquefort	2 700	42	27	2 515	40	*
Svecia	1 848	1.35	11.3	1 710	1.9	0.8

* The high amount of desoxyribosides present in the extract made impossible the determination of cyanocobalamin by the method used.

An attempt was made to determine whether the vitamin B_{12} is bound or free in the cheese. The samples of cheese were emulsified in a blender with glass-distilled water at 40—45°C, ultrafiltered and assayed for vitamin B_{12}, methionine, and deoxyribosides. As can be seen in Table 6, these last two substances are present more or less in the same amounts as in the ultrafiltrates corresponding to the determination of the total vitamin B_{12} activity. In contrast to this, the "free" vitamin B_{12} content in Cheddar and Svecia is much lower than the values obtained for the total vitamin B_{12}. This indicates that the vitamin is mainly bound in these cheeses. The determination of "free" vitamin B_{12} in Camembert and Roquefort was not possible with the method employed because of the high desoxyriboside concentration.

Table 7. Cyanocobalamin-binding capacity of four kinds of cheese. The results are given as *Lactobacillus leichmannii* activity.

	Natural activity of the ultrafiltrate mμg Cy/ml	Amount of Cy (mμg) added/ml cheese emulsion	Amount of Cy (mμg) found/ml ultra-filtrate
Cheddar	0.3	10	13.7
		20	24.0
Camembert	1.67	10	14.4
		20	23.0
Roquefort	2.5	10	13.0
		20	20.0
Svecia	0.2	10	9.6
		20	23.0

Desoxyribosides were also identified by bioautography in solvent system I and II (see Part I) with *L. delbrückii* as test-organism and thymine desoxyriboside as standard. Further investigations will be made to determine whether the activity detected with L. delbrückii is due only to desoxyribosides.

The cyanocobalamin-binding capacity of the cheeses was also investigated using the ultrafiltration technique (see Experimental).

The mean results obtained are shown in Table 7.

Hence, it can be concluded that none of the cheeses investigated has any cyanocobalamin-binding capacity.

Acknowledgements. The author wishes to thank Professor H. Lundin, Head of the Division, for his great interest in the work and for his provision of valuable laboratory facilities. The author is also indebted to his colleagues in the Institute, in particular Dr. Halina Neujahr and Dr. Arne Bolinder, for their kind advice and many interesting discussions concerning the investigations. Thanks are also due to Mjölkcentralen, Stockholm, for the various samples which were used in this work.

REFERENCES

1. Gregory, M. E. *Brit. J. Nutrition* **8** (1954) 340.
2. Gregory, M. E. and Holdsworth, E. S. *Biochem. J.* **59** (1955) 329.
3. Neujahr, H., Jonasson, M. and Lundin, H. *In press.*
4. Hartman, A. M. and Dryden, L. P. *Food Research* **21** (1956) 540.
5. Ericson, L.-E. *Unpublished.*
6. Neujahr, H. *Acta Chem. Scand.* **11** (1957) 1191.

Received November 14, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 750–757

The Structure of Ceroptene

MARTIN NILSSON

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Ceroptene, the yellow pigment of *Pityrogramma triangularis*, has been reinvestigated. The evidence of degradation and synthesis indicates that it is a methyl enol ether of 2-cinnamoyl-6,6-dimethyl*cyclo*hexane-1,3-5-trione (II), either 5-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-4-ene-1,3-dione (IV) or less probably 3-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-3-ene-1,5-dione (VI).

On the fronds of many ferns of the family *Pteridaceae* *, and especially on those belonging to the genus *Pityrogramma*, there is a white or yellow secretion on the dorsal surfaces, and these ferns are therefore sometimes cultivated for decorative purposes. In botanical literature the coating is often described as a wax layer (»ceraceous ferns»), but investigations by Blasdale [2] and by Zopf [3] at the beginning of this century show that it contains appreciable amounts of enolic compounds.

The "golden-back fern", *Pityrogramma triangularis*, Kaulf. (earlier known as *Gymnogramme triangularis* and due to some confusion in literature also as *Ceropteris triangularis*), was investigated by Blasdale, who isolated a crystalline, yellow pigment, m.p. 135°, for which he proposed the name ceroptene. He described it as an acidic compound, which analysed for $C_{18}H_{18}O_4$ and gave benzoic acid on oxidation. On treatment of ceroptene with hydroiodic acid he obtained a product, m.p. 182° ($C_{11}H_{11}IO_3$?), which also gave benzoic acid on oxidation, but only ill-defined products on treatment with alkali. Blasdale did not propose any structure for the pigment and no further work on ceroptene appears to have been reported since.

Ceroptene has now been reinvestigated. Fronds of *P. triangularis* were extracted with ether, the extract was freed from lipid material, and eventually the yellow pigment was obtained in fair yield from the alkali-soluble fraction. It melted somewhat unsharply at 137—140° and the analytical data agreed with the composition proposed by Blasdale ($C_{18}H_{18}O_4$). The mass spectrum confirmed the molecular weight as 298.

* Botanical nomenclature according to Copeland [1].

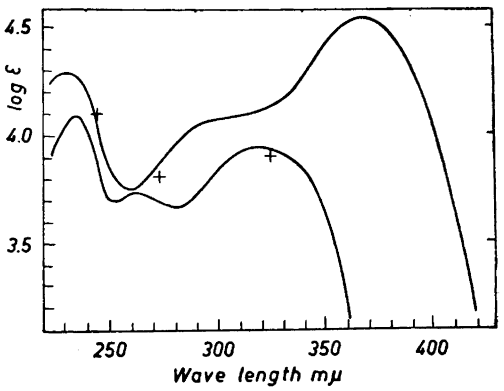


Fig. 1. Ultraviolet spectra of ceroptene (upper curve) and dihydroceroptene (lower curve) in ethanol (99.5 %). The absorption maxima reported for tasmanone [9] are indicated by crosses.

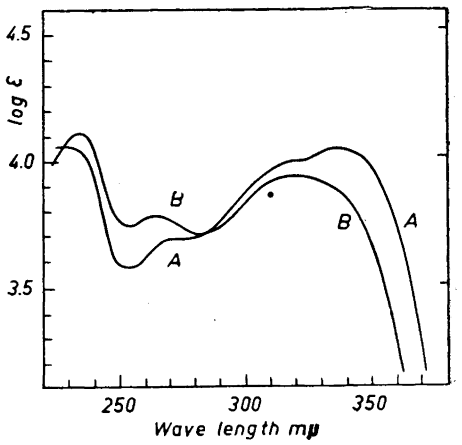


Fig. 2. Ultraviolet spectra of 3-acetylfilicinic acid (A) and O-methyl-3-acetylfilicinic acid (B) in ethanol (99.5 %).

Ceroptene proved to contain one methoxyl group and one active hydrogen atom; the large consumption of Grignard reagent (2.9 mole) in the latter determination indicated the presence of two carbonyl groups also. The C-methyl determination gave 0.4 mole of acetic acid, which suggested the presence of a *gem*-dimethyl group [4].

Ceroptene gave a strong, red colour with iron(III) chloride and a chloroform-soluble copper salt (m.p. 253—257°). This and the infrared absorption (see below) indicated it to be the chelate enol of a β-diketone.

Catalytic hydrogenation or reduction with zinc in acetic acid gave colourless dihydroceroptene, m.p. 98—100°.

The presence of a mono-substituted benzene ring, demonstrated by oxidation of ceroptene to benzoic acid, was evident also in the mass spectrum peak at $m/e = 77$ ($C_6H_5^+$, *cf.* however Ref.[5]). A peak at $m/e = 103$ was interpreted as $C_6H_5—CH=CH^+$ [6]. These peaks were weak in the mass spectrum of dihydroceroptene, which instead gave pronounced peaks at $m/e = 91$ and 105, presumably due to $C_6H_5—CH_2^+$ (rearranged [7]) and $C_6H_5—CH_2—CH_2^+$ [6]. In the infrared spectrum of ceroptene, bands near 3 100, 1 580, 1 310 and 970 cm^{-1}, which did not recur in the spectrum of dihydroceroptene, indicated the presence of a di-substituted, *trans*-ethylenic double bond conjugated with a benzene ring. The mass spectral and infrared data thus indicate that ceroptene contains a Ph—CH=CH- unit.

Degradation of dihydroceroptene by ozonolysis and subsequent oxidation with alkaline hydrogen peroxide gave hydrocinnamic acid and dimethylmalonic acid confirming the presence of the C_6C_2-grouping and also showing the presence of a *gem*-dimethyl group.

The ultraviolet spectrum of ceroptene (Fig. 1) was rather similar to those of chalcones and other arylidene ketones, but did not give very much further information. The spectrum of dihydroceroptene (Fig. 1) was of considerably greater interest because of the strong band near 320 mμ. Since the absorption of the non-conjugated benzene ring is negligible this absorption must be due to the remaining part of the molecule. Comparison with known spectra indicated a close resemblance to 3-acetylfilicinic acid (2-acetyl-6,6-dimethyl*cyclo*hexane-1,3,5-trione, I) [8]. The spectrum of this compound (Fig. 2) is the sum of contributions from various tautomers and enolate ions; the absorption above 300 mμ appears indeed to be made up of two peaks (325 and 340 mμ). The dihydroceroptene maximum is close to the first peak.

Tasmanone [9], isolated from the essential oil of *Eucalyptus risdoni*, has been supposed to be 5-methoxy-2-*iso*butyryl-4,6,6-trimethyl*cyclo*hex-4-ene-1,3- dione. Its ultraviolet absorption (maxima indicated in Fig. 1) is similar to that of dihydroceroptene. A comparison with the methyl ether(s) of 3-acetylfilicinic acid appeared however to be desirable for confirmation of the nature of the chromophore.

3-Acetylfilicinic acid was obtained, albeit in small quantity, by alkaline methylation of phloroacetophenone [8]. On treatment with methyl orthocarbonate [10] it afforded a methyl ether, m.p. 107—109°. The ultraviolet spectrum of this O-methyl-3-acetylfilicinic acid (Fig. 2) was similar to that of tasmanone and almost identical with that of dihydroceroptene. There were also striking similarities between the infrared spectra of O-methyl-3-acetylfilicinic acid and dihydroceroptene.

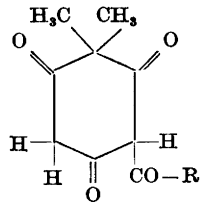
The alkaline cleavage of ceroptene proceeded very slowly, partly due to the poor solubility of the salt(s) in strong alkali and possibly also due to an unfavourable equilibrium. Prolonged heating with dilute aqueous potassium hydroxide with simultaneous, continuous extraction with toluene gave benzaldehyde and a product identical with the synthetic O-methyl-3-acetylfilicinic acid (20 %).

The reverse reaction proceeded smoothly and the condensation of O-methyl-3-acetylfilicinic acid with benzaldehyde using piperidine as a catalyst gave ceroptene in good yield.

The evidence shows that ceroptene is the methyl ether of an enol of 2-cinnamoyl-6,6-dimethyl*cyclo*hexane-1,3,5-trione (II) but does not give any definite evidence about the position of the methoxyl group.

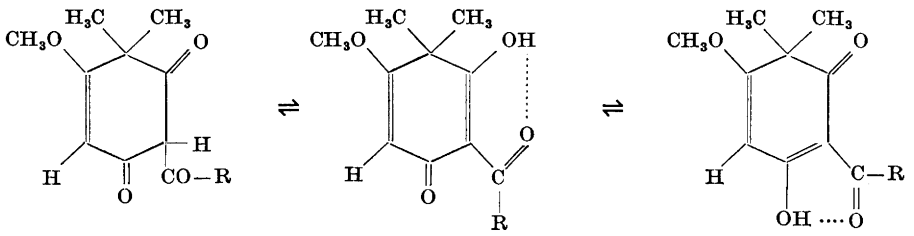
Of the possible methyl ethers of 3-acetylfilicinic acid only III and V should give conjugate chelated enols. The ultraviolet spectrum gives no direct help towards a choice between them because of lack of reference data. The position of the methoxyl group should however be the same as in tasmanone.

The infrared spectra of 2-acyl*cyclo*hexane-1,3-diones show a strong band at 1 540—1 565 cm^{-1}, which has been ascribed to a carbonyl vibration shifted to low frequency by conjugate chelation accentuated by the symmetry of the β-triketone system [11]. Ceroptene, dihydroceroptene and O-methyl-3-acetylfilicinic acid all absorb very strongly near 1 520 cm^{-1}. This band appears to have a similar origin, the shift to lower frequencies presumably being due to additional conjugation. These compounds appear to be completely enolised since the infrared absorption is independent on the concentration and since

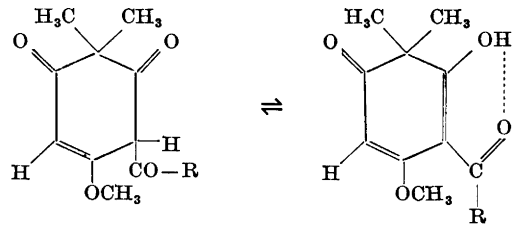


I R = CH_3–

II R = Ph–CH = CH–



III R = CH_3– IV R = Ph–CH=CH–



V R = CH_3– VI R = Ph–CH=CH–

the ultraviolet spectra in *cyclo*hexane and in ethanol are fairly similar. This and the very broad and weak OH-absorption near 2 000 cm^{-1} both support the assignment of the chelate carbonyl frequency. Further work on this subject is published elsewhere [12].

The 2-acyl*cyclo*hexane-1,3-diones also show a band near 1 660 cm^{-1} which is ascribed to the non-chelated carbonyl group [11]. This band recurs in the present spectra. The absorption of dihydroceroptene and O-methyl-3-acetylfilicinic acid at 1 625 and 1 621 cm^{-1}, respectively, is probably due to the enol ether double bond since dimedone methyl ether absorbs at 1 620 cm^{-1} [13]. In ceroptene the peak is observed at 1 625 cm^{-1} and the intensity is increased by the overlapping ethylenic absorption.

Model compounds for prediction of the infrared absorption of 3-methoxy-2-acetyl-6,6-dimethyl*cyclo*hex-3-ene-1,5-dione (V) are lacking but the striking similarity of the present compounds to the 2-acyl*cyclo*hexane-1,3-diones seems to favour structure III (5-methoxy-2-acetyl-6,6-dimethyl*cyclo*hex-4-ene-1,3-dione) for the synthetic O-methyl-3-acetylfilicinic acid.

Ceroptene is therefore probably 5-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-4-ene-1,3-dione (IV), but structure VI (3-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-3-ene-1,5-dione) is not excluded.

Ceroptene is a somewhat novel addition to the diversified pattern of naturally occurring phloroglucinol derivatives. It embraces the essential features of a $C_6C_3C_6$ compound but is C-methylated to an extent that precludes aromaticity of the phloroglucinoid nucleus and thus is reminiscent of the large group of alkylated 2-acyl*cyclo*hexane-1,3-diones and -1,3,5-triones. These have recently been reviewed by Hassall [14].

The natural order *Filicales* has given many examples of phloroglucinoid compounds. *Dryopterus Filix mas* (L.) Schott., of the family *Aspidiaceae*, which is perhaps the most carefully investigated species, contains a considerable variety of polymethylphloroacylophenones (*cf.* Ref.[14]). *Matteucia orientalis* (Hooker) Trev.[15] and *Phanerophlebia falcata* (L.) Copel. (also known as *Cyrtomium falcatum*)[16] belong to the same family. They have been found to contain matteucinol (5,7-dihydroxy-4'-methoxy-6,8-dimethylflavanone), desmethoxymatteucinol [15] and 5,7,4'-trihydroxy- and 5,7,3',4'-tetrahydroxy-6,8-dimethylflavanone [16].

Although C-alkylated phloroglucinol derivatives are also known from higher plants the wide variety of compounds of this type found in the natural order *Filicales* appears to offer particular scope for comparative biogenetic studies and for biochemical investigations on the formation and transformations of phloroglucinoid compounds.

It is tempting to speculate on the possible physiological significance of the ceroptene coating of *P. triangularis*. β-Triketones are known to exhibit antibacterial and sometimes insecticidal activity and in some cases have shown an unusual hypermetabolic activity similar to that of 2,4-dinitrophenol [17]. It is interesting to note that the young shoots of the "ceraceous ferns" are almost covered with pigment but the coating of the full-grown plants is confined to the dorsal surfaces of the fronds. Possibly the ceroptene coating acts as a protection during the early growth of the fern.

EXPERIMENTAL

Melting points were determined on a Kofler block. The infrared spectra were recorded on a Perkin Elmer No. 21 instrument fitted with a sodium chloride prism, and the ultraviolet spectra were measured on a Beckman DU instrument. The microanalyses were done in the laboratory of Dr. A. Bernhardt, Mülheim, Germany.

Isolation. Fronds and stems of *Pityrogramma triangularis*, collected in California during summer 1957 (180 g), were washed briefly with ether until the extract was almost colourless. The ether solution was filtered and evaporated giving a semi-crystalline mass, m. p. 119–135° (7.1 g). Further extraction with ether gave a greenish, waxy material (1.8 g), which was not investigated. The first extract was partitioned between dimethyl sulphoxide and light petroleum (b. p. 40–60°). The latter phase on evaporation gave a yellowish wax, m. p. 55–68° (1.5 g). The dimethyl sulphoxide solution was poured into water and the mixture was exhaustively extracted with ether. The ether solution was evaporated to a small volume, washed with water and extracted successively with sodium hydrogen carbonate (sat.), sodium carbonate (sat.) and sodium hydroxide (0.5 M) solutions. On extraction with carbonate the ether solution took on a peculiar greenish colour, probably due to trace metal chelation. If extracted with stronger sodium hydroxide solutions the sodium salt of ceroptene precipitated as an oil.

This fractionation gave: 1) Neutral material, 0.7 g; 2) Hydrogen carbonate soluble fraction, 0.02 g; 3) Carbonate soluble fraction, 0.3 g and 4) Alkali-soluble fraction, 3.9 g.

Fractions 1—3 have not been investigated. Fraction 4 was a crystalline, yellow mass and on recrystallisation from methanol gave ceroptene as lemon-coloured prisms and plates, m. p. 137—140° (3.0 g). Further recrystallisation and sublimation (130°, 0.05 mm) did not improve the m. p. In chloroform solution ceroptene was optically inactive.

Analytical data. Found: C 72.3, 72.2; H 6.4, 6.0; O 21.0; CH_3O 13.8; active H 0.44; CH_3-(C) 2.1. Calc. for $C_{18}H_{18}O_4$: C 72.5; H 6.1; O 21.5; CH_3O (one) 10.4; active H (one) 0.34; CH_3-(C) (one) 5.0; M 298.3.

C-Methylated phloroacetophenones are notorious in giving varying methoxyl analyses[8].

Infrared spectrum (measured in carbon tetrachloride solution with the exception of the italicised figures, which denote absorptions measured on a potassium bromide disc in regions of strong solvent absorption): 3 120 w, 3 080 w, 3 040 w, 3 000 m, 2 960 m, 2 900 w, 2 860 w, 1 655 vs, 1 625 vs, 1 580 s, 1 515 vs, 1 475 vs, 1 450 vs, 1 440 vs, 1 385 vs, 1 355 m, 1 335 m, 1 300 m, 1 286 w, 1 232 vs, 1 200 m, 1 165 s, 1 075 w, 1 040 w, 995 m, 985 m, 979 m, *875* w, *870* m, *838* w, *823* w, *792* w, *760* m, *728* w and 700 cm^{-1} w. In the region 3 100—1 900 cm^{-1} there was a very weak and broad absorption.

The mass spectrum was recorded by Ing. R. Ryhage, Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, with an apparatus equipped with an all-glass heated inlet system[18]. This system gave a fairly large parent peak but preliminary attempts to use a conventional metal inlet system gave extensive cracking and a very low peak for the unfragmented molecule-ion. Only major peaks and peaks relevant to the discussion are given. The figures in brackets are intensities given as *per cent* of the m/e = 221 peak. 299 (25), 298 (96), 297 (38), 283 (37), 280 (19), 227 (47), 222 (21), *221* (100), 195 (8), 131 (81), 126 (78), 125 (49), 115 (16), 111 (46), 105 (6), 104 (14), 103 (19), 102 (14), 95 (11), 91 (8), 83 (10) and 77 (46).

The copper salt was prepared in aqueous methanol and was recrystallised from chloroform-*cyclo*hexane to give bronze-glistening plates, m. p. 253—257°. (Found: C 64.2; H 5.1; residue 11.0. Calc. for $C_{36}H_{34}CuO_8$: C 65.9; H 5.2; CuO 12.0.) The salt could not be sublimed.

Ceroptene gave a strong red colour with iron(III) chloride in methanol. No reaction was obtained with diazotised benzidine even after addition of sodium carbonate or with 2,4-dinitrophenylhydrazine.

Hydrogenation. Ceroptene (0.30 g) in absolute ethanol (20 ml) on hydrogenation over palladised charcoal (0.08 g, 10 %) absorbed one mole of hydrogen in one hour. After filtration, evaporation and recrystallisation from methanol dihydroceroptene (0.21 g) was obtained as prisms and was finally purified by sublimation, m. p. 98—100°. (Found: C 72.7; H 6.7; CH_3O 10.4. Calc. for $C_{18}H_{20}O_4$: C 72.0; H 6.7; CH_3O 10.3.) Infrared spectrum (in carbon tetrachloride solution and in a potassium bromide disc): 3 070 w, 3 020 m, 2 980 m, 2 920 w, 1 665 vs, 1 625 vs, 1 525 vs, 1 495 vs, 1 480 vs, 1 462 vs, 1 390 vs, 1 360 s, 1 285 m, 1 270 w, 1 230 vs, 1 200 s, 1 165 vs, 1 082 m, 1 060 m, 1 035 w, 1 000 s, 965 m, 845 s, 835 s, *810* w, *750* s, and 695 cm^{-1} m.

Mass spectrum: 301 (21), *300* (100), 286 (16), 285 (91), 282 (13), 267 (15), 196 (11), 195 (35), 181 (17), 169 (16), 168 (98), 153 (11), 126 (22), 125 (20), 111 (16), 105 (13), 104 (6), 103 (6), 95 (7), 91 (28) and 77 (6).

When treated with copper (II) acetate in aqueous methanol dihydroceroptene gave a greenish blue copper salt, m. p. *ca.* 200°, which was not, however, obtained in a state of purity.

Oxidation. Ceroptene was oxidised with potassium permanganate in pyridine-aqueous alkali to give benzoic acid (0.7 mole), m. p. and mixed m. p. 120—123°.

Ozonolysis of dihydroceroptene. Dihydroceroptene (0.16 g) in methylene chloride (20 ml) was treated with ozone at −78° for one hour. The solvent was removed under reduced pressure below room temperature and the residue was oxidised with hydrogen peroxide (30 %, 1 ml) in aqueous alkali (2 M, 5 ml) on the water bath for 0.5 h. The cooled solution was acidified and extracted with ether and the extract was evaporated at room temperature to give an oily mixture. Extraction with cold *cyclo*hexane dissolved part of it leaving a colourless, crystalline material (28 mg), which melted at 190° with effervescence and then gave an odour of *iso*butyric acid. Mixed m. p. with dimethylmalonic acid gave no depression and the infrared spectra were identical. Evaporation of the *cyclo*hexane solution gave a low-melting solid, which was recrystallised from dilute hydro-

chloric acid to give a product with an unsharp melting point (38—48°, 30 mg), the infrared spectrum of which was identical with that of hydrocinnamic acid.

Alkaline cleavage of ceroptene. Ceroptene (50 mg), was dissolved in aqueous potassium hydroxide (1 M, 10 ml). The solution was kept on the water bath under continuous extraction with toluene. Samples were withdrawn at intervals and examined by paper chromatography in the system tetralin, acetic acid, water (10:10:1) [19]. The spots were detected by spraying with iron (III) chloride in methanol. After 50 h the ceroptene (R_F 0.7) had disappeared and the main product had R_F 0.6. There was also a faint spot at R_F 0.3, probably 3-acetyl-filicinic acid. The aqueous solution was acidified and extracted with ether and the ether extract after drying was evaporated to give a solid, m. p. 85—100° (23 mg). Sublimation gave colourless needles, m. p. 90—103° (6 mg). The ultraviolet and infrared (KBr) spectra were identical with those of the synthetic methyl ether of 3-acetylfilicinic acid described below.

The toluene solution was heated with an excess of 2,4-dinitrophenylhydrazine on the water bath for 2 h and was then extracted with dilute hydrochloric acid and evaporated to give an orange solid which was triturated with ethanol to give benzaldehyde 2,4-dinitrophenylhydrazone, m. p. and mixed m. p. 236—239° (8 mg).

Methylation of 3-acetylfilicinic acid. C-Methylation of phloroacetophenone according to Riedl and Risse [8] gave 3-acetylfilicinic acid in 5 % yield, m. p. 178—182°. (Reported yield 4 %, m. p. 174—176°.) The ultraviolet spectrum agreed with that given by these authors and a portion boiled with dilute hydrochloric acid gave filicinic acid, m. p. 216—224° (decomp.). (Reported m. p. 214—216°.)

3-Acetylfilicinic acid (63 mg) and methyl ortocarbonate [20] (0.2 ml) were heated on the water bath for 3 h. Light petroleum (2 ml) was added and after cooling the mixture deposited needles, m. p. 97—105° (47 mg). Sublimation and recrystallisation from light petroleum gave the methyl ether as needles, m. p. 107—109°. (Found: C 63.2; H 6.8; CH_3O 14.6. Calc. for $C_{11}H_{14}O_4$: C 62.8; H 6.7; CH_3O 14.7.)

Infrared spectrum (in carbon tetrachloride): 3 080 w, 2 995 m, 2 950 m, 2 860 w, 1 660 vs, 1 621 vs, 1 525 vs, 1 477 vs, 1 455 vs, 1 385 s, 1 350 m, 1 260 w, 1 230 vs, 1 200 m, 1 167 vs, 1 081 m, 1 030 w, 1 000 m, 962 m, 845 w, 740 m, 676 w.

The copper salt was obtained as blue prisms from aqueous methanol, m. p. *ca.* 240° (some decomposition from 200°). It was not obtained in a state of purity.

Synthesis of ceroptene. Synthetic O-methyl-3-acetylfilicinic acid (11 mg), benzaldehyde (50 mg) and piperidine (5 mg) were heated on the water bath for 0.5 h and then methanol (1 ml) was added. On standing ceroptene separated as prisms, m. p. and mixed m. p. 138—140° (9 mg). The infrared spectrum was identical with that of the natural product (KBr).

Acknowledgements. The present investigation was proposed by Professor H. Erdtman, who collected the initial fern material. For this and for his encouraging interest in the work I wish to express my sincere gratitude. Thanks are also due to Dr. K. Folkers, Rahway, Dr. R. W. Miller, San Francisco, Dr:s Alice and Rolla Tryon, Los Angeles and Professor E. Werdermann, Berlin-Dahlem for generous gifts of material.

I also wish to thank Ing. R. Ryhage for the determination of the mass spectra and discussions on the interpretation, Miss Gurli Hammarberg for skilful assistance in the measurements of infrared and ultraviolet spectra and Dr. B. R. Thomas for linguistic comments.

REFERENCES

1. Copeland, E. B. *Genera Filicum*, Waltham, Mass. 1947.
2. Blasdale, W. C. *J. Am. Chem. Soc.* **25** (1903) 1141.
3. Zopf, W. *Ber. deut. botan. Ges.* **24** (1906) 264.
4. Roth, H. in Müller, E. (Ed.) *Methoden der organischen Chemie (Houben-Weyl)*, Band *2*, Thieme, Stuttgart 1953, p. 273.
5. Meyerson, S. and Rylander, P. N. *J. Am. Chem. Soc.* **79** (1957) 1058.
6. *Am. Petrol. Inst. Research Project 44, "Catalog of Mass Spectral Data"*, Carnegie Inst. of Technology, Pittsburgh, Pa., 1947—1956, Nos. 359, 177.
7. Rylander, P. N., Meyerson, S. and Grubb, H. M. *J. Am. Chem. Soc.* **79** (1957) 842.

8. Riedl, W. and Risse, K. H. *Ann.* **585** (1954) 209.
9. Birch, A. J. and Elliott, P. *Austr. J. Chem.* **9** (1956) 95.
10. Smith, B. *Acta Chem. Scand.* **10** (1956) 1006.
11. Chan, W. R. and Hassall, C. H. *J. Chem. Soc.* **1956** 3495.
12. Forsén, S. and Nilsson, M. *Acta Chem. Scand.* **13** (1959) *In press.*
13. Heymann, H., Bhatnagar, S. S. and Fieser, L. F. *J. Am. Chem. Soc.* **76** (1954) 3689.
14. Hassall, C. H. *Progr. in Org. Chem.* **4** (1958) 115.
15. Fujise, S. *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* **11** (1929) 111, after *Chem. Zentr.* **1930** I 236.
16. Kishimoto, Y. *Pharm Bull (Tokyo)* **4** (1956) 24.
17. Söderberg, U. and Wachtmeister, C. A. *J. Pharmacol. Exptl. Therap.* **117** (1956) 298.
18. Ryhage, R. *Arkiv Kemi.* **13** (1959) 475.
19. Brockmann, H. and Müller, W. *Unpublished results* communicated by H. Vorbrüggen.
20. Smith, B. and Delin, S. *Svensk Kem. Tidskr.* **65** (1953) 10.

Received January 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 758–775

Equilibrium Studies of Polyanions

IV. Silicate Ions in NaCl Medium

NILS INGRI

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Equilibria in silicate solutions have been studied in 0.5 m (molal) Na(Cl) at 25°C, using emf's with a hydrogen electrode. The data, which cover total silicate concentrations, B, from 0.0025 to 0.080 m, are given in the form of curves Z (log $[OH^-]$)$_B$ as previously. The data indicate, besides the mononuclear $Si(OH)_4$, $SiO(OH)_3^-$, and $SiO_2(OH)_2^{2-}$, the presence of polynuclear species with a fairly low number, three or four, of Si-atoms. Assuming a single predominating polynuclear species in the range studied, the best agreement was obtained with $Si_4O_6(OH)_6^{2-}$, as in previous work (Part III [1]). The equilibrium constants deduced are given in Table 2, and the agreement obtained with them is shown in Fig. 2.

In Part III [1] of this series, Lagerström investigated equilibria in silicate solutions, using 0.5 m and 3.0 m Na(ClO_4) as ionic medium. These data could be explained assuming the mononuclear species $Si(OH)_4$, $SiO(OH)_3^-$, $SiO_2(OH)_2^{2-}$, and one or two tetranuclear complexes. A satisfactory agreement was obtained between calculated and experimental data. Although this was perhaps the most accurate series of equilibrium measurements by modern principles that is as yet available for silicates, it was noted that the range of the data was not as broad as desirable, and that the conclusions thus needed support from other data.

The present work is a direct continuation of Lagerström's work, and the aim has been to find out what difference (if any) it makes if Na(Cl) is substituted for Na(ClO_4) as ionic medium. Thus, an investigation very similar to that in Part III has been made using 0.5 m Na(Cl) as ionic medium, and then the data have been treated by mathematical and graphical methods independent of previous knowledge of the results in Part III. It may be considered as satisfactory that, in the end, the conclusions came out much the same.

For many of the experimental details, and for a survey of pertinent previous work on silicate solutions, the reader is referred to Part III[1]. In this paper, however, possible structures for the complexes will also be discussed.

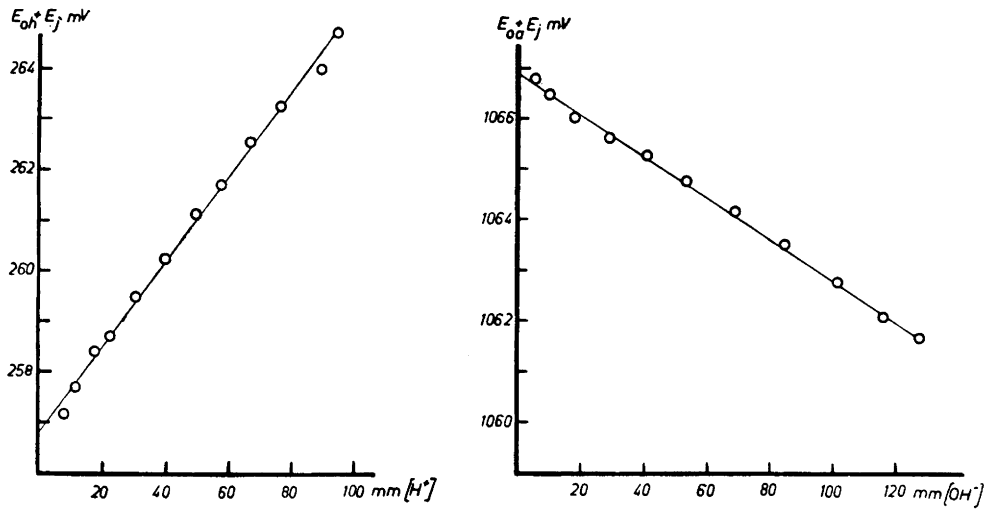


Fig. 1. Diagrams for calculating E_{oa}, E_j and K_w for 0.5 m NaCl medium at 25°C. The values in the two diagrams correspond to the same acid-base titration. The slopes give $E_j h^{-1} = 83$ and $E_j\,[OH^-]^{-1} = 41$ and the intercepts E_{oa} and E_{oh} give $-\log K_w = 13.70$. (See Part II.)

SYMBOLS

The symbols are in general the same as in Parts II[2] and III[1]. Frequently it will be convenient to use the symbol $a = [OH^-]$.

When normalized curves and projection maps have been applied, the normalized quantities B, u and v; corresponding to B, $a = [OH^-]$, and b, have been introduced and defined.

The total concentration B is, in some of the treatments, divided into different parts B_1, $B_2 \ldots B_q \ldots$, each corresponding to complexes A_pB_q with a certain "nuclearity" q. Correspondingly, BZ (the total number of OH^- bound to the $Si(OH)_4$ per liter, or the total charge on the silicate ions per liter), is also divided into the contributions B_1Z_1, $B_2Z_2 \ldots B_qZ_p \ldots$ for the various "nuclearities" present. The functions $f_1 = (1 + \beta_1 a + \beta_2 a^2)$ and $f_Q = \Sigma\beta_{pQ}a^p$ are defined in eqns (3a) and (4a).

Chemical symbols are in Roman type, concentrations in italics. Concentrations and equilibrium constants are expressed in m or mm (moles or millimoles in 1 000 g water).

EXPERIMENTAL

Reagents and apparatus

Sodium chloride, Merck p. a. was used. No Br^- could be detected using the eosin method [3], and the sodium chloride was used after drying at 400°C without further purification.

Hydrochloric acid, Kebo p. a., was standardized against $KHCO_3$ and standard NaOH.

Sodium hydroxide, silicic acid and *alkaline silicate* solutions were prepared and analysed as described in Parts II[2] and III[1], except that solid NaCl was added instead of $NaClO_4$ solution to obtain the ionic medium desired.

Commercial hydrogen gas was used and purified as described in Part II[2]. The *silver-silver chloride* electrodes were prepared [4] by electro-depositing Ag on a Pt foil and afterwards chloridising the silver as anode in dilute HCl.

Following Lagerström's recommendations [1], Jena Geräte glass was used for titration vessels and burets. According to him, during a representative titration, the amount of silica dissolved from the glass-ware will correspond to at most 0.03 mm.

CELL AND PROCEDURE

The same type of *electrode vessel* and *salt bridge* were used as in Part II, except that a different reference electrode was used:

0.5 m NaCl/0.5 m NaCl, saturated with AgCl(s)/AgCl, Ag = SE.

The cell used was thus:

SE/silicate solution/H_2(1 atm), Pt.

The cell including the titration vessel, was kept in an oil thermostat at 25.0 ± 0.1°C, and the whole equipment kept in a thermostated room at 25°C. The emf was measured with a Vernier potentiometer.

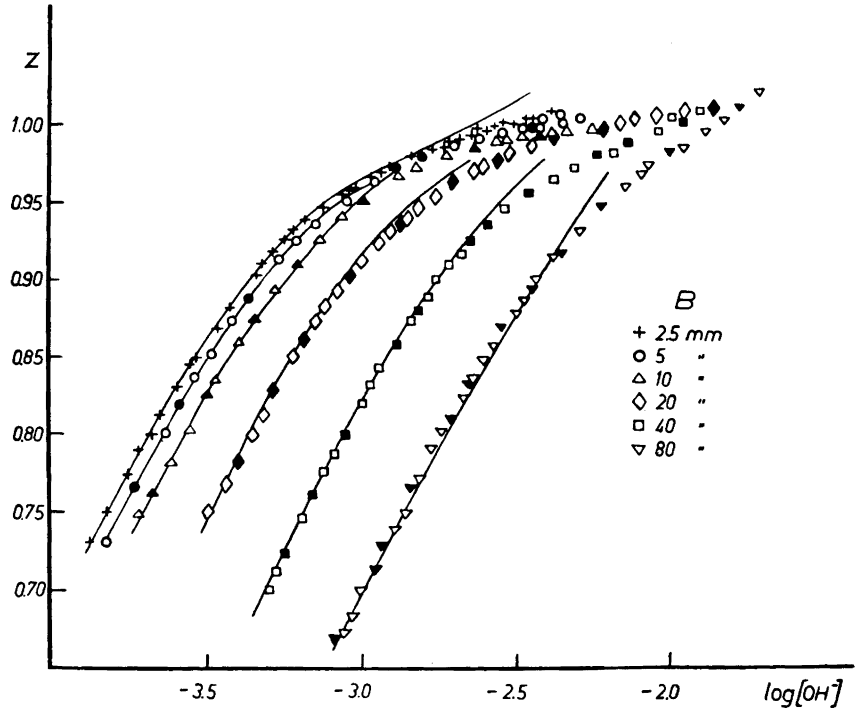


Fig. 2. Experimental data plotted as $Z(\log [OH^-])_B$. Experimental data: points with different symbols for different total concentration B. (See Table 1.) Titrations according to "procedure 1" have filled symbols, those according to procedure 2 open symbols; mm is millimoles in 1000 g water.

Table 1. Data Z (log [OH^-]) for silicates at various total concentrations, B, at 25°C, 0.5 m Na(Cl) ionic medium.

$B = 0.0025$ *m.* Z, $-\log[OH^-]$: 0.731, 3.877; 0.773, 3.7533; 0.801, 3.6777; 0.831, 3.5953; 0.845, 3.5522; 0.868, 3.4634; 0.882, 3.4246; 0.902, 3.3355; 0.931, 3.2202; 0.968, 2.9344; 1.006, 2.3856; (proc. 2) — 0.750, 3.8252; 0.812, 3.6501; 0.917, 3.2902; 0.954, 3.0608; 0.965, 2.9683; 0.996, 2.6238; 0.998, 2.5652; 1.001, 2.4669; (proc. 2) — 0.910, 3.3203; 0.980, 2.8393; 0.983, 2.7702; 0.989. 2.6819; 1.002, 2.4457; (proc. 2) — 0.789, 3.7142; 0.938, 3.1843; 0.956, 3.0409; 0.962, 3.0162; 0.976, 2.8514; 0.994, 2.6755; 0.998, 2.5086; (proc. 2.) — 0.849, 3.5321; 0.925, 3.2501; 0.958, 3.0157; 0.992, 2.6435; 1.000, 2.5401; (proc. 2) — 0.946, 3.1232; 0.972, 2.9115; 0.984, 2.7168; 0.994, 2.5943.

$B = 0.005$ *m.* Z, $-\log[OH^-]$: 0.730, 3.8221; 0.837, 3.5385; 0.913, 3.2663; 0.949, 3.0444; 0.962, 2.9563; 0.993, 2.5421; 0.996, 2.4465; 1.004, 2.3623; 1.000, 2.3010; (proc. 2) — 0.766, 3.7314; 0.819, 3.5858; 0.888, 3.3675; 0.971, 2.8864; 0.978, 2.8006; 0,995, 2.4207; (proc. 1) — 0.801, 3.6313; 0.852, 3.4838; 0.873, 3.4167; 0.924, 3.2092; 1.001, 2.4129; (proc. 2) — 0.935, 3.1485; 0.985, 2.7012; 0,996, 2.4778; 1.002, 2.3501.

$B = 0.010$ *m.* Z, $-\log[OH^-]$: 0.748, 3.7221; 0.803, 3.5608; 0.894, 3.2764; 0.940, 3.6402; 0.972, 2.8233; 0.991, 2.4849; 0.994, 2.3353; (proc. 2) — 0.762, 3.6762; 0.875, 3.3463; 0.983, 2.6367; (proc. 1) — 0.782, 3.6177; 0.835, 3.4703; 0.926, 3.1343; 0.966, 2.8849; 0.997, 2.3645; (proc. 2) — 0.859, 3.4021; 0.979, 2.7504; 0.989, 2.6668; 0.991, 2.4268; (proc. 2) — 0.826, 3.5022; 0.910, 3.2076; 0.951, 2.9958; 0.993, 2.3859; (proc.1).

$B = 0.020$ *m.* Z, $-\log[OH^-]$: 0.749, 3.5001; 0.799, 3.3501; 0.839, 3.2513; 0.872, 3.1427; 0.902, 3.0323; 0.923, 2.9428; 0.939, 2.8465; 0.962, 2.7010; 0.979, 2.5202; (proc. 2) — 0.766, 3.4344; 0.861, 3.1842; 0.911, 2.9964; 0.945, 2.8133; 0.976, 2.5583; 0.992, 2.3133; 0.485, 2.2054; 0.990, 2.1549; 1.009, 1.7766; (proc. 2) — 0.781, 3.3962; 0.828, 3.2856; 0.851, 3.2181; 0.892, 3.0721; 0.930, 2.9023; 0.952, 2.7581; 0.972, 2.6004; 0.985, 2.4441; 0.989, 2.3772; 1.009, 1.8681; (proc. 1) — 0.812, 3.3121; 0.882, 3.1109; 0.935, 2.8704; 0.969, 2.6326; 0.996, 2.2554; 1.002, 2.1123; 1.008, 1.9462; (proc. 2).

$B = 0.040$ *m.* Z, $-\log[OH^-]$: 0.700, 3.3012; 0.711, 3.2818; 0.746, 3.1931; 0.788, 3.0994; 0.819, 3.0010; 0.842, 2.9482; 0.873, 2.8418; 0.900, 2.7643; 0.945, 2.5385; 0.970, 2.3183; 0.980, 2.1821; 0.994, 2.0385; (proc. 2.) — 0.723, 3.2545; 0.761, 3.1603; 0.801, 3.0631; 0.858, 2.8903; 0.880, 2.8169; 0.924, 2.6501; 0.934, 2.5912; 0.955, 2.4581; 0.979, 2.2382; 0.987, 2.1389; 1.000, 1.9534; (proc.1) — 0.777, 3.1246; 0.832, 2.9701; 0.888, 2.7851; 0.909, 2.7184; 0.916, 2.6752; 0.964, 2.3787; 1.007, 1.9010; (proc. 2).

$B = 0.080$ *m.* Z, $-\log[OH^-]$: 0.669, 3.0981; 0.713, 2.9601; 0.810, 2.7121; 0.869, 2.5501; 0.917, 2.3553; 0.982, 1.9991; (proc.1) — 0.672, 3.0593; 0.700, 3.0075; 0.749, 2.8595; 0.791, 2.7763; 0.824, 2.6398; 0.848, 2.6123; 0.886, 2.4756; 0.914, 2.3819; 0.967, 2.0998; 0.984, 1.9545; 1.002, 1.8243; (proc.2) — 0.728, 2.9404; 0.765, 2.8406; 0.833, 2.6519; 0.894, 2.4501; 0.947, 2.2255; 1.010, 1.7761; (proc.1) — 0.683, 3.0344; 0.739, 2.8983; 0.772, 2.8152; 0.802, 2.7444; 0.836, 2.6421; 0.857, 2.5742; 0.878, 2.5015; 0.900, 2.4378; (proc. 2) — 0.930, 2.3004; 0.959, 2.1427; 0.974, 2.0683; 0.994, 1.8864; 1.019, 1.7182 (proc. 2).

Assuming the activity factors to be constant, the emf of the cell can be written

$$E = E_{oa} + 59.15 \log[OH^-] + E_j = E_{oa} + 59.15 \log K_w - 59.15 \log h + E_j \quad (1)$$

Using solutions with known [OH^-], and [H^+], $K_w = 10^{-13.70 \pm 0.02}$ m^2 and $E_j = 41$ [OH^-] mV were obtained. The constant E_{oa} was determined before every titration; for unknown reasons it is slightly different for different hydrogen electrodes. The diagrams in Fig. 1 illustrate this part of the work, which was otherwise quite similar to Part II and Part III.

In the titrations with silicate solutions, the two procedures described by Lagerström as procedure 1 and procedure 2 were used (of course, with Cl^-

instead of ClO_4^-). Thus in each titration, B was kept constant. From the known analytical excess of hydrogen, H, and from the measured $[OH^-]$, Z was obtained from

$$BZ = h - H - K_w h^{-1} = h - H - [OH^-] \quad (2)$$

The data obtained are listed in Table 1, and plotted in Fig. 2 in the usual form, $Z(\log [OH^-])_B$. Within the range shown in Fig. 2, reversible equilibria were obtained, as shown by the fact that points on the same curve were obtained with procedure 1 ($[OH^-]$ decreasing, black points), and procedure 2 ($[OH^-]$ increasing, open symbols).

The experimental range ($Z \sim 0.7$ to 1.0) was limited downwards by the "instability range", and upwards by the fact that at high $[OH^-]$, BZ is obtained as the difference between two large quantities, and thus the accuracy decreases (see eqn. (2)).

The term "instability range", introduced by Lagerström [1], denotes the range where a colloidal precipitate is formed so that stable equilibria, and thus stable emf's cannot be obtained. For $Z < 0.75$, the instability range covers the intermediate total concentrations, B, so that only at low and high values of B, could equilibria be studied.

With titration procedure 1, where, on acidification, part of the solution enters the instability range for a moment, equilibrium was obtained only after some time, whereas with procedure 2 it was obtained practically immediately.

SURVEY OF DATA. CALCULATION OF EQUILIBRIA

In Fig. 2, the data $Z(\log [OH^-])_B$ are seen to give points on different curves for different values of the total concentration, B. This shows that polynuclear complexes must be present. However, with decreasing B the curves seem to approach a limiting curve and one may reasonably assume that the limiting curve has approximately the same shape as the curve for the low B-values. This curve is easily found to fit into a family of curves calculated for a two-step equilibrium [5]. There is every reason to assume that the limiting curve is the one corresponding to equilibria between the three mononuclear species: $Si(OH)_4$, $SiO(OH)_3^-$, $SiO_2(OH)_2^{2-}$, or, in the shorter notation used in Part II and Part III, B, AB and A_2B.

The calculations may be divided into two parts: firstly, the "mononuclear" equilibrium constants were determined as accurately as possible, and secondly an attempt was made to find the formulas and formation equilibrium constants of the most important polynuclear species.

THE EQUILIBRIA WITH MONONUCLEAR SPECIES

It did not prove practicable to get a better approximation to the "mononuclear" curve by making experiments at lower total concentration, B, than about 0.0025 m. At lower B-values, the experimental uncertainty made the data spread so that Z_1 could not be obtained more accurately.

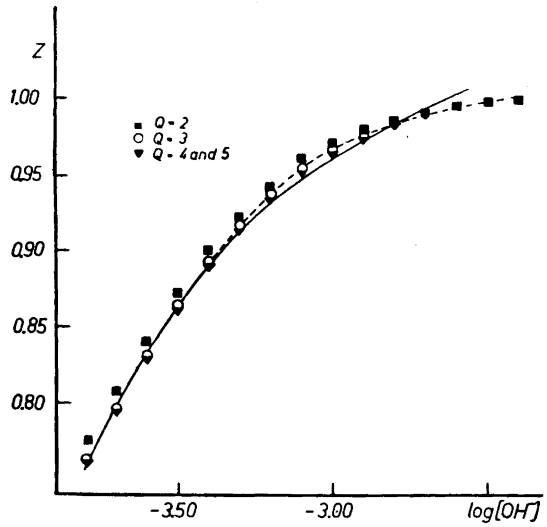


Fig. 3. "Mononuclear curves" $Z_1(\log [OH^-])$ calculated using equation (7) and different values for Q. A full curve: calculated with equilibrium constants in Table 2; a dotted curve, experimental $Z(\log [OH^-])$ curve for $B = 0.0025$ m. The full curve calculated with the constants in Table 2 agrees very well with the points for $Q=4$,

To find the mononuclear curve, $Z_1(\log [OH^-])$, use was made of a method of extrapolation which will now be outlined: It was assumed that the only species formed are the mononuclear B, AB, and A_2B, and the Q-nuclear A_pB_Q, where Q is constant but p may assume several values.

The law of mass action then gives:

$[AB] = \beta_1 ab$; $[A_2B] = \beta_2 a^2 b$; $[A_pB_Q] = \beta_{pQ} a^p b^Q$; and introducing the functions

$$f_1 = 1 + \beta_1 a + \beta_2 a^2 \text{ (3a)} \; ; \; f_1 Z_1 = \beta_1 a + 2\beta_2 a^2 \text{ (3b)} \tag{3}$$

$$f_Q = \Sigma \beta_{pQ} a^p \text{ (4a)} \; ; \; f_Q Z_Q = Q^{-1} \Sigma p \beta_{pQ} a^p \text{ (4b)} \tag{4}$$

we find for the total concentration of B and the total amount of A bound to B per liter:

$$B = b f_1 + b^Q Q f_Q \tag{5a}$$

$$BZ = b f_1 Z_1 + b^Q Q f_Q Z_Q \tag{5b}$$

We notice that Z_Q is the average number of A bound per B in the Q-nuclear complexes. If these are regarded separately, Z_Q, like Z_1 is a function of a only and independent of B.

We may combine (5a) and (5b) to get

$$Z = Z_1 + b^Q Q f_Q (Z_Q - Z_1)(b f_1 + b^Q Q f_Q)^{-1} \tag{6}$$

If a is constant, f_1, f_Q, Z_1 and Z_Q are constant; if B is small, $b \sim B f_1^{-1} \sim B$.constant. Thus, for small B's and constant a we find

$$Z \sim Z_1 + \text{constant } B^{Q-1} \tag{7}$$

Thus one may assume a certain value for Q, and extrapolate a series of plots $Z(B^{Q-1})_a$ to $B = 0$; the intercepts may be used to construct the $Z_1(\log a)$ curve. In Fig 3, the "mononuclear" curve $Z_1(\log a)$ so obtained is plotted for $Q = 2$, 3, 4, and 5. As one extreme, the curve for the lowest B-value used is also given ($B = 0.0025$ m). The equilibrium constants, β_1 and β_2, may be obtained from these curves by the fitting of normalized curves [5]. The following values were obtained (the limits of error indicate the region which might have given an acceptable fit):

	2.5 mM	$Q = 2$	$Q = 3$	$Q = 4$	$Q = 5$	average value
log β_1	4.28	(4.30)	4.29	4.29	4.29	4.29 ± 0.05
log β_2	5.22	(5.34)	5.26	5.24	5.23	5.24 ± 0.15
log K_2	0.94	(1.04)	0.97	0.95	0.94	0.95 ± 0.15

As seen from Fig. 3 a better fit is obtained with $Q=4$ than with $Q=2$ or 3.

POLYNUCLEAR SPECIES

Now that the formulas and equilibrium constants of formation for the mononuclear species were settled, it would have been desirable to apply some general method, such as the integration method used for the borates [2], that would give the average p and q of the polynuclear complexes. Unfortunately, just as in Part III [1], the range of the data is too narrow to allow this method to be applied.

In order to get some information on the formulas of the polynuclear species, an investigation was made to see to what extent the present data could be explained by each of the following three simple assumptions, namely that in addition to the mononuclear species, B, AB, and A_2B, the only species which need be considered are a) a single polynuclear species A_pB_Q, b) a series of complexes A_pB_Q, with p varying but Q constant, and c) a series of uncharged complexes B_q, with q variable.

The assumption c) would mean that all that is formed is a series of polysilicic acids, an idea which has sometimes been suggested; we shall see that it certainly cannot explain the data.

Assumption a) is, of course, a special case of assumption b). The idea that a single value, Q, would predominate is not quite unreasonable, considering that in several hydrolysis systems studied in this Institute (vanadium(V), Rossotti [6]; molybdate, Sasaki [7]; bismuth, Olin [8]) the data quite clearly indicate that in one range the predominating species are a series of complexes with the same number of simple atoms (same Q) but different charges. (In other cases, however, a number of complexes of varying q values have been indicated).

Single polynuclear species, A_pB_Q

Approach with normalized curves. Assuming the species B, AB, A_2B and A_pB_Q, we find from the law of mass action and the definitions of B and BZ:

$$[A_PB_Q] = \beta_{PQ}a^Pb^Q \tag{8}$$

$$B = b + \beta_1 ab + \beta_2 a^2 b + Q\beta_{PQ}a^Pb^Q \tag{9}$$

$$BZ = \beta_1 ab + 2\beta_2 a^2 b + P\beta_{PQ}a^Pb^Q \tag{10}$$

These expressions contain three "unknown" equilibrium constants, two of which (β_1 and β_2) are, however, approximately known. Fixing the ratio

$$l = \beta_2\beta_1^{-2} = 10^{-3.34}$$

at the best value obtained earlier, the two remaining unknowns may be obtained using normalized projection maps (Sillén [9]) obtained by transforming (9) and (10) to the normalized form

$$B = v + uv + lu^2v + Qu^Pv^Q \tag{11}$$

$$BZ = uv + 2lu^2v + Pu^Pv^Q \tag{12}$$

where the normalized variables are

$$u = \beta_1 a \text{ (13a)}; \quad v = b(\beta_1^P\beta_{PQ}^{-1})^{1/1-Q} \text{ (13b)}; \quad B = B(\beta_1^P\beta_{PQ}^{-1})^{1/1-Q} \text{ (13c)} \tag{13}$$

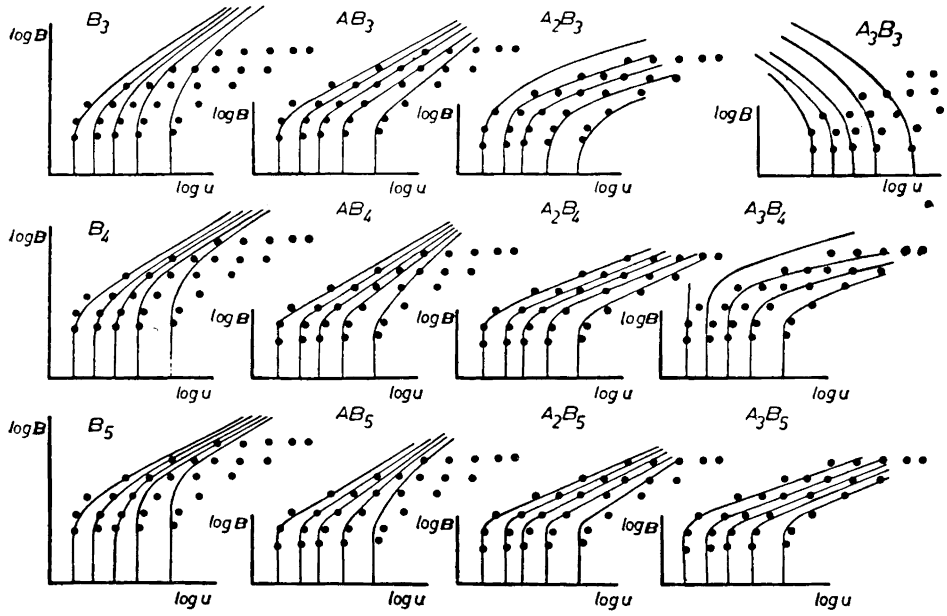


Fig. 4. Projection maps, $B(\log u)_Z$, calculated assuming the single complex A_PB_Q, and various values for P and Q. The experimental data log B (log $[OH^-]$)$_Z$ are in the position of best fit with the calculated projection maps. The experimental points are given for B = 0.0025, 0.005, 0.010, 0.020, 0.040 and 0.080 molal at constant Z. The Z values are from left to right 0.75, 0.80, 0.85, 0.90 and 0.95.

Eliminating v from (11), and (12) we obtain

$$(Q—1)\ \log B = \log [Z(1 + u + lu^2) — (u + 2lu^2)] +$$
$$(Q—1)\ \log [P + (P—Q)\,u + lu^2(P — 2Q)] — P \log u — Q \log (P — QZ) \quad (14)$$

Using eqn. (14) and the value $l = 10^{-3.34}$ obtained earlier, normalized curves log B(log $u)_Z$ have been calculated for a number of sets (P, Q) and moved across the experimental data log B(log [OH^-])$_Z$. Fig. 4 shows the calculated projection maps compared with the experimental data in the position of the best fit, or at least in a position where it is seen that no good fit at all can be obtained. Note that the calculated log B(log $u)_Z$ is vertical for $Z = P/Q$, ($Z = 0.75$ for A_3B_4), and bends to the left for $Z<P/Q$ (all curves for A_3B_3).

The best over-all fit is no doubt obtained with A_2B_4; small deviations at $Z = 0.90$ and $Z = 0.95$ indicate that other polynuclear species may also occur. With A_2B_3 a reasonable fit is obtained for $Z = 0.90$ and 0.95, and with A_2B_5 for $Z = 0.75$ and partly, for $Z = 0.80$; however, neither of these complexes alone would give an acceptable fit for the other end of the diagram. Observing how changes in P and Q affect the slope (Δ log B/Δ log $u)_Z$ and the spacing ($\Delta \log u/\Delta Z)_B$, it is easily seen that no complex outside the range of the (P, Q) sets given in Fig. 4 will be able to give a reasonable agreement with the experimental data.

Our conclusion is thus, just as in Part III[1], that the only single complex which might explain the data in this range is A_2B_4. Assuming this complex, we obtained from log B — log B, and log u — log [OH^-] in the position of best fit, using eqns. (13a) and (13c)

log $\beta_1 = 4.29 \pm 0.05$; log $\beta_{24} = 15.03 \pm 0.20$, log $\beta_2 = 5.24 \pm 0.15$.

The latter comes from the assumed value for l.

Sections at log a = constant. Another approach used the fact that Z_1 and f_1, defined in (3), are functions of a only, and moreover are known approximately. We may write (9) and (10) in the form

$$B = bf_1 + Q\beta_{PQ}a^Pb^Q\ ;\ BZ = bf_1Z_1 + P\beta_{PQ}a^Pb^Q \quad (15)$$

which gives by combination

$$B(Z_1 — Z) = Q(Z_1 — PQ^{-1})\beta_{PQ}a^Pb^Q \quad (16)$$

$$b = Bf_1^{-1}\,(Z — PQ^{-1})\,(Z_1 — PQ^{-1})^{-1} \quad (17)$$

Eliminating b we find

$$B^{Q-1}(Z — PQ^{-1})^Q(Z_1 — Z)^{-1} = f_1^QQ^{-1}\beta_{PQ}^{-1}a^{-P}(Z_1 — PQ_1^{-1})^{Q-1} = \text{function of } a$$

Thus at constant a we would have

$$Q \log (B(Z — PQ^{-1})) = \log [B(Z_1 — Z)] + \text{constant} \quad (18)$$

Plots of log ($B(Z — PQ^{-1})$) against log [$B(Z_1 — Z)$] were made assuming $PQ^{-1} = 0.00$, 0.25, 0.33, 0.50, and 0.67. These plots are given in Fig. 5, except that for $PQ^{-1} = 0.33$.

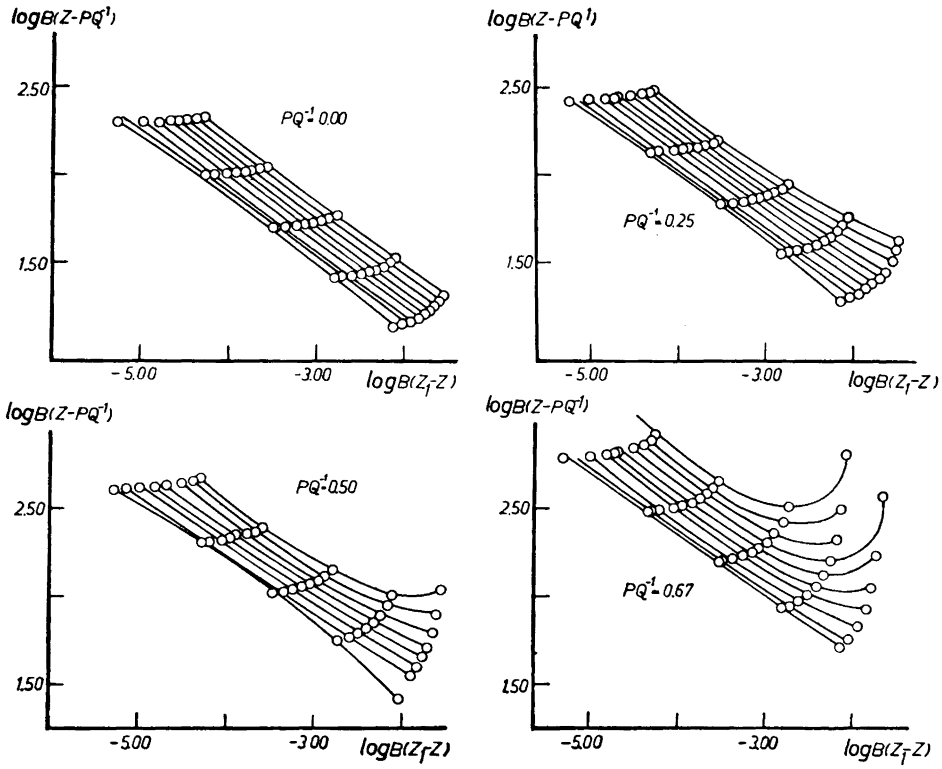


Fig. 5. Plots of $\log(B(Z-PQ^{-1}))$ *versus* $\log(B(Z_1-Z))$ assuming a single complex with various values of PQ^{-1} (compare eqn (18)). The experimental data are given for $-\log [OH^-] = 2.40, 2.50, 2.60, 2.70, 2.80, 2.90, 3.00, 3.10$ and 3.20. For $PQ^{-1} = 0.25$ and 0.67 values for $-\log [OH^-] = 3.30$ are also given and for $PQ^{-1} = 0.5$, $-\log [OH^-] = 2.30$. For $PQ^{-1} = 0.50$ the values for $-\log [OH^-] = 3.10$ and 3.20 have not been calculated.

The complex A_2B_4 would correspond to $PQ^{-1} = 0.50$, and a slope of $Q^{-1} = 0.25$ in the diagrams. As a matter of fact, reasonably straight lines are obtained for all PQ^{-1} lower than 0.67, and the slope of the lines is somewhere between 1/3 and 1/4. This type of plot is less conclusive than the normalized curves, since it pays no attention to the information contained in the distances between the lines. At any rate, it indicates that if any complex predominates, it is a rather small one, with 3 or 4 Si, and with an average charge of less than -1 per Si.

Series of homonuclear complexes A_pB_Q

We have introduced the quantities f_1, Z_1, f_Q, and Z_Q, referring to the mono- and Q-nuclear complexes separately, in connection with the extrapolation method (3), (4). They are all functions of a only. Using them, as before, (9) and (10) take the form

$$B = bf_1 + Qb^Q f_Q \text{ (19a)} ; BZ = Z_1 bf_1 + Z_Q Qb^Q f_Q \text{ (19b) which give} \quad (19)$$

$$b = B(Z - Z_Q)(Z_1 - Z_Q)^{-1} f_1^{-1} \quad (20)$$

Using (19b) and (20) to eliminate b we obtain

$$-Q \log (Z - Z_Q) + \log (Z_1 - Z) + (Q - 1) \log (Z_1 - Z_Q) = \log Q + \log f_Q f_1^{-Q} + (Q - 1) \log B \quad (21)$$

We may normalize (21) to the form

$$\log B = (Q - 1)^{-1} \log Z - Q(Q - 1)^{-1} \log(1 - Z) \quad (22)$$

with the normalized variables

$$\log B = \log B + (Q - 1)^{-1} \log Q + (Q - 1)^{-1} \log f_Q f_1^{-Q} \quad (23)$$

$$\log Z = \log(Z_1 - Z) - \log (Z_1 - Z_Q) \quad (24)$$

If a is constant, Z_1, Z_Q, f_1 and f_Q should keep constant, so that $\log B = \log B +$ constant, $\log Z = \log (Z_1 - Z) +$ constant. For a series of a values experimental curves $\log B(\log(Z_1 - Z))$ were plotted and compared with normalized curves $\log B(\log Z)$, calculated for various values for Q. As shown in Fig. 6, a satisfactory fit could be obtained for $Q = 3$ and 4 (except for $B = 10.00$ mm). For $Q = 2$ and >4, the fit was always poor. For $B \leq 0.010$ m, the curves came too close to the mononuclear one, and the spread in log $(Z_1 - Z)$ made it hard to use (23) and (24). As seen from (23) and (24) the difference $\log B - \log B$, and $\log Z - \log Z$ might be used, for each value of a, to calculate f_Q and Z_Q; in this special case, the accuracy did not permit this to be used as an independent method for determining the several equilibrium constants β_{pQ}; in some other systems it might perhaps prove useful. At any rate, these calculations, like the previous ones, indicate a fairly small complex, with 3 or 4 Si atoms.

Series of uncharged complexes, B_q

If the only species present are A, AB, A_2B, and a series B_q, we would have

$$B = f_1 b + \Sigma q \beta_{oq} b^q ; BZ = f_1 Z_1 b \quad (25)$$

$$\text{Thus } b = BZ f_1^{-1} Z_1^{-1} \quad (26)$$

For each experimental point, BZ, $f_1(a)$, and $Z_1(a)$ are known, so that b can be calculated. A plot $b(B)_a$ was constructed and used for calculating, for a series of round b-values, the function $B - bf_1(a)$.

If the hypothesis were correct, $B - bf_1$ would be $= \Sigma q \beta_{oq} b^q$, and a function of b only. In practice it is found to vary greatly with a, for constant b. Thus it may be concluded that the data cannot be explained assuming the uncharged complexes, B_q, as the only polynuclear complexes.

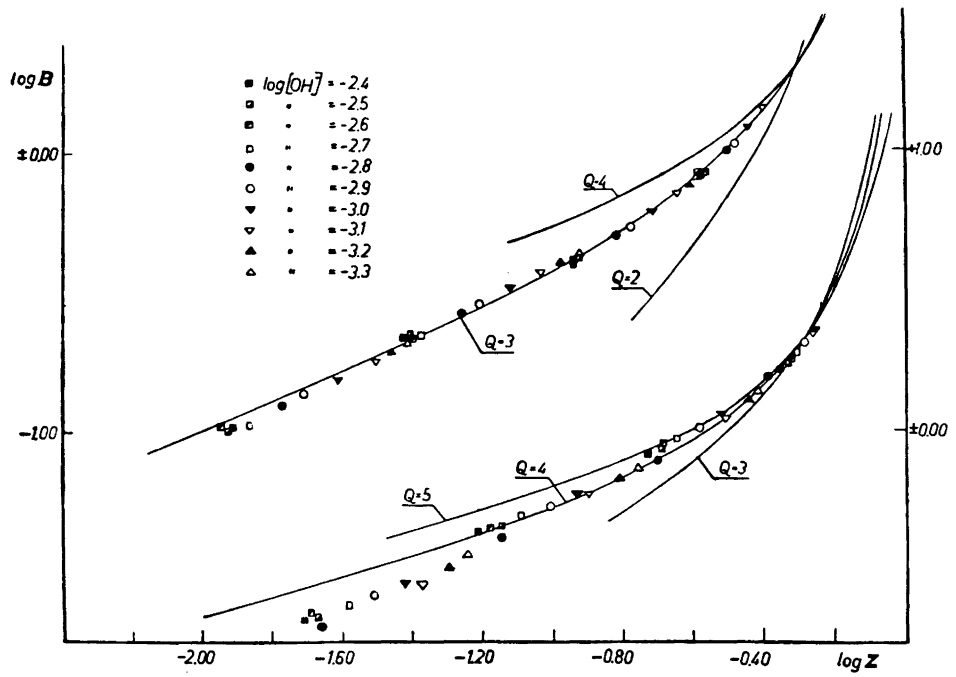


Fig. 6. Series of complexes A_pB_Q. The experimental data log $B(\log (Z_1-Z))_a$ are compared with normalized curves, log B (log $Z)_a$, calculated from (22). In the upper left part of the figure the experimental data are compared with the calculated curve for $Q = 3$ and in the lower right part for $Q = 4$. For comparison, calculated curves for $Q = 2$ and 5 are also given.

CONCLUSIONS

Thus the present data for silicate equilibria in 0.5 NaCl medium, for Z between 0.7 and 1.0, have again given evidence for the existence of the three mononuclear species B, AB, and A_2B, and have given equilibrium constants for their formation. For the polynuclear species, no final and complete picture can be drawn. It is concluded, as in Lagerström's work, that the equilibria with polynuclear species are rapidly obtained, as long as all parts of the solution are kept out of the "instability range". It is also concluded that most or all of the polynuclear ions present are fairly small, with only 3 or 4 Si-atoms. The only single complex that can give a satisfactory agreement with the data is A_2B_4, the same complex as was indicated by Lagerström [1] in $NaClO_4$ medium.

The equilibrium constants are given in Table 2, and the agreement obtained may be studied in Fig. 2.

It seems reasonable to assume, as a working hypothesis, that A_2B_4 is the most important polynuclear silicate ion in the range studied. The small deviations observed indicate that other polysilicate ions, such as A_pB_3 or A_pB_4, are present; the data allow no definite conclusion on their formula

Table 2. Equilibrium constants for silicate reactions at 25°C, 0.5 m Na(Cl) medium, deduced from present data. For comparison, Lagerström's data [1] for 25°C and 0.5 m $Na(ClO_4)$ medium are given.

		0.5 m Na(Cl)	0.5 m $Na(ClO_4)$
$H_2O \rightleftharpoons H^+ + OH^-$	log K_w	$= -13.70 \pm 0.02$	−13.73
$A + B \rightleftharpoons AB$; $OH^- + Si(OH)_4 \rightleftharpoons SiO(OH)_3^- + H_2O$	log β_1	$= 4.29 \pm 0.05$	4.27
$2A + B \rightleftharpoons A_2B$; $2OH^- + Si(OH)_4 \rightleftharpoons SiO_2(OH)_2^{2-} + 2H_2O$	log β_2	$= 5.28 \pm 0.15$	5.44
$A + AB \rightleftharpoons A_2B$; $OH^- + SiO(OH)_3^- \rightleftharpoons SiO_2(OH)_2^{2-} + H_2O$	log K_2	$= 0.99 \pm 0.15$	1.17
$2A + 4B \rightleftharpoons A_2B_4$; $2OH^- + 4Si(OH)_4 \rightleftharpoons Si_4O_6(OH)_6^{2-} + 6H_2O$	log β_{24}	$= 15.03 \pm 0.20$	14.89
$A + B \rightleftharpoons AB$; $Si(OH)_4 \rightleftharpoons SiO(OH)_3^- + H^+$	log $^*\beta_1$	$= -9.51 \pm 0.05$	−9.46
$2A + B \rightleftharpoons A_2B$; $Si(OH)_4 \rightleftharpoons SiO_2(OH)_2^{2-} + 2H^+$	log $^*\beta_2$	$= -22.12 \pm 0.15$	−22.02
$A + AB \rightleftharpoons A_2B$; $SiO(OH)_3^- \rightleftharpoons SiO_2(OH)_2^{2-} + H^+$	log $^*K_2$	$= -12.74 \pm 0.15$	−12.56
$2A + 4B \rightleftharpoons A_2B_4$; $4Si(OH)_4 \rightleftharpoons Si_4O_6(OH)_6^{2-} + 2H^+ + 4H_2O$	log $^*\beta_{24}$	$= -12.37 \pm 0.20$	−12.57

although, of course, an improved agreement probably would be obtained by assuming one or two more complexes, with suitable formation constants.

It seems most desirable to extend the studies of silicate to other media, and also to apply other experimental methods. Work in these directions has been started.

POSSIBLE STRUCTURES FOR SILICATE IONS

Some thought should perhaps be given to the question of what the structures of the silicate ions in solution may be, especially since rather divergent opinions have recently appeared in literature. A short review of the pertinent evidence will be given.

Principles of silicate structures. Beginning with the classical work of the Braggs, the crystal structures have been worked out for a very large number of natural and artificial silicates. They have proved to be built up according to a few simple principles, which are very different from those one might have guessed in the pre-X-ray period of chemistry. This subject has been treated very well in a number of standard text books [10–12].

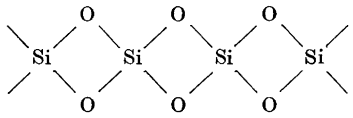
Rule 1: Each Si atom is surrounded by a tetrahedron of 4 O-atoms. The exceptions are very few.

A structure with octahedral coordination Si-6 O has been suggested in SiP_2O_7 (Levi and Peyronel [13]). It is also possible that Si is coordinated to six O's in some organic complexes, such as those with pyrocatechol [14], $Si(O_2C_6H_4)_3^{2-}$, and in a complex with three acetylacetone [15] ($CH_3COCH_2COCH_3 = HA$) acting like a positive ion, SiA_3^+, and forming salts like SiA_3AuCl_4, SiA_3FeCl_4, and $(SiA_3)_2PtCl_6$, soluble in ethanol and acetic acid. A very clear case of 6-coordination around Si is the hexafluorosilicate ion, SiF_6^{2-}, which is octahedral [16].

Although carbon is 3-coordinated in a number of compounds (C_2H_4, CO_3^{2-}, $COCl_2$ etc), no case has been observed with Si coordinated with three O in a crystal structure.

Rule 2: Two SiO_4-tetrahedra can be joined by sharing an oxygen corner. The tetrahedra do not share edges, neither is an oxygen atom shared between more than two Si-atoms.

The only known case of sharing edges seems to be the fibrous form of SiO_2 described by Weiss and Weiss [17]. It seems to have a crystal structure of the same type as SiS_2 (Zintl and Loosen [18]), the SiO_4-tetrahedra forming infinite chains by sharing opposite edges with their neighbors:



We shall disregard these few exceptions and consider instead the overwhelming number of normal structures. In accordance with the two principles given, the SiO_4-tetrahedra can join to form isolated groups, chains (sometimes double chains), sheets and three-dimensional networks. As to detailed descriptions of the structures and references, the reader is referred to the standard texts (*e.g.* Wells [12], p. 569—591).

Anhydrous silicates

Separate SiO_4^{4-} groups are found in a number of minerals and artificial silicates, *e.g.* Mg_2SiO_4. Separate groups, $Si_2O_7^{6-}$, $O_3SiOSiO_3$, are less common, but are found in a few minerals (thortveitite $Sc_2Si_2O_7$; hemimorphite $Zn_4(OH)_2Si_2O_7$; tilleyite [19]). Three-silicon rings, $Si_3O_9^{6-}$, have been reported in the crystal structures of α-and β-wollastonite, $CaSiO_3$, of benitoite, $BaTiSi_3O_9$ and catapleite. Some doubt has been cast upon these rings as Dornberger-Schiff, Liebau and Thilo [20] have reinvestigated the structure of β-wollastonite and found it to contain infinite chains, $(SiO_3)_n$, and not separate rings, $(SiO_3)_3$.

Four-silicon rings, $Si_4O_{12}^{8-}$, seem to exist as independent units in the crystal structure of axinite [21], $Ca_2(Fe, Mn)Al_2(BO_3)(Si_4O_{12})OH$. In the sheet structure of apophyllite, alternate four- and eight-silicon rings occur.

Separate six-silicon rings, $Si_6O_{18}^{12-}$, have been found in beryl, $Be_3Al_2Si_6O_{18}$, and in tourmaline [22]. In the sheet structures of clays, minerals and micas, six-silicon rings are joined to form infinite sheets.

Silicates containing hydrogen

Although a large number of crystalline hydrated silicates are known (see, *e.g.*, Vail [23], *p.* 144—150), very few crystal structures have hitherto been determined. The existence of tetrahedral groups, $HSiO_4^{3-}$, in afwillite [24], ($Ca_3(SiO_3OH)_2(H_2O)_2$) and in $Ca_2(SiO_3OH)OH$ (Heller [25]) seems fairly certain. Thilo and coworkers [26] have concluded from reaction studies that some hydra-

ted Ca and Ca-Na silicates contain $H_2SiO_4^{2-}$, and $HSiO_4^{3-}$ groups, and that crystalline NaH_3SiO_4 contains $H_3SiO_4^-$ groups [27]. Also the latter result was corroborated by the great similarity between the X-ray powder photographs of NaH_3SiO_4 and LiH_2PO_4.

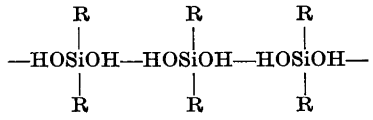
According to a private communication by Machatschki and Preisinger [28] the crystal structure for "sodium metasilicate enneahydrate", really $Na_2(H_2SiO_4)(H_2O)_8$, has been investigated. It contains isolated $H_2SiO_4^{2-}$ groups, and no condensed silicate ions, in contrast to anhydrous Na_2SiO_3 which contains chains, $(SiO_3)_n$, (Grund and Pizy [29]). According to Heide *et al.*[30], dioptase, $Cu_6Si_6O_{18}(H_2O)_6$ contains isolated Si_6O_{18}-rings and H_2O molecules.

Organic Si-O compounds

There are two main series of organic Si compounds, namely silicones and polysilicic acid esters, both of which contain an Si-O skeleton, with R or RO (R = hydrocarbon radical) attached to the remaining corners of the Si tetrahedra.

Silicones are known with straight Si-O chains (2 to 11 Si), with Si-O rings, and also with two rings having one Si atom in common (spiro structures). Rings $(R_2SiO)_n$ with n between 3 and 8 have been prepared and isolated. The 4- and 5-rings seem to be the most stable (Wells [12], p. 562—566). Ring structures $(SiO)_8$ and $(SiO)_4$ have been proved by X-ray studies [31,32] on $((CH_3)_2SiO)_8$ and $((CH_3)_2SiO)_4$. For *polysilicic acid esters*, no structure determination seems to have been made as yet. The compounds $((RO)_2SiO)_n$ with $n = 3$ or 4 are in all probability ring-shaped (Signer and Gross [33]; R = C_6H_{11}, *cyclo*hexyl). Iler [34] claims to have prepared even larger polysilicate ester rings with up to 8 Si (R = C_4H_9). There are also esters known with a lower proportion of R, say $(ROSiO_{1.5})_n$, which may contain a dense Si-O core (several condensed rings), surrounded by R groups (Iler [35], p. 77—78, n around 10 or 12; Sprung and Guenther [36], $n = 6$ and 8).

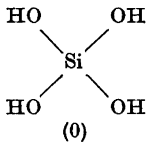
It may be of interest that the methyl and ethyl silanols, R_3SiOH, are, like alcohols, strongly associated in the liquid state and in *cyclo*hexane solution (Grubb and Osthoff [37]). In ROH solutions, R_3SiOH reacts with the ultimate formation of an equilibrium mixture of $R_3SiOSiR_3$, R_3SiOR, and H_2O (Grubb [38]). In crystals, molecules of $R_2Si(OH)_2$ form chains by means of hydrogen bonds (Kakudo and Watase [39], Kasai and Kakudo [40]).



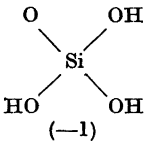
Structures in solutions

It seems plausible that the silicate ions which occur in aqueous solutions are built up on the same principles as have been found in practically all crystalline silicates, namely as SiO_4-tetrahedra, isolated or sharing O atoms, and with

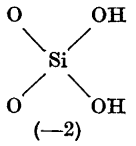
a certain number of protons attached to the unshared O atoms, forming OH groups. The structures of the mononuclear silicate ions would then be,



(0)

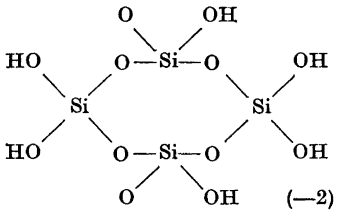


(—1)



(—2)

The present data indicate that complexes with a certain number of Si atoms, namely 4, predominate in the solutions. It seems likely that they contain a ring, so that the structure of A_2B_4 would be

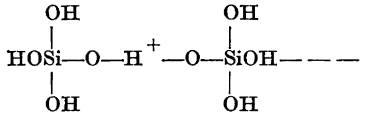


(—2)

If the polysilicate ions had been chain-like, it is hard to understand why, for instance, 4 should be preferred to 2 or 3. One might suggest a still more condensed, tetrahedral structure, $Si_4O_{10}H_2^{2-}$, like P_4O_{10}, however this group has not been found in crystal structures.

Two other hypotheses have been proposed and as far as we can find, are based on insufficient evidence.

Miedreich [41] (p. 14) finds it "very improbable" that Si-O-Si bonds are formed in aqueous solutions, since the equilibria are reached so rapidly in clear solutions. He prefers to think (p. 89) that the polysilicate ions are formed by hydrogen bridging

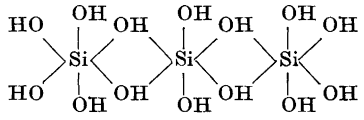


The argument might seem plausible if one makes a comparison only with the polyphosphates; the P-O-P bonds are certainly hydrolyzed only slowly. On the other hand, the formation of O bonds in aqueous solution is a rapidly reversible process for several neighbors of Si in the periodic table. For instance, the polyborate ions undoubtedly contain B-O-B bonds and still give very rapid equilibria [2]. The same is true with the Be-OH-Be bonds formed in the hydrolysis of Be^{2+}, and with quite a number of other O or OH bonds met with in the hydrolysis of positive ions (Kakihana and Sillén[42], Sillén [43]).

With Miedreich's hypothesis it also seems hard to explain the composition of the "X-ray amorphous" precipitates found by Thilo, Wodtcke and Funk [44] on adding Ag^+ to silicate solutions. The general formula, according to them, is $Ag_{n+2}H_nSi_nO_{3n+1}$, with preference for $n = 1$, 2, or 4, whereas Miedreich's

hypothesis demands an average number of 4 O per Si. The formula given by Thilo, Wodtcke and Funk would seem to indicate open-chain ions with 2 or 4 Si. However, as these authors note themselves, it then seems hard to explain why chains with exactly these numbers would be preferred.

Iler [35] (p. 18—30) has followed up a proposal by Weyl [45] that Si is six-coordinated when present as silicate ions in solutions, and that polysilicate ions are formed by sharing two OH's between two adjacent Si-atoms, *e.g.*,



Although the octahedral SiF_6^{2-} ion is well established, there seems to be no structural evidence for the existence of an $Si(OH)_6^{2-}$ ion. On the contrary, as mentioned above, the crystal structure of "$Na_2SiO_3(H_2O)_9$" seems to contain $H_2SiO_4^{2-}$ tetrahedra rather than $Si(OH)_6^{2-}$ octahedra. Nor is there any other evidence to make the existence of such ions very plausible.

The similarity of the Raman spectra of the A_2B silicate ion with $H_2PO_4^-$, (Fortum and Edwards [46]) also suggests strongly a tetrahedral structure $SiO_2(OH)_2^{2-}$.

On the other hand, it may well be that the condensation and breaking of the Si-O-Si bonds in aqueous solution proceeds through some unstable intermediate state with a higher coordination than 4 around Si, and that the rapidity of the reactions is due to the relative ease with which Si can assume a higher coordination number.

Acknowledgements. The present work was carried out thanks to the generous support of Columbia-Southern Chemical Corporation. I am greatly indebted to Professor Lars Gunnar Sillén, who introduced me to these types of equilibrium measurements and for his valuable help during the course of the work. I am also indebted to Drs Franklin Strain and Björn Holmström for valuable advice and support in many ways. I would like to thank Dr. Gösta Lagerström for help and guidance in the beginning of this work, and Mrs. Mina Frydman and Mr. Frans Ericson for valuable help in the experimental parts.

REFERENCES

1. Lagerström, G. *Acta Chem. Scand.* **13** (1959) 722. (Part III.)
2. Ingri, N., Lagerström, G., Frydman, M. and Sillén, L. G. *Acta Chem. Scand.* **11** (1957) 1034. (Part II.)
3. Bates, R. and Pinching, G. D. *J. Research Natl. Bur. Standards* **37** (1946) 311; Güntelberg, E. *Thesis,* Copenhagen 1938.
4. Brown, A. S. *J. Am. Chem. Soc.* **56** (1934) 646.
5. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 186.
6. Rossotti, F. J. C. and Rossotti, H. *Acta Chem. Scand.* **10** (1956) 957. (Part I.)
7. Sasaki, Y. *To be published;* Sasaki, Y., Lindqvist, I. and Sillén, L. G. *J. Inorg. & Nuclear Chem.* **5** (1959) 93.
8. Olin, Å. *Acta Chem. Scand.* **13** (1959). *In press.*
9. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 803.
10. Bragg, W. L. *Structures of minerals,* Ithaca, N.Y. 1937.
11. Pauling, L. *The nature of the chemical bond,* 2nd Ed. Ithaca, N.Y. 1940.
12. Wells, A. F. *Structural inorganic chemistry,* 2nd Ed., Oxford 1950.

13. Levi, G. R. and Peyronel, G. *Z. Kryst.* **A 92** (1935) 190.
14. Rosenheim, A., Raibmann, B. and Schendel, G. *Z. anorg. allgem. Chem.* **196** (1931) 160.
15. Dilthey, W. *Ber.* **36** (1903) 923.
16. Bozorth, R. M. *J. Am. Chem. Soc.* **44** (1922) 1066.
17. Weiss, A. and Weiss, A. *Z. anorg. allgem. Chem.* **276** (1954) 95.
18. Zintl, E. and Loosen, K. *Z. physik. Chem.* **A174** (1935) 301.
19. Smith, J. V. *Acta Cryst.* **6** (1953) 9.
20. Dornberger-Schiff, K., Liebau, F. and Thilo, E. *Naturwiss.* **41** (1954) 551.
21. Ito, T. and Takéuchi, Y. *Acta Cryst.* **5** (1952) 202.
22. Ito, T. and Sadanaga, R. *Acta Cryst.* **4** (1951) 385.
23. Vail, I. G. *Soluble silicates*, Reinhold Publishing Comp. New York, 1952.
24. Megaw, H. D. *Acta Cryst.* **5** (1952) 477.
25. Heller, L. *Acta Cryst.* **5** (1952) 724.
26. Thilo, E., Funk, H. and Wichmann, E. M. *Abhandl. deut. Akad. Wiss. Berlin, Math-Naturw. Kl.* **No 4**, 1950.
27. Thilo, E. and Miedreich, W. *Z. anorg. Chem.* **267** (1951) 76.
28. Machatschki, F. and Preisinger, A. *Private communication* to L. G. Sillén 1955.
29. Grund, A. and Pizy, M. *Acta Cryst.* **5** (1952) 937.
30. Heide, H. G., Boll-Dornberger, K., Thilo, E. and Thilo, E. M. *Acta Cryst.* **8** (1955) 425.
31. Frevel, L. K. and Hunter, M. I. *J. Am. Chem. Soc.* **67** (1945) 2275.
32. Steinfink, H., Post, B. and Fankuchen, I. *Acta Cryst.* **8** (1955) 420.
33. Signer, R. and Gross, H. *Ann.* **488** (1931) 56.
34. Iler, R-K. *Ind. Eng. Chem.* **39** (1947) 1384.
35. Iler, R. *The colloidal chemistry of silica and silicates*, Cornell University Press, Ithaca, N.Y. 1955.
36. Sprung, M. M. and Guenther, F. O. *J. Am. Chem. Soc.* **77** (1955) 3990, 3996.
37. Grubb, W. T. and Osthoff, R. C. *J. Am. Chem. Soc.* **75** (1953) 2233.
38. Grubb, W. T. *J. Am. Chem. Soc.* **76** (1954) 3408.
39. Kakudo, M. and Watase, T. *J. Chem. Phys.* **21** (1953) 1894.
40. Kasai, N. and Kakudo, M. *Bull. Chem. Soc. Japan* **27** (1954) 605.
41. Miedreich, W. *Thesis*, Frankfurt am Main 1954.
42. Kakihana, H. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 985.
43. Sillén, L. G. *Quart. Rev.* (1959). *In print.*
44. Thilo, E., Wodtcke, F. and Funk, H. *Z. anorg. allgem. Chem.* **278** (1955) 225.
45. Weyl, V. A. *A new approach to surface chemistry and to heterogeneous catalysis* (Mineral Industries Exp. Sta. Bull. No 57, State College, Pa:Pennsylvania State College, 1951), p. 47.
46. Fortnum, D. and Edwards, J. O. *J. Inorg. & Nuclear Chem.* **2** (1956) 264.

Received January 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 776–783

On the Bile Acid Metabolism in the Pig

Bile Acids and Steroids 81

SUNE BERGSTRÖM, HENRY DANIELSSON
and ÅKE GÖRANSSON

Department of Physiological Chemistry, University of Lund, Lund, and the Department of Chemistry, Karolinska Institutet, Stockholm, and Djursjukhuset, Hälsingborg, Sweden

Pig bile, collected from bile fistulas at different times after fistulation, has been analyzed. The main bile acids found in samples collected during the first day after fistulation were chenodeoxycholic, hyocholic and hyodeoxycholic acid, conjugated with glycine and to a small extent with taurine. Bile samples collected later than 24 h after fistulation did not contain hyodeoxycholic acid, indicating that this acid is formed by the action of intestinal microorganisms during the enterohepatic circulation of the bile acids.

This was confirmed by the results of studies on the metabolism of labelled chenodeoxycholic, hyodeoxycholic and hyocholic acid in intact and bile-fistulated pigs.

24-^{14}C-chenodeoxycholic acid was 6α-hydroxylated to hyocholic acid in the fistulated pig, while 24-^{14}C-hyodeoxycholic acid was not metabolized except for conjugation. After circulating enterohepatically in intact pigs for 3 days, 50 % of tritium-labelled hyocholic acid had been transformed into hyodeoxycholic acid.

The metabolism of the main bile acids in the pig could then be summarized as follows:

$$\text{Chenodeoxycholic acid} \xrightarrow{\text{liver}} \text{hyocholic acid}$$

$$\text{Chenodeoxycholic acid} \xrightarrow[\text{microorganisms}]{\text{intestinal}} \text{hyodeoxycholic acid}$$

The main bile acids found in gall-bladder bile of pigs are hyocholic, hyodeoxycholic and chenodeoxycholic acid. They are present as conjugates with glycine and to a small extent with taurine [1]. Hyodeoxycholic (3α, 6α-dihydroxycholanic) acid was identified in 1923 by Windaus and Bohne [2]; the presence of chenodeoxycholic (3α, 7α-dihydroxycholanic) acid was observed in 1939 by Ido and Sakurai [3], but it was not until 1954 that Haslewood and Sjövall [4] demonstrated the presence of considerable amounts of a trihydroxy bile acid. Its structure was shortly thereafter shown to be 3α, 6α, 7α-trihydroxycholanic acid [5,6].

The present investigation is concerned with the metabolism of these three bile acids in the pig. We have prepared labelled hyocholic, hyodeoxycholic and chenodeoxycholic acid and injected them intraperitoneally into pigs. The bile acids were isolated from the bile by chromatography and the identity of the labelled compounds was established by repeated chromatography and cocrystallization with authentic material.

MATERIAL AND METHODS

Labelled compounds. 24-^{14}C-labelled chenodeoxycholic and hyodeoxycholic acid were prepared according to Bergström *et al.*[7] Hyocholic acid was labelled with tritium gas using the method of Wilzbach [8] in the apparatus described by Bergström and Lindstedt [9]. The labelled bile acids were purified by reversed phase partition chromatography in suitable systems (see below) and crystallized to constant specific activity with unlabelled material.

Chromatographic procedures. Reversed phase partition chromatography as described by Sjövall [10] and Norman [11] was used for the separations of the bile acids. The following solvent systems were employed:

	Moving phase	Stationary phase
C.	150 ml methanol	15 ml chloroform
	150 ml water	15 ml *iso*-octanol
D.	300 ml water	100 ml *n*-butanol
F.	165 ml methanol	45 ml chloroform
	135 ml water	5 ml heptane

Hyflo Supercel (Johns Manville and Co., USA) or Hostalene (Farbwerke Hoechst, G.m.b.H., West-Germany) [12] was used as supporting material. The procedure for making Hyflo Supercel hydrophobic has been described [10]. Prior to use, Hostalene was extracted with 95 % ethanol for 48 h in a Soxhlet extractor and then dried at 75°. Columns were prepared as described by Norman [11]. When Hostalene was used as supporting material only 3 ml of stationary phase were used per 4.5 g of this material.

Radioactivity assay. ^{14}C was determined with a Tracerlab end-window counter, tritium with a Tracerlab gas-flow counter.

Animal experiments and procedures for analysis of the bile. The bile acid was injected intraperitoneally in the form of its sodium salt, dissolved in about 10 ml of saline.

Young female pigs weighing about 20 kg were used. Bile fistulas were prepared using trichloroethylene anaesthesia by inserting a polyethylene cannula into the gall-bladder and tying off the ductus choledochus. During the collection of bile from the fistula the animals were restrained in a cage, to which they had previously been conditioned for approximately a week. The animals had free access to drinking water and were fed their normal diet. Bile was collected for 4 h periods into 95 % ethanol. After determination of their radioactivity, the bile samples were diluted with water, acidified with hydrochloric acid and extracted twice with water-saturated butanol. The combined butanol extracts were washed with small volumes of water until neutral and evaporated to dryness *in vacuo*. The residue was chromatographed in phase system C, the effluent was titrated with 0.02 N methanolic sodium hydroxide, and suitable aliquots were assayed for radioactivity. For purpose of further identification the conjugated bile acids were hydrolyzed with N NaOH in a closed steel tube for 6 h at 120°. The saponification mixtures were diluted with water, acidified with hydrochloric acid and extracted twice with ether. The combined ether-extracts, that contained the free bile acids, were washed until neutral with water, and evaporated to dryness. The residue was chromatographed on phase systems C or F, depending upon the acid being purified. The labelled bile acids were diluted with carrier to a weight of approximately 100 mg and crystallized four times from different solvents to a final weight of crystals of about one quarter the original weight. Hyocholic acid was crystallized from ethyl acetate / methanol / heptane, acetone/water, acetic acid/water and acetone/water; hyodeoxycholic acid from ethyl acetate / methanol / heptane, ethyl acetate, acetic acid / water and acetone / water; chenodeoxycholic acid from ethyl acetate / light petroleum, acetic acid / water and ethyl acetate / light petroleum twice.

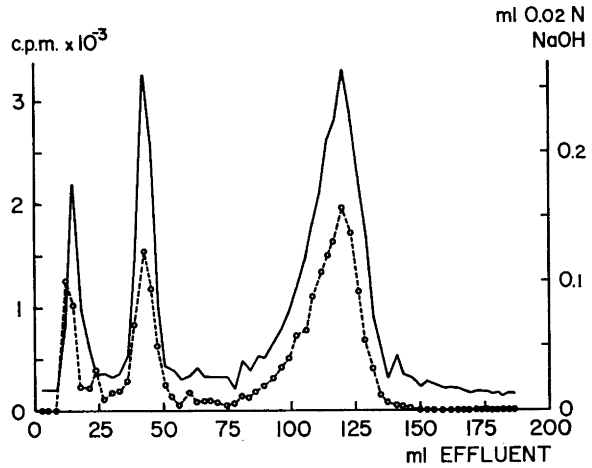


Fig. 1. Chromatogram of unhydrolyzed fistula bile from pig injected i. p. with 24-^{14}C-chenodeoxycholic acid. Fistulation 48 h before the injection. Phase system C. 4.5 g hydrophobic Hyflo Supercel. Solid line: titration. Broken line: c.p.m.

RESULTS

The composition of fistula bile. A sample of pig fistula bile, collected 48 h after the preparation of the fistula, was chromatographed on phase system C, and was found to contain three distinct titration peaks, as illustrated in Fig. 1. The bile acids constituting the first peak were identified as a mixture of taurochenodeoxycholic and taurohyocholic acid by rechromatography on phase system D and after saponification on C and F. The second titration peak was due to glycohyocholic acid, identified in the same manner as described above.

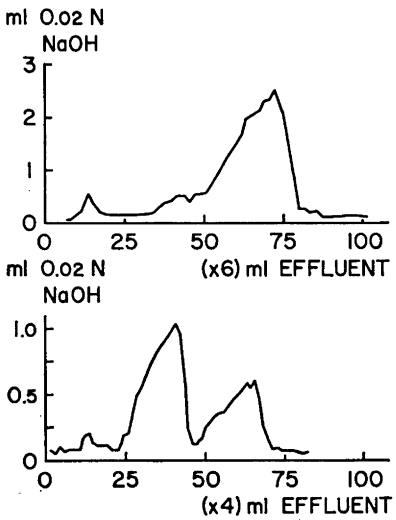


Fig. 2. Chromatogram of hydrolyzed dihydroxycholanic acid band from bile collected 48 h after fistulation (upper curve) and from bile collected immediately after fistulation (lower curve). Phase system F. 27 g hostalene (upper curve), 18 g hostalene (lower curve).

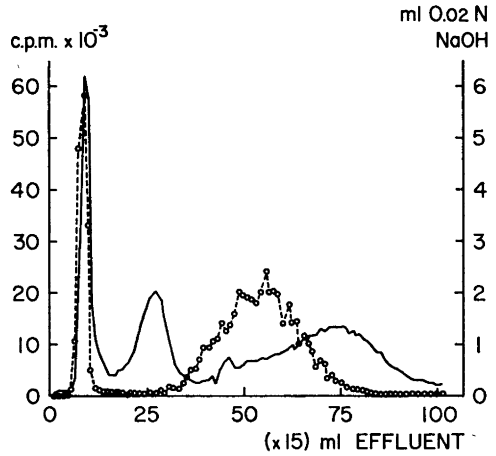


Fig. 3. Chromatogram of unhydrolyzed fistula bile from pig injected i.p. with 24-^{14}C-hyodeoxycholic acid. Fistulation 4 days before injection. Phase system C. 67 g hostalene. Solid line: titration. Broken line: c.p.m.

The third peak, appearing in the usual location for glycodihydroxycholanic acids was hydrolyzed and rechromatographed on phase system F and found to contain only chenodeoxycholic acid, *cf.* Fig. 2. For comparison, there is included in this figure a titration curve of the hydrolyzed glycodihydroxycholanic acids from fistula bile, collected immediately after fistulation, when the bile acids present in the entero-hepatic circulation were being excreted. The presence of a second titration peak, due to hyodeoxycholic acid, is clearly seen. This acid, however, is not present in the bile excreted through the fistula two days later.

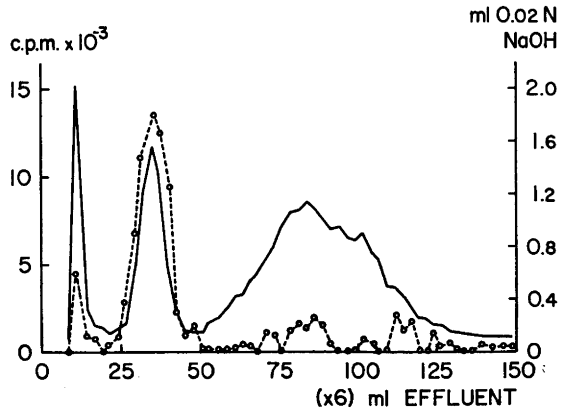


Fig. 4. Chromatogram of unhydrolyzed bile from pig injected i. p. with tritium-labelled hyocholic acid 24 h before sacrifice. Phase system C. 27 g hostalene. Solid line: titration. Broken line: c.p.m.

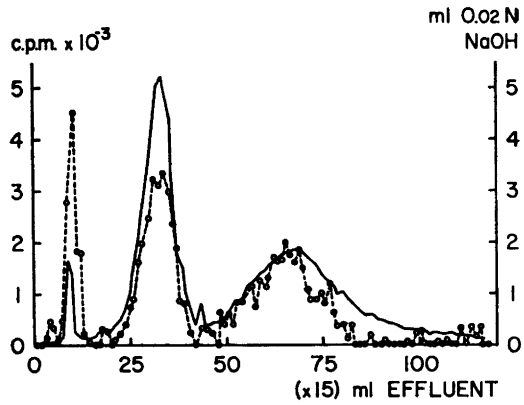


Fig. 5. Chromatogram of unhydrolyzed bile from pig injected i. p. with tritium-labelled hyocholic acid 72 h before collection of bile. Phase system C. 67 g hostalene. Solid line: titration. Broken line: c.p.m.

Injection of 24-^{14}C-chenodeoxycholic acid. Fig. 1. shows a chromatogram of a sample of unhydrolyzed fistula bile from an animal, where the fistula was prepared 2 days prior to the injection of 6×10^6 c.p.m. 24-^{14}C-chenodeoxycholic acid (approx. 40 μC, 2 mg). The radioactivity was found to be distributed in all three titration peaks. 5 % of the radioactivity was retained in the stationary phase and consisted of unchanged chenodeoxycholic acid. More than 90 % of the radioactivity of the first peak was present as taurochenodeoxycholic acid. After hydrolysis and rechromatography on phase system F, this material was diluted with carrier chenodeoxycholic acid, and the specific activity remained constant through four crystallizations. The second radioactive peak was shown to be glycohyocholic acid by rechromatography after saponification on phase system C and crystallization with unlabelled material. The radioactive compound appearing in the third titration peak was in a similar manner shown to be glycochenodeoxycholic acid.

Injection of 24-^{14}C-hyodeoxycholic acid. In Fig. 3 is shown a chromatogram of the unhydrolyzed bile acids from an animal, that had been injected with 9×10^6 c.p.m. 24-^{14}C-hyodeoxycholic acid (3 mg) four days after the preparation of the bile fistula. The radioactivity in the first peak was identified by rechromatography as taurohyodeoxycholic acid. After additional chromatography as the free acid, it was crystallized four times with unlabelled hyodeoxycholic acid, the specific activity remaining constant. The second radioactive elution band was found to be glycohyodeoxycholic acid, the identity of which was confirmed by cocrystallization with authentic hyodeoxycholic acid. 12 % of the radioactivity was retained in the column and consisted of unchanged free hyodeoxycholic acid.

Injection of tritium-labelled hyocholic acid. Two animals were injected each with 1×10^7 c.p.m. hyocholic acid (2 mg). One animal was sacrificed 24 h later and the bile was collected from the gall-bladder. In the other animal a bile fistula was prepared 72 h after the injection and bile was collected for a

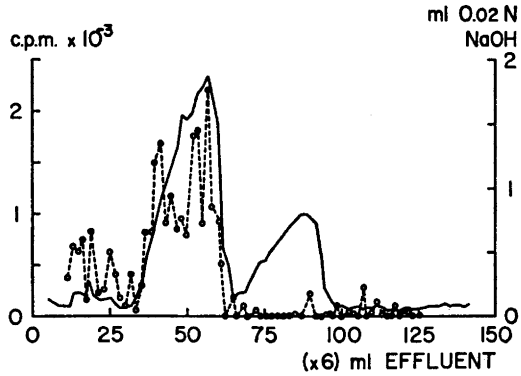


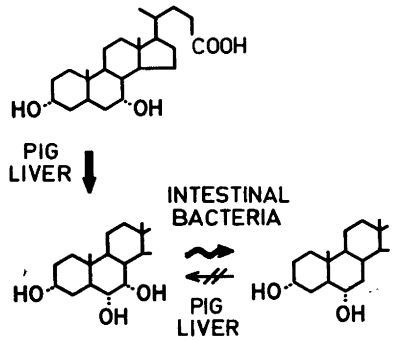
Fig. 6. Rechromatography of dihydroxycholanic acid band (*cf.* fig. 5) after hydrolysis. Phase system F. 27 g hydrophobic Hyflo Supercel. Solid line: titration. Broken line: c.p.m.

few hours. In Fig. 4 is shown a chromatogram of the non-saponified bile from the former animal, where the labelled hyocholic acid had been present in the entero-hepatic circulation of the bile for a maximum period of 24 h. The injected acid had been conjugated with glycine (second radioactive peak), and to a lesser degree with taurine (first peak). Small amounts of labelled material were found in the glycodihydroxycholanic acid band.

In the chromatogram (Fig. 5) of unhydrolyzed bile from the second animal, that had an intact entero-hepatic circulation for 72 h after the injection of the labelled hyocholic acid, it is seen, that almost half of the radioactivity is present in a band corresponding to a glycine conjugated dihydroxycholanic acid. This material was subsequently saponified and rerun with phase system F (Fig. 6). The radioactivity was found to coincide with the titration peak of hyodeoxycholic acid. The identity of the above-mentioned radioactive compounds was confirmed by cocrystallization with unlabelled material, where the specific activity remained constant through four recrystallizations.

DISCUSSION

The results obtained can be summarized in the following scheme:



The observation that hyodeoxycholic acid was present only in the first portions of the fistula bile but later disappeared, indicated that this acid might be a secondary acid not produced by the liver cells. This was further strengthened by the results with labelled bile acids, that showed that chenodeoxycholic acid was a precursor of hyocholic acid, whereas hyodeoxycholic acid remained unchanged except for its conjugation when injected intraperitoneally into pigs with a functioning bile fistula.

It was established that hyodeoxycholic acid indeed was a secondary product when labelled hyocholic acid was injected into intact pigs. After 24 h some labelled hyodeoxycholic acid had been formed, and after three days about half of the activity was present in the hyodeoxycholic acid formed by elimination of the 7α-hydroxyl group from hyocholic acid.

These results are thus in certain respects very similar to the bile acid metabolism in man [13] and rabbit [14]. In the latter cases intestinal microorganisms eliminate the 7α-hydroxyl from cholic acid resulting in the formation of deoxycholic acid, and in both instances deoxycholic acid — normally present in gallbladder bile of man and rabbit — disappears from the fistula bile, when the bile acids present in the enterohepatic circulation have been excreted.

In the rat the situation is also similar in that deoxycholic acid is formed from cholic acid in the intestine [15,16], but in this case the deoxycholic acid formed is rehydroxylated at position 7α to cholic acid in the liver [17].

In all these cases the intestinal microorganisms thus eliminate a 7α hydroxyl irrespective of whether the other hydroxyls are in position 3α and 12α or 3α and 6α.

A common feature in each of these cases, the rabbit excluded, is that chenodeoxycholic acid occurs in the primary bile, *i. e.* 3α and 7α hydroxyls are present. In most animals the third hydroxyl is then at position 12α, but in the pig it is at 6α, as is apparent from the reviews published by Haslewood [18,19]. There are some exceptions, for example the seals which are reported to have the third hydroxyl at C_{23} (β-phocaecholic acid, 3α, 7α, 23-trihydroxycholanic acid) [20]. Among the animals with trihydroxylated C_{24}-bile acids the only species known to have bile acids unsubstituted at C_7 are the Python and Constrictor snakes. They have pythocholic acid (3α, 12α, 16-trihydroxycholanic acid) as their main bile acid [21]. In these cases the primary bile acids are not known, but considering the long duration of the entero-hepatic circulation in these animals, there appears to be unusually favorable conditions for secondary changes by the intestinal microorganisms, possibly followed by further changes by the liver enzymes.

Earlier work has indicated, that at least some of the changes on the ring system of cholesterol take place before the side chain is degraded. Furthermore, Lindstedt [22] has found that 7α-hydroxycholesterol is transformed rapidly into cholic acid in the rat. It therefore appears possible, that the 7α-hydroxylation of cholesterol is a common and early step in the degradation of cholesterol to bile acids in most higher animals. Further work is needed to clarify the details of these reactions and their possible rôle as rate limiting steps in the degradation of cholesterol to bile acids.

Acknowledgements. We are greatly indebted to Dr. Fritz Sevelius, Djursjukhuset, Hälsingborg, for the facilities and help placed at our disposal. The technical assistance of Mrs. Birgitta Bengtsson and Miss Berit Persson is gratefully acknowledged.

This work is part of investigations supported by *Statens Medicinska Forskningsråd*, *Knut och Alice Wallenbergs Stiftelse* and the *National Heart Institute* (H 2842), *U. S. Public Health Service*, Bethesda, Maryland.

REFERENCES

1. Haslewood, G. A. D. and Ogan, A. U. *Biochem. J.* **67** (1957) 30P.
2. Windaus, A. and Bohne, A. *Ann.* **433** (1923) 278.
3. Ido, T. and Sakurai, R. *J. Biochem. Tokyo* **29** (1939) 51.
4. Haslewood, G. A. D. and Sjövall, J. *Biochem. J.* **57** (1954) 126.
5. Haslewood, G. A. D. *Biochem. J.* **62** (1956) 637.
6. Ziegler, P. *Can. J. Chem.* **34** (1956) 523.
7. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
8. Wilzbach, K. E. *J. Am. Chem. Soc.* **79** (1957) 1013.
9. Bergström, S. and Lindstedt, S. *Acta Chem. Scand.* **11** (1957) 1275.
10. Sjövall, J. *Acta Physiol. Scand.* **29** (1953) 232.
11. Norman, A. *Acta Chem. Scand.* **7** (1953) 1413.
12. Wiss, O. and Gloor U. *Z. physiol. Chem. Hoppe-Seyler* **310** (1958) 260.
13. Lindstedt, S. *Arkiv Kemi 11* (1957) 145.
14. Lindstedt, S. and Sjövall, J. *Acta Chem. Scand.* **11** (1957) 421.
15. Norman, A. and Sjövall, J. *Biochim. et Biophys. Acta* **29** (1958) 467; *J. Biol. Chem.* **233** (1958) 872.
16. Lindstedt, S. and Samuelsson, B. *J. Biol. Chem. In press.*
17. Bergström, S., Rottenberg, M. and Sjövall, J. *Z. physiol. Chem. Hoppe-Seyler* **295** (1953) 278.
18. Haslewood, G. A. D. and Wootton, V. *Biochem. J.* **47** (1950) 584.
19. Haslewood, G. A. D. *Physiol. Revs.* **35** (1955) 178.
20. Igarashi, H. and Kakubata, H. *Mem. Fac. Agr. Hokkaido Univ.* **1** (1952) 181.
21. Haslewood, G. A. D. *Biochem. J.* **49** (1951) 67.
22. Lindstedt, S. *Acta Chem. Scand.* **11** (1957) 417.

Received February 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 784–802

The Kinetics of Crystal Growth in Barium Sulfate Precipitation

II. Temperature Dependence and Mechanism *

ARNE E. NIELSEN

Universitetets fysisk-kemiske institut, Copenhagen, Denmark

The chronometric integrals (chronomals) for precipitation processes with time-independent particle number concentrations are solved, and a table of their numerical values is calculated.

The diffusion coefficient of $BaSO_4$ and the fourth order rate constant of crystal growth in $BaSO_4$ precipitation are determined from experiments at a range of temperatures between 0 and 70°C.

The dissociation of 2–2 electrolytes in aqueous solution is discussed with special regard to $BaSO_4$.

A number of formally possible rate-determining mechanisms are discussed and four of them found to be in agreement with the experiments.

The results are compared with the Volmer-Becker-Döring and the Frank-Cabrera-Burton theories of crystal growth and are found to be in agreement with the former, but not the latter of these, *i. e.* the rate is governed by the formation of surface nuclei requiring a supersaturation ratio of about 10 or more in order to proceed at appreciable speed.

I. THEORETICAL PART; PRECIPITATION CHRONOMALS

1. Introduction. During the precipitation from a supersaturated solution the number of particles may either be constant or change with time. Here we shall only discuss cases where the number of particles is constant [1–10]. It follows that provided the solution is properly stirred the particles will grow with the same rate and be equally large. This has been observed with many sparingly soluble salts.

We shall first derive kinetic equations for precipitation, stating explicitely the constants of proportionality in terms of quantities such as the number of particles, the initial concentrations and the molar volume of the precipitating matter, and fundamental rate-constants. Then we will discuss how these quantities may be obtained from experiment and from theory.

* Part I. of this work, Ref.[1].

These methods will then be applied to the data from experiments on barium sulfate. Several mechanisms are suggested and compared to the experimental results. At last the results of this investigation will be compared to the accepted theories of crystal growth.

2. Derivation of the kinetic equations. If the rate of particle growth during precipitation from a supersaturated solution is controlled by the diffusion from the bulk of the solution to the surface of the particles we have [1,5,6,11].

$$\mathrm{d}r/\mathrm{d}t = vD(\mathrm{d}c/\mathrm{d}\varrho)_{\varrho=r}$$

where r is the radius of the particle or, if it is not a sphere, of a sphere of approximately the same volume as the particle, t the time, v the molar volume of the material, D the diffusion coefficient, c the concentration in mole/cm^3, and ϱ the distance from the center of the particle. Smoluchowski [12] showed that

$$(\mathrm{d}c/\mathrm{d}\varrho)_{\varrho=r} = (c-s)\,/\,r$$

where s is the solubility of the material. If we introduce the degree of reaction α by means of

$$1-\alpha = (c-s)\,/\,(c_0-s)$$

where c_0 is the concentration at $t = 0$, we may write

$$\mathrm{d}r/\mathrm{d}t = vD(c-s)\,/\,r = vD(c_0-s)\;(1-\alpha)\,/\,r$$

Since now

$$r = r_\infty \cdot \alpha^{1/3}$$

where r_∞ is r at $t = \infty$, we have

$$\begin{aligned}
&(\mathrm{d}r/\mathrm{d}t =)\\
&(1/3)r_\infty\alpha^{-2/3}\mathrm{d}\alpha/\mathrm{d}t = vD(c_0-s)\;(1-\alpha)/r_\infty\alpha^{1/3}\\
&\mathrm{d}\alpha/\mathrm{d}t = 3vD(c_0-s)\alpha^{1/3}\;(1-\alpha)/r_\infty^2\\
&\therefore t = r_\infty^2 I_\mathrm{D}/3v(c_0-s)
\end{aligned}$$

where

$$I_\mathrm{D} \equiv \int_0^\alpha \alpha^{-1/3}(1-\alpha)^{-1}\mathrm{d}\alpha$$

If the rate of growth is controlled by a reaction of the order p in the surface of the particle [13–15], with the rate constant k_p (mole/cm^2 · s) (mole/cm^3)$^{-p}$

$$\mathrm{d}r\,/\,\mathrm{d}t = vk_pc^p$$

We then have (when $c \gg s$)

$$\begin{aligned}
&(\mathrm{d}r/\mathrm{d}t =)\;(1/3)r_\infty\alpha^{-2/3}\mathrm{d}\alpha/\mathrm{d}t = vk_pc_0^p(1-\alpha)^p\\
&\therefore t = r_\infty I_p/3vk_pc_0^p
\end{aligned}$$

where

$$I_p \equiv \int_0^\alpha \alpha^{-2/3}(1-\alpha)^{-p}\mathrm{d}\alpha$$

Frisch and Collins [11] solved the problem of mixed diffusion and first order surface reaction. In our terminology the solution may be obtained thus: let the concentration be c' at the surface of the crystal; then

$$\mathrm{d}r/\mathrm{d}t = (1/3)r_\infty\alpha^{-2/3}\mathrm{d}\alpha/\mathrm{d}t = vD(c_0(1-\alpha)-c')/r_\infty\alpha^{1/3} = vk_pc'^p$$

By elimination of c' one gets, for all values of p

$$\frac{d\alpha}{dt} = 3vk_p r_\infty^{-1}\alpha^{1/3}\left[c_0(1-\alpha) - \frac{r_\infty^2}{3vD\alpha^{1/3}}\cdot\frac{d\alpha}{dt}\right]^p$$

For $p = 1$ this can be reduced to

$$\frac{dt}{d\alpha} = \frac{r_\infty^2}{3vDc_0(1-\alpha)\alpha^{1/3}} + \frac{r_\infty}{3vk_1c_0(1-\alpha)\alpha^{1/3}}$$

with the solution

$$t = \frac{r_\infty^2 I_D}{3vDc_0} + \frac{r_\infty I_1}{3vk_1c_0}$$

which is the sum of the solutions to the "pure" problems. For $p \neq 1$ the solution is not simply the sum of the corresponding expressions.

Sometimes it is convenient to use the length l of the edge in a cube of the same volume as the particle rather than r. In case of diffusional rate control it is reasonable to substitute r_∞ by means of

$$(4/3)\pi r^3 = l^3$$

giving

$$t = l_\infty^2 I_D \,/\, \sqrt[3]{48\pi^2}\; vD(c_0-s)$$

In case of a surface reaction we may use

$$dl/dt = 2vk_p c^p$$
$$l = l_\infty \alpha^{1/3}$$

from which follows

$$(dl/dt =)\ (1/3)l_\infty \alpha^{-1/3} d\alpha/dt = 2vk_p c_0^p (1-\alpha)^p$$
$$d\alpha/dt = 6vk_p l^{-1} c_0^p \alpha^{1/3}(1-\alpha)^p$$

and

$$t = l_\infty I_p / 6vk_p c_0^p$$

If the number of particles N per unit volume is known in stead of r_∞ or l_∞ we may use

$$l_\infty^3 = v(c_0-s) \,/\, N$$

and obtain

$$t = I_D \,/\, D\sqrt[3]{48\pi^2 v(c_0-s)N^2}$$

and, respectively (for $c \gg s$)

$$t = I_p \,/\, 6v^{1/3}k_p c_0^{p-1/3}N^{1/3}$$

These two formulae are only exact for spheres and cubes, respectively, but may be applied without serious errors to particles of highly compact shape. In case of thin plates, needles or "star-like" crystals as are often met with in electrolyte precipitation experiments the formulae can be expected to give only the right order of magnitude.

3. Formulae, numerical values. The integrals I_D and I_p have been calculated by numerical integration [5,8]. I_D was expressed by analytic functions by LaMer and Dinegar [6]. The mixed case of I_D and I_1 was similarly solved by Frisch and Collins [11]. Formulae for I_D and I_p (with any positive integer p) may be found by substituting a new variable for $\alpha^{1/3}$, decomposing, and using standard methods of integration. We introduce for brevity the two functions of α

$$L = \ln \frac{\sqrt{1 + \alpha^{1/3} + \alpha^{2/3}}}{1-\alpha^{1/3}} = \tfrac{1}{2} \ln \frac{1-\alpha}{(1-\alpha^{1/3})^3}$$

$$T = \sqrt{3} \operatorname{arctg} \frac{\sqrt{3}}{1 + 2\alpha^{-1/3}}$$

(arctg is the inverse tangent, sometimes denoted $\tan^{-1}$). The integrals can then be written

$$I_D = L - T$$

$$I_1 = L + T$$

$$I_2 = \frac{\alpha^{1/3}}{1-\alpha} + \frac{2}{3} I_1$$

$$I_3 = \frac{\alpha^{1/3}(8-5\alpha)}{6(1-\alpha)^2} + \frac{5}{9} I_1$$

$$I_4 = \frac{\alpha^{1/3}(41-52\alpha + 20\alpha^2)}{27(1-\alpha)^3} + \frac{40}{81} I_1$$

$$I_5 = \frac{\alpha^{1/3}(532-1\,023\alpha + 792\alpha^2-220\alpha^3)}{324(1-\alpha)^4} + \frac{110}{243} I_1$$

$$I_6 = \frac{\alpha^{1/3}(4\,210-10\,885\alpha + 12\,705\alpha^2-7\,084\alpha^3 + 1540\alpha^4)}{2\,430(1-\alpha)^5} + \frac{308}{729} I_1$$

Numerical values of I_D, I_1, I_2, I_3 and I_4 are given in Table 1. L and T may be found from the table by using

$$L = (I_1 + I_D)/2$$
$$T = (I_1 - I_D)/2$$

The coefficients in the general expression for I_p,

$$I_p = \frac{a_{p,1}\alpha^{1/3} + a_{p,2}\alpha^{1+1/3} + \ldots + a_{p,p-1}\alpha^{p-2+1/3}}{(1-\alpha)^{p-1}} + b_p I_1$$

can be found by solving the linear equations

$$\tfrac{1}{3} a_{p,1} = 1 - b_p$$

$$(i-\tfrac{2}{3})a_{p,i} + (p-i+\tfrac{2}{3})a_{p,i-1} = (-1)^i \binom{p-1}{i-1} b_p, \quad i = 2,3 \ldots p-1$$

$$\tfrac{2}{3} a_{p,p-1} = (-1)^p b_p$$

where

$$\binom{p-1}{i-1} = \frac{(p-1) \ldots (p-i+1)}{2 \cdot 3 \ldots (i-1)}$$

Table 1. The precipitation chronomals.

a	I_D	I_1	I_2	I_3	I_4
0.0001	0.00323	0.13925	0.1393	0.1393	0.1393
0.0003	0.00672	0.20084	0.2009	0.2009	0.2009
0.001	0.01501	0.30008	0.3001	0.3002	0.3003
0.003	0.03124	0.43299	0.4333	0.4337	0.4340
0.01	0.06990	0.64795	0.6496	0.6512	0.6529
0.03	0.14659	0.93928	0.9466	0.9540	0.9614
0.05	0.20778	1.11943	1.1341	1.1492	1.1647
0.10	0.33696	1.42943	1.4686	1.5104	1.5548
0.15	0.45155	1.65949	1.7314	1.8106	1.8978
0.20	0.56005	1.85382	1.9669	2.0960	2.2438
0.25	0.66620	2.02854	2.1922	2.3868	2.6194
0.30	0.77234	2.19183	2.4175	2.6977	3.0485
0.35	0.88009	2.34863	2.6500	3.0423	3.5596
0.40	0.99114	2.50263	2.8965	3.4370	4.1922
0.45	1.10686	2.65657	3.1644	3.9036	5.0051
0.50	1.22901	2.81312	3.4628	4.4731	6.0927
0.55	1.35960	2.97499	3.8040	5.1931	7.6131
0.60	1.50122	3.14530	4.2055	6.1403	9.8509
0.65	1.65737	3.32791	4.6936	7.4469	13.3541
0.675	1.74238	3.42542	4.9827	8.3047	15.8998
0.700	1.83304	3.52814	5.3118	9.3593	19.2811
0.725	1.93048	3.63722	5.6915	10.6825	23.8943
0.750	2.03596	3.75396	6.1369	12.3826	30.3893
0.775	2.15128	3.88019	6.6692	14.6297	39.8844
0.800	2.27882	4.01828	7.3204	17.7043	54.4171
0.825	2.42192	4.17160	8.1404	22.0962	77.9742
0.850	2.58542	4.34506	9.2118	28.7269	119.0924
0.875	2.77694	4.54623	10.6825	39.5090	198.357
0.900	3.00909	4.78777	12.8467	58.9801	374.256
0.925	3.30564	5.09345	16.3869	100.2645	858.979
0.950	3.71983	5.51652	23.3387	216.058	2 813.51
0.960	3.94639	5.74658	28.4932	332.021	5 433.07
0.970	4.23748	6.04112	37.0240	580.797	12 737.23
0.980	4.64633	6.45340	53.9667	1 286.58	42 530.6
0.990	5.34284	7.15329	104.4344	5 070.31	336 725
0.995	6.03767	7.84980	204.899	20 137.4	2 680 115
0.999	7.64846	9.46189	1 005.97	500 672	333 667 227

The first and the last equation are *not* obtained by letting $i = 1$ and $i = p$ in the formula given for $i = 2, 3, \ldots p-1$.

It is easily proved that for $p > 1$

$$\sum_{i=1}^{p-1} a_{p,i} = \frac{1}{p-1}$$

which constitutes a check on the numerical calculations.

4. The evaluation of kinetic constants. The best method of treating the data is probably to plot I_D or I_p as a function of the (through a) corresponding experimental time [8]. If the measurements are in accordance with the assump-

tions behind the '*I*' chosen a straight line through origo is obtained. In some cases the reaction is diffusion controlled in the beginning and becomes, *e. g.*, fourth order later on. Then a curved curve is obtained which gradually straightens and eventually approaches an asymptote of well defined slope. Both when the whole curve and when only a part of it is a straight line the diffusion coefficient or the rate constant may be calculated by means of the following equations.

$$\begin{aligned} D &= (\mathrm{d}I_D/\mathrm{d}t)l_\infty^2 \,/\, (48\pi^2)^{1/3} v(c_o - s) \\ &= (\mathrm{d}I_D/\mathrm{d}t) \,/\, (48\pi^2 v(c_o - s)\, N^2)^{1/3} \\ &= (\mathrm{d}I_D/\mathrm{d}t) \,/\, 7.795(v(c_o - s)N^2)^{1/3} \\ k_p &= (\mathrm{d}I_p/\mathrm{d}t)l_\infty \,/\, 6vc_o^p \\ &= (\mathrm{d}I_p/\mathrm{d}t) \,/\, 6v^{1/3}c_o^{p-1/3}N^{1/3} \end{aligned}$$

l_∞ may be found by measuring the particles under microscope. In the experiments reported below N was found by counting at least 100 particles on a blood cell counting chamber under microscope. Another possible procedure would be to determine the sedimentation velocity according to Odén [16] or Andreasen [17]. In a certain range of sizes the turbidity may be taken proportional to the surface [18], which would also give the required information if the constant of proportionality is known. It is safest, however, in all cases to examine the particles under microscope in order to be sure whether they are compact enough to justify the assumptions made in deriving the formulae.

II. EXPERIMENTAL PART. THE DIFFUSION COEFFICIENT AND THE RATE CONSTANT FOR $BaSO_4$

1. Introduction. Previously it was found [1] that at 22°C the rate of growth of $BaSO_4$ may be expressed by

$$\mathrm{d}l \,/\, \mathrm{d}t = 630\ c^4\ \mathrm{m}\mu/\mathrm{s} \text{ for } c < 0.3\ \mathrm{mM}$$

where l is the length of the edge in a cube of the same volume as the crystal. Dividing by twice the molar volume we get the rate of depositing matter on a unit of surface. Transforming into c-g-s-units we have

$$k_4 = 6.3 \times 10^{19} \,/\, 2 \times 51.9 = 6.1 \times 10^{17}\ (\mathrm{mole/cm^2 s})\ (\mathrm{cm^3/mole})^4$$

In Table 2 are shown some new and probably more accurate data for a range of temperatures. It was determined to use $c_o = 0.5$ mM because this gave the rates that could be measured best with the apparatus. With this c_o the rate is diffusion controlled in the first part and "surface-reaction"-controlled in the latest part of the experiment. Both D and k_4 can therefore be determined from the same run.

2. Experimental. The reacting aqueous solutions, 40 ml 1.00 mM $BaCl_2$ and 40 ml 1.00 mM Na_2SO_4 *(pro analysi)* were placed in an apparatus shown in Fig. 1, equilibrated in a thermostated water bath, and mixed by pressing the rubber ball. The electric conductance was measured as a function of time. The first reading was obtained 5 s after the moment of mixing. From measurements on solutions having the compositions of the reacting mixture at the degree of reaction a equal to 0.5 and 1.0, together with the value

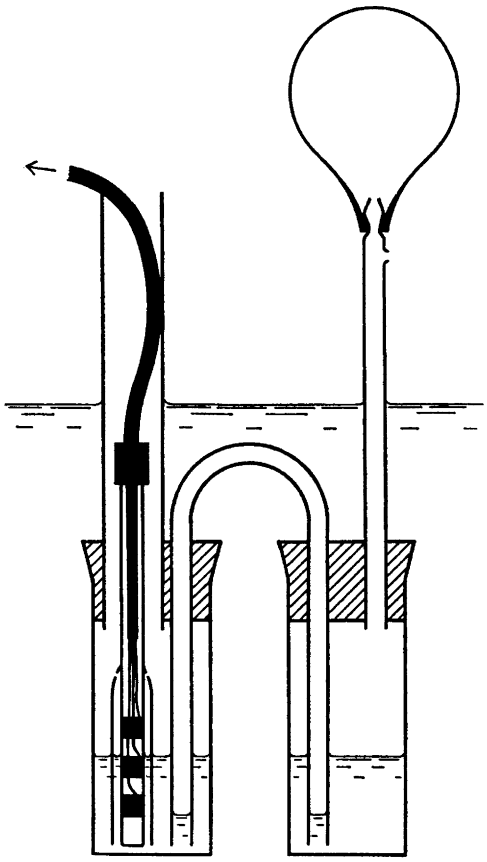


Fig. 1. The apparatus. The solutions were mixed by pressing the rubber ball. The arrow indicates the connection from the conductivity cell to the conductivity meter.

at $a = 0$ obtained in the kinetic experiment, a calibration curve was constructed for converting the electric conductance into a. The calibration curve was almost linear. The solution was frequently stirred by transferring it from one to the other of the two glasses. Afterwards a sample was placed on a counting plate and at least 100 particles were counted under microscope.

3. *Numerical treatment.* D was calculated by means of

$$\begin{aligned} D &= (\mathrm{d}I_\mathrm{D}/\mathrm{d}t) \,/\, (48\pi^2 v(c_\mathrm{o}\text{—}s)N^2)^{1/3} \\ &= (\mathrm{d}I_\mathrm{D}/\mathrm{d}t) \,/\, 7.795(vc_\mathrm{o}N^2)^{1/3} \\ &= (\mathrm{d}I_\mathrm{D}/\mathrm{d}t) \,/\, 0.231\ N^{1/3} \end{aligned}$$

For $\mathrm{d}I_\mathrm{D}/\mathrm{d}t$ was used the initial slope on a graph of I_D *versus* t. See Fig. 2 and Table 3.

k_4 was calculated by means of

$$\begin{aligned} k_4 &= (\mathrm{d}I_4/\mathrm{d}t) \,/\, 6v^{1/3}c_\mathrm{o}^{4-1/3}N^{1/3} \\ &= (\mathrm{d}I_4/\mathrm{d}t) \,/\, 6.58 \times 10^{-22}N^{1/3} \end{aligned}$$

Table 2. Time in seconds (interpolated) as a function of a, from experiments with $c_0 = 0.5$ mM, at different temperatures.

a	$t=0$	25	35	50	60	70°C
0.05	25	14		10.5		
0.1	50	24	17	15.5	22.5	11
0.2	102	37	43	25.5	38	16
0.3	154	49	62	37	53	20
0.4	217	64	85	49	68	24
0.5	293	85	116	65	89	31
0.55	330	105	138	75	103	36
0.6	376	132	169	91	120	42
0.65	450	170	210	111	143	52
0.675	500	193	232	124	160	57
0.70	560	215	268	143	180	64
0.725	640	243	310	165	198	72
0.75	740	283	366	205	225	80
0.775		340	444	250	300	97
0.80		430	560	420	360	125

where dI_4/dt was found graphically on a plot of I_4 *versus* t, using the straight-line part of the curve for large t. See Fig. 2 and Table 3.

4. Results. In Fig. 3 $\log D$ is plotted as a function of $1/T$. From the slope $d\log D/d(1/T) = -1\,080$ one finds the heat of activation for the diffusion process $\Delta H_D^{\ddagger} = 5\,000 \pm 500$ cal/mole[‡]. In the same figure is also plotted the logarithm of the fluidity of pure water. It is seen that the heat of activation of diffusion is a little greater than that of the fluidity. This is what one would expect, because of the hydration of the ions.

Fig. 3 also shows $\log k_4$ against $1/T$. $-d\log k_4/d(1/T) = 1\,100$ which corresponds to the apparent heat of activation

$$\Delta H_A = 5\,000 \pm 500 \text{ cal/mole.}$$

A ten degree rise in temperature increases k_4 about one-third.

This may be compared to the value for crystal growth in magnesium oxalate precipitation obtained by Lichstein and Brescia [19], $\Delta H_A = 3$ kcal/mole, although they used a different theory [20] involving a first order constant.

Table 3. D and k_4, from N and data in Table 2 (see Fig. 2).

t °C	N $10^6 cm^{-3}$	dI_D/dt s^{-1}	dI_4/dt s^{-1}	D $10^{-6} cm^2/s$	k_4 $10^{18} cm^{10}/s \cdot mole^3$
0	1.81	0.0070	0.051	2.04	0.69
25	3.38	0.0157	0.160	3.02	1.68
35	0.66	0.0125	0.110	7.15	1.92
50	2.16	0.028	0.170	7.25	2.01
60	0.30	0.0146	0.160	14.1	3.63
70	2.76	0.050	0.544	11.0	5.88

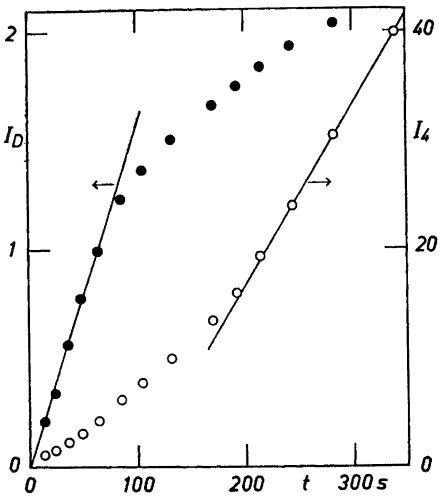


Fig. 2. I_D and I_4 as function of the experimentally determined times at 25°C. From the slope of the straight-line parts of the curves the diffusion coefficient and the fourth-order velocity constant were determined.

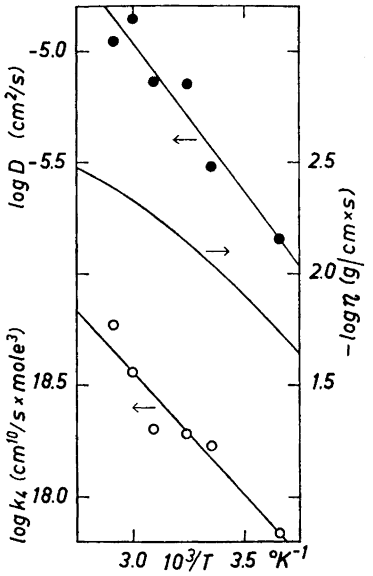


Fig. 3. Diagram for determination of the apparent heat of activation for diffusion of $BaSO_4$ and for the surface reaction of $BaSO_4$ crystal growth. In the middle is plotted for comparison the logarithm of the fluidity ($1/\eta$) of pure water.

III. THE DISSOCIATION OF 2—2 ELECTROLYTES

Before discussing these results we will consider the composition of the system. It is generally recognized that 2—2 electrolytes are only partly dissociated in aqueous solution [21,22]. For sulfates of bivalent metals we define

$$K_1 = (M^{++})\ (SO_4^{--})\ /\ (MSO_4)$$
$$K_2 = (MSO_4)\ (SO_4^{--})\ /\ (M(SO_4)_2^{--})$$
$$K_3 = (M^{++})\ (M(SO_4)_2^{--})\ /\ ((MSO_4)_2)$$
$$K_4 = (M^{++})^2(SO_4^{--})^2\ /\ ((MSO_4)_2) = K_1K_2K_3$$

The interpretation of experimental data from activity-, conductance- or cryoscopic measurements on dilute solutions for finding the K's is very uncertain, since the effects of the interionic forces masks the effects of the incomplete dissociation, and only approximate values of K_1 have been obtained this way.

The difficulty has however been solved by Kenttämaa [23] who measured the freezing point depression of $CuSO_4$, $ZnSO_4$, $NiSO_4$ and $MgSO_4$ in eutectic (cryohydratic) mixtures of water and a salt; the salts used were KNO_3, $KClO_3$ and $KClO_4$. Because of the presence of electrolytes in a roughly constant concentration even in the "pure solvent" the depressions may be extrapolated to infinite dilution of the 'MSO_4' in question without taking the interionic forces

into account. From the law of mass action it follows that if m_s denotes the stoichiometric concentration of the added electrolyte and Σm_i the sum of the concentrations of all the ions and molecules that are not common with those arising from the saturating electrolyte, the function $\bar{\varphi}$ defined by

$$\bar{\varphi} = (2m_s - \Sigma m_i) / (\Sigma m_i - m_s)^2$$

satisfies

$$\bar{\varphi} = 1/K_1 + \Sigma m_i / K_1 K_2$$

Σm_i and thereby also $\bar{\varphi}$ are found from the freezing point depressions.

From a plot of $\bar{\varphi}$ *versus* Σm_i K_1 and K_2 may be obtained. K_1 is strongly dependent on the ionic strength I, whereas K_2 may be regarded — in a first approximation — as independent of the salt concentration. Kenttämaa did not give K_2 explicitely, but it is easily found from the reported values of the coefficient in the linear expression for $\bar{\varphi}$.

All values of K_2 lie between 0.1 and 0.25, most of them close to 0.15 M. At $I = 0$, pK_1 ranges between 2.186 and 2.294 for the four sulfates of bivalent metals investigated by Kenttämaa. In our experiments on $BaSO_4$ precipitation $I = 0.002$ in the part of the experiment which is used for determination of k_4. At $I \cong 0.002$ the correction to pK_1 is about —0.16; we may take $pK_1 = 2.1$, $K_1 = 0.008$ mole/l as a representative value.

Since the dissociation constants seem to be the same for all sulfates of bivalent metals, we may apply them to $BaSO_4$ as well.

This conclusion may seem a little too risky. Since $BaSO_4$ is only 10^{-5} as soluble as the sulfates on which K_1 and K_2 were measured one would perhaps expect it to be appreciably less dissociated. This is however not the case. The calibration curves mentioned in the experimental part of this article turned out to be very near to straight lines. Actually the conductivity at $\alpha = 0.5$ was higher than the arithmetic mean of the conductivities at $\alpha = 0$ and $\alpha = 1$ by about 2.5 % of the total change, *i. e.*

$$e \equiv (\varkappa_{0.5} - (\varkappa_0 + \varkappa_1) / 2) / (\varkappa_0 - \varkappa_1) = 0.025$$

The curvature is due partly to interionic forces and partly to the incompleteness of the dissociation

$$BaSO_4 = Ba^{++} + SO_4^{--}$$

with the dissociation constant

$$K_1 = (Ba^{++})\ (SO_4^{--}) / (BaSO_4) = c\delta^2 / (1-\delta) = c_0\ (1-\alpha)\delta^2 / (1-\delta)$$

where δ is the degree of dissociation. If we neglect for the moment the interionic forces but not the incompleteness of the dissociation we get

$$\begin{aligned}
\varkappa_\alpha &= \Lambda^\circ_{BaSO_4} c_0 (1-\alpha)\delta + \varkappa_1 \\
e &\equiv (\delta_{0.5} - \delta_0) / 2\delta_0 \\
\delta_{0.5}/\delta_0 &= q \equiv 1 + 2e \\
K_1 / c_0 &= \delta_0^2 / (1-\delta_0) = 0.5\delta_{0.5}^2 / (1-\delta_{0.5}) \\
&= q^2\delta_0^2 / 2(1-q\delta_0) \\
\delta_0 &= (2-q^2) / (2q-q^2) \\
K_1 &= c_0(2-q^2)^2 / (2q-q^2)\ (2q-2) \\
&= c_0(1-4e-4e^2)^2 / (1-4e^2)4e \\
&\cong c_0(1/4e-2) = 0.0005 \times 8 = 0.004 \text{ mole/l}
\end{aligned}$$

Since the interionic forces also tend to diminish the electric conductivity and thus contribute positively to e, the value found for K_1 is a minimum value; we therefore only know that for $BaSO_4$

$$K_1 > 0.004 \text{ mole/l}$$

but this is entirely in agreement with the assumption that K_1 has the same value for $BaSO_4$ as for the highly soluble sulfates of bivalent metals, *i. e.* $K_1 = 0.008$ mole/l. It is less certain to put $K_2 = 0.15$ for $BaSO_4$ because it is that much in case of the highly soluble sulfates, but there is no reason to assume it is wrong by more than, *e. g.*, a factor of ten. K_3 is entirely unattainable from Kenttämaas experiments since the function $\bar{\varphi}$ was found to depend lineary on Σm_i. If we assume that the experimental uncertainty allows for a maximum concentration of $(MSO_4)_2$ equal to $M(SO_4)_2^{--}$ at the highest concentration of MSO_4 used, we find from the mass action law

$$K_3 = (M^{++})(M(SO_4)_2^{--}) / ((MSO_4)_2) > (M^{++}) = 0.2 \text{ mole/l}$$

For $BaSO_4$ of course K_3 may be less. For the highly soluble sulfates we have now

$$K_4 = K_1K_2K_3 > 0.008 \times 0.15 \times 0.2 = 2 \times 10^{-4}M^3 = 2 \times 10^{-13} \text{ mole}^3/\text{cm}^9$$

IV. THE RATE-DETERMINING MECHANISM IN $BaSO_4$ CRYSTAL GROWTH

Since the activation energy of the fourth order crystallization reaction is nearly the same as the activation energies of $BaSO_4$ diffusion and of viscous flow of pure water, the rate-determining step is a reaction in which almost any close approach between two molecules of the reactants leads to reaction.

If the collision number of the reactants — when supposed not to react — is Z collisions per unit of volume (or surface) and time, calculated from simple gas theory, we may write the rate of the reaction

$$J = Z\exp(-\Delta G^{\neq}/RT)$$

where $\Delta G^{\neq}$ is the standard activation free energy change, and $\exp(-\Delta G^{\neq}/RT)$ is the ratio between the number of collisions leading to reaction and the number of collisions between the reactant molecules if no reaction took place.

In a liquid two molecules subject to an encounter have a rather large probability of repetitive encounters, performing some sort of oscillations, before a third molecule comes in between. If a reaction of activation free energy less than the activation free energy of diffusion can occur between the two colliding molecules, it is almost certain that it will happen rather than the two molecules depart. Therefore the free energy of activation of the reaction shall be replaced by the free energy of activation for diffusion, and the temperature dependence of the velocity is determined entirely by the enthalpy of activation for diffusion, and the change of Z with temperature. If we neglect any entropy change of activation for diffusion we have

$$J = Z\exp(-\Delta H^{\neq}/RT)$$

where empirically $\Delta H^{\neq} \cong 5\,000$ cal/mole$^{\neq}$ if Z is not strongly temperature dependent; $\exp(-\Delta H^{\neq}/RT) = 10^{-4}$ at 0°C.

We will now discuss the following types of possible rate determining mechanisms.

(i) transport of matter from the solution to the crystal.
(ii) collision between molecules (or ions) from the solution and adsorbed molecules (ions).
(iii) triple collisions between two molecules (ions) and the crystal.
(iv) collision between adsorbed molecules.

Above $c = ca.\ 4 \times 10^{-7}$ mole/cm^3 (= 0.4 mM) the rate determining mechanism has already been shown [1] to be the diffusion of $BaSO_4$ — mostly as ions — from the bulk of the solution to the surface. In the following we are going to discuss $c < 4 \times 10^{-7}$ mole/cm^3. As an example we will estimate the concentrations in a 0.1 mM = 10^{-7} mole/cm^3 solution of $BaSO_4$. (The solubility is *ca.* 10^{-8} mole/cm^3 at 25°C and *ca.* 8×10^{-9} at 0°C; see Seidell [24] for references.)

If we assume the following orders of magnitude (at 0°C):

$$K_1 = 10^{-5},\ K_2 = 10^{-4},\ K_3 = 10^{-4} \text{ mole/cm}^3$$

and, consequently $K_4 = 10^{-13}$ (mole/cm^3)^3 we find for $c = 10^{-7}$ mole/cm^3 the following concentrations:

$$(Ba^{++}) = (SO_4^{--}) = c = 10^{-7},\ (BaSO_4) = 10^{-9},$$
$$(Ba(SO_4)_2^{--}) = 10^{-12} \text{ and } ((BaSO_4)_2) = 10^{-15} \text{ mole/cm}^3.$$

At this concentration we found experimentally $J = k_4 c^4 = 7 \times 10^{-11}$ mole/cm^{2}s.

If the rate is determined by the frequency of collisions between the crystal and molecules of concentration C and molecular weight M (~200 g/mole) the reaction rate per cm^2 is

$$J = C\sqrt{RT/\pi M}\ \exp(-\Delta H^{\neq}/RT) = 0.6 \times C$$

It follows that if this mechanism shall be rate determining

$$C = 7 \times 10^{-11}/0.6 = 10^{-10} \text{ mole/cm}^3$$

Since $((BaSO_4)_2)$ is only 10^{-15} mole/cm^3 it can not be the diffusion of $(BaSO_4)_2$ that is rate determining. It may however be the diffusion of $BaSO_4$-molecules. In order to get the right order (4) of the reaction only collisions with another $BaSO_4$-molecule at the surface may lead to growth. If one-tenth of the surface is covered by adsorbed $BaSO_4$-molecules in adsorption equilibrium with the ionic contents of the solution at $c = 10^{-7}$ mole/cm^3, the observed rate is obtained by this mechanism. Since the molar volume of $BaSO_4$ is 52 cm^3 this corresponds to a concentration of 1/520 mole/cm^3; which is $1/(520 \times 10^{-9}) = 2 \times 10^6$ times as high as in the solution. Another possible mechanism of the same sort is the reaction between a colliding Ba^{++} ion and an adsorbed $Ba(SO_4)_2^{--}$ ion; only $10^{-10}/10^{-7} = 10^{-3}$ of the surface needs be covered by the reactive ionic species. Since the atoms of the (probably) linear $^-SO_4BaSO_4^-$ shall rearrange rather appreciably forming the (probably) square $(BaSO_4)_2$, there must be a rather low entropy of activation so that much more than 10^{-3}

of the surface must be covered, and the concentration at the surface must be more than about $(1/100) \times 10^{-3}/10^{-12} = 10^7$ times the concentration in the solution.

Triple collisions are difficult to define in a generally acceptable way. If we however count any encounter between two molecules or ions closer than a distance λ from the surface of the crystal as a triple collision, we may find the required λ by comparison with the experiment. The encounter may either occur between two $BaSO_4$ molecules or between a Ba^{++} and a $Ba(SO_4)_2^{--}$-ion. We have, respectively, the (simple gas theory) collision numbers

$$Z_{11} = 2n^2d^2\sqrt{\pi RT/M}$$

and

$$Z_{12} = n_1n_2d_{12}^2\sqrt{8\pi RT/\mu_{12}}$$

where

$$d_{12} = (d_1 + d_2)/2, \quad \mu_{12} = M_1M_2 / (M_1 + M_2)$$

With $M = 200$, $\mu = 100$, $d = d_{12} = 5$ Å $= 5 \times 10^{-8}$ cm we find at 0°C

$$Z_{11} = C^2 \times 5 \times 10^{13}$$
$$Z_{12} = C_1C_2 \times 10^{14} \text{ mole/cm}^2\text{s}$$

where C, C_1 and C_2 are measured in mole/cm^3. At $c = 10^{-7}$, $J_{exp} = 7 \times 10^{-11}$ mole/cm^{2}s; but

$$J = \lambda \times Z_{11} \times \exp(-\Delta H^{\neq}/RT)$$
$$= \lambda \times C^2 \times 5 \times 10^{13} \times 10^{-4} \text{ or } = \lambda \times C_1C_2 \times 10^{14} \times 10^{-4}$$

With $C = (BaSO_4) = 10^{-9}$ we find

$$\lambda = 7 \times 10^{-11}/5 \times 10^{-9} = 0.01 \text{ cm}$$

With $C_1 = (Ba^{++}) = 10^{-7}$, $C_2 = (Ba(SO_4)_2^{--}) = 10^{-12}$ we find

$$\lambda = 7 \times 10^{-11}/10^{-9} = 0.1 \text{ cm}$$

None of these mechanisms can therefore give the observed rate with a reasonable value of λ (which must, of course, be of molecular dimensions, *i. e.* $\lambda \sim 1$ Å).

In a two dimensional gas

$$Z_{11} = n^2d\sqrt{\pi RT/M}$$
$$Z_{12} = n_1n_2d_{12}\sqrt{2\pi RT/\mu}$$

where Z_{11} and Z_{12} are the numbers of collisions per cm^2 and s, between similar and different molecules, respectively, and n, n_1, n_2 are the surface concentrations in molecules per cm^2, other symbols having the same meaning as in the case of a three-dimensional gas. With $d = 5 \times 10^{-8}$ cm, $M = 200$, $\mu = 100$ we have

$$Z_{11} = n^2 \times 10^{-3}$$
$$Z_{12} = n_1n_2 \times 2 \times 10^{-3}$$
$$J = 7 \times 10^{-11} = Z_{11}\exp(\Delta H^{\neq}/RT)/N_A = n^2 \times 10^{-3-4}/6 \times 10^{23}$$
$$\therefore n = 2 \times 10^{10} \text{ cm}^{-2}$$

or from Z_{12}

$$\therefore n_1n_2 = 10^{20} \text{ cm}^{-4}$$

In a compact layer of $BaSO_4$ molecules n = thickness × contents in 1 $cm^3 = d \times (1/v) \times N_A = 5 \times 10^{-8} \times 0.02 \times 6 \times 10^{23} = 6 \times 10^{14}$ cm^{-2}. The surface concentration necessary for the rate observed is only the fraction $2 \times 10^{10}/6 \times 10^{14} = 3 \times 10^{-3}$ of that of a compact layer. Calculated as volume concentration it is $2 \times 10^{10}/5 \times 10^{-8} = 4 \times 10^{17}$ molecules/cm^3 = $4 \times 10^{17}/6 \times 10^{23} = 10^{-6}$ mole/cm^3, *i. e.* 1 000 times as high as in the solution. This mechanism seems thus quite reasonable.

Finally, $n_1 n_2 = 10^{20}$ cm^{-4} corresponds to $C_1 C_2 = 10^{20}$ $(6 \times 10^{23})^{-2} = 3 \times 10^{-28}$ $(mole/cm^2)^2 = 3 \times 10^{-28}/(5 \times 10^{-8})^2 = 10^{-11}$ $(mole/cm^3)^2$; in the solution $C_1 C_2 = (Ba^{++})$ $(Ba(SO_4)^{--}) = 10^{-7} \times 10^{-12} = 10^{-19}$ $(mole/cm^3)^2$; we see that if the concentrations in the surface are 10^4 as large as in the solution, this mechanism can give the right rate.

Conclusion. Four of the discussed mechanisms could give a fourth order reaction with the right rate constant.

Reaction			c_{ads}/c_{soln}
(1) $BaSO_4$(soln) + $BaSO_4$(ads)	→	crystal	2×10^6
(2) Ba^{++}(soln) + $Ba(SO_4)_2^{--}$(ads)	→	»	$> 10^7$
(3) 2 $BaSO_4$(ads)	→	»	10^3
(4) Ba^{++}(ads) + $Ba(SO_4)_2^{--}$(ads)	→	»	$> 10^4$

If the differences between the four values of c_{ads}/c_{soln} are significant and if we rate a reaction as more probable than any other requiring a higher adsorption affinity to the crystal surface, in order to have the same velocity, we may point out reaction (3) as the dominating one, (4) as less important and (1) and (2) as practically negligible.

We have assumed that the ("apparent") activation energy $-R\mathrm{d}\ln k_4/\mathrm{d}(1/T)$ was due entirely to the diffusion process. Since

$$k_4 = J/c^4 = (Z/c^4)\exp(-\Delta G^{\ddagger}/RT)$$

any dependence of Z on T will contribute as well. Since the collisions involve complex molecules or ions the enthalpy of association (= minus enthalpy of dissociation) is a part of the "apparent" activation energy. A change by a factor 10 in the ration c_{ads}/c_{soln} is necessary for every $RT\ln 10 = 1\,250$ cal of dissociation enthalpy. The heat of precipitation is about 5 000 cal/mole $BaSO_4$ (Muller [25] found 5 455 at 25°C; from National Bureau of Standards' "Selected Values . . ."[26] one finds 4 630 cal/mole $BaSO_4$ (evolved).) The heat of dissociation of $BaSO_4$ is probably numerically less than this number. It may be zero if the energy change of "hydration" of the ions just compensates the work of the electric attraction forces.

V. COMPARISON WITH THE VOLMER-BECKER-DÖRING AND THE FRANK-CABRERA-BURTON THEORIES OF CRYSTAL GROWTH

1. *Introduction.* The theories of crystal growth have been developed primarily by Volmer and Weber [27,28], Farkas [29], Kaischew and Stranski [30], Becker and Döring [31], Cabrera and Burton [32,33] and Frank [33,34]. If the rate

of crystal growth is controlled by surface two-dimensional nucleation the rate is, according to Cabrera and Burton [32]

$$J = \nu ac \exp(-(U + \tfrac{1}{3}A)/kT)$$

where

ν = "frequency of vibration" or $kT/h = 6 \times 10^{12}$ s^{-1},
a = a molecular dimension (diameter of an ion or thickness of an adsorbed layer),
c = concentration in the supersaturated solution,
U = energy of activation for diffusion,
$A = \gamma'^2\pi/kT\ln\alpha$ = free energy of formation of critical (circular!) surface nucleus,
γ' = edge (free) energy per molecule of the surface nucleus,
k = Boltzmann's constant,
T = temperature,
α = supersaturation ratio (ratio of c to the solubility).

The reason why the energy of activation shall enter in the formula with only one-third of its full value A, is that the bottle-neck of crystal growth rate controlled by surface nucleation is not the nucleus of maximum free energy, because this is formed by collisions between smaller embryos, but rather the formation of embryos of energy about one-third of the nucleus.

Usually an electrolyte crystal will grow with a measurable speed when α is greater than about 1.01 and this observation does not agree at all with the quoted formula. Therefore another mechanism with lower activation energy had to be found, and as shown by Frank [31] this is provided by dislocations. In the case of $BaSO_4$, however, the rate of growth is extremely slow when α is smaller than about 10. This indicates clearly that in $BaSO_4$ growth, surface nucleation may have a chance to be rate determining in stead of the Frank dislocation mechanism.

2. The surface nucleus. In order to examine this possibility we calculate the value of γ' consistent with the experimental J and α.

From the Cabrera-Burton-expression for J we derive

$$\ln J = \ln \nu ac - U/kT - \gamma'^2\pi/3k^2T^2\ln(c/s)$$
$$p = d\ln J/d\ln c = 1 + \gamma'^2\pi/3(kT\ln\alpha)^2 = 1 + n/3$$

where p is the order of the reaction and n the number of ions in the (equilibrium) surface nucleus. With $p = 4$, $T = 298°K$, $\alpha = 25$ we find

$$n = 3(p-1) = 9$$
$$\gamma' = \sqrt{n/\pi}kT\ln\alpha = 2.0 \times 10^{-13} \text{ erg}$$

3. The surface tension. It is crucial to this hypothesis whether the value of γ' is reasonable. This quantity is impossible to measure except through J, and difficult to calculate because the structures of the surface and of the surface nuclei are rather unknown. But perhaps it may be estimated from the surface tension γ by means of

$$\gamma' = a^2\gamma$$

where $a = 3.5$ Å is the distance between the centres of two neighbouring ions in the $BaSO_4$-lattice. This formula corresponds to the view that the free energy of the surface + nucleus can be calculated as the surface tension times the total surface area including that of the vertical wall of the nucleus of heigth a.

The surface tension of an ionic crystal in vacuum may be estimated from Born and Stern's formula [35]

$$\gamma = 0.1166 e^2/\delta^3$$

(e = ionic charge, δ = lattice constant) although it was only derived for a NaCl-type crystal with electrostatic (Coulomb) forces of attraction and repulsion, and with a general repulsion between ions corresponding to a potential energy term proportional to the minus 9th power of distance. Substituting $e = z\varepsilon$, $\delta^3 = 4v/N$, where z = charge number, ε = electronic charge, v = molar volume and N = Avogadro's number we get

$$\gamma = 0.02915\ z^2\varepsilon^2 N/v$$

and for $BaSO_4$ ($z = 2$, $v = 51.9$ cm^3/mole) we obtain

$$\gamma = 312 \text{ erg/cm}^2$$

This value (or, to precise 310) was claimed to agree with experiments on $BaSO_4$ by Bruzs [36], but it is not clear from the article how the agreement is substantiated.

The surface tension of $BaSO_4$ was determined by Hulett [37] and Dundon [38] from the dependence of solubility on particle size. In Hulett's article there were several errors in the calculation, as pointed out by Freundlich [39]. (Freundlichs recalculated value, $\gamma = 4\,000$ erg/cm^2 is, however, also in error. The correct value was given by Dundon [38] and in later editions of Freundlichs book.[40]) According to Dundon, Hulett's measurements give 1 250 and her own 3 000 erg/cm^2, both numbers being rather uncertain, mainly because it is difficult to measure the particle size accurately.

Both Hulett and Dundon did only approach equilibrium through decreasing concentrations (measured by electric conductance), therefore the results may be too high by an unknown amount. There are at least two causes of experimental errors that can keep a too high concentration constant in time, 1) the stirring motion may "wear" on the crystals, so that small bits of higher solubility are broken off leading to a stationary non-equilibrium state with constant concentration, and 2) there may be a practical limiting supersaturation for growth, below which no further precipitation occurs. At low supersaturation $1/\ln a$ in the formulae for nucleus size and for the nucleation free energy becomes very large and the crystal growth becomes extremely slow. This must also lead to higher values than $p = 4$ in the rate equation, at lower concentrations than investigated in the present work.

(One might expect that the critical size and the kinetic order of the reaction both change gradually during the experiment; but since we are dealing with small numbers (< 20) we must pay attention to the fact that certain numbers (*e. g.* 4, 6 or 12) of ions can be arranged in configurations of much lower energy than other numbers (*e. g.* 5). Therefore the critical size must

be a discontinuous function (with large jumps) of the concentration, and may very well be a constant in the whole range investigated kinetically).

The works of Hulett and Dundon have been criticized by several authors; for references see the articles of Cohen and Blekkingh [41], who tried to approach equilibrium from "the other side". They dissolved finely powdered very pure barium sulfate in conductivity water, and claim that they did not succeed in transgressing the solubility of large crystals. (Perhaps they removed the supersaturation during the filtrations before the final measurements?)

As a consequence of these facts we will assume that the best choice for the surface tension of barium sulfate (even in contact with solution) is the theoretical value in vacuum 312 erg/cm^2. Therefore we find

$$\gamma' = 312 \times (3.5 \times 10^{-8})^2 = 3.8 \times 10^{-13} \text{ erg}$$

The disagreement between this value and the 2.0×10^{-13} erg required by the nucleation theory is not sufficient to reject the theory, since both values are calculated using very simplifying assumptions.

4. The temperature dependence. We still have to check whether the temperature dependence is in accord with the surface nucleation theory. The temperature dependence is expressed in the total (apparent) heat of activation.

Assuming γ' but not s independent of T we derive

$$\begin{aligned} \Delta H_A &= k\mathrm{dln}k_p \,/\, \mathrm{d}(1\,/\,T) = -k\mathrm{dln}(J\,/\,c^p) \,/\, \mathrm{d}(1\,/\,T) \\ &= U + 2A\,/\,3 + (p-1)k\mathrm{dln}s \,/\, \mathrm{d}(1\,/\,T) \\ &= kT\ln(vaca^{p-1}\,/\,J) - (p-1)\Delta H_s\,/\,2 \\ &= kT\ln(va\,/\,s^{p-1}k_p) - (p-1)\Delta H_s\,/\,2 \end{aligned}$$

ΔH_s is the heat of solution per molecule of $BaSO_4$. With $a = 3.5 \times 10^{-8}$ cm, $s = 10^{-8}$ mole/cm^3, $p = 4$, $T = 298°$K $k_4 = 1.7 \times 10^{18}$ cm^{10}/s · mole^3, $\Delta H_s = 4\,630$ cal/mole (Rossini *et al.*[26]) we find

$$\begin{aligned} kT\ln(va\,/\,s^{p-1}k_p) &= 7.8 \times 10^{-13} \text{ erg} = 11\,000 \text{ cal/mole} \\ \Delta H_A &= 11\,000 - 7\,000 = 4\,000 \text{ cal/mole} \end{aligned}$$

in good agreement with the measured value, 5 000 cal/mole.

5. The factor 1/3 on the activation energy. As was mentioned earlier in this paper the reason why the energy of activation for surface nucleation shall enter in the formulae with only one-third of its value, is that the bottle-neck of crystal growth rate controlled by surface nucleation is not the formation of the nuclei of maximum free energy, but only of embryos with one-third of the critical energy, because these have greater probability for encounters with similar or larger molecules than for being dissolved. Cabrera and Burton [32] do not give all details of the proof of this essentiel theorem. Since however $BaSO_4$ provides an example of this sort of growth mechanism it is of interest to investigate if the theorem is obeyed. We identify the 'one-third surface nuclei' with adsorbed $(BaSO_4)_2$-molecules. Since adsorbed ionic species such as Ba^{++} and $Ba(SO_4)_2^{--}$ seem to have concentrations 10^4 as high in the adsorption layer as the same ions in the solution, we assume that the concentration of $(BaSO_4)_2$ is 10^8 times as high in the adsorption layer (of thickness 5 Å) as in

the solution. In the case subject to numerical estimates ($c_o = 10^{-7}$ mole/cm^3, 0°C) this amounts to

$$10^8 \times 10^{-15} = 10^{-7} \text{ mole/cm}^3$$

this is equivalent to

$c' = 5 \times 10^{-8} \times 10^{-7} = 5 \times 10^{-15}$ mole per cm^2 of the crystal surface.

The formula for collisions between like molecules in a two-dimensional layer is

$$Z_{11} = n^2 d \sqrt{\pi RT/M}$$

where n = number of molecules per cm^2 of the layer, d = collision diameter $\cong 5 \times 10^{-8}$ cm, M = molecular weight $\cong 200$ g/mole. Substituting $n = Nc'$, dividing by N (Avogadros' number) and multiplying by the activation energy exponential function $\exp(-U/RT)$ for diffusion ($U \cong 5\,000$ cal/mole) we get the rate of collisions between $(BaSO_4)_2$-molecules in the adsorption layer:

$$J = (Z_{11}/N) \exp(-U/RT) = c'^2 \, Nd\sqrt{\pi RT/M} \exp(-U/RT)$$
$$= (5 \times 10^{-15})^2 \times 6 \times 10^{-16} = 1.5 \times 10^{-12} \text{ mole}(BaSO_4)_2/\text{cm}^2\text{s}$$

Since this is of the same order of magnitude as the experimental rate of growth (7×10^{-11} mole $BaSO_4$/cm^{2}s) the theorem has been verified.

VI. CONCLUSION

We have arrived at the following view of crystal growth of barium sulfate. At low concentrations ($c <$ about 0.4 mM) the rate-determining step is the formation of adsorbed double-molecules $(BaSO_4)_2$ on the crystal surface. This is a fourth order reaction, and its rate is determined by the rate of diffusional (or "Brownian") movement of the smaller ionic or molecular species ($Ba^{++} + Ba(SO_4)_2^{--}$ or $BaSO_4 + BaSO_4$) against each other in an adsorbed layer on the crystal surface.

The critical size of the surface nucleus is calculated from simplifying assumptions to be about 9 ions; this is critical with respect to free energy, but not to rate. In contrast to most other crystals whose rate of growth has been investigated, $BaSO_4$ does not grow by the Frank dislocation mechanism, and requires consequently much higher supersaturation ratios ($\alpha > 10$) than the latter ($\alpha > 1.01$).

At high concentrations ($c >$ about 0.4 mM) the rate of growth is controlled by the diffusion of Ba^{++} and SO_4^{--} ions from the bulk of the solution to the crystal surfaces as found in the first part [1] of this work.

REFERENCES

1. Nielsen, A. E. *Acta Chem. Scand.* **12** (1958) 951.
2. Odén, S. and Werner, D. *Arkiv Kemi, Mineral. Geol.* **9** (1925) No. 23.
3. Tovborg Jensen, A. *Z. physik. Chem.* **A180** (1937) 3.
4. Andreasen, A. H. M., Skeel-Christensen, K. and Kjær, B. *Kemisk Maanedsblad* **24** (1943) 93.
5. Wert, C. and Zener, C. *J. Appl. Phys.* **21** (1950) 5.

6. LaMer, V. K. and Dinegar, R. H. *J. Am. Chem. Soc.* **72** (1950) 4847.
7. Turnbull, D. *Acta Met.* **1** (1953) 684.
8. Nielsen, A. E. *J. Colloid Sci.* **10** (1955) 576.
9. Pacter, A. *J. Phys. Chem.* **62** (1958) 1025.
10. Doremus, R. H. *J. Phys. Chem.* **62** (1958) 1068.
11. Frisch, H. L. and Collins, F. C. *J. Chem. Phys.* **21** (1953) 2158.
12. v. Smoluchowski, M. *Z. physik. Chem.* **92** (1918) 129.
13. Christiansen, J. A. and Nielsen, A. E. *Z. Elektrochem.* **56** (1952) 465.
14. Johnson, R. A. and O'Rourke, J. D. *J. Am. Chem. Soc.* **76** (1954) 2124.
15. O'Rourke, J. D. and Johnson, R. A. *Anal. Chem.* **27** (1955) 1699.
16. Odén, S. *Kolloid-Z.* **18** (1916) 33.
17. Andreasen, A. H. M. and Lundberg, J. J. V. *Ber. deut. keram. Ges.* **11** (1930) 249.
18. Wells, P. V. *Chem. Revs.* **3** (1927) 331, p. 351.
19. Lichstein, B. and Brescia, F. *J. Am. Chem. Soc.* **79** (1957) 1591.
20. Peisach, J. and Brescia, F. *J. Am. Chem. Soc.* **76** (1954) 5946.
21. Bjerrum, N. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **7** (1926) No. 9.
22. Davies, C. W. *Trans. Faraday Soc.* **23** (1927) 354.
23. Kenttämaa, J. *Acta Chem. Scand.* **12** (1958) 1323.
24. Seidell, A. *Solubilities of Inorganic and Metal-organic Compounds*, 3rd Ed. Vol. 1, D. Van Nostrand, New York 1940.
25. Muller, J.-A. *Bull. soc. chim. France* **23** (1918) 13.
26. Rossini, F. D., Wagman, D. D., Ewans, W. H., Levine, S. and Jaffe, I. *Selected Values of Chemical Thermodynamical Properties*, Natl. Bur. Standards (U.S.) Circ. No. 500 Washington 1952, p. 419.
27. Volmer, M. and Weber, A. *Z. physik. Chem.* **119** (1926) 277.
28. Volmer, M. *Kinetik der Phasenbildung*, Steinkopff, Dresden and Leipzig 1939.
29. Farkas, L. *Z. physik. Chem.* **125** (1927) 236.
30. Kaischew, R. and Stranski, I. N. *Z. physik. Chem.* **B26** (1934) 317.
31. Becker, R. and Döring, W. *Ann. Physik* **24** (1935) 719.
32. Cabrera, N. and Burton, W. K. *Discussions Faraday Soc.* **5** (1949) 40.
33. Burton, W. K., Cabrera, N. and Frank, F. C. *Trans. Roy. Soc. London*, Ser. **A 243** (1951) 299.
34. Frank, F. C. *Discussions Faraday Soc.* **5** (1949) 48.
35. Born, M. and Stern, O. *Sitz.-ber. preuss. Akad. Wiss. Physik. math. Kl.* **1919** 901, p. 908.
36. Bruzs, B. *J. Phys. Chem.* **34** (1930) 621.
37. Hulett, G. A. *Z. physik. Chem.* **37** (1901) 385.
38. Dundon, M. L. *J. Am. Chem. Soc.* **45** (1923) 2658.
39. Freundlich, H. *Kapillarchemie*, Akademische Verlagsgesellschaft, Leipzig 1909, p. 145.
40. Freundlich, H. *Kapillarchemie*, 4th ed. Akademische Verlagsgesellschaft, Leipzig 1930, Vol. 1, p. 220.
41. Cohen, E. and Blekkingh Jr., J. J. A. *Koninkl. Ned. Akad. Wetenschap. Proc.* **43** (1940) 32, 189, 334.

Received January 28, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 803–813

The Bacteriostatic Action of Benzoic and Salicylic Acids

I. The Effect on the Oxidation of Glucose and Pyruvic Acid by *Proteus vulgaris*

INGMAR BOSUND*

Swedish Institute for Food Preservation Research, Gothenburg, Sweden

The effect of benzoic and salicylic acids on glucose and pyruvate oxidation by *Proteus vulgaris* was studied by means of Warburg experiments. Neither benzoic nor salicylic acid had any inhibitory influence on the rate of oxygen consumption until the oxidation level of acetate was reached. Further oxidation was specifically stopped in the presence of 0.01 M benzoic, or 0.003 M salicylic acid, at pH 6.0. These concentrations are close to those required for a bacteriostatic effect.

The antimicrobial action of benzoic and salicylic acids has long been known and studied in various respects. Much work [1–17] has been done concerning the effect of these acids on different enzymes, or complex of enzymes, isolated or in intact cells, but the ultimate cause of the growth-inhibiting effect, in terms of metabolic reactions, is still open to discussion. The present work is an attempt to trace the mode of action somewhat further by determining what reactions, vital to the cell, are susceptible to the inhibitors at concentrations suppressing the growth of microorganisms. The experiments presented below are respiration studies with resting cells of *Proteus vulgaris*, in which the conventional Warburg technique has been used.

EXPERIMENTAL

Organism. A strain of *Proteus vulgaris* was selected as testorganism, mainly on account of its rapid growth on different carbon sources in synthetic media. Washed cells of this organism readily oxidize members of the tricarboxylic acid cycle and related compounds, besides glucose and pyruvic acid, especially when aerated during cultivation and harvested in the logarithmic growth phase. Moreover, such suspensions have a very low endogenous respiration, a fact of great value in the present studies. The culture was maintained on nutrient agar and transferred to new agar slants every month.

* Present address: State Dairy Research Station, Alnarp, Sweden.

Medium and growth conditions. The cultivation medium had the following composition, unless otherwise stated: glucose, 10.0 g; NH_4Cl, 1.0 g; K_2HPO_4, 9.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; casein hydrolysate (Difco vitamin-free casamino acids), 1.0 g; nicotinamide, 0.2 mg; distilled water to 1 l; adjusted to pH 7.0. The proper amount of glucose was dissolved in part of the water and autoclaved as a separate solution. Cultures were grown at 37°C, either in Erlenmeyer flasks without shaking, or in Kluyver flasks with sufficient aeration to ensure maximum growth rate. The growth media were inoculated with actively growing cells from a medium of the same composition. In most experiments the cells were harvested at the end of the logarithmic phase, or just after cessation of growth. In these cases, the growth was followed 1—2 h before harvesting by optical density measurements in a Beckman spectrophotometer at 425 mμ with sterile culture medium in the blank cell. Units of cell mass were determined by means of a standard curve for the organism, relating dry weight to optical density. In some experiments the cells were harvested at the beginning of the logarithmic phase of growth. In these cases, the culture medium was inoculated with a heavy suspension of washed cells, the optical density measurements being immediately started and the cells harvested, as soon as the logarithmic phase was established.

Preparation of cell suspensions. The cells were harvested by centrifugation and washed once by resuspension in the approximate growth volume of M/15 phosphate buffer of the same pH as in the actual experiment, usually 6.0. After repeated centrifugation, the cells were again suspended in the buffer, and the cell concentration adjusted to appropriate density. Repeated washings had no effect on cell activity, or endogenous respiration. Of this suspension 2.0 ml were added to each Warburg vessel.

Respiration studies. Oxygen consumption was measured by the conventional Warburg technique. The cell suspension was added to the main compartment of the Warburg vessels, and neutralized solutions of inhibitors and substrates to the side arms. The center well of the flasks contained 0.2 ml of 10 % KOH, absorbed on filter paper. The additions of substrate were so limited that the oxygen uptake could be followed until it had decreased to the value of the endogenous respiration. When necessary, cell activity was terminated by the addition of 0.3 ml of 5 N H_2SO_4 at the end of the experiment. CO_2 evolution, when determined, was measured according to the direct method of Warburg. The data recorded in the figures and tables have been corrected for the endogenous respiration which in no case was more than 10 % of that obtained in the presence of added substrate. All respiration measurements were made at 37°C and pH 6.0, if not otherwise stated.

Analytical procedures. Analyses of the contents of the Warburg vessels were made after removal of the cells by centrifugation. Pyruvic acid was determined as total keto acids with 2,4-dinitrophenylhydrazine, according to Friedemann and Haugen [18]. In the present experiments this method proved to give the same results as polarographic analysis. Acetic acid was determined as volatile acid by fractional steam-distillation. The titration was carried out with 0.1 N NaOH by means of a micrometer syringe. Benzoic acid, when present, distilled over together with acetic acid, and was corrected for by measuring the optical density of the distillate at 230 mμ in a Beckman DU spectrophotometer.

RESULTS

Growth experiments

The first step in this investigation was to determine the concentration of benzoic and salicylic acids required to prevent growth of *P. vulgaris*. As in the case of all weak acids, the biological activity of these compounds depends on the amount of substance present as neutral molecules, probably because these alone, not the ions, can penetrate the semipermeable membrane assumed to surround the bacterial cell. Thus, the activity of a given acid will depend on pH in a predictable manner, increasing with decreasing pH. Most of the

experiments in this investigation were carried out at pH 6.0, since a lower pH in itself would have led to unfavourable conditions for the organism.

The growth-inhibiting effect of benzoic acid on *P. vulgaris* was studied in the medium adjusted to pH 6.0 (Table 1). The main effect seemed to be a lengthening of the lag phase, but at concentrations approaching those required for a bacteriostatic effect, the growth rate in the logarithmic phase was also influenced. A characteristic feature in these experiments was the fact that benzoic acid, even at rather low concentrations, reduced the total amount of growth as compared with the amount obtained without inhibitor. This is easily accounted for by the formation of acid during growth, which resulted in an increasing effect of the benzoic acid and ended with growth being completely suppressed. In fact, a calculation of the actual concentrations of undissociated benzoic acid present at the moment when growth ceased in cultures with varying total amounts of the inhibitor gave the same value in all cases. In cultivation media where pH did not change during the experiment, no effect of benzoic acid on the total amount of growth could be demonstrated. It is evident that benzoic acid at concentrations about 0.01 M completely inhibited growth of *P. vulgaris* at pH 6.0. The same experiment with salicylic acid proved this inhibitor, producing bacteriostasis at concentrations about 0.002 M, to be more effective. However, the good correspondence between the amounts of undissociated acid present when growth ceased, as shown for benzoic acid in Table 1, did not appear when salicylic acid was used as inhibitor.

Table 1. Effect of benzoic acid on the growth of *P. vulgaris*. 50 ml of growth medium in 100-ml Erlenmeyer flasks were inoculated with cells actively growing in medium of the same composition without inhibitor. Different inocula were used in the two experiments. Initial pH, 6.0. Temp. 35°C.

Expt. No.	Total concn. of benzoic acid, M × 10^3	Growth	pH-values of the culture media when growth had ceased	Calculated * concn. of undissociated benzoic acid when growth had ceased, M × 10^3
1	—	+	4.51	—
	0.7	+	4.65	0.18
	1.7	+	5.09	0.19
	3.5	+	5.63	0.13
	5.3	+	5.82	0.12
	7.0	+	5.86	0.15
	10.5	—	5.98	—
2	—	+	4.62	—
	0.7	+	4.80	0.14
	1.7	+	5.28	0.13
	3.5	+	5.66	0.12
	5.3	+	5.81	0.13
	7.0	+	5.91	0.13
	10.5	—	5.98	—

* $10^{-4.20}$ has been used as dissociation constant for benzoic acid in this calculation.

The effect of benzoic and salicylic acids on glucose oxidation

Preliminary respiration studies at pH 6.0, with glucose as substrate, showed that neither inhibitor, at the concentrations mentioned above, had any effect on the rate of oxygen consumption during the first stage of glucose oxidation, and that lower concentrations generally increased the rate somewhat. The results, however, varied with growth conditions and the age of the cells at the time of harvesting.

Experiments with cells grown without shaking or aeration. The cultures were harvested in the stationary phase, generally within 1—2 h after growth had ceased. Warburg experiments with such cells showed that oxygen consumption during glucose oxidation proceeded at a constant rate until a sudden break occurred, after which it continued at a lower, though still constant rate, which, however, was significantly higher than that of the endogenous respiration. At the time of the break, all added glucose had disappeared from the medium. Pyruvate accumulated during the first part of the oxidation and disappeared again after the break (Fig. 1). The maximum amount of pyruvate in the medium, always appearing simultaneously with the break, varied in magnitude with the cell preparations according to some unknown factor, although the experimental conditions were kept as constant as possible.* In

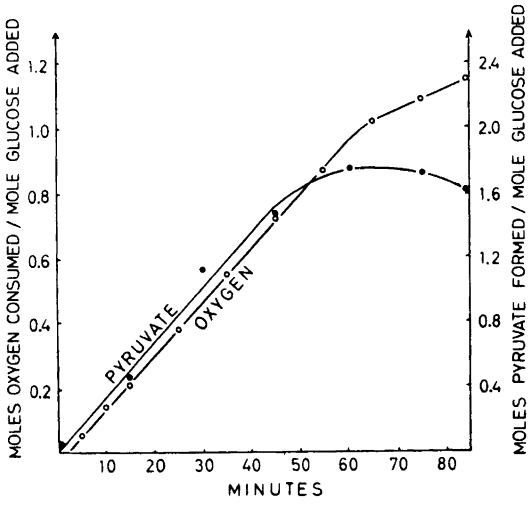


Fig. 1. Pyruvate accumulation during glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate in a total volume of 2.5 ml.

* After these experiments were performed, related results have been reported by Jackson[19] who investigated the accumulation of pyruvate in cultures of *P. vulgaris* grown with limiting concentrations of nicotinic acid. Although the concentrations of nicotinamide (0.2 μg/ml) used in the present work hardly can have had a limiting effect on growth, it is still possible that this factor might have been of some significance even in this case.

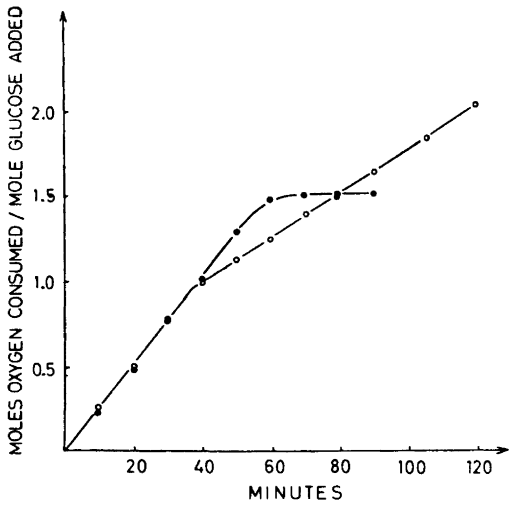


Fig. 2. Effect of benzoic acid on the oxygen consumption during glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate. O———O no benzoate, ●———● 0.01 M sodium benzoate. Final volume 2.5 ml.

some experiments, about 90 % of the maximum amount of pyruvate possible, as calculated from the amount of glucose added, had accumulated before the break. In such cases, the break occurred when almost exactly one mole of oxygen had been consumed per mole of glucose added, which seemed to indicate that the oxidation took place in two separate steps, first a conversion of all glucose added into pyruvate, and next a further oxidation of this metabolite.

This picture was completely changed when the oxidation took place in the presence of benzoic acid (Fig. 2). The essential effect of this drug consisted in postponing the break, after which the oxygen consumption was strongly inhibited. This inhibition was complete at the same concentration of benzoic acid as that required to prevent growth of the organism at the pH in question. Under these conditions, accumulation of pyruvate was prevented.

Experiments with cells aerated during growth. The two characteristic features of glucose oxidation with old cells of *P. vulgaris*, the break in the rate of oxygen consumption at a point where about one mole of oxygen had been taken up per mole of glucose added, and the accumulation of pyruvate, seemed to be intimately linked with some factors in the growth conditions. These signs of a disturbed oxidative ability did not appear in cells aerated during growth and harvested in the logarithmic growth phase. The oxygen consumption during glucose oxidation by such cells, and the effect of varying concentrations of benzoic acid, are illustrated in Fig. 3. 0.0035 M benzoic acid slightly stimulated the rate of oxygen uptake, while higher concentrations decreased the rate. The results obtained indicate a specific effect of benzoic acid on the later stages of glucose oxidation, and the amount of oxygen consumed in presence

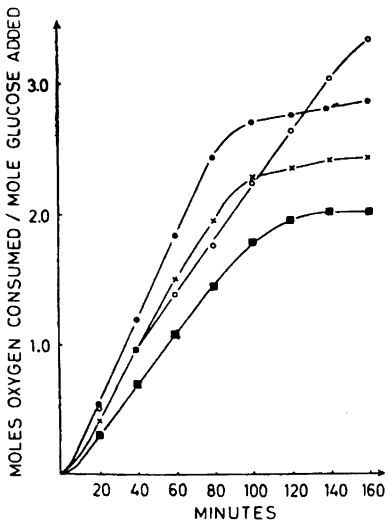


Fig. 3. Effect of benzoic acid on the glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 2.5 μmoles of glucose and 133 μmoles of potassium phosphate. O——O no benzoate, ●——● 0.0035 M, ×——× 0.0070 M, ■——■ 0.0140 M sodium benzoate. Final volume 2.5 ml.

of 0.014 M benzoic acid, about 2 moles per mole glucose added, suggests that the sensitive point lies at the oxidation level of acetate. Higher concentrations of benzoic acid inhibited the rate of oxygen consumption still more, but had no influence on this minimum amount of oxygen taken up per mole glucose. Essentially the same results were obtained with salicylic acid. This inhibitor, however, was more effective, reducing the oxygen consumption per mole glucose to 2 moles at concentrations about 0.003 M (Fig. 4). The close resemblance between these figures for the two acids and the bacteriostatic concentrations given on page 00 should be noticed. Large amounts of volatile acid were demonstrated in vessels where the oxidation occurred in the presence of benzoic or salicylic acid, whereas only traces were found in vessels without inhibitor.

The inhibition of ammonia assimilation during glucose oxidation by benzoic and salicylic acids

It has already been shown by Bernheim and DeTurk [10] that salicylic acid, benzoic acid, and related compounds, inhibit ammonia assimilation during succinate oxidation in *Pseudomonas aeruginosa*, more than they inhibit the oxidation of succinate itself. A similar specific effect on ammonia assimilation seemed to exist even in *P. vulgaris* (Table 2) when glucose was used as an energy source. Benzoic acid, at a concentration of 0.007 M, actually had a stimulating effect on the rate of oxygen consumption during glucose oxidation in the absence of ammonium ions. The degree of this stimulation varied with the age of the cells, being greater the older the cells were at the time of harvesting. The main results of the four experiments with benzoic acid

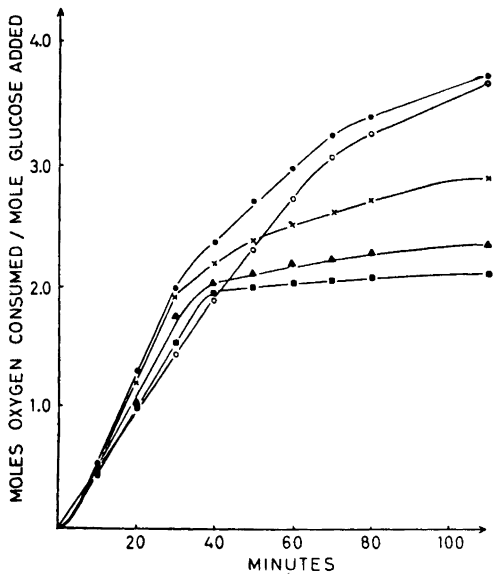


Fig. 4. Effect of salicylic acid on the glucose oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 5 μmoles of glucose and 133 μmoles of potassium phosphate. O———O no salicylate, ●———● 0.00125 M, ×———× 0.00188 M, ▲———▲ 0.00313 M, ■———■ 0.00500 M sodium salicylate. Final volume 2.5 ml.

were the same, being independent of the growth conditions, *i.e.* the increase in oxidation rate accompanying the presence of ammonium ions was completely abolished. Similar results were obtained with salicylic acid.

The effect of benzoic and salicylic acids on pyruvate oxidation

Bacteria from aerated cultures were used in these experiments. The cells were harvested at the end of logarithmic phase, or just after they had reached

Table 2. Influence of benzoic acid on the rate of glucose oxidation in the presence and absence of ammonium ions (four experiments). Each vessel contained 133 μmoles of potassium phosphate, 5 μmoles of glucose and 5 μmoles of $(NH_4)_2SO_4$ (when added). The final concentration of sodium benzoate was 0.007 M when present.

Additions	Relative rates of oxygen consumption. Rates with glucose as the only addition = 1			
	I	II	III	IV
Glucose	1	1	1	1
Glucose + $(NH_4)_2SO_4$	1.5	1.4	1.6	1.5
Glucose + sodium benzoate	1.3	1.1	1.2	1.4
Glucose + $(NH_4)_2SO_4$ + sodium benzoate	1.3	1.1	1.2	1.4

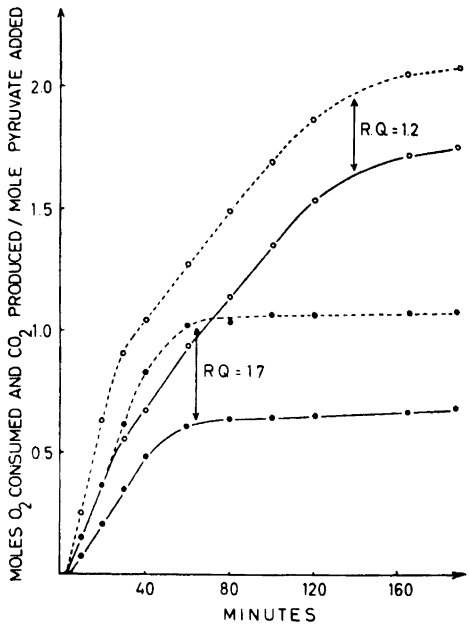


Fig. 5. Effect of benzoic acid on the pyruvate oxidation by *P. vulgaris*. Each vessel contained 2.0 ml of cell suspension, 5 μmoles of sodium pyruvate and 133 μmoles of potassium phosphate. Final volume 2.5 ml. O———O no benzoate, ●———● 0.0083 M sodium benzoate. Solid line, oxygen; dash line, carbon dioxide.

the stationary phase. The growth phase at harvesting did not seem to be critical for the results. As in the case of glucose oxidation, the results (Fig. 5) strongly suggest that the main effect of benzoic acid is close to the oxidation of acetate, or some »active» form of it. The theoretical amounts of oxygen consumed and carbon dioxide produced at a conversion of pyruvate into acetate (or some of its »active» forms) are 0.5 and 1.0 mole per mole pyruvate, respectively. As shown in Fig. 5, the actual values obtained in the presence of 0.0083 M benzoic acid agreed well with these figures. Determination of accumulated volatile acid in the vessels, after the oxygen consumption had practically ceased, supported this interpretation of the data. The results of one such determination are shown in Fig. 6 B, and the corresponding manometric data in Fig. 6 A. Similar experiments with salicylic acid gave corresponding results. The distillation constant calculated from the experimental data agreed with that obtained by distilling a similar solution with known amounts of acetic and benzoic acids, and acetate was demonstrated in the distillate by a method described by Feigl [20]. In other experiments the contents of the Warburg vessels were analysed by chromatographic methods. Apart from benzoic and salicylic acids, only acetic acid could be demonstrated. When the oxidation was carried out without inhibitor, only traces of acid were found (Fig. 6 C).

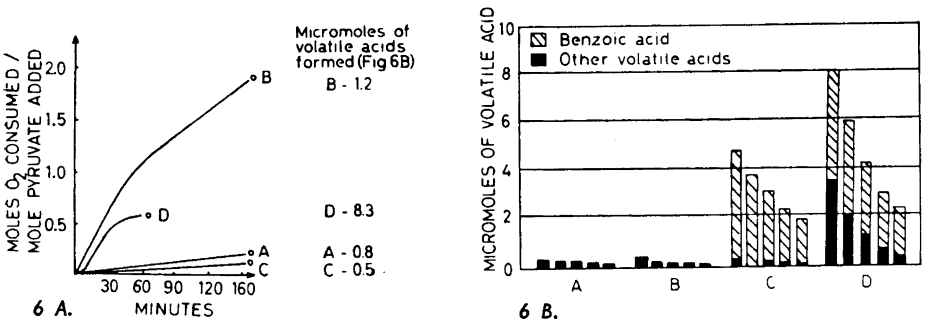


Fig. 6 A. Effect of benzoic acid on oxygen consumption and formation of volatile acids during pyruvate oxidation by *P. vulgaris*. The vessels contained besides 2.0 ml of cell suspension and 133 μmoles of potassium phosphate: A, no further additions; B, 10 μmoles of sodium pyruvate; C, 0.01 M sodium benzoate; D, 10 μmoles of sodium pyruvate and 0.01 M sodium benzoate. Final volume 2.5 ml. The oxidation was stopped, at points indicated, by addition of H_2SO_4 and the amounts of volatile acids determined as shown in Fig. 6B.

Fig. 6 B. Formation of volatile acids during pyruvate oxidation by *P. vulgaris*. Each group of columns represents the amount of volatile acids in the first five fractions of distillate from one Warburg vessel. Indications as in Fig. 6 A. The volume of the fractions was equal to that of the distilling solution.

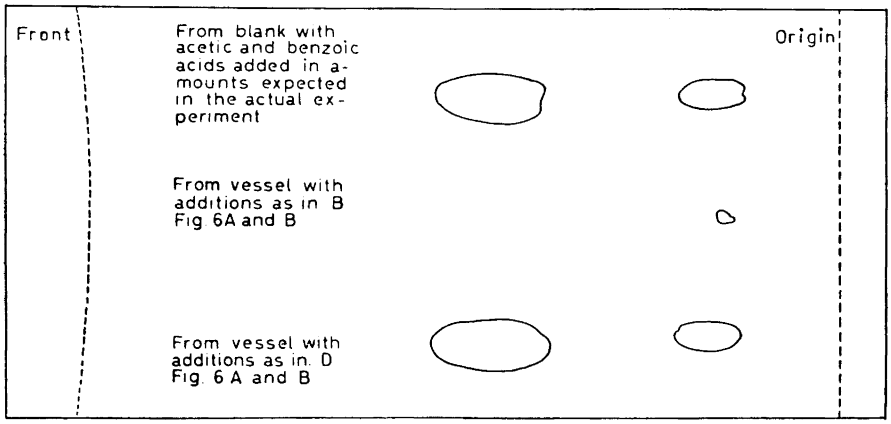


Fig. 6 C. Chromatographic separation of reaction products after an experiment similar to that in Fig. 6 A. The oxidation was stopped by addition of H_2SO_4, the contents in the vessels neutralized and concentrated by evaporation, acidified and extracted with *n*-butanol. Descending solvent: water-saturated *n*-butanol.

DISCUSSION

The experiments described show that the presence of benzoic or salicylic acid during glucose and pyruvate oxidation by *P. vulgaris* causes an accumulation of acetate in the medium. The utilization of this compound, as acetyl-

S-CoA, or in some other activated form, in synthesis of cell material and as an energy source is thereby prevented. The central position of such intermediates in the metabolism of living cells and the fact that benzoic and salicylic acids in the present experiments caused the accumulation of acetate at the same concentrations as those required to suppress growth of *P. vulgaris* suggest that the bacteriostatic effect, at least in part, may be due to an interference with the metabolism of the acetyl group.

Although the net result of the action of the two acids on the oxidation processes studied is relatively clear, the data presented give no information as to the way in which the inhibition is accomplished. Nevertheless, the results may be considered in view of what is known about the pyruvate metabolism in *P. vulgaris*. Apart from the enzyme system transforming pyruvate into metabolic active C_2-compounds, this organism seems to possess a pyruvate oxidase of quite another type. This enzyme has been thoroughly investigated by Stumpf [21] and by Moyed and O'Kane [22] and shown to give rise to acetate from pyruvate, without intermediate formation of any detectable acyl compound. The only cofactors required for the action of this enzyme are divalent ions and cocarboxylase. If the normal way of pyruvate metabolism in *P. vulgaris* is sensitive to benzoic and salicylic acids, and the acetate generating system is not, the presence of these acids during oxidation should lead to the withdrawal of great amounts of C_2-compounds as acetate from the normal metabolic route.

Irrespective of the mechanism of the acetate accumulation, the mere fact that it occurs indicates that the oxidation of acetate in the present experiments is much more sensitive to benzoic and salicylic acids than that of glucose and pyruvate. As the first step in acetate oxidation involves activation in an energy-requiring process, it is possible that the inhibition of this oxidation by benzoic and salicylic acids is related to the effect of these acids on another energy-requiring process, *e.g.* assimilation of ammonium ions. The work by Bernheim and DeTurk [10] already referred to and the experiments described in this paper show that benzoic and salicylic acids specifically inhibit the increased rate of substrate oxidation observed during simultaneous assimilation of ammonium ions. Bernheim and DeTurk suggest that their results are due to an inhibition of the formation, or utilization, of high energy compounds in the presence of salicylic or benzoic acid. In the present experiments such an interpretation would agree well with the stimulation of glucose oxidation, caused by the two acids, as an increase in oxidation rate is often observed in the presence of substances disturbing the energy conservation process. In recent years, inhibition of oxidative phosphorylation in isolated mitochondria by salicylic acid (Penniall, Kalnitsky and Routn [23]) and by salicylic and benzoic acids (Bosund [24]) has been reported. These findings, especially in the case of salicylic acid, bear out the hypothesis that some of the growth-inhibiting effect may be due to an interference with the formation of high energy compounds.

This work is part of an investigation supported by the *Swedish Natural Science Research Council* and the *Swedish Technical Research Council.*

I am greatly indebted to Professor G. Lindeberg, Agricultural College of Norway, Vollebekk, Norway, for introducing me into this field of investigation and for his

patient advice and encouragement throughout the work. I also wish to thank Professor E. Adler, Chalmers University of Technology, Gothenburg, Sweden, for continued interest and helpful criticism.

REFERENCES

1. Jowett, M. and Quastel, J. H. *Biochem. J.* **29** (1935) 2159.
2. Klein, J. R. and Kamin, H. *J. Biol. Chem.* **138** (1941) 507.
3. Iványovics, G. *Z. physiol. Chem. Hoppe-Seyler* **276** (1942) 33.
4. Iványovics, G. *Z. Immunitätsforsch.* **102** (1942) 238.
5. Adler, E. von Euler, H. and Skarzynski, B. *Arkiv Kemi, Mineral. Geol.* **16 A** (1943) No. 9.
6. von Euler, H. and Ahlström, L. *Z. physiol. Chem. Hoppe-Seyler* **279** (1943) 175.
7. Herner, B. *Acta Physiol. Scand.* **8** (1944) Suppl. XXIII.
8. Bartlett, G. R. *J. Am. Chem. Soc.* **70** (1948) 1010.
9. Barbour, R. G. H. and Vincent, J. M. *J. Gen. Microbiol.* **4** (1950) 110.
10. Bernheim, F. and DeTurk, W. E. *J. Pharmacol. Exptl. Therap.* **103** (1951) 107.
11. Klein, J. R. *J. Biol. Chem.* **205** (1953) 725.
12. Kuttner, R. and Wagreich, H. *Arch. Biochem. Biophys.* **43** (1953) 80.
13. Kaplan, E. H., Kennedy, J. and Davis *J. Arch. Biochem. Biophys.* **51** (1954) 47.
14. Avigan, J., Quastel, J. H. and Scholefield, P. G. *Biochem. J.* **60** (1955) 329.
15. Brody, T. M. *J. Pharmacol. Exptl. Therap.* **117** (1956) 39.
16. Smith, M. J. H. and Jeffrey, S. W. *Biochem. J.* **63** (1956) 524.
17. Smith, M. J. H. and Jeffrey, S. W. *Biochem. J.* **64** (1956) 589.
18. Friedemann, T. E. and Haugen, G. E. *J. Biol. Chem.* **147** (1943) 415.
19. Jackson, F. L. *J. Gen. Microbiol.* **16** (1957) 711.
20. Feigl, F. *Spot tests in organic analysis*, 5th ed., Elsevier publishing company, 1956, p. 342.
21. Stumpf, P. K. *J. Biol. Chem.* **159** (1945) 529.
22. Moyed, H. S. and O'Kane, D. J. *J. Biol. Chem.* **218** (1956) 831.
23. Penniall, R., Kalnitsky, G. and Routh, J. I. *Arch. Biochem. Biophys.* **64** (1956) 390.
24. Bosund, I. *Acta Chem. Scand.* **11** (1957) 541.

Received January 8, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 814–818

Structure of Elastin

2. Yellow Coloration

AIMO KÄRKELÄ and EINO KULONEN

Department of Medical Chemistry, University of Turku, Finland

For the investigation of its yellow pigment, elastin was partially hydrolyzed with acids, bases and enzymes under varying conditions and the hydrolyzates submitted to several fractionations. The best result was obtained by hydrolysis in 2 N sulfuric acid for 96 h at +95°C with subsequent adsorption on aluminium oxide and elution with ammonia.

Data on the infra-red absorption spectra of elastin and an enriched yellow preparation are presented. This preparation could not be degraded further without loss of the colour. The yellow pigment is tightly bound to the peptide chain and could not be identified as a bile pigment or by ultraviolet absorption spectrum.

The yellow colour was intensified in an unexplained manner by dialysis or treatment with tungstate.

The yellow pigment of elastin has recently attracted considerable interest [1-3] and several classes of compounds have been suggested as possible chromophores.

The first aim was to isolate the yellow component, but only enriched preparations could be obtained. The elastin was broken down with several enzymes, acids and bases. All methods of hydrolysis were handicapped by the formation of humins. In this respect barium hydroxide and papain were the most favourable agents. A complete hydrolysis of elastin was not achieved without concurrent destruction of the pigment.

The partial hydrolysates were fractionated by dialysis, by ion-exchange chromatography (Dowex-50) using different buffer systems, by preparative silica gel electrophoresis, by adsorption chromatography and by peptide-precipitating agents.

The best yellow fractions were obtained by adsorption on aluminium oxide and elution with ammonia. The yellow color seems to be tightly bound to the peptide chain (*cf.* Partridge and Davis [4]) and it could not be separated from the amino acids. Under certain conditions the colour is intensified, for example during dialysis or after addition of tungstates. This

effect is quite unexplained. An unsaturated bond suggested by Labella [2] is possible.

The main results are the preparation of the enriched fraction, the infra-red data of elastin and analysis of the peptide to which the yellow component is attached. It seems that there exists a yellow "core" which is gradually broken down to smaller non-coloured fragments, of which some amino acids were identified.

The attempts to identify the yellow component as a bile pigment or porphyrin derivative were not successful.

Material and preparation

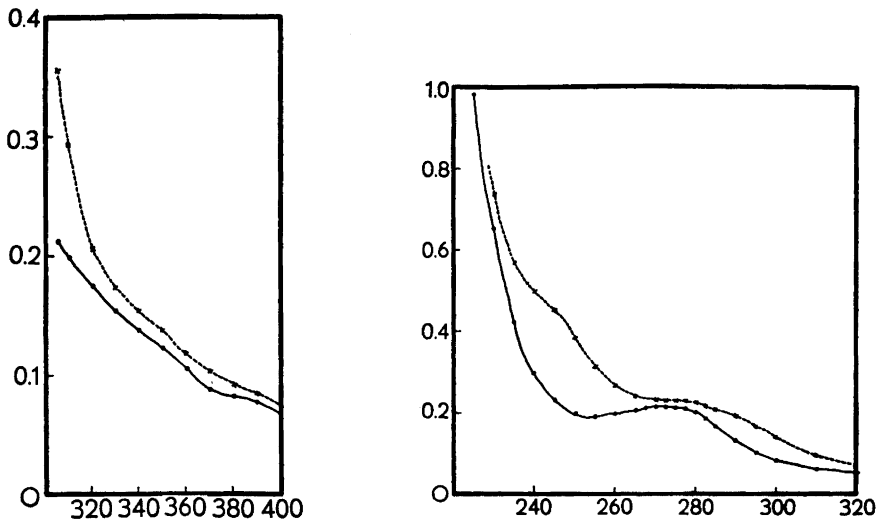
The preparation of elastin has been described in our earlier paper on the structure of elastin [5].

The *best enriched yellow preparation* was designated as R-5/Al_2O_3 and obtained as follows: Fifty g of elastin was suspended in 1 000 ml of 2 N sulfuric acid and kept at +95°C for 48 h with occasional shaking. Already after the first hours the fluid acquired a reddish-brown colour and at the end of the heating the fluid was rather dark. After filtration the fluid was neutralized with solid barium hydroxide. The precipitate adsorbed a part of the dark-brown colour but the filtrate was still rather brown, like concentrated urine.

The hydrolysis was continued at +95°C for an additional 48 h in 2 N sulfuric acid and more dark-brown colour was formed. The precipitation with barium hydroxide was repeated and the supernatant centrifuged clear. For removal of the dark colour additional barium sulfate was suspended and after 2 ½ h agitation the supernatant was collected by centrifugation. It resembled normal urine in colour. At a lower temperature (+37°C)

Table 1. The intensity of the yellow colour in some elastin preparations.

Method of preparation	Nitrogen mg/ml in solutions with $E^{1\,cm}_{330} = 1$
Oxalic acid-soluble elastin	0.82
Hydrolyzate with $Ba(OH)_2$	2.36
fraction obtained with Dowex-50, pH 3.42	0.57
Hydrolyzate (partial) with 4 N HCl,	
fraction obtained with Dowex-50, pH 3.42	0.42
» » » » pH 6.7	0.23
Hydrolyzate (partial) with 4 N H_2SO_4	0.85
outside the bag after dialysis	0.52
R-5	1.60
R-5/Al_2O_3 (adsorbed on Al_2O_3 and eluted with NH_3)	0.12
Hydrolyzate with papain	1.05
supernatant after precipitation with 10 % trichloroacetic acid	0.81
supernatant after precipitation with tungstic acid (final conc. 1.67 % sodium tungstate in 0.11 N H_2SO_4, supernatant neutralized to pH 5; the colour not quite elastin-like)	0.14
Humic acid (Light & Co., Ltd., in 0.1 N HCl)	0.093



Figs. 1—2. The absorption spectra of elastin in 0.1 N hydrochloric acid (solid) and in 0.1 N sodium hydroxide (dotted).

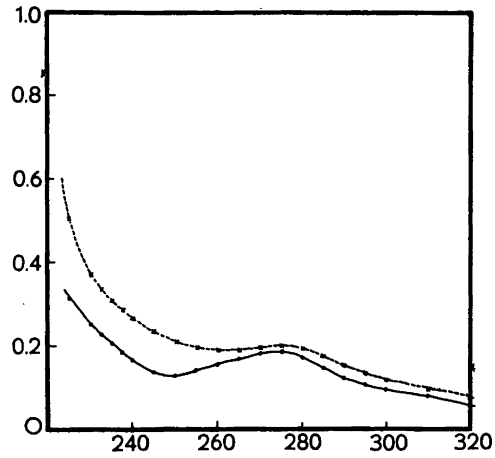


Fig. 3. The ultraviolet absorption spectrum of the preparation R-5/Al_2O_3 in 0.1 N hydrochloric acid (solid) and in 0.1 N sodium hydroxide (dotted).

less brown and red substances were formed, but hydrolysis lasted much longer and was more incomplete.

The hydrolysate was allowed to drain through a 2 cm layer of aluminium oxide (Merck, "nach Brockmann"). The yellow colour was retained in the surface of the aluminium oxide, which was washed with water until the washings were ninhydrin-negative. Only very little of the yellow colour was removed. The bulk of the yellow substance was eluted with 1 M ammonia, which was removed by evaporation in vacuum. The residue was brownish-yellow and the yield was 0.7 g.

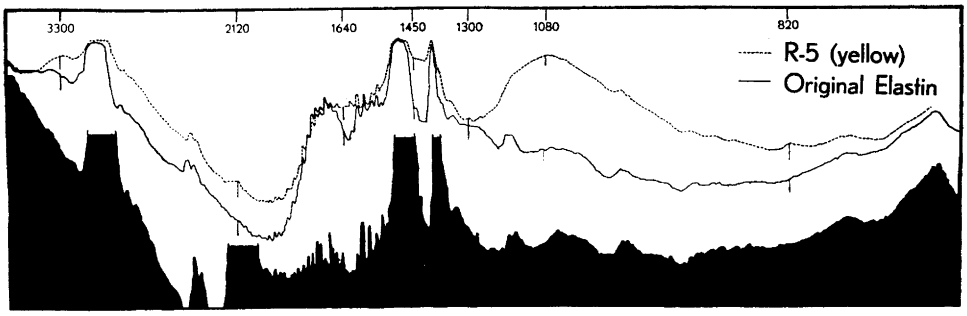


Fig. 4. The infra-red absorption spectra of oxalic acid-soluble elastin and of the enriched preparation R-5/Al_2O_3. (Perkin-Elmer single-beam instrument, sodium chloride cell of 0.05 mm, concn. 10 mg in 0.05 ml liquid paraffin, the borderline of the black area indicates the spectrum of the paraffine.) The differences in the spectra are pointed out with their wave numbers (cm^{-1}).

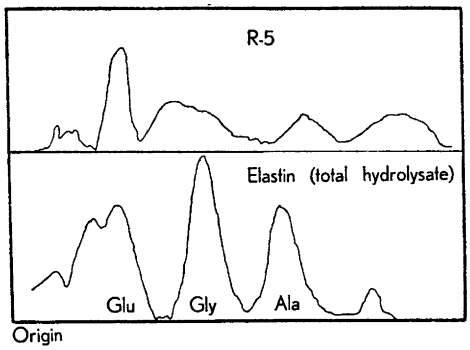


Fig. 5. Comparison of "total hydrolyzates" of elastin and of R-5/Al_2O_3 (densitograms from one-dimensional paper chromatograms run with phenol/NH_3). Note the absence of glycine in R-5.

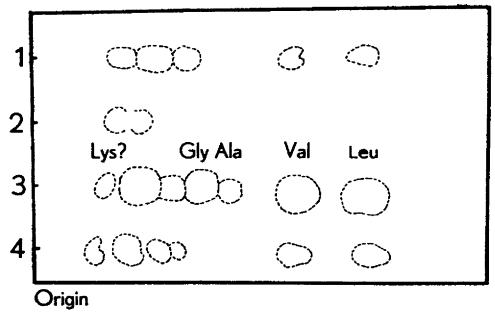


Fig. 6. Comparison of the butanol/acetic acid chromatograms of R-5 and original elastin. 1 = R-5/Al_2O_3, "total hydrolyzate"; 2 = Asp + Glu; 3 = elastin, "total hydrolyzate"; 4 = yellow "start" spot of No. 1 (the hydrolyzed R-5), rehydrolyzed.

Hydrolysis with papain was carried out at +37—38° C for 24 h. The digest contained 40 ml of 2.5 % elastin solution, 5 ml of 0.25 M citrate buffer (pH 5) and 5 ml of H_2S-activated papain (0.626 mg N/ml). The liberated amino nitrogen amounted to 18 % of the total.

Hydrolysis with saturated Ba (OH)$_2$ was performed in a solution containing 5 % elastin which was kept for 3—4 days at +37°C. The fluid remained "purely" yellow.

The *R-4-hydrolysate* was made at +37°C in 4 N sulfuric acid for 14 days and finally the mixture was boiled under a reflux condenser for 24 h.

The intensity of the yellow colour was expressed as the concentration of nitrogen per ml solution having the extinction coefficient $E^{1\,cm}_{330} = 1$.

Some data are presented in the Table 1. Specimens of the absorption spectra are given in Figs. 1—3. There was no evidence to support the hypothesis of bile pigment origin (attempts were made with chloroform extraction and oxidation with hydrogen peroxide.) The comparison with a "humic acid" sample remained inconclusive.

In the many fractionation experiments with Dowex-50 it was observed that after the change of any buffer a yellow fraction appeared. Two sources of error were recognized: (1) some yellow colour can be eluted from the resin itself and (2) other proteins, for example gelatin, form during the hydrolysis yellow artefacts which appear similarly. The significance of these yellow fractions remains unknown.

Analysis of the preparation R-5/Al_2O_3

Infra-red absorption (Fig. 4). Absorptions at 3 300 cm^{-1} and 1 640 cm^{-1} are due to amide groups, at 2 120 cm^{-1} to amino acids and at 1 450 cm^{-1} perhaps to carboxyl groups. Peaks at 1 300 cm^{-1} and 820 cm^{-1} are so small that no conclusions, for example concerning double bonds, seem warranted. The broad peak at 1 080 cm^{-1} may be interpreted in several different ways (C—O of ethers, alcohols, or anhydrides, molecular size).

Amino-acid composition. Although the yellow substance was of such lowmolecular weight that it was dialysable, it did not move in the chromatograms as a compact spot but the colour faded in these manipulations as also in column chromatography and in preparative electrophoresis. The comparison of the amino acids of R-5/Al_2O_3 and elastin is presented in Fig. 5.

It was found that on the origin of the chromatographed R-5-hydrolyzates there always remained a yellow ninhydrin-positive spot. When this "start" spot was rehydrolyzed, a pattern resembling original R-5 hydrolysate was obtained (Fig. 6). From this it is concluded that the yellow component is during acid hydrolysis gradually broken to non-coloured products and that the R-5/Al_2O_3 represents the smallest yellow unit for the present.

Acknowledgements. We are greatly indebted to Mr. Tapio Salo, M. A., from the Central Laboratories of Finnish State Alcohol Monopoly, for the infra-red spectrometry. This work was generously supported by *Finnish Medical Society "Duodecim"* and *Sigrid Jusélius Foundation.*

REFERENCES

1. With, T. *Acta Med. Scand.* **128** (1947) 25.
2. Labella, F. S. *Nature* **180** (1957) 1360.
3. Loomeijer, F. J. *Nature* **182** (1958) 182.
4. Partridge, S. M. and Davis, A. F. *Biochem. J.* **61** (1955) 11.
5. Kärkelä, A. and Kulonen, E. *Acta Chem. Scand.* **11** (1957) 1434.

Received January 9, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 819–858

Short Communications

On the Amino Acid Sequence of Bovine Fibrinopeptides

BIRGER BLOMBÄCK and PER WALLÉN

Chemistry Department II, Karolinska Institutet, Stockholm,

JOHN SJÖQUIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Fibrinopeptides are split off from the fibrinogen of various species during its conversion to fibrin. Summaries of the results achieved in this field have recently been given by Scheraga and Laskowski [1] and by Blombäck [2]. From ox fibrinogen, at least two peptides, tentatively named A and B, are split off. These peptides have been isolated by different methods, and chemical studies on their amino acid and N-terminal composition have been performed in our laboratories, as well as in others [3-7]. These studies confirm that glutamic acid is N-terminal in peptide A, whereas peptide B seems to lack any N-terminal amino acid residue. With carboxypeptidase, however, arginine can be split off as C-terminal from both peptides A and B [8,9]. The sequence of the three first amino acid residues from the N-terminal end of peptide A has recently been described by Blombäck and Sjöquist [2]. Sequence studies of bovine peptides A and B have later been reported by Folk *et al.*[9]

Digestion of peptides A and B with a variety of proteolytic enzymes, *i. e.*, papain, chymotrypsin, subtilisin and trypsin, released several fragments which were separated either by high-voltage electrophoresis, or on ion-exchange columns of 2 % cross-linked Dowex-50 [5]. The N-terminal amino acids were determined with the phenylthiocarbamyl method of Edman, as applied by Sjöquist [10]. The amino acids were determined quantitatively according to the method of Sjöquist [7,11,12].

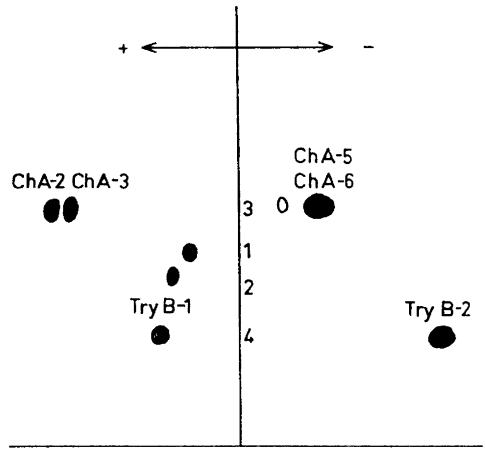


Fig. 1. Paper electrophoresis of peptides A and B after digestion with proteolytic enzymes. The experiments were carried out in 0.1 M pyridine adjusted to pH 4.1 with acetic acid. The potential gradient was approximately 20 V/cm. Each run lasted for about 3 hours.
1. Peptide B
2. Peptide A
3. Peptide A after digestion with chymotrypsin
4. Peptide B after digestion with trypsin

By means of chymotrypsin digestion, peptide A was split into four main fragments (Figs. 1 and 2). It can be seen from Table 1 that fragments ChA-2 and ChA-3 are similar in composition with respect to all amino acids except leucine (or *iso*leucine). Fragments ChA-5 and ChA-6 also differ only with regard to this particular amino acid. The latter peptides were not separated during electrophoresis at pH 4.1 in pyridine acetate buffer (Fig. 1). Separation could, however, be achieved by ion-exchange chromatography.

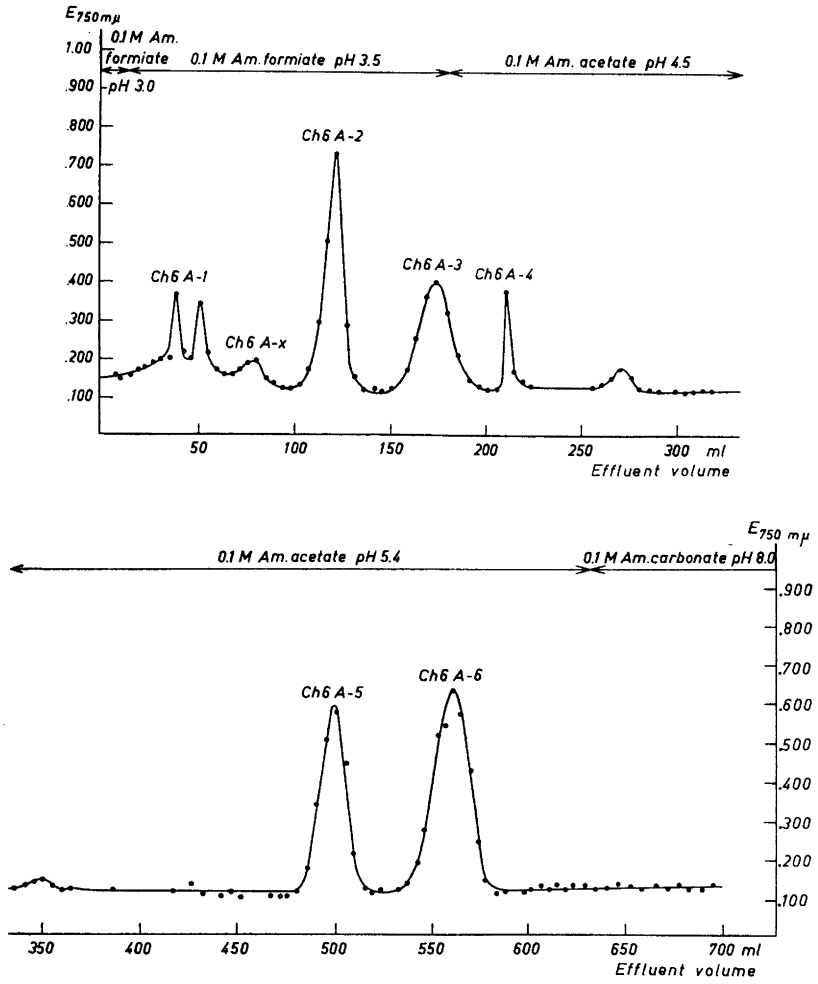


Fig. 2. Separation of peptides from a chymotryptic hydrolysate of peptide A by chromatography.

Assuming that peptide A consists of only one peptide chain, chymotrypsin had apparently split the chain on both sides of leucine (or *iso*leucine). The amino acid sequence determination of the fragments from the N-terminal end gave the following results: *ChA-2*: glu.asp.gly.ser.asp.asp., *ChA-3:* glu.asp.gly.ser.asp.asp., *ChA-5:* thr.glu.gly.gly.gly.val.arg., *ChA-6:* leu. (or ileu.) thr.glu.gly.gly.gly.val.arg. Since chymotrypsin has a specificity for peptide linkages near aromatic residues, the C-terminal residue in ChA-2 is most probably phenylalanine. An attempt was also made to investigate the C-terminal part of fragment ChA-2 and of the undigested A peptide, by means of the hydrazinolysis method of Akabori, as modified by Wallén and Sjöholm [14]. It was found that the C-terminal amino acid in fragment ChA-2 is phenylalanine, and that the residue next to the C-terminal amino acid is probably glycine. Furthermore, it was possible to confirm that the C-terminal sequence of the undigested A peptide is gly.val.arg. The amino acid

Table 1. Amino acid residues of peptide A and of peptides isolated from a chymotryptic digest of peptide A.

Amino acids	Number of residues						
	ChA-2	ChA-3	ChA-5	ChA-6	Peptide A	ChA-2+ ChA-6	ChA-3+ ChA-5
Arginine	0	0	1	1	1	1	1
Aspartic acid	3	3	0	0	3	3	3
Glycine	2	2	3	3	5	5	5
Glutamic acid	1	1	1	1	2	2	2
*Iso*leucine / Leucine	0	1	0	1	1	1	1
Phenylalanine	1	1	0	0	1	1	1
Proline	2	2	0	0	2	2	2
Serine	2	2	0	0	2	2	2
Threonine	0	0	1	1	1	1	1
Valine	0	0	1	1	1	1	1
Total amino acid residues	11	12	7	8	19	19	19

residues found in the fragments are in fairly good agreement with the residues found in the undigested peptide A (Table 1). From the N-terminal end, the following amino acid sequence was found in peptide A on direct determination: glu. asp.gly.ser.asp. However, when the sequence determination was performed on the undigested A peptide, an unidentified compound with the same R_F value as proline appeared in the second, third, fourth and fifth degradation steps. This compound is believed to be a side-reaction product formed during cyclization.

Digestion of peptide A with papain gave a fragment with the N-terminal sequence pro.pro.ser.gly. Thus, the studies so far seem to indicate that peptide A consists of a single peptide chain, with the following amino acid sequence: glu.asp.gly.ser.asp. asp.pro.pro.ser.gly.phe.leu.thr.glu.gly.gly. gly.val.arg.

Peptide B lacks an N-terminal residue which reacts with fluorodinitrobenzene, or which can form a phenylthiohydantoin derivative after coupling with phenyl*iso*thiocyanate. This indicates that at least this part of the molecule may be involved in a cyclic structure. Peptide B, however, has a C-terminal arginine residue as determined by means of carboxypeptidase or with the aforementioned hydrazinolysis method. This peptide has also been found to contain tyrosine-O-sulphate [5,13].

Peptide B could be split into two fragments by digestion with trypsin chymotrypsin or papain (Fig. 1). The different enzymes yielded different peptides, one of which was the main part of the original peptide, whereas the others contained fewer amino acid residues. The amino acid composition of the peptides (Try B-1 and Try B-2) obtained from a tryptic hydrolysate is shown in Table 2. The main fragment, Try B-1, containing tyrosine-O-sulphate, failed to give any N-

Table 2. Amino acid residues of peptide B and of peptides isolated from a tryptic hydrolysate of peptide B.

Amino acids	Number of residues			
	Try B-1	Try B-2	Peptide B	Try B-1+ Try B-2
Alanine	0	1	1	1
Arginine	1	1	2	2
Aspartic acid	4	0	4	4
Glutamic acid	3	0	3	3
Glycine	1	2	3	3
*Iso*leucine / Leucine	0	1	1	1
Lysine	1	0	1	1
Phenylalanine	1	0	1	1
Proline	2	0	2	2
Serine	0	0	0	0
Threonine	1	0	1	1
Tyrosine	1	0	1	1
Valine	0	1	1	1
Total amino acid residues	15	6	21	21

terminal residue. In the peptide Try B-2, valine is N-terminal. The amino acid sequence of this fragment is val.gly.leu. (or ileu.)gly.ala.arg. As is seen from Table 2, the residues found in the fragments are in fairly good conformity with the residues found in peptide B. In the preliminary report on the amino acid composition of peptide B (cf. Ref.[2]), threonine was stated to be absent. In subsequent analysis with a somewhat modified procedure [7], threonine was recovered from the hydrolysate of peptide B. This is in agreement with the original finding by Bettelheim [3].

For further elucidation of the structure of peptide B, digestion of this peptide by means of subtilisin has given promising results. After digestion with this enzyme, 6 or 7 small peptides were isolated by chromatography or by electrophoresis.

The complete paper on the amino acid sequence of bovine fibrinopeptides A and B will be published elsewhere.

The authors' thanks are due to Mr. Nils Gröndahl for the performance of the electrophoretic analysis.

1. Scheraga, H. A. and Laskowski, M. *Advances in Protein Chem.* **12** (1957) 1.
2. Blombäck, B. *Acta Physiol. Scand.* **43** (1958) Supplementum 148.
3. Bettelheim, F. R. *Biochim. et Biophys. Acta* **19** (1956) 121.
4. Lorand, L. *Physiol. Revs.* **34** (1954) 742.
5. Blombäck, B. and Vestermark, A. *Arkiv Kemi* **12** (1958) 173.
6. Gladner, J. A., Folk, J. E., Laki, K. and Carroll, W. R. *J. Biol. Chem.* **234** (1959) 62.
7. Sjöquist, J. *Acta Chem. Scand.* (1959) *in press.*
8. Laki, K., Gladner, J. E. and Kominz, D. R. *Seventh Annual Symposium on Blood,* Wayne State University, U.S.A. 1958.
9. Folk, J. E., Gladner, J. A. and Laki, K. *J. Biol. Chem.* **234** (1959) 67.
10. Sjöquist, J. *Arkiv Kemi* (1959) *in press.*
11. Sjöquist, J. *Arkiv Kemi* **11** (1957) 129.
12. Sjöquist, J. *To be published.*
13. Bettelheim, F. R. *J. Am. Chem. Soc.* **76** (1954) 2838.
14. Wallén, P. and Sjöholm, I. *To be published.*

Received January 22, 1959.

Synthesis of (—)-Methyl 2D, 4D,6D-Trimethylnonacosanoate and Identification of C_{32}-Mycocerosic Acid as a 2,4,6,8-Tetramethyloctacosanoic Acid

CECILE ASSELINEAU and JEAN ASSELINEAU

Institut de Biologie physico-chimique, Paris, France,

RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden,

STINA STÄLLBERG-STENHAGEN

Institute of Medical Biochemistry, Göteborg, Sweden,
and

EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala, Sweden

A mass spectrometric study [1] of the methyl ester of mycocerosic acid isolated by Anderson and co-workers [2-4] from the lipids of tubercle bacilli, indicated a molecular weight of 494, corresponding to the methyl ester of a C_{32}-acid. The mass spectrum furthermore indicated the presence of methyl side chains at positions 2, 4, and 6. These methyl group positions were the same as deduced by Polgar [5] for the closely related or identical compound called mycoceranic acid [6,7]. The levo-rotation of mycoceranic acid and the optical rotations recorded by Polgar for the products obtained in the step-wise degradation suggested an all D optical configuration. For comparison we therefore synthesized (—)-methyl 2D,4D,6D-trimethylnonacosanoate. The synthesis was performed as follows.

n-Eicosylmalonic acid, m. p. 122.8—124.2°, was prepared in a yield of 90 % from ethyl *n*-docosanoate and ethyl oxalate [8]. The reaction of the acid chloride derived from (—)-6-methoxycarbonyl-3L, 5L-dimethylhexanoic acid [9] with the sodium derivative of di-(tetrahydropyranyl) *n*-eicosylmalonate [10] gave (+)-methyl 3D,5D-dimethyl-7-oxooctacosanoate, m. p. 42.8—43.6°, $[\alpha]_D^{25}$ +0.4° (c = 19.3) *,

* All optical rotations were measured in chloroform solution using a 1 dm tube.

terminal residue. In the peptide Try B-2, valine is N-terminal. The amino acid sequence of this fragment is val.gly.leu. (or ileu.)gly.ala.arg. As is seen from Table 2, the residues found in the fragments are in fairly good conformity with the residues found in peptide B. In the preliminary report on the amino acid composition of peptide B (cf. Ref.[2]), threonine was stated to be absent. In subsequent analysis with a somewhat modified procedure [7], threonine was recovered from the hydrolysate of peptide B. This is in agreement with the original finding by Bettelheim [3].

For further elucidation of the structure of peptide B, digestion of this peptide by means of subtilisin has given promising results. After digestion with this enzyme, 6 or 7 small peptides were isolated by chromatography or by electrophoresis.

The complete paper on the amino acid sequence of bovine fibrinopeptides A and B will be published elsewhere.

The authors' thanks are due to Mr. Nils Gröndahl for the performance of the electrophoretic analysis.

1. Scheraga, H. A. and Laskowski, M. *Advances in Protein Chem.* **12** (1957) 1.
2. Blombäck, B. *Acta Physiol. Scand.* **43** (1958) Supplementum 148.
3. Bettelheim, F. R. *Biochim. et Biophys. Acta* **19** (1956) 121.
4. Lorand, L. *Physiol. Revs.* **34** (1954) 742.
5. Blombäck, B. and Vestermark, A. *Arkiv Kemi* **12** (1958) 173.
6. Gladner, J. A., Folk, J. E., Laki, K. and Carroll, W. R. *J. Biol. Chem.* **234** (1959) 62.
7. Sjöquist, J. *Acta Chem. Scand.* (1959) *in press.*
8. Laki, K., Gladner, J. E. and Kominz, D. R. *Seventh Annual Symposium on Blood,* Wayne State University, U.S.A. 1958.
9. Folk, J. E., Gladner, J. A. and Laki, K. *J. Biol. Chem.* **234** (1959) 67.
10. Sjöquist, J. *Arkiv Kemi* (1959) *in press.*
11. Sjöquist, J. *Arkiv Kemi* **11** (1957) 129.
12. Sjöquist, J. *To be published.*
13. Bettelheim, F. R. *J. Am. Chem. Soc.* **76** (1954) 2838.
14. Wallén, P. and Sjöholm, I. *To be published.*

Received January 22, 1959.

Synthesis of (—)-Methyl 2D, 4D,6D-Trimethylnonacosanoate and Identification of C_{32}-Mycocerosic Acid as a 2,4,6,8-Tetramethyloctacosanoic Acid

CECILE ASSELINEAU and JEAN ASSELINEAU

Institut de Biologie physico-chimique, Paris, France,

RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden,

STINA STÄLLBERG-STENHAGEN

Institute of Medical Biochemistry, Göteborg, Sweden,
and

EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala, Sweden

A mass spectrometric study [1] of the methyl ester of mycocerosic acid isolated by Anderson and co-workers [2-4] from the lipids of tubercle bacilli, indicated a molecular weight of 494, corresponding to the methyl ester of a C_{32}-acid. The mass spectrum furthermore indicated the presence of methyl side chains at positions 2, 4, and 6. These methyl group positions were the same as deduced by Polgar [5] for the closely related or identical compound called mycoceranic acid [6,7]. The levo-rotation of mycoceranic acid and the optical rotations recorded by Polgar for the products obtained in the step-wise degradation suggested an all D optical configuration. For comparison we therefore synthesized (—)-methyl 2D,4D,6D-trimethylnonacosanoate. The synthesis was performed as follows.

n-Eicosylmalonic acid, m. p. 122.8—124.2°, was prepared in a yield of 90 % from ethyl *n*-docosanoate and ethyl oxalate [8]. The reaction of the acid chloride derived from (—)-6-methoxycarbonyl-3L, 5L-dimethylhexanoic acid [9] with the sodium derivative of di-(tetrahydropyranyl) *n*-eicosylmalonate [10] gave (+)-methyl 3D,5D-dimethyl-7-oxooctacosanoate, m. p. 42.8—43.6°, $[\alpha]_D^{25}$ +0.4° (c = 19.3) *,

* All optical rotations were measured in chloroform solution using a 1 dm tube.

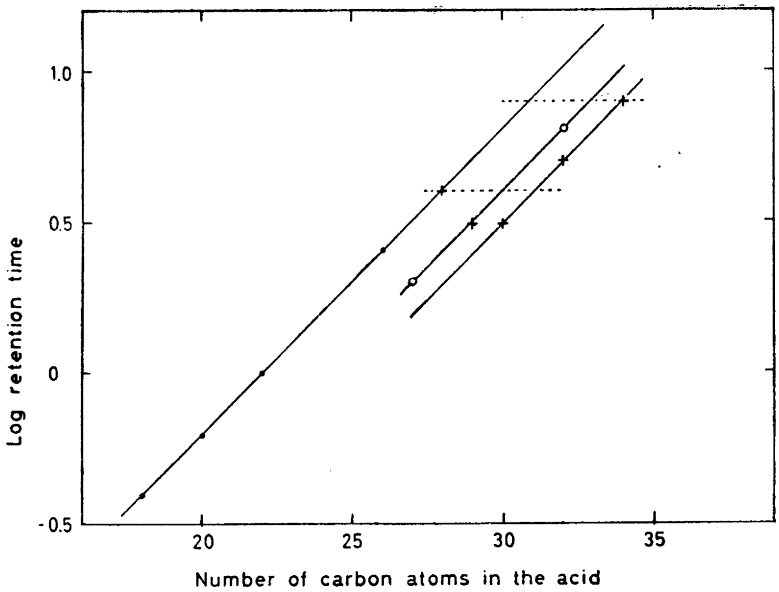
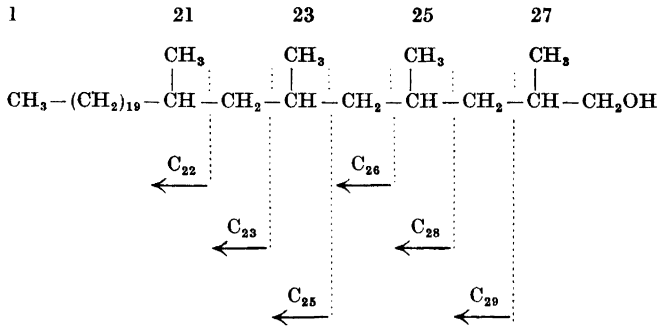


Fig. 1. The relations between the logarithm of the retention time and the number of carbon atoms in the acid in the gas chromatography of the methyl esters on silicone at 260°. ●, Methyl esters of normal chain acids; O, methyl esters of 2,4,6-trimethyl-substituted acids; +, fractions from methyl mycocerosate. Dotted horizontal lines are drawn through points corresponding to fractions of unknown molecular weight.

in a yield of 52 %. The reduction of the oxo-group was carried out by the desulphurization of the ethylene mercaptal [11]. (+)-Methyl 3D,5D-dimethyloctacosanoate, m. p. 41.2—42.0°, $[\alpha]_D^{25}$ +1.5° (c = 6.0), was obtained in a yield of 95 %. Saponification gave the free acid, m. p. 59.9—60.2°.

Hunsdiecker degradation [12] of the silver salt of this acid gave (—)-1-bromo-2D,4D-dimethyl-heptacosane, m. p. 40—41°, $[\alpha]_D^{21}$ —0.5° (c = 5.8), in a yield of 81 %. The bromide was converted into the iodide by means of sodium iodide in acetone. The reaction between the iodide and the sodium derivative of diethyl methylmalonate, followed by saponification and decarboxylation, gave a mixture of 2D, 4D,6D- and 2L,4D,6D-trimethylnonacosanoic acids in a yield of 72 %. The methyl esters were prepared by means of diazomethane, and the two stereo-isomers separated by means of chromatography on magnesium trisilicate. The separation was followed by measuring the optical rotation. Repeated chromatography finally yielded a levo-rotatory fraction, the rotation of which was unchanged on further chromatography. (—)-Methyl 2D,4D,6D-trimethylnonacosanoate thus obtained had m. p. 37.9—38.2°, $[\alpha]_D^{20}$ —7.2° (c = 5.5).

The melting point of the synthetic ester was about 17° higher than that of methyl mycocerosate (m.p. 20.8—21.2° [1]), and the mass spectra, although very similar, were not identical. It therefore became necessary to reinvestigate the natural product. Gas chromatography on silicone at a temperature of 260° showed the presence of two major and four minor components. The former (designated A and B in the following) had shorter retention times (10.2 and 16.9 min, respectively) than the methyl ester of the synthetic 2,4,6-trimethyl-substituted C_{32}-acid (21.3 min). The mass spectrometric examination of the isolated fractions A and B showed that A was a mixture of two compounds with molecular weights of 452 and 466, respectively. Fraction B (65 % of the total) appeared to consist of a single compound of molecular weight 494. In the diagram of Fig. 1



the point for the compound of molecular weight 452 falls on the same line as those for the methyl esters of the C_{27} and C_{32}-2,4,6-trimethyl-substituted acids. The positions of the compounds of molecular weights 466 and 494 in the diagram indicate that they possess still more branched structures. The mass spectrum of fraction B (M = 494) showed high peaks at m/e = 88 and 101, and very small peaks at m/e = 74, 115, and 157, which indicates methyl side chains at positions 2, 4, and 6. The mass spectrum of the ester suggested the presence of a methyl group also at position 8, but the relevant peaks were small. A comparison between the mass spectra given by the alcohol derived from synthetic methyl 2,4,6-trimethylnonacosanoate and that derived from fraction B provided conclusive evidence, however, that the main component (B) of methyl mycocerosate is the methyl ester of a 2,4,6,8-tetramethyloctacosanoic acid. The mass spectrum of 2,4,6-trimethylnonacosanol-1 shows a series of marked peaks due to hydrocarbon fragments formed by breaking the bonds to the tertiary carbon atoms. The C_{25}, C_{26}, C_{28}, and C_{29} groups of peaks are thus marked, whereas the C_{24}, C_{27}, and C_{30} peaks, which can only arise from fragments formed by simultaneous cleavage of two bonds, are of low height. In the alcohol derived from fraction B, the C_{22}, C_{23}, C_{25}, C_{26}, C_{28}, and C_{29} groups are marked, and the C_{21}, C_{24}, C_{27}, and C_{30} groups of peaks are low. The observed peak height distribution indicates the presence of methyl side chains at positions 21,23,25, and 27 from the hydrocarbon end of the molecule.

The alcohols were prepared on the milligram scale by reduction of the methyl esters by means of lithium aluminium hydride [13]. The alcohol (crude product) from fraction B had m.p. $\sim$ 35°, and alcohol derived from a partially separated mixture of the two stereo-isomeric methyl 2,4,6-trimethylnonacosanoates melted at 53°.

Components corresponding to fractions A and B are present also in the methyl esters derived from the *Test* and the *Canetti* strains of the tubercle bacilli. Our results indicate the occurrence of normal chain acids with 22, 24, 26, and 28 carbon atoms, 2,4,6-trimethyl-substituted acids with 25, 27, and 29 carbon atoms, and 2,4,6,8-tetramethyl-substituted acids with 30, 32, and 34 carbon atoms in the strains studied.

Full details of this work will be published later.

1. Asselineau, J., Ryhage, R. and Stenhagen, E. *Acta Chem. Scand.* **11** (1957) 196.
2. Anderson, R. J. *J. Biol. Chem.* **97** (1932) 639.
3. Wieghard, C. W. and Anderson, R. J. *J. Biol. Chem.* **126** (1938) 505.
4. Ginger, L. G. and Anderson, R. J. *J. Biol. Chem.* **157** (1945) 203.
5. Polgar, N. *J. Chem. Soc.* **1954** 1011.
6. Chanley, J. D. and Polgar, N. *J. Chem. Soc.* **1954** 1003.
7. Asselineau, J. *Compt. rend.* **237** (1953) 1804.
8. Org. Synth. **34** 13.
9. Ahlquist, L., Asselineau, J., Asselineau, C., Serck-Hanssen, K., Ställberg-Stenhagen, S. and Stenhagen, E. *Arkiv Kemi. In press.*
10. Bowman, R. E. and Fordham, W. D. *J. Chem. Soc.* **1952** 3945.
11. Wolfrom, M. L. and Karabinos, J. V. *J. Am. Chem. Soc.* **66** (1944) 909.
12. Hunsdiecker, H. and Hunsdiecker, C. *Ber.* **75** (1942) 291.
13. Nystrom, R. F. and Brown, W. G. *J. Am. Chem. Soc.* **69** (1947) 2548.

Received March 3, 1959.

On the Accuracy of Moving-Bomb Calorimetry

III. The Accuracy of Heat of Combustion Data of Sulfur Compounds

STIG SUNNER

Thermochemistry Laboratory, University of Lund, Lund, Sweden*

The method of moving-bomb calorimetry has been shown to give highly reproducible results for sulfur compounds [1]. It has been more difficult, however, to assess the absolute accuracy of the bomb calorimetric data since there may be systematic errors present that defy detection. A reliable check can only be ascertained by a comparison of results obtained with the bomb calorimetric method with those determined in an independent way.

Hubbard and Waddington [2] compared the heat of formation value of propanethiol-2, obtained from heat of combustion experiments with that derived from equilibrium measurements. They found an excellent agreement, indicating the absence of systematic errors in the moving-bomb technique.

Sunner and Wadsö published a check based purely on thermochemical measurements [3]. They took advantage of the fact that thiolacetic acid can be quantitatively hydrolyzed to acetic acid. The heat of hydrolysis was determined as well as appropriate heats of solution and dilution of reactants and reaction products. Thus, the enthalpy change accompanying the idealized reaction

$$CH_3{\cdot}COSH(l) + H_2O(l) \rightarrow CH_3{\cdot}COOH(l) + H_2S(g)$$

was determined to -0.64 ± 0.07 kcal.mole^{-1}.

By combining this value with heat of formation data of water, acetic acid and hydrogen sulfide, a value for the heat of formation of thiolacetic acid could be derived and compared with that obtained from combustion experiments [4]. Unfortunately, the heat of formation of acetic acid was not unambiguously known at the time when Ref.[3] was published. A precise check of the moving-bomb technique by this method had therefore to await an accurate redetermination of the heat of combustion of acetic acid.

Recently, Evans and Skinner have determined the heat of combustion of acetic acid accurately [5]. They calculated therefrom the heat of formation of acetic acid to $\Delta Hf^\circ_{298.16} = -115.72 \pm 0.1$ kcal.mole^{-1}. Using this value, the well-known heat of formation data of water and hydrogen sulfide and the heat of hydrolysis value given above, the heat of formation of thiolacetic acid is found to be

$$\Delta Hf^\circ_{298.16} = -51.58 \pm 0.1 \text{ kcal.mole}^{-1}.$$

This datum has to be compared with the heat of formation of thiolacetic acid obtained from combustion calorimetry:

$$\Delta Hf^\circ_{298.16} = -51.5 \pm 0.2 \text{ * kcal.mole}^{-1}.$$

The close agreement between the two results is additional strong evidence to support the contention that the moving-bomb calorimetric method as used for sulfur compounds does not involve significant, systematic errors.

Acknowledgement. The spontaneous co-operation of Drs Skinner and Evans is gratefully appreciated.

1. Waddington, G., Sunner, S. and Hubbard, W. N. *Combustion in a Bomb of Organic Sulfur Compounds.* Chapter 7 in *Experimental Thermochemistry*, Interscience Publishers, Inc., New York 1956.
2. Hubbard, W. N. and Waddington, G. *Rec. trav. chim.* **73** (1954) 910.
3. Sunner, S. and Wadsö, I. *Trans. Faraday Soc.* **53** (1957) 455.
4. Sunner, S. *Acta Chem. Scand.* **9** (1955) 847.
5. Evans, F. W. and Skinner, H. A. *Trans. Faraday Soc.* **55** (1959) 260.

* Sponsored by *The Swedish Natural Science Research Council* and by *The Swedish Technical Research Council.*

* The overall standard deviation of the mean.

Received March 11, 1959.

Oxidation by Partially Hydrolyzed Polysaccharides

KALE JUVA and EINO KULONEN

Department of Medical Chemistry, University of Turku, Finland

When certain polysaccharides are hydrolyzed partially with hydrochloric acid in sealed tubes compounds are formed which catalyze the oxidation of many aromatic substances (*e.g.* phenol, *p*-phenylenediamine), especially in the presence of hydrogen peroxide [1]. The chemical nature of the catalysts is unknown, but they are acidic and contain a carbonylic group [2]. Hydrogen peroxide degrades hyaluronic acid [3] in the presence of ascorbic acid but no catalyst is formed after this "oxidative depolymerization" (see below). The oxidizing ability may contribute in the production of humins and other artefacts in hydrolyzates. For example, an increased oxidation of methionine in acid hydrolysis in the presence of carbohydrates has been described [4].

For *testing* equal volumes of the solutions in question, 1.5 % hydrogen peroxide and ammoniacal water-saturated phenol or fresh aqueous 1 % *p*-phenylenediamine (Merck) were mixed in test tubes. Controls were run with water instead of hydrolyzate. The results were recorded after 1 h at room temperature, either visually or with a spectrophotometer at 500 mμ.

The *partial hydrolyzate* was made with 2 N HCl (0.33 % of polysaccharide) keeping the sealed tubes overnight at 100°C. The acid was removed by repeated evaporations on the water bath. The catalyzing agent was dialysable. The catalytic activity is dependent on the degree of polymerization.

Ascorbic acid did not influence the reaction in this system, even when added 2 h before the *p*-phenylenediamine.

The *hyaluronate* complex used in the present investigation was prepared from umbilical cords by 1 % NaCl extraction, precipitation with "Cetavlon", solution in 1.25 M sodium chloride and fractional precipitation by lowering the salt concentration below 0.3 M. Cetavlon was removed with Fuller's earth and the solution lyophilized [5]. Before the lyophilization the fluid contained dry matter 2.6 mg/ml, uronic acid (Dische) 1.07 mg/ml, glucosamine (Blix) 0.45 mg/ml, and protein 0.62 mg/ml.

The *depolymerization with ascorbic acid* was performed in 0.2 % solution of hyaluronate, buffered to pH 6.8 with 0.0067 M phosphate and containing 0.8 % NaCl. After addition of 0.020 % H_2O_2 no change occurred, but after further addition of 0.050 % ascorbic acid the relative viscosity fell in 20 min to a tenth ($\eta_{rel} = 1.5$). The fluid was dialyzed against tap water and lyophilized. The partial hydrolyzate from this degraded preparation had no catalytic activity.

Purification of the catalyst was carried out from 82.2 mg of HCl-treated material using a cellulose powder (Whatman) column (28 × 2 cm) and butanol (almost saturated with water):acetic acid (19:1) as the solvent [6]. Five ml fractions were collected and screened with spot tests. Fractions No. 20—30 and 170—185 were positive with aniline phthalate, but with ammoniacal phenol brown spots were obtained

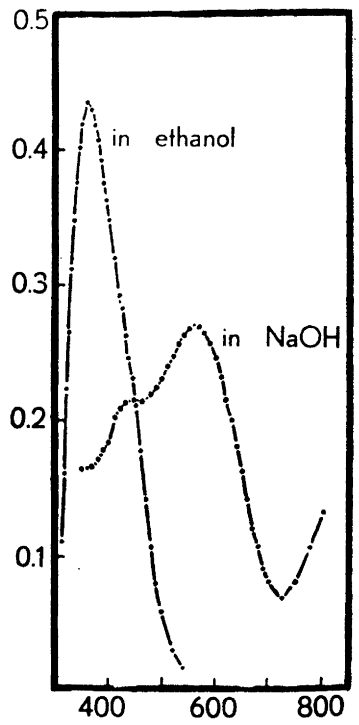


Fig. 1. The absorption spectra of the 2,4-dinitrophenylderivative of the isolated preparation. Abscissa: wavelength in mμ, ordinate: extinction.

only from fractions 30—50. They were combined and evaporated.

The residue weighed 5.6 mg and was hydrolyzed in 0.56 ml N sulfuric acid for 5 h at 100°C. The digest was neutralized with $Ba(OH)_2$ to pH 5.8. The supernatant was evaporated, the residue dissolved in 0.05 ml of water and chromatographed with butanol:pyridine:water (5:3:2). No distinct carbohydrate spots were obtained with aniline phthalate, but with ninhydrin several spots, one coinciding with that of glucosamine. The test with aniline for furfurol [7] was negative. 2,4-Dinitrophenylhydrazine (dissolved into 2 N H_2SO_4) gave a precipitate, which was collected and submitted to spectroscopic examination (Fig. 1). The sharp change to violet in alkali seems worthy of mention.

Financial support from *Sigrid Jusélius Foundation* is gratefully acknowledged.

1. Kulonen, E. *Suomen Kemistilehti B* **25** (1952) 19.
2. Kulonen, E. *Ibid.* **28** (1955) 103.
3. Skanse, B. and Sundblad, L. *Acta Physiol. Scand.* **6** (1943) 37.
4. Osono, K., Mukoi, I. and Tominaga, F. *Nagasaki Igakkai Zassi* **30** (1955) 156; *Chem. Abstracts* **49** (1955) 8344.
5. Scott, J. E. *Biochem. J.* **62** (1956) 31P.
6. Hirst, E. L., Hough, L. and Jones, J. K. V. *J. Chem. Soc.* **1959** 3145.
7. Feigl, F. *Spot Tests* II, 4th Ed. Elsevier, Amsterdam, 1954.

Received March 13, 1959.

Studies on Ester Sulfates

1. Application of Two-Dimensional Paper Chromatography in Studies on the Biosynthesis of Ester Sulfates

ANDERS VESTERMARK and HARRY BOSTRÖM

Department of Metabolic Research, the Wenner-Gren Institute of Experimental Biology, Stockholm, Sweden

In several recent biochemical studies on ester sulfate compounds, great advantage has been taken of the selective incorporation of ^{35}S-sulfate in these compounds. Mainly on the grounds of their high sensitivity, different isotope methods, such as autoradiography of electropherograms on body fluids and tissue extracts, have been used successfully in this field.

A somewhat more detailed picture of the sulfatation of different compounds in various *in vitro* systems could be expected to result from the use of two-dimensional paper chromatography combined with autoradiography. For this reason, a screening study along these lines was undertaken. Some observations made during this work will be briefly reported in the following.

In one type of experiment, 2-mg slices of liver from albino rats were incubated in small test tubes in 100 µl of Krebs-Ringer bicarbonate solution and 10 µl of a solution of ^{35}S-labeled sulfate containing 10 mc/ml. A gas mixture consisting of 93.5 % of oxygen and 6.5 % of carbon dioxide was insufflated into each test tube during incubation.

In another type of experiment, 40 µl of a cell-free supernatant of liver homogenate prepared according to Roy [1] was mixed with 50 µl of a buffer solution, containing equal parts of 0.3 M KH_2PO_4/K_2HPO_4 buffer, pH 6.8, 0.03 M K_2SO_4 and 0.005 M $MgCl_2$. This mixture was combined with 10 µl of 0.04 M ATP and 10 µl of the ^{35}S-labeled sodium sulfate solution.

When these types of sulfatation system were used for studies on sulfatation of different substances, 10 µl of a 10 mM solution of the substance in alcohol or water was added to an empty test tube. The solvent was evaporated *in vacuo*. The slices or supernatant systems were added to this tube.

In each series of experiments, one tube with 10 µl of water and another with 10 µl of alcohol, to which the relevant sulfatation system was added, were run as controls.

In all experiments, 5 µl-samples of the medium were taken 45 and 120 min after starting incubation, for two-dimensional ascending chromatography according to Datta, Dent and Harris [2]. Solvent I: Phenol-water (400 g + 100 g). A beaker containing concentrated H_3N was placed in the chromatographic tank. Solvent II: Butanol — 2 N H_3N (250 ml + 250 ml).

After drying the papers in a stream of hot air from an electric hair-drying fan, the chromatograms were subjected to autoradiography on Gevaert Curix X-ray film, exposed for one week in a wooden screw press and then developed with Gevaert G 150.

only from fractions 30—50. They were combined and evaporated.

The residue weighed 5.6 mg and was hydrolyzed in 0.56 ml N sulfuric acid for 5 h at 100°C. The digest was neutralized with $Ba(OH)_2$ to pH 5.8. The supernatant was evaporated, the residue dissolved in 0.05 ml of water and chromatographed with butanol:pyridine:water (5:3:2). No distinct carbohydrate spots were obtained with aniline phthalate, but with ninhydrin several spots, one coinciding with that of glucosamine. The test with aniline for furfurol [7] was negative. 2,4-Dinitrophenylhydrazine (dissolved into 2 N H_2SO_4) gave a precipitate, which was collected and submitted to spectroscopic examination (Fig. 1). The sharp change to violet in alkali seems worthy of mention.

Financial support from *Sigrid Jusélius Foundation* is gratefully acknowledged.

1. Kulonen, E. *Suomen Kemistilehti B* **25** (1952) 19.
2. Kulonen, E. *Ibid.* **28** (1955) 103.
3. Skanse, B. and Sundblad, L. *Acta Physiol. Scand.* **6** (1943) 37.
4. Osono, K., Mukoi, I. and Tominaga, F. *Nagasaki Igakkai Zassi* **30** (1955) 156; *Chem. Abstracts* **49** (1955) 8344.
5. Scott, J. E. *Biochem. J.* **62** (1956) 31P.
6. Hirst, E. L., Hough, L. and Jones, J. K. V. *J. Chem. Soc.* **1959** 3145.
7. Feigl, F. *Spot Tests* II, 4th Ed. Elsevier, Amsterdam, 1954.

Received March 13, 1959.

Studies on Ester Sulfates

1. Application of Two-Dimensional Paper Chromatography in Studies on the Biosynthesis of Ester Sulfates

ANDERS VESTERMARK and HARRY BOSTRÖM

Department of Metabolic Research, the Wenner-Gren Institute of Experimental Biology, Stockholm, Sweden

In several recent biochemical studies on ester sulfate compounds, great advantage has been taken of the selective incorporation of ^{35}S-sulfate in these compounds. Mainly on the grounds of their high sensitivity, different isotope methods, such as autoradiography of electropherograms on body fluids and tissue extracts, have been used successfully in this field.

A somewhat more detailed picture of the sulfatation of different compounds in various *in vitro* systems could be expected to result from the use of two-dimensional paper chromatography combined with autoradiography. For this reason, a screening study along these lines was undertaken. Some observations made during this work will be briefly reported in the following.

In one type of experiment, 2-mg slices of liver from albino rats were incubated in small test tubes in 100 µl of Krebs-Ringer bicarbonate solution and 10 µl of a solution of ^{35}S-labeled sulfate containing 10 mc/ml. A gas mixture consisting of 93.5 % of oxygen and 6.5 % of carbon dioxide was insufflated into each test tube during incubation.

In another type of experiment, 40 µl of a cell-free supernatant of liver homogenate prepared according to Roy [1] was mixed with 50 µl of a buffer solution, containing equal parts of 0.3 M KH_2PO_4/K_2HPO_4 buffer, pH 6.8, 0.03 M K_2SO_4 and 0.005 M $MgCl_2$. This mixture was combined with 10 µl of 0.04 M ATP and 10 µl of the ^{35}S-labeled sodium sulfate solution.

When these types of sulfatation system were used for studies on sulfatation of different substances, 10 µl of a 10 mM solution of the substance in alcohol or water was added to an empty test tube. The solvent was evaporated *in vacuo*. The slices or supernatant systems were added to this tube.

In each series of experiments, one tube with 10 µl of water and another with 10 µl of alcohol, to which the relevant sulfatation system was added, were run as controls.

In all experiments, 5 µl-samples of the medium were taken 45 and 120 min after starting incubation, for two-dimensional ascending chromatography according to Datta, Dent and Harris [2]. Solvent I: Phenol-water (400 g + 100 g). A beaker containing concentrated H_3N was placed in the chromatographic tank. Solvent II: Butanol — 2 N H_3N (250 ml + 250 ml).

After drying the papers in a stream of hot air from an electric hair-drying fan, the chromatograms were subjected to autoradiography on Gevaert Curix X-ray film, exposed for one week in a wooden screw press and then developed with Gevaert G 150.

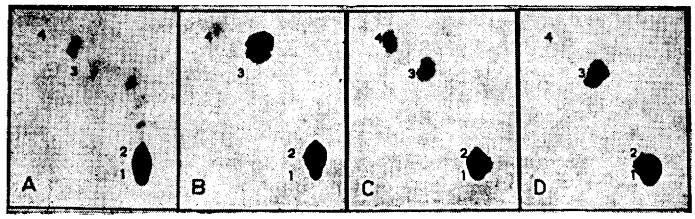


Fig. 1. Chromato-autoradiogram on 5-μl samples of the incubation mixtures after 2 h incubation. A. Slices system (see text) + 10 μl of water. B. Slices system + 10 μl of ethanol. C. Cell-free system + 10 μl of water. D. Cell-free system + 10 μl of ethanol.

Paper electrophoresis (0.075 M sodium acetate-acetic acid buffer, pH 5.5, 30 V/cm, 1 h) was also performed on 5-μl samples of the incubation medium.

In the control experiments with the slices system (Fig. 1, chromatograms A and B), the following basic pattern was obtained. In addition to a spot, No. 1, obtained where the sample was placed on the paper, and the inorganic sulfate spot, No. 2, two other spots, Nos. 3 and 4, were consistently present. The control with water (A) showed a weak No. 3 spot and a distinct No. 4. The control with ethanol (B) showed the reverse picture, a weak No. 4 and a very strong No. 3.

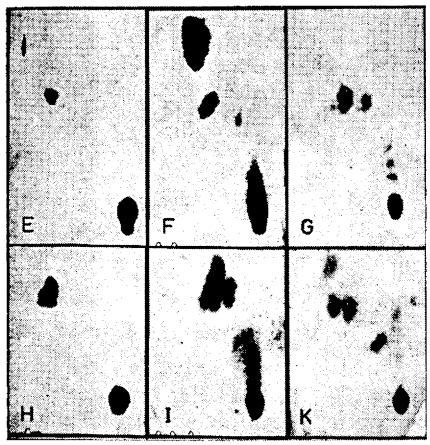


Fig. 2. Ester sulfate pattern of the incubation media after addition to the slices system of androsterone (E), α-naphthylamine (G) and β-naphthylamine (I) as compared to the corresponding pattern obtained with the same substances in the cell-free system (F, H, K), respectively.

In the supernatant system as well, the presence of small amounts of ethanol (D) resulted in increased strength of No. 3 and a decrease in concentration of No. 4, as compared to the water controls (C).

Addition of amines, steroids or phenols to either of the sulfatation systems used was generally followed by the appearance of one or several new spots in the chromatograms, as exemplified in Figs. 2 and 3 (chromatograms E—S).

Thus, both in the slices system (Fig. 2 E) and in the cell-free medium (F), one new distinct spot appeared when androsterone was added to the incubation mixtures. In similar studies, ester sulfates of several other steroids as well have been obtained [3].

Of the two isomeric amines tested, α-naphthylamine gave one new single spot in the cell-free medium (G), whereas at least two ester sulfates were formed in the presence of liver slices. More complex patterns were obtained in both systems with β-naphthylamine.

A series of new unidentified ester sulfates (Fig. 3) was also obtained when adrenaline (L) or noradrenaline (N) was added to the slices. Somewhat less complicated patterns were produced by these substances in the cell-free system (M and O).

A marked difference between the two systems was also observed in the presence of thymol, which gave two well-marked ester sulfates in the slices system (P), but only one in the cell-free medium (Q).

No evidence was obtained of the formation of tyrosine-O-sulfate, known to occur in human urine [4], in either of the sulfatation systems used (R and S).

When high voltage electrophoresis was applied to samples of all the incubation media mentioned above, separation of some of the

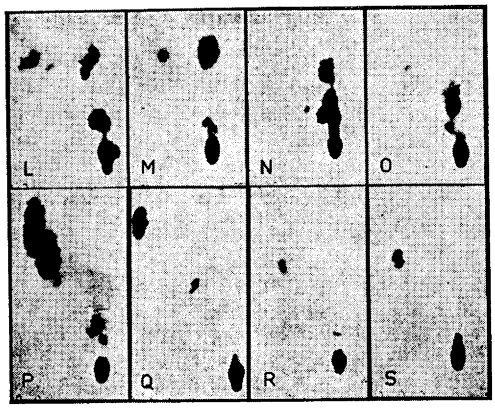


Fig. 3. Ester sulfate pattern found in the presence of adrenaline (L), noradrenaline (N), thymol (P) and tyrosine (R), in the slices system and the cell-free system (M, O, Q and S), respectively.

Fig. 4. Formation of different ^{35}S-labeled compounds *in vitro* in a cell-free or a slices system of rat liver, in the presence of various possible sulfate acceptors, as studied by high voltage electrophoresis and autoradiography. The black zone at the top of the picture corresponds to the position of inorganic sulfate. The various experiments are marked as in Figs. 1—3.

labeled compounds was also obtained. Thus, in the basic liver supernatant pattern, three distinct spots (C) were visualized, as compared to only two in the chromatographic system (Fig. 1 C). In the presence of ethanol (D), a shift occurred, consisting of a decrease in concentration of the slowest moving fraction, and an increase in the fastest moving compound. A similar phenomenon was observed in the slices system (A and B).

When adrenaline was present in the supernatant system (M), one radioactive spot, probably corresponding to a monosulfate of adrenaline, moved very slowly. Other compounds, which might have been derivatives of adrenaline containing two or three sulfate groups, moved faster. In the slices system (L), the slow moving spot was found, in addition to two other ^{35}S-labeled compounds.

With noradrenaline as well, a slow moving compound was synthesized in both systems (O:cell-free, N:slices). A few other labeled compounds were also recognized in these cases.

The results of this screening study on the ability of a system of liver slices, or of a cell-free supernatant of liver homogenates, to activate sulfate groups and transfer them to different types of acceptors are in good agreement with those reported by other authors on phenols [5-7], steroids [1,8,9] and amines [10].

Features of special interest in our study were the complicated ester sulfate pattern found in the presence of catechol amines, the failure of sulfatation of free tyrosine in either of the systems, and the interesting shift in the basic ester sulfate pattern in both systems in the presence of small amounts of ethanol (Fig. 1).

The particular value of the chromato-autoradiographic method used in the present study seems to be that a good overall picture of the occurrence of different types of transferring enzymes and sulfate acceptors in different sulfatation systems is obtained in small-scale experiments.

This study was supported by research grants from *The National Institutes of Health*, Bethesda, Md (A-2568 M & N), *The Swedish Medical Research Council* and *The Helen Hay Whitney Foundation*, New York.

1. Roy, A. B. *Biochem. J.* **63** (1956) 294.
2. Datta, S. P., Dent, C. E. and Harris, H. *Science* **112** (1950) 621.
3. Boström, H. and Vestermark, A. *Unpublished observations.*
4. Tallan, H. H., Bella, S. T., Stein, W. H. and Moore, S. *J. Biol. Chem.* **217** (1955) 703.
5. Arnolt, R. I. and De Meio, R. H. *Rev. soc. argentina biol.* **17** (1941) 570.
6. De Meio, R. H. and Tkacz, L. *Arch. Biochem.* **27** (1950) 242.
7. Gregory, J. D. and Lipmann, F. *J. Biol. Chem.* **229** (1957) 1081.
8. De Meio, R. H. and Lewycka, C. *Endocrinology* **56** (1955) 489.
9. Schneider, J. J. and Lewbart, M. L. *J. Biol. Chem.* **222** (1956) 787.
10. Roy, A. B. *Biochem. et Biophys. Acta* **30** (1958) 193.

Received March 14, 1959.

Mass Transfer by Forced Laminar Convection at Plane Plate Electrodes

GÖSTA WRANGLÉN

Division of Applied Electrochemistry, Royal Institute of Technology, Stockholm 70, Sweden

The investigation of the influence of liquid flow on electrode processes is of great interest for industrial electrolysis and for the theory of metallic corrosion in flowing liquids. Heat and mass transfer at a plane plate in longitudinal flow under laminar conditions have been treated by various authors [1-3] under the assumption of a uniform surface temperature and concentration, respectively. As far as electrolysis is concerned, this postulate probably applies only for limiting current conditions at the electrodes. In normal electrolysis with a uniform current density, the actual boundary condition is a uniform concentration gradient at the electrode surface. This case or the corresponding heat transfer problem does not seem to have been considered earlier.

The notation used in an earlier communication [4] is employed, except that x is taken to mean the distance in the flow direction from the leading edge of the electrodes and u denotes the flow velocity in the x-direction in the boundary layer. Furthermore, l is the length of the electrode in the flow direction and $\mathrm{Re}_x = \frac{u_b x}{\nu}$ is the local Reynolds number. The relevant boundary layer equations in integrated form are

labeled compounds was also obtained. Thus, in the basic liver supernatant pattern, three distinct spots (C) were visualized, as compared to only two in the chromatographic system (Fig. 1 C). In the presence of ethanol (D), a shift occurred, consisting of a decrease in concentration of the slowest moving fraction, and an increase in the fastest moving compound. A similar phenomenon was observed in the slices system (A and B).

When adrenaline was present in the supernatant system (M), one radioactive spot, probably corresponding to a monosulfate of adrenaline, moved very slowly. Other compounds, which might have been derivatives of adrenaline containing two or three sulfate groups, moved faster. In the slices system (L), the slow moving spot was found, in addition to two other ^{35}S-labeled compounds.

With noradrenaline as well, a slow moving compound was synthesized in both systems (O:cell-free, N:slices). A few other labeled compounds were also recognized in these cases.

The results of this screening study on the ability of a system of liver slices, or of a cell-free supernatant of liver homogenates, to activate sulfate groups and transfer them to different types of acceptors are in good agreement with those reported by other authors on phenols [5-7], steroids [1,8,9] and amines [10].

Features of special interest in our study were the complicated ester sulfate pattern found in the presence of catechol amines, the failure of sulfatation of free tyrosine in either of the systems, and the interesting shift in the basic ester sulfate pattern in both systems in the presence of small amounts of ethanol (Fig. 1).

The particular value of the chromato-autoradiographic method used in the present study seems to be that a good overall picture of the occurrence of different types of transferring enzymes and sulfate acceptors in different sulfatation systems is obtained in small-scale experiments.

This study was supported by research grants from *The National Institutes of Health*, Bethesda, Md (A-2568 M & N), *The Swedish Medical Research Council* and *The Helen Hay Whitney Foundation*, New York.

1. Roy, A. B. *Biochem. J.* **63** (1956) 294.
2. Datta, S. P., Dent, C. E. and Harris, H. *Science* **112** (1950) 621.
3. Boström, H. and Vestermark, A. *Unpublished observations.*
4. Tallan, H. H., Bella, S. T., Stein, W. H. and Moore, S. *J. Biol. Chem.* **217** (1955) 703.
5. Arnolt, R. I. and De Meio, R. H. *Rev. soc. argentina biol.* **17** (1941) 570.
6. De Meio, R. H. and Tkacz, L. *Arch. Biochem.* **27** (1950) 242.
7. Gregory, J. D. and Lipmann, F. *J. Biol. Chem.* **229** (1957) 1081.
8. De Meio, R. H. and Lewycka, C. *Endocrinology* **56** (1955) 489.
9. Schneider, J. J. and Lewbart, M. L. *J. Biol. Chem.* **222** (1956) 787.
10. Roy, A. B. *Biochem. et Biophys. Acta* **30** (1958) 193.

Received March 14, 1959.

Mass Transfer by Forced Laminar Convection at Plane Plate Electrodes

GÖSTA WRANGLÉN

Division of Applied Electrochemistry, Royal Institute of Technology, Stockholm 70, Sweden

The investigation of the influence of liquid flow on electrode processes is of great interest for industrial electrolysis and for the theory of metallic corrosion in flowing liquids. Heat and mass transfer at a plane plate in longitudinal flow under laminar conditions have been treated by various authors [1-3] under the assumption of a uniform surface temperature and concentration, respectively. As far as electrolysis is concerned, this postulate probably applies only for limiting current conditions at the electrodes. In normal electrolysis with a uniform current density, the actual boundary condition is a uniform concentration gradient at the electrode surface. This case or the corresponding heat transfer problem does not seem to have been considered earlier.

The notation used in an earlier communication [4] is employed, except that x is taken to mean the distance in the flow direction from the leading edge of the electrodes and u denotes the flow velocity in the x-direction in the boundary layer. Furthermore, l is the length of the electrode in the flow direction and $\mathrm{Re}_x = \frac{u_b x}{\nu}$ is the local Reynolds number. The relevant boundary layer equations in integrated form are

$$\frac{d}{dx}\int_0^{\delta_h} u(u_b-u)\mathrm{d}y = \nu\left(\frac{\mathrm{d}u}{\mathrm{d}y}\right)_{y=0} \quad (1)$$

$$\frac{\mathrm{d}}{\mathrm{d}x}\int_0^{\delta_d} u/c_b - c/\mathrm{d}y = j \quad (2)$$

with the boundary conditions

$$u = 0,\ \frac{dc}{\mathrm{d}y} = \frac{j}{D} \text{ for } y = 0$$

$$u = u_b \text{ for } y \geq \delta_h,\ c = c_b \text{ for } y \geq \delta_d$$

For the velocity profile we assume [1]

$$\frac{u}{u_b} = \frac{3}{2}\frac{y}{\delta_h} - \frac{1}{2}\frac{y^3}{\delta_h^3} \quad (3)$$

and obtain from (1)

$$\delta_h = \left(\frac{280\ \nu\ x}{13\ u_b}\right)^{\frac{1}{2}} \quad (4)$$

The concentration profile is approximated by the equation

$$/c_b - c/ = \frac{2}{3}\frac{j\delta_d}{D}\left[1 - \frac{3}{2}\frac{y}{\delta_d} + \frac{1}{2}\frac{y^3}{\delta_d^3}\right] \quad (5)$$

With $\frac{\delta_d}{\delta} = r$ and considering that $r < 1$ for high Schmidt numbers one obtains

$$\int_0^{\delta_d} u/c_b - c/\mathrm{d}y = \frac{u_b j}{10D}\, r^3\ \delta_h^2 \quad (6)$$

and hence

$$\frac{u_b}{10D}\frac{\mathrm{d}}{\mathrm{d}x}\,(r^3\,\delta_h^2) = 1 \quad (7)$$

Although δ_h and δ_d are functions of x, r may be considered to be independent of x. Hence, using eqn. (4)

$$r^3 = \frac{13}{28}\frac{D}{\nu} \quad (8)$$

Finally, we obtain for *uniform current density:*

$$= 0.774\ \mathrm{Sc}^{-1/3},\ \delta_d = 3.59\ D^{1/3}\,\nu^{1/6}\left(\frac{x}{u_b}\right)^{\frac{1}{2}},\ \delta_\mathrm{N} = \frac{2}{3}\,\delta_d$$

$$\Delta c_x = 2.39\ j\ D^{-2/3}\,\nu^{1/6}\left(\frac{x}{u_b}\right)^{\frac{1}{2}},\ \Delta c_\mathrm{av} = \frac{2}{3}\,\Delta c_l$$

$$\mathrm{Nu}_x = 0.417\ \mathrm{Re}_x^{\frac{1}{2}}\,\mathrm{Sc}^{1/3},\ \mathrm{Nu}_\mathrm{av} = 0.625\ \mathrm{Re}_l^{\frac{1}{2}}\,\mathrm{Sc}^{1/3}$$

An analogous treatment for *limiting current density* gives:

$$r = 0.976\ \mathrm{Sc}^{-1/3},\ \delta_d = 4.53\ D^{1/3}\,\nu^{1/6}\left(\frac{x}{u_b}\right)^{\frac{1}{2}},\ \delta_\mathrm{N} = \frac{2}{3}\,\delta_d$$

$$i_{\mathrm{lim},x} = 0.331\ \frac{zF}{1-t}\,\Delta c\ D^{2/3}\ \nu^{-1/6}\left(\frac{u_b}{x}\right)^{\frac{1}{2}},\ i_{\mathrm{lim,av}} = 2\,i_{\mathrm{lim},l} \quad (2)$$

$$\mathrm{Nu}_x = 0.331\ \mathrm{Re}_x^{\frac{1}{2}}\ \mathrm{Sc}^{1/3},\ \mathrm{Nu}_\mathrm{av} = 0.662\ \mathrm{Re}_l^{\frac{1}{2}}\ \mathrm{Sc}^{1/3}$$

According to an exact numerical solution [3], the coefficient for $i_{\mathrm{lim},x}$ and Nu_x is 0.332.

In both cases, one obtains for the concentration difference of an inert conducting electrolyte, such as sulphuric acid in copper refining,

$$/\Delta c^1/ = \frac{t^1}{1-t}\left(\frac{D}{D^1}\right)^{2/3} /\Delta c/ \quad (9)$$

where t^1 and D^1 are the transference number and diffusion coefficient of the inert electrolyte.

Forced laminar flow without disturbing free convection is probably best realized with bipolar horizontal electrodes, which act as anodes on the upper side and as cathodes on the under side. Vertical plate electrodes are, however, of greater practical interest in view of the attempts that are being made to develop processes for the electrodeposition of metals at high current densities in a flowing electrolyte [5]. In this latter case, free convection cannot be neglected, except at very high velocities for the forced flow.

1. Eckert, E. R. G. *Introduction to the Transfer of Heat and Mass*, New York 1950, p. 88.
2. Tobias, C. W., Eisenberg, M. and Wilke, C. R. *J. Electrochem. Soc.* **99** (1952) 359 C.
3. Schlichting, H. *Grenzschicht-Theorie*, Karlsruhe 1958, p. 289.
4. Wranglén, G. *Acta Chem. Scand.* **12** (1958) 1143.
5. Walldén, S. J., Henriksson, S. T., Arbstedt, P. G. and Miöen, Th. *Trans. AIME. In press.*

Received March 19, 1959.

A Surface-balance Study of the Structural Relationships between Cryptopimaric, Dextropimaric, and *Iso*dextropimaric Acids

HENRIK H. BRUUN

Institute of Wood Chemistry, Åbo Akademi, Åbo, Finland
and

EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala, Sweden

In a surface-balance study of monomolecular layers of rosin acids carried out by one of us in 1953 [1], it was found that in a compressed monolayer a molecule of dextropimaric acid at 2.5° occupied an area approximately 1.6 Å^2 larger than that taken up by an *iso*dextropimaric acid molecule. Since, according to the opinion held at that time, these acids were believed to differ from each other only in the steric orientation of the vinyl and methyl groups at carbon-7 [2-4] *, the observed difference in molecular area was taken to justify certain conclusions about the configurations at this position in the two acids. The configuration that may be expected to occupy the larger area in the monolayer and which can be established by reference to molecular models was that in which the vinyl group is quasi-axially, and the methyl group quasi-equatorially, oriented. This configuration must thus be ascribed to dextropimaric acid and the opposite configuration to *iso*dextropimaric acid [1]. During recent years, however, convincing evidence has been collected which indicates that the acids also differ in configuration at carbon-13 [8-12]. The true C-7 epimer of dextropimaric acid seems to be cryptopimaric acid[11,12], an acid first described in 1937 [13]. These new developments, which are illustrated in Fig. 1, do not invalidate the conclusions drawn about the orientation of the groups at carbon-atom 7 in dextro- and *iso*dextropimaric acids, since the molecular areas taken up by the *iso*-dextropimaric-acid molecules with the old and new configurations cannot be expected to differ appreciably. Molecular models of the structures in Fig. 1 are shown in Fig. 2. In view of the importance of the structure of cryptopimaric acid when determining the structural relationships of the pimaric acids, it was considered desirable to supplement the previous monolayer data with similar data for cryptopimaric acid. Through the courtesy of Prof. H. Imamura of Tokyo in providing us with a sample of cryptopimaric acid, we have been able to measure the monolayer characteristics of the acid. At the same time we have repeated the measurements on the other two pimaric acids, using pure samples obtained from Dr. R. V. Lawrence, Olustee, Florida.

The surface-balance pressure-area curves of the three pimaric acids were recorded under identical conditions, and are reproduced in Fig. 3. The monolayers are seen to be similar in their mechanical properties (they are typical liquid condensed monolayers), and in all cases the monolayer collapses at approximately the same surface pressure, *viz.* 19—20 dyn/cm. The areas (Å^2) per molecule at the collapse point are:

	3°	20°
Dextropimaric acid	42.4	42.9
*Iso*dextropimaric acid	39.2	40.5
Cryptopimaric »	39.2	41.0

* See also Refs.[5-7]

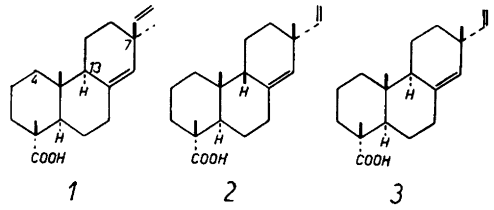


Fig. 1. Structures of *1* dextropimaric, *2 iso*dextropimaric and *3* cryptopimaric acids (see text).

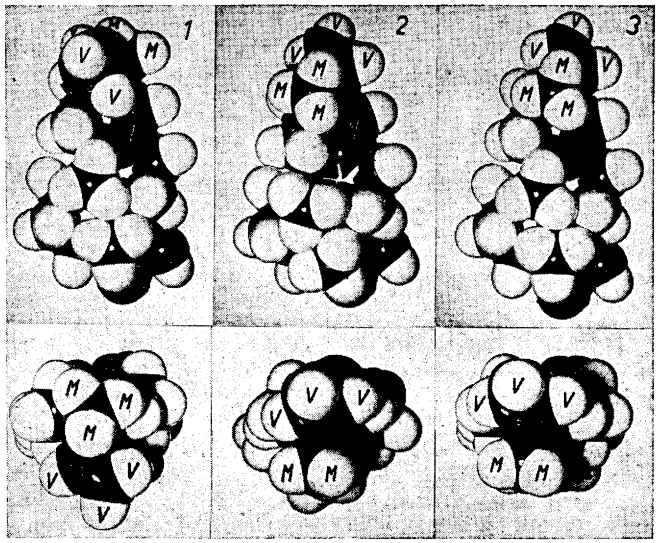


Fig. 2. Molecular models of *1* dextropimaric, *2* *iso*dextropimaric and *3* cryptopimaric acids based on the structures shown in Fig. 1. Upper row, viewed from the front. Lower row, viewed from above. The vinyl and methyl groups at C—7 are indicated by V and M, respectively.

The molecular areas taken up by crypto- and *iso*dextropimaric acid at 3° and 20° are not appreciably different, but they are 2—3 Å² smaller than the areas taken up by dextropimaric acid. These results confirm the previous findings that dextropimaric acid requires a distinctly greater area than *iso*dextropimaric acid in compressed monolayers [1].

The monolayer measurements suggest that cryptopimaric and *iso*dextropimaric acid both possess the same configuration at C-7, *i. e.* they have a quasi-equatorial vinyl group and a quasi-axial methyl group. This is in agreement with recent infra-red data [12], which have shown that the vinyl group has a different orientation in dextropimaric acid than in crypto- and *iso*dextropimaric acids.

Some of the area values found in the present study are slightly lower than those reported in the earlier study [1]. This is

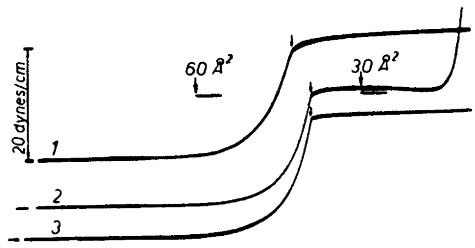


Fig. 3. Surface-balance (pressure-area) curves of *1* dextropimaric, *2* *iso*dextropimaric, and *3* cryptopimaric acids. Substrate, 3 M NaCl, pH 2 (HCl). Temperature, 3.0°C. The points from which the collapse areas were read are indicated by small arrows.

probably not due to the different type of surface balance used (for comparative data obtained with the Wilhelmy-Dervichian and the Mikrokator surface-balances, see Ref.[15]), but due to the fact that the samples of rosin acids used in the present study had been purified by chromatography and were exceptionally pure.

In contrast to the pressure-area curves for the other pimaric acids, the curve for *iso*dextropimaric acid exhibits an ascending part due to a relatively rapid solidification of the film under further compression after the collapse of the monolayer (Fig. 3). For further details relating to this interesting phenomenon, which is obviously related to some peculiarity in the structure of the *iso*dextropimaric acid molecule, see Ref.[16]

Experimental part. The pressure-area curves were obtained with the recording Mikrokator Balance [14]. The rate of compression was approximately 2 cm/min. The substrate was a solution of 3 M sodium chloride (Merck, pro analysi, ignited) brought to pH 2 by addition of hydrochloric acid. The importance of these conditions, which reduce the solubilities of the acid monolayers and the tendency of the monolayers to undergo collapse, has been demonstrated earlier [1]. The area values were reproducible within ± 0.2 Å^2. Spreading from benzene and from light petroleum (b.p. 60–80°) gave identical results.

For the physical properties of the rosin acids studied, the reader is referred to Ref.[11]

Acknowledgements. The authors are indebted to Mrs. Karin Nilsson for skilled assistance in the monolayer work.

1. Bruun, H. H. *Acta Acad. Aboensis, Math. & Phys.* **19** (1954).
2. Harris, G. G. and Sanderson, T. F. *J. Am. Chem. Soc.* **70** (1948) 2081.
3. Brossi, A. and Jeger, O. *Helv. Chim. Acta* **33** (1950) 722.
4. Harris, G. C. in Wiese, L. E. and Jahn, E. C. *Wood Chemistry*, New York 1952, p. 590.
5. Harper, S. H. *Annual Rep. Chem. Soc. London* **45** (1948) 174.
6. Barton, D. H. R. *Quart. Revs. London* **3** (1949) 46.
7. Simonsen, J. and Barton, D. H. R. *The Terpenes III*, Cambridge 1952, p. 374.
8. Wenkert, E. *Chem. & Ind. London* **1955** 282.
9. Green, B., Harris, A. and Whalley, W. B. *Chem. & Ind. London* **1958** 1084.
10. Green, B., Harris, A. and Whalley, W. B. *J. Chem. Soc.* **1958** 4715.
11. Bruun, H. H., Ryhage, R. and Stenhagen, E. *Acta Chem. Scand.* **12** (1958) 789.
12. Bruun, H. H., Fischmeister, I. and Stenhagen, E. *Acta Chem. Scand.* **13** (1959) 379.
13. Keimatsu, S., Ishiguro, T. and Fukui, G. *J. Pharm. Soc. Japan* **57** (1957) 92.
14. Ställberg-Stenhagen, S. and Stenhagen, E. *Nature* **159** (1947) 814.
15. Ställberg-Stenhagen, S. and Stenhagen, E. *Acta Chem. Scand.* **3** (1949) 1035.
16. Bruun, H. H. *Arkiv Kemi* **8** (1955) 412.

Received March 20, 1959.

Isolation of Polynuclear Aromatic Hydrocarbons from the Roots of *Chrysanthemum vulgare* Bernh.

ERLING GUDDAL*

Institutt for organisk kjemi, Norges tekniske högskole, Trondheim, Norway

During the investigation of the roots of tansy (*Chrysanthemum vulgare* Bernh.) for the presence of acetylenic compounds [1], one sample collected in the vicinity of the local Gas Works gave a small hydrocarbon fraction with a characteristic ultraviolet spectrum and strong fluorescence, which turned out to be a complex mixture of polynuclear aromatic hydrocarbons.

15 kg of fresh roots were extracted with acetone, and the extracted lipids transferred to light petroleum. From the petroleum soluble material a mixture of sparingly soluble compounds were removed by crystallisation from ether at −10°C. The mother liquor was evaporated to dryness and the residue dissolved in a minimum of hot methanol. This was added to a hot saturated solution of trinitrotoluene in methanol. A yellow crystalline precipitate was formed immediately, and the crystallisation was completed by cooling to −10°C. The mixture of addition compounds was dissolved in benzene and filtered through a

* Present addres: Research Dept., Leo Pharmaceutical Products, Copenhagen, Denmark.

probably not due to the different type of surface balance used (for comparative data obtained with the Wilhelmy-Dervichian and the Mikrokator surface-balances, see Ref.[15]), but due to the fact that the samples of rosin acids used in the present study had been purified by chromatography and were exceptionally pure.

In contrast to the pressure-area curves for the other pimaric acids, the curve for *iso*dextropimaric acid exhibits an ascending part due to a relatively rapid solidification of the film under further compression after the collapse of the monolayer (Fig. 3). For further details relating to this interesting phenomenon, which is obviously related to some peculiarity in the structure of the *iso*dextropimaric acid molecule, see Ref.[16]

Experimental part. The pressure-area curves were obtained with the recording Mikrokator Balance [14]. The rate of compression was approximately 2 cm/min. The substrate was a solution of 3 M sodium chloride (Merck, pro analysi, ignited) brought to pH 2 by addition of hydrochloric acid. The importance of these conditions, which reduce the solubilities of the acid monolayers and the tendency of the monolayers to undergo collapse, has been demonstrated earlier [1]. The area values were reproducible within ± 0.2 Å^2. Spreading from benzene and from light petroleum (b.p. 60–80°) gave identical results.

For the physical properties of the rosin acids studied, the reader is referred to Ref.[11]

Acknowledgements. The authors are indebted to Mrs. Karin Nilsson for skilled assistance in the monolayer work.

1. Bruun, H. H. *Acta Acad. Aboensis, Math. & Phys.* **19** (1954).
2. Harris, G. G. and Sanderson, T. F. *J. Am. Chem. Soc.* **70** (1948) 2081.
3. Brossi, A. and Jeger, O. *Helv. Chim. Acta* **33** (1950) 722.
4. Harris, G. C. in Wiese, L. E. and Jahn, E. C. *Wood Chemistry*, New York 1952, p. 590.
5. Harper, S. H. *Annual Rep. Chem. Soc. London* **45** (1948) 174.
6. Barton, D. H. R. *Quart. Revs. London* **3** (1949) 46.
7. Simonsen, J. and Barton, D. H. R. *The Terpenes III*, Cambridge 1952, p. 374.
8. Wenkert, E. *Chem. & Ind. London* **1955** 282.
9. Green, B., Harris, A. and Whalley, W. B. *Chem. & Ind. London* **1958** 1084.
10. Green, B., Harris, A. and Whalley, W. B. *J. Chem. Soc.* **1958** 4715.
11. Bruun, H. H., Ryhage, R. and Stenhagen, E. *Acta Chem. Scand.* **12** (1958) 789.
12. Bruun, H. H., Fischmeister, I. and Stenhagen, E. *Acta Chem. Scand.* **13** (1959) 379.
13. Keimatsu, S., Ishiguro, T. and Fukui, G. *J. Pharm. Soc. Japan* **57** (1957) 92.
14. Ställberg-Stenhagen, S. and Stenhagen, E. *Nature* **159** (1947) 814.
15. Ställberg-Stenhagen, S. and Stenhagen, E. *Acta Chem. Scand.* **3** (1949) 1035.
16. Bruun, H. H. *Arkiv Kemi* **8** (1955) 412.

Received March 20, 1959.

Isolation of Polynuclear Aromatic Hydrocarbons from the Roots of *Chrysanthemum vulgare* Bernh.

ERLING GUDDAL*

Institutt for organisk kjemi, Norges tekniske högskole, Trondheim, Norway

During the investigation of the roots of tansy (*Chrysanthemum vulgare* Bernh.) for the presence of acetylenic compounds [1], one sample collected in the vicinity of the local Gas Works gave a small hydrocarbon fraction with a characteristic ultraviolet spectrum and strong fluorescence, which turned out to be a complex mixture of polynuclear aromatic hydrocarbons.

15 kg of fresh roots were extracted with acetone, and the extracted lipids transferred to light petroleum. From the petroleum soluble material a mixture of sparingly soluble compounds were removed by crystallisation from ether at −10°C. The mother liquor was evaporated to dryness and the residue dissolved in a minimum of hot methanol. This was added to a hot saturated solution of trinitrotoluene in methanol. A yellow crystalline precipitate was formed immediately, and the crystallisation was completed by cooling to −10°C. The mixture of addition compounds was dissolved in benzene and filtered through a

* Present addres: Research Dept., Leo Pharmaceutical Products, Copenhagen, Denmark.

column of highly activated alumina. Evaporation of the benzene left 185 mg of a slightly yellow crystalline mixture of aromatic hydrocarbons. This mixture was dissolved in light petroleum and chromatographed on deactivated alumina.

The mixture showed strong fluorescence even when adsorbed, and the zones could readily be made visible by irradiating the column with an ultraviolet lamp. The fractions obtained were rechromatographed several times, and by this procedure it was possible to obtain spectroscopically pure fractions of anthracene, pyrene, and fluoranthene, the ultraviolet spectra of which were identical with those given in the literature [2]. 2.5 mg of anthracene was obtained as colourless plates with a strong violet fluorescence. It melted at 212–213°C in a sealed tube, not depressed on admixture with authentic anthracene. Further evidence of their identity was furnished by their infrared spectra.

To ensure that these compounds did not originate from adhering soil, a sample of 10 kg of the soil of the same location was extracted with acetone and treated as above. In this case, the splitting of the trinitrotoluene-complexes left 200 mg of a yellow oil, showing no tendency of crystallisation even after prolonged cooling to −10°C. Its ultraviolet spectrum, however, was very similar to that of the crude mixture of aromatic hydrocarbons isolated from the plant material. Since the contamination of the plant material with soil hardly exceeds a few per cent by weight, these findings indicate that some polynuclear aromatic hydrocarbons, although highly toxic to most higher organisms, are actually resorbed by the plant. By extraction of material from areas which are not polluted by the smoke from the Gas Works, it has been shown that these compounds are not normal metabolites of the plant.

Polynuclear aromatic hydrocarbons have previously been isolated from animal tissues in a few cases. Zechmeister and Koe [3] isolated a great number of these compounds from the barnacle *Tetraclita squarosa rubescens*, and Cahnmann and Kuratsune [4] reported similar results from oysters growing in polluted waters. Sula [5] isolated anthracene and benzpyrene from human lung tissue.

The occurrence of naphthalene in plant material has been reported in several cases. Soden and Rojahn [6] found "appreciable amounts" of naphthalene in oil of cloves and storax oil, and Schimmel & Co. isolated the same compound from iris oil [7], and from two samples of Indian costus root oil [8]. These findings have not been confirmed, and may have arisen as a result of a roasting of the roots before distillation [9]. Stoll and Scherrer isolated naphthalene from oakmoss from several localities, and noticed accompanying changes in the amount and composition of terpenes in the lichens [10]. Treibs [11] found naphthalene as a normal constituent of oil of leaf buds of *Betula lenta*, and Sebe [12] found it in rice embryo together with methyl substituted homologues. Regarding the two last findings, nothing can be said concerning the possibilities of smoke contamination, as nothing is said of the origin of the material. It appears that the isolation of higher aromatic hydrocarbons from plant material has not been reported previously.

The author is glad to thank Professor N. A. Sörensen for his interest in this work and for his constant encouragement. This work was carried out under the tenure of a Research Fellowship from *Norges tekniske högskole*.

1. Guddal, E. and Sörensen, N. A. *Acta Chem. Scand.* **13** (1959). *In press.*
2. Friedel, R. H. and Orchin, M. *Ultraviolet Spectra of Aromatic Compounds.* New York 1951.
3. Zechmeister, L. and Koe, B. K. *Arch. Biochem. Biophys.* **69** (1952) 1.
4. Cahnmann, H. J. and Kuratsune, M. *Anal. Chem.* **29** (1957) 1312.
5. Sula, J. *Chem. Abstr.* **49** (1955) 486.
6. Soden, H. v. and Rojahn, W. *Chem. Zentr.* **1907** *I* 1117.
7. *Schimmel & Co., Berichte* **1907** 55.
8. Gildemeister, E. and Hoffmann, F. *Die Ätherischen Öle.* Dritte Aufl. Schimmel & Co. Leipzig 1931, **III** 1047.
9. Guenther, E. *The Essential Oils*, D. Van Nostrand, New York 1952, **Vol. 5** 446.
10. Stoll, M. and Scherrer, W. *Chem. Zentr.* **1958 II** 2512.
11. Treibs, W. *Ber.* **71** (1938) 612, p. 616.
12. Sebe, Y. *Chem. Abstr.* **41** (1947) 3510.

Received March 11, 1959.

Nucleotides Containing Amino Acids in Bacteria

J. JONSEN, S. LALAND and I. SMITH-KIELLAND

Department of Biochemistry, The University of Oslo, Blindern, Norway

The importance of nucleic acids in protein synthesis is well established [1,2]. In later years Hoagland *et al.*[3] and others [4-9] have presented evidence for the existence of amino acyl-adenylate compounds and amino acid — RNA complexes [10,11] as intermediates in the biosynthesis of proteins.

Since low molecular nucleotides containing amino acids may act as participants or carriers of amino acids to the active site of protein or peptide synthesis, our laboratory has for some time been actively engaged in the search for such substances in different bacteria, yeasts and mammalian cells under various conditions of growth.

The recent publications by Brown [12] on nucleotidebound peptides from *S.faecalis* and of similar reports [13-15] prompt us to describe some of our work.

As nucleotides containing amino acids might be labile and difficult to extract we have used different extraction procedures.

The present report concerns the isolation of nucleotides containing amino acids from *E. coli* and *B. subtilis* grown under aerobic condition and harvested in the logarithmic growth phase. In the case of *E.coli* the rapidly frozen cells were disrupted in a Hughes bacterial press and extracted with cold 0.6 M perchloric acid at 0°C. The perchloric acid was removed as the potassium salt. The cells of *B. subtilis* were extracted with 90 % phenol at room temperature. The extracted material was displaced into an aqueous solution by the addition of ether. Both extracts were adsorbed on a Dowex 1 column in the formate form and fractionated by gradient elution. Since the presence of weakly charged substances was anticipated, better resolution of these would be obtained by elution with weaker concentrations of formic acid than those usually employed. Details of the gradient chromatography are shown in Figs. 1 and 2.

Fraction III from *E. coli* (Fig. 1) was separated into 4 U.V. absorbing bands by paper chromatography in *iso*butyric acid-N ammonia-EDTA (100:60:1.6). The slowest band was DPN. The second slowest band (III_2) had the same R_F value as AMP-5′

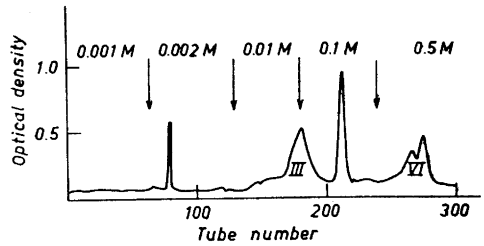


Fig. 1. Gradient elution of *E. coli* extract. Mixing volume 500 ml. Dowex 1 × 8 in the formate form (1.2 cm × 10 cm). Elution was started with 0.001 M formic acid followed by 0.002 M, 0.01 M, 0.1 M and 0.5 M as indicated by the arrows. Volume of individual fractions 5 ml. 2 190 optical units put on column.

in this solvent system and was further investigated. The substance had the same R_F value as AMP-5′ in *n*-butanol-conc. ammonia-water (30:5:15) and *n*-butanol-acetic acid-water (50:25:25). By paper electrophoresis in 0.2 M ammonium acetate buffer pH 5.8 and in borate buffer pH 10, the mobility was identical to that of AMP-5′. In 0.05 M phosphate buffer pH 7.5, AMP-5′ moved slightly ahead of the substance. By elution with 0.1 N HCl from a paper chromatogram in butanol-acetic acid-water the absorption maxima and minima were the same as for AMP-5′, whereas the ratios 280/260 and 290/260 were 0.44 (AMP-5′ 0.25) and 0.22 (AMP-5′ 0.09), respectively. The U. V. absorbing

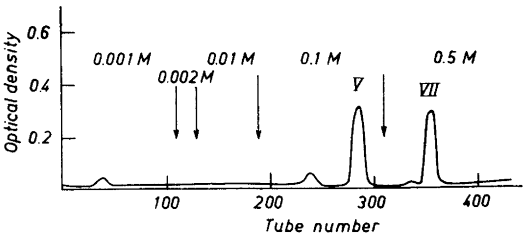


Fig. 2. Gradient elution of *B.subtilis* extract. Volume of mixing flask 500 ml. Dowex 1 × 4 in the formate form (20 cm × 1 cm). Elution was started with 0.001 M-formic acid in the reservoir flask followed by 0.002 M, 0.01 M, 0.1 M and 0.5 M as indicated by the arrows. Volume of individual fractions 5 ml. 216 optical units put on column.

substance gave a ninhydrine negative reaction. Chromatography in *n*-butanol-acetic acid-water (4:1:5) of material hydrolyzed with 6 N HCl for 18 h at 120°C gave four ninhydrine positive spots with the R_F values 0.43 (alanine 0.47), 0.35 (glutamic acid 0.36), 0.29 (glycine 0.29) and 0.15.

After hydrolysis with N HCl for one hour at 100°C adenine was identified and three ninhydrine spots (R_F 0.31, 0.26 and 0.14) were found by chromatography in *n*-butanol-acetic acid-water.

The elution diagram of *B. subtilis* is seen in Fig. 2. Each of the fractions V and VII were purified by successive chromatography in *iso*butyric acid-N ammonia-EDTA (100:60:1.6), ethanol-M ammonium acetate pH 7.5 (70:30), 0.1 M phosphate buffer pH 6.8-ammonium sulphate-*n*-propanol (Pabst) and gave only one U.V. absorbing spot with the same R_F value as AMP-5′ in these solvent systems.

After purification in the third solvent, the eluates were absorbed on charcoal and eluted with ethanol-ammonia. After freeze-drying, their spectra were determined in 0.1 N HCl and compared with AMP-5′ treated in the same way.

Fractions V, VII and AMP-5′ had the same absorption maxima (257 mμ) and minima (232 mμ), whereas the 280/260 ratios were: 0.33, 0.32, and 0.25, respectively. By paper electrophoresis in 0.2 M ammonium acetate buffer pH 5.8 fraction V had 33 % and fraction VII 85 % of the mobility of AMP-5′.

After hydrolysis with 6 N HCl for 20 h at 120°C 10 ninhydrine positive spots were found by chromatography in *n*-butanol-acetic acid-water (50:25:25).

The following amino acids were tentatively identified: lysine, aspartic acid, glycine, glutamic acid, alanine and valine.

Hydrolysate from substance VII gave four ninhydrine positive spots by chromatography in *n*-butanolformic acid-water (75:13:12). Three of these were tentatively identified as glycine, aspartic acid and alanine.

After hydrolysis in N HCl at 100°C for one hour adenin was identified.

Since the two substances from *E.coli* behave electrophoretically similar to AMP-5′ it is surprising that they are eluted more easily from the column than AMP-5′ (identified in fraction VI). The reason for this is not known for the present time, but the structure of the substances is being investigated.

The substances are possible intermediates in peptide or protein synthesis, but may also be formed by degradation of more complex substances containing nucleotides and amino acids.

We wish to thank *Norges Almenvitenskapelige Forskningsråd* and *Norsk Varekrigsfond* for financial support.

1. Caspersson, T. O. *Cell Growth and Cell Function*. W. W. Norton Company Inc., New York.
2. Gale, E. F. in McElroy, W. D. and Glass, B. H. *Amino Acid Metabolism*. Baltimore 1955, p. 171.
3. Hoagland, M. B., Keller, E. B. and Zamecnik, P. C. *J. Biol. Chem.* **218** (1956) 345; Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. J. and Zamecnik, P. C., *J. Biol. Chem.* **231** (1958) 241; Hecht, L. J., Stephenson, M. L. and Zamecnik, P. C. *Biochim. et Biophys. Acta* **29** (1958) 460.
4. Bernlohr, R. W. and Webster, G. C. *Nature* **182** (1958) 531.
5. Holly, R. W. and Prock, P. *Federation Proc.* **17** (1958) 244.
6. Berg, P. and Ofengand, E. J. *Proc. Natl. Acad. Sci. U.S.* **44** (1958) 78.
7. Schweet, R. S., Bovard, F. C., Allen, E. and Glassman, E. *Proc. Natl. Acad. Sci U.S.* **44** (1958) 173.
8. Weiss, S. B., Acs, G., Lipmann, F. *Proc. Natl. Acad. Sci. U.S.* **44** (1958) 189.
9. Ogata, K. and Nohara, H. *Biochim. et Biophys. Acta* **25** (1957) 659.
10. Habermann, V. *Biochim. et Biophys. Acta* **32** (1958) 297.
11. Potter, J. L. and Dounce, A. L. *J. Am. Chem. Soc.* **78** (1956) 3078.
12. Brown, A. D. *Biochim. et Biophys. Acta.* **30** (1958) 448; Brown, A. D. *Biochem. J.* **71** (1959) 5 p.
13. Weinstein, C., Hammond, D., Berkman, J. I., Gallop, P. M. and Seifter, S. *Federation Proc.* **17** (1958) 332.
14. Harris, G., Davis, J. W. and Parsons, R. *Nature* **182** (1958) 1565.
15. Gilbert, D. A. and Yemm, E. W. *Nature* **182** (1958) 1746.

Received March 21, 1959.

A New Acidic Ultraviolet Absorbing Substance Containing Amino Acids in Bacteria, Yeasts and Spore Preparations

J. JONSEN, S. LALAND, I. SMITH-KIELLAND and R. SÖMME

Department of Biochemistry The University of Oslo, Blindern, Oslo, Norway

In recent years nucleotides containing amino acids have been isolated from extracts of mammalian tissues [1] and bacterial cells [2]. In a previous paper [3] we have described such substances from *E. coli* and *B. subtilis* harvested in the logarithmic growth phase. We now wish to report the occurrence and properties of an unidentified substance containing amino acids and with a characteristic ultraviolet absorption spectrum.

The substance has been isolated by gradient elution of cell extracts adsorbed on a Dowex 1 × 8 column in the formate form. Fig. 1 shows a typical elution of an extract from *B. cereus*. The unknown substance was eluted in fraction VII close to CMP-5′ when using 0.5 M formic acid in the reservoir. It was purified by successive paper chromatography and paper electrophoresis.

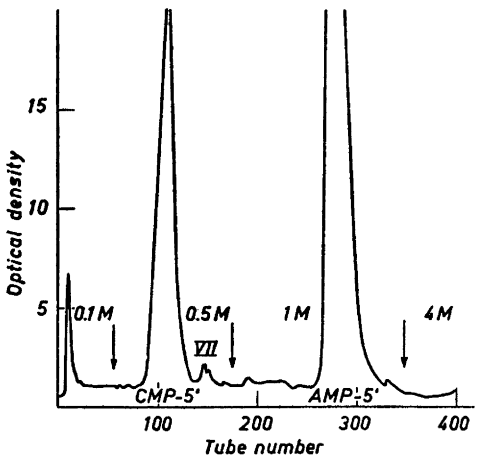


Fig. 1. Part of the gradient elution diagram of *B. cereus* extract. Volume of mixing flask 750 ml. Dowex 1 × 8 in the formate form (3 cm × 18 cm). Elution was started with 0.001 M formic acid in the reservoir flask followed by 0.002 M, 0.02 M, 0.1 M. The arrows indicate concentrations of 0.5 M, 1.0 M and 4 M formic acid. Volume of individual fractions. 29 600 optical units put on column.

The substance has been found in *E.coli*, *B. cereus* spores and vegetative cells and in *Candida tropicalis* var. *lambica*. It was present in acid-soluble extracts from cells disrupted in the Hughes bacterial press and was also released by boiling the cells in distilled water for 10 min. The substance could be adsorbed on activated charcoal and again eluted at 0°C with 50 % ethanol containing 2 % conc. ammonia. The amount varied from 0.3—0.6 % of the total U.V. absorption at 260 mμ fo the crude extracts.

The most conspicuous feature of the ultraviolet absorption spectrum of the highly purified material (Figs. 2 and 3) is the ratio 280/260 in 0.1 N HCl. This is on an average 0.1 and is therefore lower than that of known nucleotides. The ratio 250/260 is usually 0.60. While the point of maximum absorption has constantly been found at 261—262 mμ, the inflection points at about 258 mμ and 268 mμ (Fig. 2) have been more or less pronounced and may be almost missing as shown in Fig. 3. Figs. 2 and 3 represent the extremes of the variation in the absorption spectrum and the variation is not caused by U.V. absorbing impurities since it can be experienced in eluates from two identical chromatograms.

No significant change in the U.V. absorption was found after hydrolysis with formic acid at 175°C for 2 h or with 6 N HCl at 120°C for 18 h.

In paper electrophoresis the unidentified substance moved towards the anode at pH values above pH 4.0. In acetate buffer at pH 5.9 it had approximately the same mobility as orotic acid. In phosphate buffer of pH 7.4 the mobility was about 20 % greater than that of AMP-5′.

The R_F values in paper chromatography was greater than those of the usual nucleotides. For instance in *iso*butyric acid-N-ammonia-EDTA (100:60:1.6) it had R_F 0.78 whereas the R_F for AMP-5′ is 0.52. The mobility was unchanged after hydrolysis. The U.V. absorption of the substance and its hydrolysate could not be reduced by treatment with sodium amalgam [4].

No ribose was found by the orcinol test using 20 min of heating at 100°C. Tests on phosphorus on material hydrolyzed with 6 N HCl were negative.

The purified U.V. absorbing substance gave a negative ninhydrine test while

A New Acidic Ultraviolet Absorbing Substance Containing Amino Acids in Bacteria, Yeasts and Spore Preparations

J. JONSEN, S. LALAND, I. SMITH-KIELLAND and R. SÖMME

Department of Biochemistry The University of Oslo, Blindern, Oslo, Norway

In recent years nucleotides containing amino acids have been isolated from extracts of mammalian tissues [1] and bacterial cells [2]. In a previous paper [3] we have described such substances from *E. coli* and *B. subtilis* harvested in the logarithmic growth phase. We now wish to report the occurrence and properties of an unidentified substance containing amino acids and with a characteristic ultraviolet absorption spectrum.

The substance has been isolated by gradient elution of cell extracts adsorbed on a Dowex 1 × 8 column in the formate form. Fig. 1 shows a typical elution of an extract from *B. cereus*. The unknown substance was eluted in fraction VII close to CMP-5′ when using 0.5 M formic acid in the reservoir. It was purified by successive paper chromatography and paper electrophoresis.

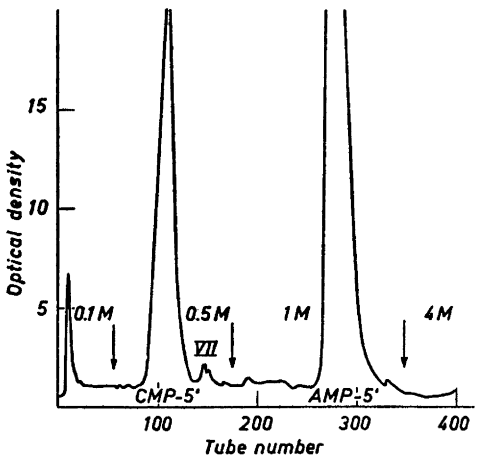


Fig. 1. Part of the gradient elution diagram of *B. cereus* extract. Volume of mixing flask 750 ml. Dowex 1 × 8 in the formate form (3 cm × 18 cm). Elution was started with 0.001 M formic acid in the reservoir flask followed by 0.002 M, 0.02 M, 0.1 M. The arrows indicate concentrations of 0.5 M, 1.0 M and 4 M formic acid. Volume of individual fractions. 29 600 optical units put on column.

The substance has been found in *E.coli*, *B. cereus* spores and vegetative cells and in *Candida tropicalis* var. *lambica*. It was present in acid-soluble extracts from cells disrupted in the Hughes bacterial press and was also released by boiling the cells in distilled water for 10 min. The substance could be adsorbed on activated charcoal and again eluted at 0°C with 50 % ethanol containing 2 % conc. ammonia. The amount varied from 0.3—0.6 % of the total U.V. absorption at 260 mμ fo the crude extracts.

The most conspicuous feature of the ultraviolet absorption spectrum of the highly purified material (Figs. 2 and 3) is the ratio 280/260 in 0.1 N HCl. This is on an average 0.1 and is therefore lower than that of known nucleotides. The ratio 250/260 is usually 0.60. While the point of maximum absorption has constantly been found at 261—262 mμ, the inflection points at about 258 mμ and 268 mμ (Fig. 2) have been more or less pronounced and may be almost missing as shown in Fig. 3. Figs. 2 and 3 represent the extremes of the variation in the absorption spectrum and the variation is not caused by U.V. absorbing impurities since it can be experienced in eluates from two identical chromatograms.

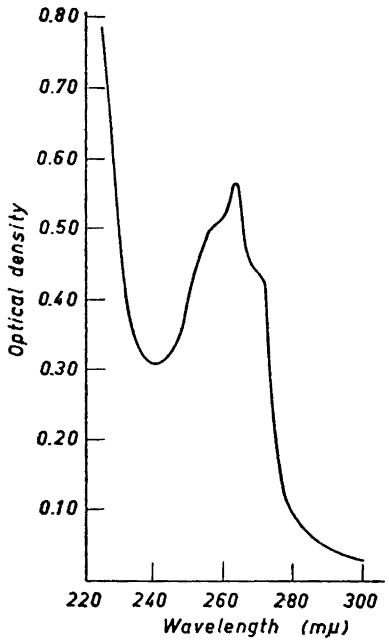
No significant change in the U.V. absorption was found after hydrolysis with formic acid at 175°C for 2 h or with 6 N HCl at 120°C for 18 h.

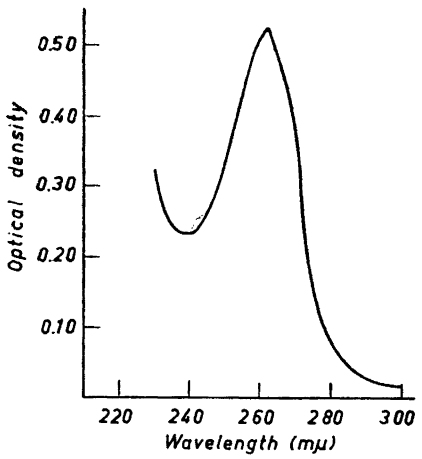
In paper electrophoresis the unidentified substance moved towards the anode at pH values above pH 4.0. In acetate buffer at pH 5.9 it had approximately the same mobility as orotic acid. In phosphate buffer of pH 7.4 the mobility was about 20 % greater than that of AMP-5′.

The R_F values in paper chromatography was greater than those of the usual nucleotides. For instance in *iso*butyric acid-N-ammonia-EDTA (100:60:1.6) it had R_F 0.78 whereas the R_F for AMP-5′ is 0.52. The mobility was unchanged after hydrolysis. The U.V. absorption of the substance and its hydrolysate could not be reduced by treatment with sodium amalgam [4].

No ribose was found by the orcinol test using 20 min of heating at 100°C. Tests on phosphorus on material hydrolyzed with 6 N HCl were negative.

The purified U.V. absorbing substance gave a negative ninhydrine test while





Figs. 2 and 3. Ultra violet absorption spectra in 0.1 N HCl of eluates of spots of the unknown substance from two identical chromatograms developed in ethanol-conc. ammonia-water (80:5:15)

several ninhydrine positive spots split off with varying rates when chromatographed. This seemed to occur particularly when using *n*-butanol-acetic acid-water (40:10:50) as a solvent. After hydrolysis for 18 h at 120°C in 6 N HCl, two dimensional chromatography in *n*-butanol-acetic acid-water (40:10:50) followed by phenol-*m*-cresol-borate buffer pH 8.3 [5] showed ninhydrine positive spots corresponding to the position of serine, glutamic acid, aspartic acid, glycine and alanine together with an unidentified spot.

The stability of the substance to hydrolysis makes the possibility of it being a purine unlikely and the failure of reducing it with sodium amalgam seems to exclude the possibility of a pyrimidine.

The unidentified substance is interesting for several reasons. So far it seems to be distributed widely in the microbial world. The fact that it seems to be a constituent of a bacterial spore may suggest an important function in essential processes of cell development.

The liberation of amino acids by paper chromatography in an acidic solvent suggests a very labile form of amino acid linkages not commonly found in peptides.

Large scale preparation of this substance, together with structural studies and its possible participation in the biosynthesis of proteins or cell wall substances are being carried out.

We wish to thank *Norges Almenvitenskapelige Forskningsråd* and *Det Norske Vitenskapsfond av 1919* for financial support.

1. Hoagland, M. B., Zemecnik, P. C. and Stephenson, M. L. *Biochim. et Biophys. Acta.* **24** (1957) 215.
2. Koningsberger, V. V., Van der Grinten, C. O. and Overbeck, J. T. G. *Biochim. et Biophys. Acta.* **26** (1957) 483.
3. Jonsen, J., Laland, S. and Smith-Kielland, I. *Acta Chem. Scand.* **13** (1959) 836.
4. Laland, S., McKinley McKee and Roth, E. *Acta. Chem. Scand.* **11** (1957) 1081.
5. Levy, A. L. and Chung, D. *Anal. Chem.* **25** (1953) 397.

Received March 21, 1959.

1,2-Dithiane-4-carboxylic Acid

GÖRAN CLAESON and
ARNE LANGSJOEN*

Chemical Institute, University of Uppsala, Uppsala, Sweden

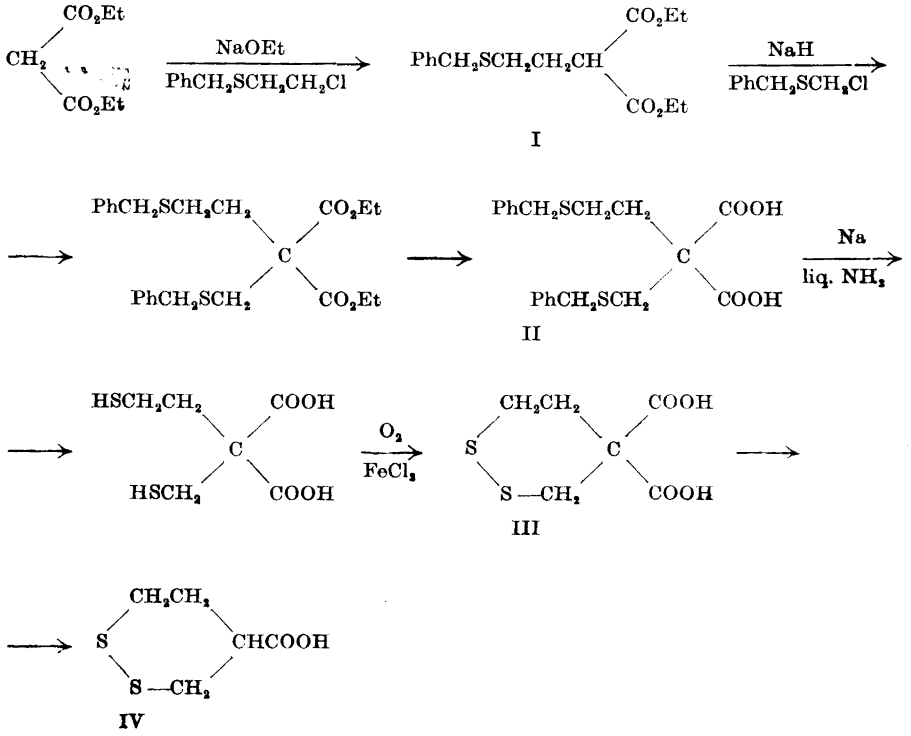
Studies of the stereochemistry of cyclic disulfides presently underway in these laboratories have dictated a need for the compound, 1,2-dithiane-4-carboxylic acid. We wish to report the unequivocal synthesis and preliminary resolution of this compound.

Using the reaction sequence illustrated below, pure 1,2-dithiane-4-carboxylic acid (IV) was obtained in 15 % overall yield from the readily prepared diethyl β-benzylthioethyl-malonate (I).

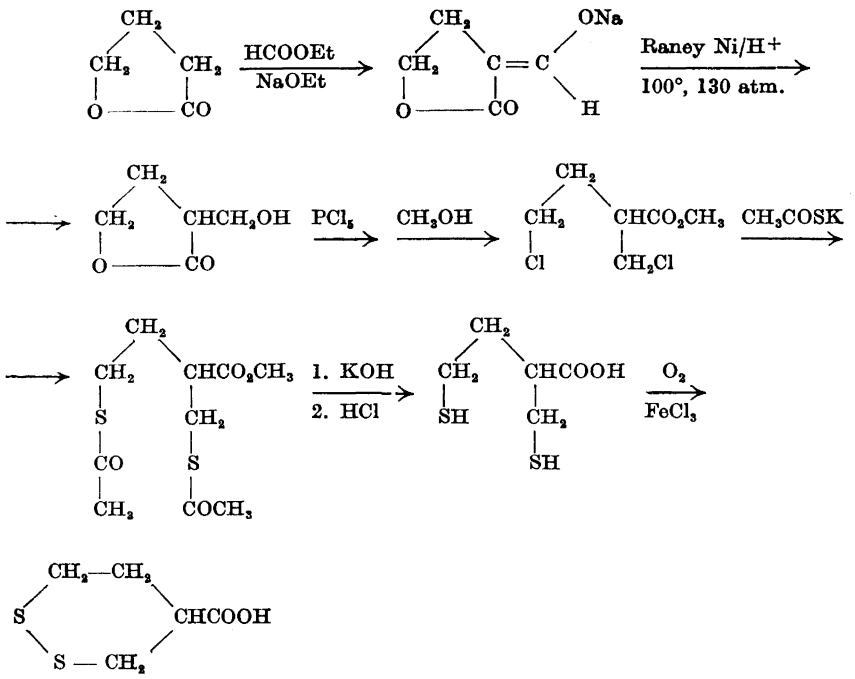
A small amount of IV was also isolated from among the products resulting when γ-butyrolactone was subjected to the following series of reactions (see p. 841). The presence of active hydrogen in β-position to sulphur, however, is known to favor disproportionation reactions under alkaline conditions [1], and these, together with thiolactone formation and polymerization reactions permitted very meager success with this synthesis.

A partial resolution of 1,2-dithiane-4-carboxylic acid has been accomplished.

Experimental. β-Benzylthioethyl-benzylthiomethylmalonic acid (II). β-Benzylthioethyl chloride and diethyl α-benzylthioethylmalonate (I) were prepared by the methods of du Vigneaud and Brown [2]. The pure monoalkyl ester (I), b.p. 176—178°/1 mm, was obtained in 56 % yield from diethyl malonate. (No physical constants were reported for compound I in Ref.[2]) Benzylthiomethyl chloride (b.p. 116.5—117.5°/8 mm) was obtained in 54 % yield using the method of Böhme, Fisher and Frank [3] at room temperature.



* Present address, Gustavus Adolphus College, St. Peter, Minnesota.



IV

A solution of 23.5 g (0.0755 mole) of diethyl β-benzylthioethylmalonate (I) in 20 ml of dry toluene was added dropwise and with stirring to 1.9 g (0.0793 mole) of sodium hydride suspended in 40 ml of dry toluene. The evolution of hydrogen had stopped after 5.5 h at 70°, and a solution of 13 g (0.0755 mole) of benzylthiomethyl chloride in 20 ml of toluene was added. The reaction mixture was stirred at 75° for 63 h (shorter reaction times proved to be insufficient). The toluene was removed under reduced pressure, and the crude ester saponified directly by refluxing for 4 h with a solution of 16 g (0.4 mole) of sodium hydroxide in 80 ml of water and 50 ml of ethanol. The alcohol was removed under reduced pressure, and the cooled, heterogeneous residue extracted with three 50 ml portions of ether. The water soluble remainder was acidified to pH 4 with 50 % ice-hydrochloric acid and the resulting yellow oil extracted with two 100 ml portions of benzene. The crude yield of (II) after removal of the benzene under reduced pressure at room temperature was 21 g (71 %). White radial clusters of needles were obtained after digestion in 85 % formic acid and repeated crystallization from petroleum ether-ether. M. p. 101.8–102.5° with evolution of CO_2 at 122°. Equiv. wt. calc. 195.3; found 201.0.

It is interesting to note that the ether extracts, upon evaporation and recrystallization from alcohol produced 2.8 g (28 % from benzylthiomethyl chloride) of dibenzylthiomethane, m. p. 47.5–48.2°. The structure was established through analyses, melting point and mixed melting point with an authentic specimen [4]. (Found: C 69.27; H 6.20; S 24.59. Calc for $C_{15}H_{16}S_2$ (260.41): C 69.18; H 6.19; S 24.62.)

1,2-Dithiane-4,4-dicarboxylic acid (III). A solution of 19 g (0.046 mole) of crude II in 30 ml of dry toluene was added dropwise over a period of one hour to 500 ml of liquid ammonia to which a small piece of sodium had been added. The blue colour was maintained by appropriate additions of sodium. After a total of 3 h, and the addition of 8.2 g (0.357 mole) of sodium, the reaction was complete. The excess sodium was neutralized with powdered ammonium chloride, the ammonia allowed to evaporate, and the remaining salts extracted with 1 500 ml of water. The pH was adjusted

to 7.5 with hydrochloric acid, 2 ml of 1 % ferric chloride added, and air was bubbled through *via* a sintered glass filter. After 27 h, the solution became clear yellow, and it was acidified to pH 3. An unidentified yellow oil was first removed by extraction with five 100 ml portions of benzene, and then the product (III) was isolated by extraction with three 100 ml portions of ether. The crude yield of 1,2-dithiane-4,4-dicarboxylic acid was 5 g (52 %). Recrystallization from alcohol-water produced white crystals which melted with decarboxylation at 152°. Equiv. wt. calc. 104.1; found 107.0.

1,2-Dithiane-4-carboxylic acid (IV). 4 g (0.0192 mole) of crude 1,2-dithiane-4,4-dicarboxylic acid (III) was heated at 150—170° for half an hour. Recrystallization from alcohol-water and subsequently from petroleum ether-benzene produced 1.85 g (59 %) of pure 1,2-dithiane-4-carboxylic acid melting at 115.2—116.2°. (Found: C 37.10; H 4.90; S 38.78; equiv. wt. 165.0. Calc. for $C_5H_8S_2O_2$ (164.2); C 36.81; H 4.91; S 39.05; equiv. wt. 164.2.)

Preliminary resolution of 1,2-dithiane-4-carboxylic acid. Quinine and cinchonidine salts were found to be suitable for the resolution of racemic IV. Brucine, α-phenyl-ethylthiuronium chloride and α-(2-naphthyl)-ethylamine proved to be ineffective as resolving agents. The impure dextro enantiomorph, $[\alpha]_D^{25} = +71.5°$ in ethanol solution; m. p. 109—113°, was obtained after four crystallizations of the cinchonidine salt from abs. ethanol. The impure levo antipode, $[\alpha]_D^{25} = -123.5°$; m. p. 106.5—108°, was obtained after four crystallizations of the quinine salt from abs. ethanol. The UV spectra of the two enantiomorphs were essentially identical, with absorption maxima at 211 and 298 mμ (in ethanol).

The authors are indebted to Professor Arne Fredga for constructive discussions and for the facilities placed at our disposal. The second author also gratefully acknowledges a Science Faculty fellowship from the *National Science Foundation, Washington, DC*, which has made his part of this work possible.

1. Böhme, H. and Greve, H.-G. *Ber.* **85** (1952) 409.
2. du Vigneaud, V. and Brown, G. B. *Biochem. Preparations* **5** (1958) 84.
3. Böhme, H., Fischer, H. and Frank, R. *Ann* **563** (1949) 65.
4. Fromm, E., Forster, A. and von Scherschewitzki, B. *Ann.* **394** (1912) 343.

Received March 23, 1959.

A Note on the Constitution of Rhodopin

SYNNÖVE LIAAEN JENSEN

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

The carotenoid rhodopin was first isolated in 1936 by Karrer and Solmssen [1] from mixed cultures containing *Rhodovibrio* species and was also shown to be present in *Thiocystis* [2]. The elementary analysis was in good agreement with a molecular formula $C_{40}H_{56}O$, although Karrer *et al*[3]. did not exclude the possibility of $C_{40}H_{56}O$. The oxygen function was shown to be a hydroxyl group by quantitative determination of active hydrogen [2,3]. Because of its resistance to acetylation rhodopin was assumed to have a tertiary hydroxyl group [3]. Upon catalytic hydrogenation an uptake of 11.93 and 11.71 moles of hydrogen per mole rhodopin was recorded, and Karrer and Solmssen [3] assumed that twelve carbon-carbon double bonds were present in rhodopin. Rhodopin occurred in complex mixture with other carotenoids including a carotene which these workers named rhodopurpurin[1], and they suggested that rhodopin might be a derivative of rhodopurpurin [1]. They also pointed out the possible identity of lycopene and rhodopurpurin [3] *, but no structural formula for rhodopin was suggested.

More recently Goodwin *et al.*[4,5] have isolated from a number of photosynthetic bacteria *(Athiorhodaceae* and *Thiorhodaceae)* a carotenoid which has the lycopene chromophore and which according to partition tests and chromatographic behaviour was assumed to be a mono-OH-lycopene. Taking into account the source of isolation and the visible spectra and chromatographic behaviour Goodwin and Land concluded that this pigment was identical with rhodopin.

A mono hydroxy derivative of lycopene, lycoxanthin, had previously been isolated by Zechmeister and Cholnoky [6] from the berries of *Solanum dulcamara*. Lycoxan-

* This suggestion has since been verified by Goodwin and Land [5] by mixed chromatography and comparison of spectroscopic properties. It should, however, be kept in mind that lycopene from photosynthetic bacteria has so far not been isolated in crystalline state.

to 7.5 with hydrochloric acid, 2 ml of 1 % ferric chloride added, and air was bubbled through *via* a sintered glass filter. After 27 h, the solution became clear yellow, and it was acidified to pH 3. An unidentified yellow oil was first removed by extraction with five 100 ml portions of benzene, and then the product (III) was isolated by extraction with three 100 ml portions of ether. The crude yield of 1,2-dithiane-4,4-dicarboxylic acid was 5 g (52 %). Recrystallization from alcohol-water produced white crystals which melted with decarboxylation at 152°. Equiv. wt. calc. 104.1; found 107.0.

1,2-Dithiane-4-carboxylic acid (IV). 4 g (0.0192 mole) of crude 1,2-dithiane-4,4-dicarboxylic acid (III) was heated at 150—170° for half an hour. Recrystallization from alcohol-water and subsequently from petroleum ether-benzene produced 1.85 g (59 %) of pure 1,2-dithiane-4-carboxylic acid melting at 115.2—116.2°. (Found: C 37.10; H 4.90; S 38.78; equiv. wt. 165.0. Calc. for $C_5H_8S_2O_2$ (164.2); C 36.81; H 4.91; S 39.05; equiv. wt. 164.2.)

Preliminary resolution of 1,2-dithiane-4-carboxylic acid. Quinine and cinchonidine salts were found to be suitable for the resolution of racemic IV. Brucine, α-phenyl-ethylthiuronium chloride and α-(2-naphthyl)-ethylamine proved to be ineffective as resolving agents. The impure dextro enantiomorph, $[\alpha]_D^{25} = +71.5°$ in ethanol solution; m. p. 109—113°, was obtained after four crystallizations of the cinchonidine salt from abs. ethanol. The impure levo antipode, $[\alpha]_D^{25} = -123.5°$; m. p. 106.5—108°, was obtained after four crystallizations of the quinine salt from abs. ethanol. The UV spectra of the two enantiomorphs were essentially identical, with absorption maxima at 211 and 298 mμ (in ethanol).

The authors are indebted to Professor Arne Fredga for constructive discussions and for the facilities placed at our disposal. The second author also gratefully acknowledges a Science Faculty fellowship from the *National Science Foundation, Washington, DC*, which has made his part of this work possible.

1. Böhme, H. and Greve, H.-G. *Ber.* **85** (1952) 409.
2. du Vigneaud, V. and Brown, G. B. *Biochem. Preparations* **5** (1958) 84.
3. Böhme, H., Fischer, H. and Frank, R. *Ann* **563** (1949) 65.
4. Fromm, E., Forster, A. and von Scherschewitzki, B. *Ann.* **394** (1912) 343.

Received March 23, 1959.

A Note on the Constitution of Rhodopin

SYNNÖVE LIAAEN JENSEN

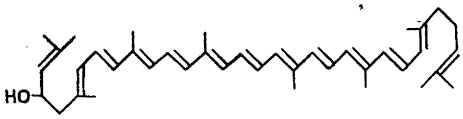
Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

The carotenoid rhodopin was first isolated in 1936 by Karrer and Solmssen [1] from mixed cultures containing *Rhodovibrio* species and was also shown to be present in *Thiocystis* [2]. The elementary analysis was in good agreement with a molecular formula $C_{40}H_{56}O$, although Karrer *et al*[3]. did not exclude the possibility of $C_{40}H_{56}O$. The oxygen function was shown to be a hydroxyl group by quantitative determination of active hydrogen [2,3]. Because of its resistance to acetylation rhodopin was assumed to have a tertiary hydroxyl group [3]. Upon catalytic hydrogenation an uptake of 11.93 and 11.71 moles of hydrogen per mole rhodopin was recorded, and Karrer and Solmssen [3] assumed that twelve carbon-carbon double bonds were present in rhodopin. Rhodopin occurred in complex mixture with other carotenoids including a carotene which these workers named rhodopurpurin[1], and they suggested that rhodopin might be a derivative of rhodopurpurin [1]. They also pointed out the possible identity of lycopene and rhodopurpurin [3] *, but no structural formula for rhodopin was suggested.

More recently Goodwin *et al.*[4,5] have isolated from a number of photosynthetic bacteria *(Athiorhodaceae* and *Thiorhodaceae)* a carotenoid which has the lycopene chromophore and which according to partition tests and chromatographic behaviour was assumed to be a mono-OH-lycopene. Taking into account the source of isolation and the visible spectra and chromatographic behaviour Goodwin and Land concluded that this pigment was identical with rhodopin.

A mono hydroxy derivative of lycopene, lycoxanthin, had previously been isolated by Zechmeister and Cholnoky [6] from the berries of *Solanum dulcamara*. Lycoxan-

* This suggestion has since been verified by Goodwin and Land [5] by mixed chromatography and comparison of spectroscopic properties. It should, however, be kept in mind that lycopene from photosynthetic bacteria has so far not been isolated in crystalline state.



(I)

thin was easily acetylated, and Zechmeister and Cholnoky suggested the formula (I). The hydroxyl group was allocated to the 3-position in accordance with the normal, smooth formation of acetates from secondary alcohols, and the widespread occurrence of 3-OH-substituted carotenoids.

Goodwin and Land [5] reisolated lycoxanthin from *Solanum dulcamara*, and compared it with the rhodopin isolated from a photosynthetic sulphur-bacterium, *Chromatium spp.* It was concluded that rhodopin was identical with lycoxanthin on the basis of their similar chromatographic behaviour, and because of the fact that the two pigments exhibited the same visible absorption spectrum in different solvents before and after iodine isomerization. Whilst the similarity of the visible absorption spectra and chromatographic behaviour indicate that lycoxanthin and rhodopin have the same chromophore and the same number of hydroxyl groups, it is by no means certain that the positions of the latter are the same in the two compounds. Goodwin and Land did, however, exclude hydroxyl in the 4-position on the grounds of a negative $HCl-CHCl_3$ test. It is surprising that these authors made no comment on the different behaviour of rhodopin and lycoxanthin [3,6] towards acetylating agents. In later work the rhodopin of the photosynthetic bacteria has been referred to mainly as lycoxanthin in the literature [7], although Stanier *et al.*[8] preferred the designation OH-lycopene until this structural problem was satisfactorily settled.

It has been established by Stanier *et al.*[8] that OH-lycopene is an intermediate between lycopene and spirilloxanthin in the biosynthesis of carotenoids in *Rhodospirillum rubrum*. Since the structure of OH-lycopene is of importance in this connection, the properties of this carotenoid have been investigated in more detail.

OH-Lycopene was isolated from *Rhodospirillum rubrum* in the exponential growth phase and from *Rhodopseudomonas palustris*. According to van Niel [9] the genetic name *Rhodopseudomonas* is synonymous with *Rhodovibrio* and *Rhodobacillus*, from which Karrer and Solmssen isolated rhodopin.

As already reported by Goodwin and Land [5] no difference in structure for lycoxanthin and rhodopin can be predicted from comparison of visible spectra in different solvents, nor from partition tests and chromatographic behaviour. Also the melting points recorded are so close that they yield no useful information. The melting point reported for lycoxanthin is 168°C, whilst that for rhodopin varies from 159—160°C [1] to 171°C [3] with increasing purity. The OH-lycopene used in the present studies melted at 166—168°C.

The outstanding difference between lycoxanthin and rhodopin was in their behaviour towards acetylating agents, and this aspect was therefore studied first. Numerous attempts were made using different methods to acetylate OH-lycopene from *Rhodospirillum rubrum* and *Rhodopseudomonas palustris*. After standing with acetic anhydride in pyridine at room temperature for periods of up to two days the OH-lycopene could be completely recovered. A parallel experiment with lutein (3,3′-di-OH-α-carotene) resulted in the formation of lutein diacetate in quantitative yield. Treatment with freshly distilled acetyl chloride in pyridine at room temperature for periods up to four days gave a product with properties corresponding to an acetate. However, the yield did not exceed 8 % of the OH-lycopene used for the test. Upon saponification of this acetate with 15 % methanolic KOH about 30 % was converted to OH-lycopene. This behaviour of OH-lycopene towards acetylating agents is similar to that reported by Karrer *et al.*[3] for rhodopin, but not for lycoxanthin [6].

Further support for the tertiary character of the hydroxyl group in OH-lycopene from photosynthetic bacteria is obtained from the IR absorption data presented in Table 1.

Carotenoids with the hydroxyl groups in 3- or 4-positions show strong absorption in the 1 030 cm^{-1} region. Chloroxanthin and OH-lycopene, however, have no absorption band in this region. Chloroxanthin is a mono- or di-OH-neurosporene which was isolated by Nakayama [12] from a green mutant of *Rhodopseudomonas spheroides*. Less than 5 % of chloroxanthin is acetylated on treatment with acetic anhydride in pyridine, and this led Nakayama to the conclusion that the hydroxyl group(s) of chloroxanthin must be tertiary. Both chloroxanthin and OH-lycopene exhibit a band of somewhat weaker intensity around 1 140 cm^{-1} which might be due to tertiary hydroxyl groups [13]. It might be argued that this region is also typical for *iso*propyl groups (1 170—1 140 cm^{-1}) [13], but an IR-spectrum of lycopene isolated from tomatoes showed no band of similar intensity in this region. The

Table 1.

Carotenoid	IR-absorption connected with the hydroxyl group Frequency cm^{-1}
Cryptoxanthin (3-OH-β-carotene) $CHCl_3$[10]	*ca.* 1 035
Isozeaxanthin (4,4′-di-OH-β-carotene) CCl_4[11]	*ca.* 1 035
Zeaxanthin (3,3′-di-OH-β-carotene) KBr	1 038
Violaxanthin (3,3′-di-OH-β-carotene-5,6,5′,6′-di-epoxide) $CHCl_3$	1 025
Chloroxanthin KBr	1 145
OH-Lycopene KBr	1 140

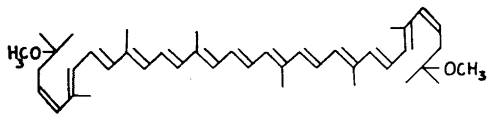
IR-spectrum of lycopene is in fact surprisingly simple, and corresponds closely to that of OH-lycopene apart from a band at 3 450 cm^{-1} (OH), a relatively weak band at 1 260 cm^{-1} and the band at 1 140 cm^{-1} already discussed.

Microdetermination of *iso*propylidene was carried out, using the method of Kuhn and Roth [14]. The result is presented in Table 2 together with the value obtained simultaneously for lycopene isolated from tomatoes.

Table 2.

Carotenoid	Found: Moles of acetone/mole	Number of *iso*-propylidene groups
Lycopene	1.70	2
OH-Lycopene	1.12	1

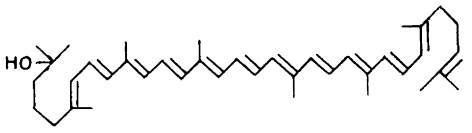
It is seen that the value obtained for OH-lycopene is much lower than that for lycopene. It has been shown that spirilloxanthin to which the formula (II) is now ascribed [15],



(II)

and which contains no *iso*propylidene groups, yields 0.42—0.73 [15,2] moles of acetone upon ozonolysis. The *iso*propylidene value obtained for OH-lycopene is therefore interpreted as being due to the presence of one *iso*propylidene end group together with a second end group which yields a small amount of acetone on ozonolysis.

Combining the chemical evidence obtained by Karrer *et al.*[1-3] for rhodopin, and the evidence presented here, the following structure (III) is suggested for the OH-lycopene from photosynthetic bacteria.



(III)

In accordance with the generally accepted nomenclature, this should now be referred to as rhodopin. The name lycoxanthin should be reserved for the hydroxy derivative of lycopene which was first isolated from *Solanum dulcamara* and for which the structure (I) has been suggested by Zechmeister and Cholnoky [6].

In view of the findings described above, it is clear that as far as the biosynthetic reactions leading from lycopene to spirilloxanthin are concerned, no definite conclusions should be drawn until the chemical structure of all the intermediates which can be isolated in reasonable amounts is firmly established.

The author wishes to express her gratitude to Professor C. B. van Niel for a generous gift of dried cells of *Rhodopseudomonas palustris*, to Dr. T. O. M. Nakayama for a sample of chloroxanthin, to Professor N. A. Sörensen for his inspiring interest and advice in this work and to *Norges Tekniske Högskole* for a maintenance grant.

1. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 1306.
2. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 1019.

3. Karrer, P., Solmssen, U. and Koenig, H. *Helv. Chim. Acta* **21** (1938) 454.
4. Goodwin, T. W. *Arch. Microbiol.* **24** (1956) 313.
5. Goodwin, T. W. and Land, D. G. *Arch. Microbiol.* **24** (1956) 305.
6. Zechmeister, L. and Cholnoky, L. v. *Ber.* **69** (1936) 422.
7. Braithwaite, G. D. and Goodwin, T. W. *Nature* **182** (1958) 477.
8. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochim. et Biophys. Acta* **29** (1958) 477.
9. van Niel, C. B. *Bacteriol. Rev.* **8** (1944) 1.
10. Isler, O., Lindlar, H., Montavon, M., Rüegg, R., Saucy, G. and Zeller, P. *Helv. Chim. Acta* **40** (1957) 456.
11. Isler, O., Montavon, M., Rüegg, R. and Zeller, P. *Helv. Chim. Acta* **39** (1956) 274.
12. Nakayama, T. O. M. *Arch. Biochem. Biophys.* **75** (1958) 352.
13. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules.* 2nd Ed. Methuen and Co. Ltd., London 1958.
14. Kuhn, R. and Roth, H. *Ber.* **65** (1932) 1285.
15. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 381.

Received March 12, 1959.

The Mass Spectra of Methyl Oleate, Methyl Linoleate, and Methyl Linolenate

BO HALLGREN

Institute of Medical Biochemistry, Gothenburg, Sweden

RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden and

EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala, Sweden

In connexion with studies on the mass spectra of esters of long-chain fatty acids we have examined a number of unsaturated esters. In general, unsaturated esters give more complex spectra than esters of saturated, normal-chain acids [1,2]. In order to interpret the spectrum of methyl oleate we have examined the isomeric 2-, 6-, 8-, 10-, 13- and 17-octadecenoates, but it has proved desirable to study also the ester of an acid with the double bond at position 4:5 or 5:6. None of these acids has been described in the literature. Synthetic work will therefore have to be undertaken and may also be required for the interpretation of the spectra of the di- and triethenoid esters. In view of the common occurrence of oleic, linoleic, and linolenic acids, however, and the analytical possibilities afforded by the mass spectrometer [3], a brief description of the mass spectra of their esters will be made.

The mass spectrum of methyl oleate (Fig. 1a) shows that the introduction of a double bond in the hydrocarbon chain leads to a fragmentation pattern considerably different from that of saturated methyl esters. Compared with methyl stearate [2], the peaks in the low-mass range due to fragments containing an intact methoxycarbonyl group are much less prominent than those due to hydrocarbon fragments. The peak at $m/e = 55$ ($C_4H_7^+$) is of the same order of magnitude as the rearrangement peak at $m/e = 74$. In the sequence of the higher-methoxycarbonyl-type fragments, the peak at $m/e = 87$ is the most prominent one (45 % of the base peak). The higher members of this series of peaks do not show the marked rhythmic variation in height which is characteristic of methyl esters of normal-chain saturated acids. In the high-mass range, marked peaks are found at $m/e = 296$, 264, 222, and 180. The peak due to the molecule-ion at $m/e = 296$ is relatively small, and the base peak at $m/e = 264$ corresponds to a fragment formed with the loss of 32 mass units. A comparison with the ethyl ester shows that the molecule-ion loses a methoxyl group and one hydrogen atom (formally, this corresponds to the loss of one molecule of methanol). The peak at $m/e = 265$ is higher than expected for the isotope peak corresponding to the peak at $m/e = 264$, which shows that ions are also formed with the loss of the methoxyl group only. The peak at $m/e = 222$ ($= M-74$) is due to a fragment formed by the loss of methylene-methoxycarbonyl, $-CH_2COOCH_3$, of mass 73, together with one hydrogen atom. The appearance of fairly marked peaks at $m/e = 180$, 222, 264, and 265 in both the methyl and the ethyl esters shows that the corresponding ions do not contain the ester alkoxy group.

The mass spectrum of methyl elaidate is practically indistinguishable from that of methyl oleate. Methyl esters of *cis-* and *trans-*petroselinic acids were both found to be indistinguishable from that of methyl oleate. Even a shift of the double bond to the 17:18

3. Karrer, P., Solmssen, U. and Koenig, H. *Helv. Chim. Acta* **21** (1938) 454.
4. Goodwin, T. W. *Arch. Microbiol.* **24** (1956) 313.
5. Goodwin, T. W. and Land, D. G. *Arch. Microbiol.* **24** (1956) 305.
6. Zechmeister, L. and Cholnoky, L. v. *Ber.* **69** (1936) 422.
7. Braithwaite, G. D. and Goodwin, T. W. *Nature* **182** (1958) 477.
8. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochim. et Biophys. Acta* **29** (1958) 477.
9. van Niel, C. B. *Bacteriol. Rev.* **8** (1944) 1.
10. Isler, O., Lindlar, H., Montavon, M., Rüegg, R., Saucy, G. and Zeller, P. *Helv. Chim. Acta* **40** (1957) 456.
11. Isler, O., Montavon, M., Rüegg, R. and Zeller, P. *Helv. Chim. Acta* **39** (1956) 274.
12. Nakayama, T. O. M. *Arch. Biochem. Biophys.* **75** (1958) 352.
13. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules.* 2nd Ed. Methuen and Co. Ltd., London 1958.
14. Kuhn, R. and Roth, H. *Ber.* **65** (1932) 1285.
15. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 381.

Received March 12, 1959.

The Mass Spectra of Methyl Oleate, Methyl Linoleate, and Methyl Linolenate

BO HALLGREN

Institute of Medical Biochemistry, Gothenburg, Sweden

RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden and

EINAR STENHAGEN

Department of Medical Biochemistry, Institute of Medical Chemistry, Uppsala, Sweden

In connexion with studies on the mass spectra of esters of long-chain fatty acids we have examined a number of unsaturated esters. In general, unsaturated esters give more complex spectra than esters of saturated, normal-chain acids [1,2]. In order to interpret the spectrum of methyl oleate we have examined the isomeric 2-, 6-, 8-, 10-, 13- and 17-octadecenoates, but it has proved desirable to study also the ester of an acid with the double bond at position 4:5 or 5:6. None of these acids has been described in the literature. Synthetic work will therefore have to be undertaken and may also be required for the interpretation of the spectra of the di- and triethenoid esters. In view of the common occurrence of oleic, linoleic, and linolenic acids, however, and the analytical possibilities afforded by the mass spectrometer [3], a brief description of the mass spectra of their esters will be made.

The mass spectrum of methyl oleate (Fig. 1a) shows that the introduction of a double bond in the hydrocarbon chain leads to a fragmentation pattern considerably different from that of saturated methyl esters. Compared with methyl stearate [2], the peaks in the low-mass range due to fragments containing an intact methoxycarbonyl group are much less prominent than those due to hydrocarbon fragments. The peak at $m/e = 55$ ($C_4H_7^+$) is of the same order of magnitude as the rearrangement peak at $m/e = 74$. In the sequence of the higher-methoxycarbonyl-type fragments, the peak at $m/e = 87$ is the most prominent one (45 % of the base peak). The higher members of this series of peaks do not show the marked rhythmic variation in height which is characteristic of methyl esters of normal-chain saturated acids. In the high-mass range, marked peaks are found at $m/e = 296$, 264, 222, and 180. The peak due to the molecule-ion at $m/e = 296$ is relatively small, and the base peak at $m/e = 264$ corresponds to a fragment formed with the loss of 32 mass units. A comparison with the ethyl ester shows that the molecule-ion loses a methoxyl group and one hydrogen atom (formally, this corresponds to the loss of one molecule of methanol). The peak at $m/e = 265$ is higher than expected for the isotope peak corresponding to the peak at $m/e = 264$, which shows that ions are also formed with the loss of the methoxyl group only. The peak at $m/e = 222$ ($= M-74$) is due to a fragment formed by the loss of methylene-methoxycarbonyl, $-CH_2COOCH_3$, of mass 73, together with one hydrogen atom. The appearance of fairly marked peaks at $m/e = 180$, 222, 264, and 265 in both the methyl and the ethyl esters shows that the corresponding ions do not contain the ester alkoxy group.

The mass spectrum of methyl elaidate is practically indistinguishable from that of methyl oleate. Methyl esters of *cis-* and *trans-*petroselinic acids were both found to be indistinguishable from that of methyl oleate. Even a shift of the double bond to the 17:18

position has little effect upon the mass spectrum. On the other hand, the mass spectra of the *cis-* and *trans-* $\Delta^{2:3}$ isomers differ considerably from each other and from those of esters in which the double bond is at position 6:7 or beyond.

The results just described show that it is in general not possible to determine the position of the double bond in a monoethenoid acid by direct mass spectrometric analysis. The position of the double bond may be determined, however, after deuteration by means of hydrazine-d_4 [4] or after hydroxylation [5].

The spectrum of methyl linoleate (Fig. 1b) shows prominent peaks due to hydrocarbon fragments at $m/e = 67$ (base peak), 81, and 95. In general, the 'hydrocarbon' peaks are much stronger than those due to oxygen-containing fragments. The peak at $m/e = 294$ due to the molecule-ion is very prominent; the

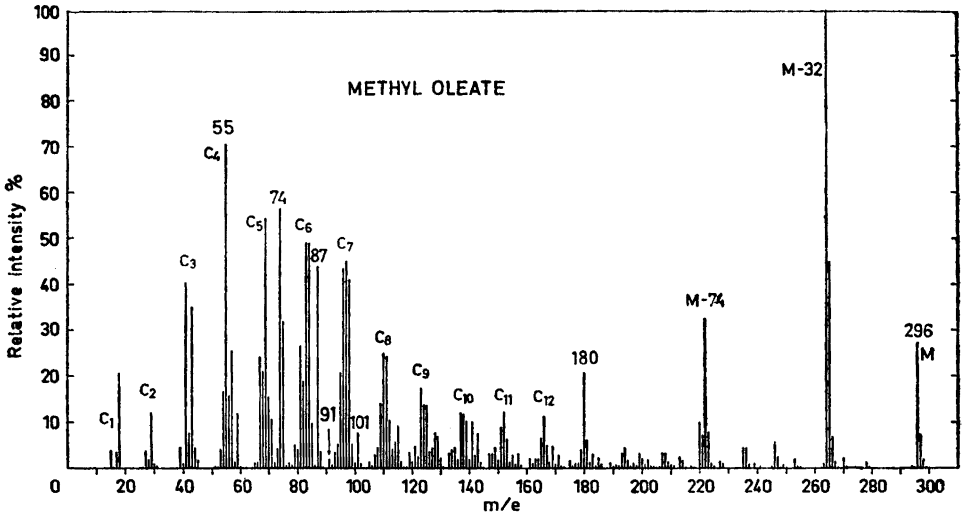


Fig. 1 a.

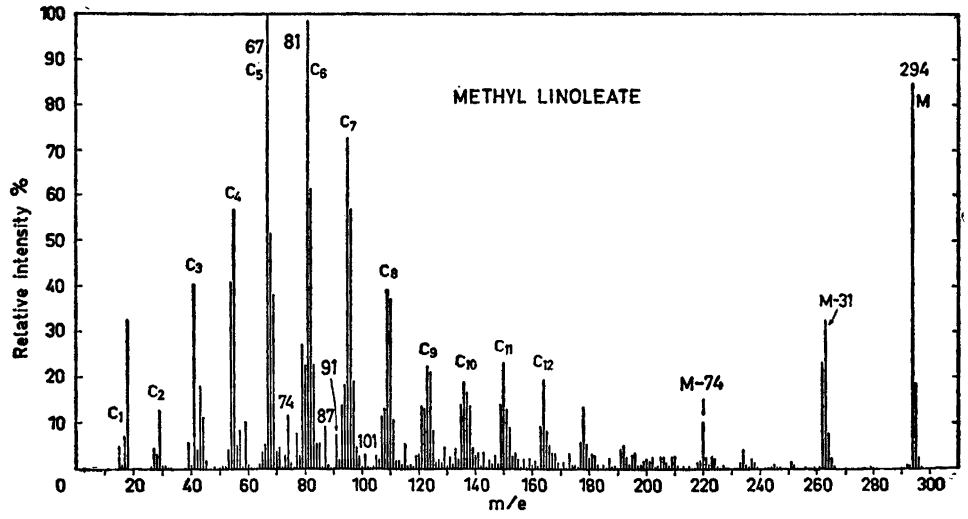


Fig. 1 b.

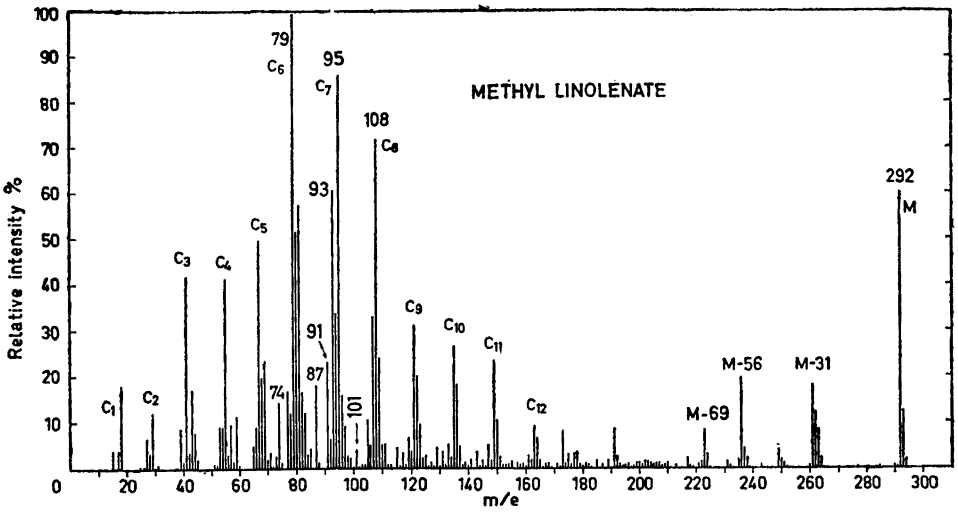


Fig. 1 c.

peak at $m/e = M-31$ is higher than that at $m/e = M-32$; and the peak at $m/e = 220$ (M−74) is obviously analogous to that at $m/e = 222$ in the spectrum of methyl oleate.

In the spectrum of methyl linolenate (Fig. 1c), the base peak is at $m/e = 79$ ($C_6H_7^+$). The 'methoxycarbonyl' peaks are relatively small. In the high-mass range, the molecule ion at $m/e = 292$ gives the most prominent peak, followed by the peaks at $m/e = M-56$ and $m/e = M-31$. The peak at $m/e = M-56$ is probably formed by cleavage between carbon atoms 14 and 15, with rearrangement of one hydrogen atom, leading to the formation of a neutral molecule of 1-butene and an ionized fragment of $m/e = 236$. In the spectrum of methyl linolenate there is a peak at $m/e = 91$ the height of which is about 20 % of that of the base peak. This peak, which may be due to tropylium ions [6] formed by cyclization and rearrangement, is very high in the spectra of methyl esters of unsaturated acids with many double bonds. It is the base peak in the case of a methyl docosahexaenoate which we have examined.

Experimental. The mass spectrometer and the general procedure have been described previously [7]. The intake system was kept at 200°, and the energy of the electrons was 70 eV.

The esters used in order to obtain the spectra shown in Fig. 1 were purchased from Mann Research Laboratories, New York. Their purity was checked by gas chromatography on a Reoplex 400 column with an efficiency approaching 5 000 plates. The oleate contained 1 % of palmitate, and the linoleate about 1 % of oleate. No significant amount of impurity was found in the linolenate. We are indebted to Dr. W. L. Courchene, The Procter & Gamble Company, Cincinnati, for samples of octadecenoic acids synthesized by Huber [8], to Dr. L. Crombie, University of London King's College, for the 2-octadecenoic acids, to Dr. R. T. Holman of the Hormel Institute of the University of Minnesota for the methyl docosahexaenoate, and to Mr. Ng. Dinh-Nguyen for the preparation of several esters. Grants from *Gustaf och Tyra Svenssons Stiftelse* and the *Swedish Medical, Natural Science,* and *Technical Research Councils* are gratefully acknowledged.

1. Asselineau, J., Ryhage, R. and Stenhagen, E. *Acta Chem. Scand.* **11** (1957) 196.
2. Ryhage, R. and Stenhagen, E. *Arkiv Kemi,* **13** (1959) 523.
3. Hallgren, B., Ryhage, R. and Stenhagen, E. *Arkiv Kemi* **11** (1957) 1064.
4. Dinh-Nguyen, Ng. *Acta Chem. Scand.* **12** (1958) 1350.
5. *To be published later.*
6. *Cf.* Rylander, P. N., Meyerson, S. and Grubb, H. M. *J. Am. Chem. Soc.* **79** (1957) 842.
7. Ryhage, R. *Arkiv Kemi* **13** (1959) 475.
8. Huber, W. F. *J. Am. Chem. Soc.* **73** (1951) 2730.

Received March 25, 1959.

Preparation of Tritium Labelled Dioctyl Adipate

GUNNAR B. LINDSTRÖM

Division of Physical Chemistry, The Royal Institute of Technology, Stockholm, Sweden

The vapour pressure of a plasticizer, di(2-ethyl-hexyl) adipate (DOA), was to be studied at relatively low temperatures. The vapour pressures of plasticizers are very low and very difficult to determine. Several methods for the determination of low pressures have been used in the past but they are not very accurate. However, radioactive tracer methods possess certain advantages and are, moreover, relatively accurate. There are two suitable isotopes for the labelling of the molecules, *viz.* ^{14}C and T. Since a high sensitivity and thus high specific activities are needed and since also large amounts were required, ^{14}C would be too expensive. Besides it is more difficult to incorporate ^{14}C into the molecule.

Tritium can be introduced into a molecule in several ways but, in this case, only two were tried, *viz.* labelling by direct exposure to tritium gas according to Wilzbach [1] and catalytic hydrogenation.

The Wilzbach method is very simple. It consists merely in placing the substance to be labelled in contact with T-gas (usually of purity > 90 %). The substance then becomes labelled with tritium due to the catalytic influence of the radiation. The product has to be carefully purified since, of course, other substances are also formed.

In order to get high specific activities, the pressure of the tritium gas should be high (this means that the volume of the reaction vessel should be relatively small) and the surface area of the sample should be large. Thus, solid samples are usually powdered and liquid samples shaken vigorously so that the liquid forms a foam during the tritiation. The DOA is quite viscous and it was found to be impossible, when working with small volumes, to produce a foam from small amounts of DOA. It was found necessary to use a volume of 30–40 ml and more than 5 g DOA to obtain the conditions desired. This meant that the pressure of the gas was reduced considerably but it was thought that the low pressure should be more than compensated for by the large surface area obtained by the vigorous shaking. This was obviously not the case since the specific activity of the product (6.3 g) was only 2.2 μC/g. This is fairly low compared with those activities obtained by other authors [2] who had employed tritium pressures of about 1 atm. It therefore seems probable that a high pressure for the tritium gas is more important than a large surface area.

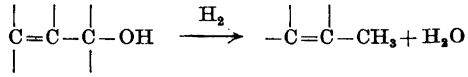
There is also another possible explanation for the poor result. Tables of G_T values seem to show that hydrogen atoms activated by polar groups in their neighbourhood are more readily exchanged by T-atoms than ordinary hydrogen atoms in saturated hydrocarbons. DOA contains some activated hydrogen atoms but most of them are not particularly activated and should thus exchange less readily.

The G_T value of this reaction is 0.0003.

Because the Wilzbach labelling failed, the conclusion was that the best way for getting high specific activities and sufficiently large amounts was to introduce the tritium gas by catalytic hydrogenation. A synthesis was required in which the tritium gas was introduced as late as possible in the synthesis run in order to avoid losses during the earlier stages. The following was considered a suitable way for doing this.

The starting material was 2-ethyl-3-propyl acrolein which is an intermediate product in the preparation of DOA. This was reduced to the corresponding alcohol which was then esterified with adipic acid. The unsaturated ester was then tritiated. However, when the product was subjected to a trial hydrogenation, it was found that 30–40 % more hydrogen was needed than the amount calculated. This was first thought to be due to the fact that the compound was not pure but, even after a very careful purification, more hydrogen was needed than expected. The following is a possible explanation.

It is known [3] that, in the catalytic hydrogenation of olefinic linkages, the following reaction occurs readily:



If the double bond is not so easily reduced, as in this case where one of the unsaturated carbon atoms is a tertiary one, then this serves to labilize the C–O linkage with the resultant reduction indicated above. Now, it is assumed that the catalyst contained some small amounts of acid impurities

which caused the hydrolysis of the ester to a certain extent. The unsaturated alcohol would then be reduced both at the double bond and the hydroxyl group. The free adipic acid, thus formed, would catalyze the further hydrolysis of other ester molecules and we would therefore get an accelerating hydrolysis of the ester, *i.e.* a chain reaction. In addition, it is possible that the double bond also activated the ester bonding, thus making the hydrolysis proceed faster.

Since neither of the two previous methods were successful, a third method for preparing this substance was chosen. Muconic acid was prepared from pyrocatecol. The muconic acid was then tritiated and esterified with 2-ethyl-hexanol. This method was successful.

Procedure. I. The tritium gas, obtained from AERE, England, was 96 % pure. It was introduced into a dispenser apparatus [4] and, from this, about 1 C was transferred to a tritium container. The transference of T-gas to the reaction vessel was somewhat more complicated since the reaction vessel must be cooled with liquid air in order to prevent evaporation during the evacuating. A simple, modified Toepler pump was designed for the transference of the T-gas to the reaction vessel (Fig. 1). The tritiating was carried out in a 50 ml bottle with 10 g of DOA of commercial grade. The flask was shaken vigorously for six days. The vessel was then connected with a U-tube (filled with activated carbon) and this was connected in turn to the Toepler pump. The vessel and the tube were cooled in liquid air. The tritium gas was then pumped back to the container. By this arrangement, the gases formed during the tritiating were either condensed in the bottle or adsorbed in the U-tube and thus the T-gas pumped back was quite clean. The DOA was then dissolved in 50 ml ether and washed three times with 10 ml saturated $NaHCO_3$-solution. After boiling off the ether, the DOA was distilled in a spinning band column at 206°C.

II. 2-Ethyl-3-propyl acrolein was distilled at 83°C, 10 mm Hg. The product was reduced with $LiAlH_4$ to the alcohol using a procedure given by Nystrom and Brown [5]. The product was distilled at 98—99°C, 23 mm Hg. The yield was 75 %. The ester was prepared by esterification with adipyl chloride. The crude ester was then distilled in a spinning band column at 206°—210°C, 6—7 mm Hg. The yield was 61 %. When the identity of the ester was investigated, it was found that 30—40 % more hydrogen was needed for the hydrogenation than the amount calculated. It was first thought that this was due to some decomposition during the distillation. The ester was molecularly distilled but there was still an over-consumption of hydrogen. This method was therefore abandoned.

III. The muconic acid [6] prepared, the identity of which was confirmed by catalytic hydrogenation (0.0596 mg muconic acid, 20.7 ml

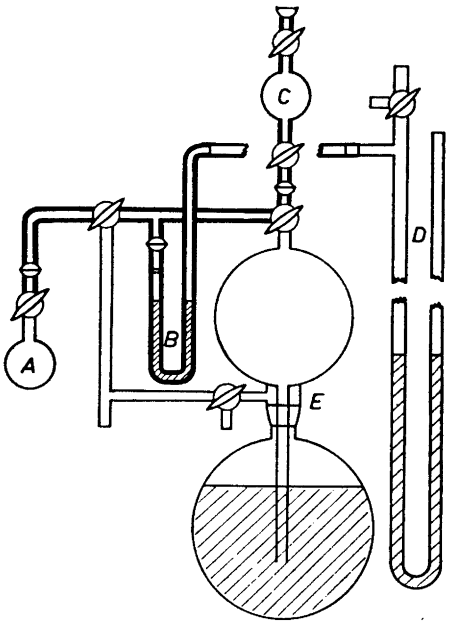


Fig. 1. Modified Toepler pump for the transference of tritium gas from the ampoule to the tritiating vessel and back again for Wilzbach labelling. Such an apparatus is particularly suitable for the labelling of liquids since the vessel can be cooled.

A) Reaction vessel, B) Zero-manometer,
C) T-container, D) Main Hg-manometer,
E) Toepler pump.

The "zero"manometer is used in order to keep the volumes between the reaction vessel and Toepler pump as small as possible. When reading the pressure, the "zero"manometer is set at zero by letting in air or pumping air out through the upper stopcock of the manometer. The pressure is read on the main manometer. The "zero" manometer is equipped with a glass filter under the ball joint in order to prevent mercury getting into the apparatus.

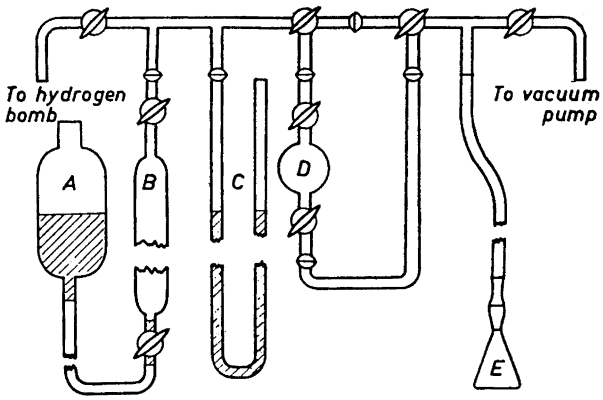


Fig. 2. Tritiating apparatus: A) mercury bottle, B) 50 ml gas burette, C) Hg manometer, D) tritium container, E) hydrogenation flask. Capillary glass tubes were used (D = 2 mm).

H_2, theor. 20.2 ml H_2) was tritiated in a modified tritiating apparatus (Fig. 2). Muconic acid (0.356 g) was dissolved in 10 ml *p*-dioxane (p.a.) in the hydrogenation flask and 25.5 mg Adams catalyst was added. The mixture was cooled with dry ice and the apparatus evacuated. The flask was the allowed to warm to room temperature and the reduction of the catalyst with hydrogen started. When ~ 8 ml H_2 had been consumed, the reduction was stopped by cooling once more with dry ice and the flask was then evacuated again. Hydrogen was introduced again into the apparatus but this time through the tritium gas container which, so to speak, forced the gas into the hydrogenation flask. The amount of tritium gas was ~ 200 mC. The total amount of hydrogen consumed was 58.7 ml, theor, calc. 57.3. Adipic acid (3 g) was then added as carrier and the combined acids were crystallized from the solution and recrystallized from dioxane. 1.00 g of the T-adipic acid and 4.85 g adipic acid were then esterified at 160°C with 13 g (theor. 10.43 g) of 2-ethylhexanol. The reaction was completed after 4 h. The crude product was dissolved in ether and washed three times with saturated $NaHCO_3$-solution. The ether-solution was dried, the ether boiled off and the residue distilled in a spinning band column at 206—208°C and 6 mm Hg. The yield was 11.85 g (78 %). The activity of the product was 0.65 mC/g. Taking into account the losses and dilution during the synthesis, this meant that the yield of tritium from the tritiating was ~ 65 %.

The author wishes to thank the head of the division, Professor Ole Lamm, for his support and encouragement. The author is also indebted to Dr. T. Westermark for putting at his disposal the facilities and the instruments of the »industrial group» and to Dr. G. Aniansson for valuable discussions and advice, to the *Swedish Atomic Committee* and the *State Technical Research Council* for financial assistance.

1. Wilzbach, K. E. *J.Am.Chem.Soc.* **79** (1957) 1013.
2. Tolbert, B. M. and Lemmon, R. M. *Radiation Research* **3** (1955) 52.
3. Gilman, H. *Organic Chemistry*, (1944) p. 799.
4. Barton, J. A. and Parrish, D. *Nucleonics* **14** (1956) 84.
5. Nystrom, R. F. and Brown, W. G. *J. Am. Chem.Soc.* **69** (1957) 1197.
6. Wacek, A. and Fiedler, R. *Monatsh.* **80** (1946) 170.

Received April 3, 1959.

*iso*Thiocyanates XXXV.* Miscellaneous *iso*Thiocyanate Glucoside Acetates

ANDERS KJÆR

Organic Chemical Laboratory of The Royal Veterinary and Agricultural College, Copenhagen, Denmark

Most naturally occurring *iso*thiocyanate glucosides possess poor crystallization properties as apparent from the fact that so far only seven individual glucosides have been reported as crystalline substances (*cf.* Ref.[1]). In contrast herewith, crystalline glucoside O-acetates are frequently obtainable as useful derivatives for purification and characterization purposes. The acetylation procedure used in this laboratory [2] and independently applied to a broader range of glucosides by Schultz and Wagner [3] is similar to that customarily employed in carbohydrate chemistry. It is the purpose of the present note to describe a few *iso*thiocyanate glucoside acetates and, in addition, present some supplementary or corrective data for previously reported derivatives.

Glucoiberin, a crystalline glucoside isolated as a potassium salt from seeds of *Iberis amara* [4], affords a crystalline tetraacetate on acetylation. A non-analyzed specimen of the latter was characterized by its m. p.[3,5] (148–149° (decomp.)), optical rotation [5] $[\alpha]_D^{20}$ −12.2° (*c* 1.4, H_2O)) and infra-red spectrum [5]. A specimen of *potassium tetraacetylglucoiberate* prepared in this laboratory separated from 96 % ethanol as a *monohydrate* in tiny, colourless needles, m. p. 145–147° (decomp.) **, $[\alpha]_D^{28}$ −16.6° (*c* 2.0, H_2O). (Found: C 35.50; H 4.87; N 2.13; S 15.25. Calc. for $C_{12}H_{26}O_{14}NS_2K$, H_2O: C 35.23; H 4.67; N 2.16; S 14.85).

The rubidium salt of glucoiberin, desired for special purposes, was prepared from the corresponding potassium salt by percolating a solution of the latter through a column containing a cation exchange resin (Dowex 50) in its rubidium ion form. *Rubidium glucoiberate* separated as a *monohydrate* from 85 % ethanol, m. p. 130–132° (decomp.), $[\alpha]_D^{23}$ −51.9° (*c* 2.1, H_2O). (Found: C 24.95; H 4.24; N 2.66; Rb 16.62. Calc. for $C_{11}H_{20}O_{10}NS_2Rb,H_2O$: C 25.12; H 4.22; N 2.66; Rb 16.25).

In 1948 Schmid and Karrer [6] reported on the isolation and structure elucidation of the mustard oil sulphoraphene, an enzymic hydrolysis product of a glycosidic progenitor in radish seeds. From seeds of *Matthiola bicornis*, Schultz and Wagner [3] isolated a crystalline glycoside tetraacetate which was tacitly assumed to be identical with the tetraacetate of the radish seed glucoside for which the name glucoraphenin was introduced [5]. Because no rotation values were reported and analytical data were inconclusive with regard to the contents of water of crystallization [5], a sample of the tetraacetate of the glucosidic progenitor of sulphoraphene was isolated in this laboratory from radish seed.

A methanolic glucoside extract of crushed and defatted radish seed was purified, first by ion exchange on acid-washed alumina and then by removal of impurities with lead acetate [3]. The glassy residue was acetylated in the usual way in pyridine with acetic anhydride. After recrystallization from ethanol, in which the tetraacetate dissolved very slowly, an almost pure product was obtained in amounts corresponding to 3.6 g per 100 g of fresh radish seed. The analytical specimen was recrystallized thrice from ethanol, and separated as a *monohydrate* in colourless needles, m. p. 155–156° (decomp.) $[\alpha]_D^{27}$ −23.5° (*c* 1.5, H_2O). (Found: C 36.45; H 4.50; N 2.13; S 14.36; K 5.69; Calc. for $C_{20}H_{38}O_{14}NS_2K$, H_2O: C 36.41; H 4.58; N 2.12; S 14.58; K 5.93). The infra-red spectrum of our specimen proved identical with that reported elsewhere [5] *. Several attempts to prepare the genuine glucoside in crystalline form after deacetylation were unsuccessful.

From seeds of the wall-flower *(Cheiranthus cheiri)* Schneider and Schütz [7] isolated, many years ago, the *iso*thiocyanate glucoside glucocheirolin as a crystalline potassium salt, affording 3-methylsulphonylpropyl mustard oil upon enzymic hydrolysis. Failure to repeat the isolation of crystalline glucoside by the reported procedure prompted the development of the following modification which has afforded crystalline glucocheirolin monohydrate with

* Part XXXIV of this series: *Acta Chem. Scand.* 13 (1959) 144.

** All melting points reported are uncorrected and determined in capillary tubes in an electrically heated block. Microanalyses were performed by Mr. P. Hansen at the Chemical Laboratory of the University of Copenhagen.

* The author is grateful to Dr. W. Wagner for kindly placing the original spectrum at his disposal for comparison.

correct analysis and a rotation value in agreement with that reported *.

A methanolic extract of commercial wallflower seeds was concentrated, dissolved in water and filtered. Addition of potassium thiocyanate caused the precipitation of the quaternary base sinapine as the slightly soluble thiocyanate. The dried residue from the filtrate was subjected to acetylation in the usual way [3] and the crude tetraacetate, in turn, subjected to deacetylation with ammonia in methanol to give the oily glucoside. On recrystallization of this from 80 % ethanol, glucocheirolin monohydrate separated as colourless needles, purified for analysis by two additional recrystallizations.

The intermediate, anhydrous *potassium tetraacetylglucocheirolinate* was obtained from water as thin colourless needles, m. p. 193—194° (decomp.), $[\alpha]_D^{24}$ −18.0° (*c* 1.0, H_2O). (Found: C 35.32; H 4.26; N 2.04; S 14.78. Calc. for $C_{19}H_{28}O_{15}NS_2K$: C 35.33; H 4.37; N 2.17; S 14.89.)

Seeds of *Lunaria annua* L. (= *L. biennis Moench*) have formerly served in this laboratory as a source of *iso*propyl *iso*thiocyanate [8]. The hypothetical glucosidic progenitor of this mustard oil, *glucoputranjivin*, which was independently suggested by Puntambekar [9] as a constituent of the Indian plant *Putranjiva Roxburghii* Wall. *(Euphorbiaceae)*, has now been isolated in form of its crystalline tetraacetate. A crude specimen, isolated from *Lunaria* seeds and acetylated in the usual way, was repeatedly recrystallized from 96 % ethanol to give colourless needles of the anhydrous *potassium tetraacetylglucoputranjivate* with m.p. 180—190° (decomp.), highly dependent on the rate of heating; $[\alpha]_D^{23}$ −15.4° (*c* 1.2, H_2O). (Found: C 38.20; H 4.77; N 2.54. Calc. for $C_{18}H_{26}O_{13}NS_2K$: C 38.08; H 4.62; N 2.47.)

Valuable assistance of Dr. R. Gmelin, Mr. Bo W. Christensen and Mrs. Dorthe Hansen in the present work is gratefully acknowledged. The work is part of investigations supported by *The Danish State Research Foundation*, *The Carlsberg Foundation* and *Kai Hansen's Fond*.

1. Kjær, A. *Handbuch der Pflanzenphysiologie*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1958, vol. IX, p. 73.
2. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
3. Schultz, O.-E. and Wagner, W. *Arch. Pharm.* **288/60** (1955) 525.
4. Schultz, O.-E. and Gmelin, R. *Arch. Pharm.* **287/59** (1954) 404.
5. Wagner, W. *Papierchromatographische Analyse der Senfölglucoside, präparative Darstellung ihrer Acetylderivate und ein Beitrag zu ihrer allgemeinen Struktur* (Diss.) University, Tübingen 1956.
6. Schmid, H. and Karrer, P. *Helv. Chim. Acta* **31** (1948) 1017.
7. Schneider, W. and Schütz, L. A. *Ber.* **46** (1913) 2634.
8. Kjær, A. and Conti, J. *Acta Chem. Scand.* **7** (1953) 1011.
9. Puntambekar, S. V. *Proc. Indian Acad. Sci.* **A 32** (1950) 114.

Received April 2, 1959.

* For our preparation a decomposition range from 140—160° was observed, rather than the m. p. 158—160° reported [7].

Electrical Conductivity of Some Palladium Sulfides and of Silver Palladium Sulfide

H. FISCHMEISTER

Institute of Chemistry, University of Uppsala, Sweden *

The System Pd-S has recently been examined by Grønvold and Røst [1] who found the following compounds stable at room temperature: Pd_4S, $Pd_{2,3}S$, PdS and PdS_2. The system Ag—Pd—S was studied, in 1954, by Raub, Wullhorst and Plate [2]. Only one ternary compound, Ag_2Pd_2S, was detected.

Specimens of $Pd_{2,3}S$ and PdS were kindly supplied by Dr. Grønvold. Pd_4S and Ag_2Pd_3S were prepared by heating briquetted mixtures of the finely powdered elements (c.p. grade) in evacuated, sealed-off silica tubes to 630°C for several days, in accordance with the conditions of formation indicated by the phase studies [2,3]. The samples were crushed, X-ray powder patterns recorded to confirm the completeness of the reaction, and the powders rebriquetted at *ca.* 250 kg/cm² in the form of small bars of 15 × 4 × 3 mm. These were sintered for a week in evacuated silica tubes at 600°C. X-Ray patterns recorded

* Present address: The Swedish Institute of Metal Research (Metallografiska Institutet), Stockholm 26.

correct analysis and a rotation value in agreement with that reported *.

A methanolic extract of commercial wallflower seeds was concentrated, dissolved in water and filtered. Addition of potassium thiocyanate caused the precipitation of the quaternary base sinapine as the slightly soluble thiocyanate. The dried residue from the filtrate was subjected to acetylation in the usual way [3] and the crude tetraacetate, in turn, subjected to deacetylation with ammonia in methanol to give the oily glucoside. On recrystallization of this from 80 % ethanol, glucocheirolin monohydrate separated as colourless needles, purified for analysis by two additional recrystallizations.

The intermediate, anhydrous *potassium tetraacetylglucocheirolinate* was obtained from water as thin colourless needles, m. p. 193—194° (decomp.), $[\alpha]_D^{24}$ −18.0° (*c* 1.0, H_2O). (Found: C 35.32; H 4.26; N 2.04; S 14.78. Calc. for $C_{19}H_{28}O_{15}NS_2K$: C 35.33; H 4.37; N 2.17; S 14.89.)

Seeds of *Lunaria annua* L. (= *L. biennis Moench*) have formerly served in this laboratory as a source of *iso*propyl *iso*thiocyanate [8]. The hypothetical glucosidic progenitor of this mustard oil, *glucoputranjivin*, which was independently suggested by Puntambekar [9] as a constituent of the Indian plant *Putranjiva Roxburghii* Wall. *(Euphorbiaceae)*, has now been isolated in form of its crystalline tetraacetate. A crude specimen, isolated from *Lunaria* seeds and acetylated in the usual way, was repeatedly recrystallized from 96 % ethanol to give colourless needles of the anhydrous *potassium tetraacetylglucoputranjivate* with m.p. 180—190° (decomp.), highly dependent on the rate of heating; $[\alpha]_D^{23}$ −15.4° (*c* 1.2, H_2O). (Found: C 38.20; H 4.77; N 2.54. Calc. for $C_{18}H_{26}O_{13}NS_2K$: C 38.08; H 4.62; N 2.47.)

Valuable assistance of Dr. R. Gmelin, Mr. Bo W. Christensen and Mrs. Dorthe Hansen in the present work is gratefully acknowledged. The work is part of investigations supported by *The Danish State Research Foundation*, *The Carlsberg Foundation* and *Kai Hansen's Fond*.

1. Kjær, A. *Handbuch der Pflanzenphysiologie*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1958, vol. IX, p. 73.
2. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
3. Schultz, O.-E. and Wagner, W. *Arch. Pharm.* **288/60** (1955) 525.
4. Schultz, O.-E. and Gmelin, R. *Arch. Pharm.* **287/59** (1954) 404.
5. Wagner, W. *Papierchromatographische Analyse der Senfölglucoside, präparative Darstellung ihrer Acetylderivate und ein Beitrag zu ihrer allgemeinen Struktur* (Diss.) University, Tübingen 1956.
6. Schmid, H. and Karrer, P. *Helv. Chim. Acta* **31** (1948) 1017.
7. Schneider, W. and Schütz, L. A. *Ber.* **46** (1913) 2634.
8. Kjær, A. and Conti, J. *Acta Chem. Scand.* **7** (1953) 1011.
9. Puntambekar, S. V. *Proc. Indian Acad. Sci.* **A 32** (1950) 114.

Received April 2, 1959.

* For our preparation a decomposition range from 140—160° was observed, rather than the m. p. 158—160° reported [7].

Electrical Conductivity of Some Palladium Sulfides and of Silver Palladium Sulfide

H. FISCHMEISTER

Institute of Chemistry, University of Uppsala, Sweden *

The System Pd-S has recently been examined by Grønvold and Røst [1] who found the following compounds stable at room temperature: Pd_4S, $Pd_{2,3}S$, PdS and PdS_2. The system Ag—Pd—S was studied, in 1954, by Raub, Wullhorst and Plate [2]. Only one ternary compound, Ag_2Pd_2S, was detected.

Specimens of $Pd_{2,3}S$ and PdS were kindly supplied by Dr. Grønvold. Pd_4S and Ag_2Pd_3S were prepared by heating briquetted mixtures of the finely powdered elements (c.p. grade) in evacuated, sealed-off silica tubes to 630°C for several days, in accordance with the conditions of formation indicated by the phase studies [2,3]. The samples were crushed, X-ray powder patterns recorded to confirm the completeness of the reaction, and the powders rebriquetted at *ca.* 250 kg/cm² in the form of small bars of 15 × 4 × 3 mm. These were sintered for a week in evacuated silica tubes at 600°C. X-Ray patterns recorded

* Present address: The Swedish Institute of Metal Research (Metallografiska Institutet), Stockholm 26.

in reflection from the surface of the bars revealed no changes during sintering.

Preliminary measurements indicated very low resistances for all samples, including PdS. Of this latter compound, only one test bar had been made, which was accidentally damaged before accurate measurements could be carried out. The preliminary experiments would put its conductivity in the same order of magnitude as that of the other palladium sulfides. For the other samples, measurements were made both with direct current and at different A. C. frequencies up to 10 kc/sec. They gave practically identical values, indicating that grain boundary resistance had no appreciable influence on the results.

The following values were recorded:

Compound	$\varrho_{20°C}$ * (Ωcm)	$d\varrho/dt$ (Ωcm/degree C)
Pd_4S	3.20×10^{-4}	$+1.05 \times 10^{-6}$
$Pd_{2.2}S$	10.00×10^{-4}	$+1.80 \times 10^{-6}$
Ag_2Pd_3S	1.93×10^{-4}	$+0.22 \times 10^{-6}$

The positive temperature coefficient as well as the low absolute values of the resistivities indicate that these compounds are metallic conductors. For the Pd sulfides, Grønvold and Røst had inferred, from magnetic susceptibility measurements, that they should be essentially nonionic, *i. e.* either metallic or covalent. The present measurements show that the former is the case. As for Ag_2Pd_3S, its high metal content suggested that this compound should be considered as an alloy phase.

I wish to thank Prof. G. Hägg for the facilities placed at my disposal. Thanks are due to Mr. S. Djurle for his assistence with the preparation of the samples, and to Telefon-AB L. M. Ericsson, Stockholm, for the loan of a conductivity bridge.

1. Grønvold, F. and Røst, E. *Acta Chem. Scand.* **10** (1956) 1620.
2. Raub, E., Wullhorst, B. and Plate, W. *Z. Metallk.* **45** (1954) 533.
3. Weibke, F. and Laar, J. *Z. anorg. Chem.* **228** (1935) 49.

* Without correction for porosity, which was of the order of 10 %.

Received April 3, 1959.

A New Crystalline Modification of Cellulose as Revealed by X-Ray Diffractograms of Hydrolyzed Cotton and Wood Pulp

Ø. ELLEFSEN

The Norwegian Pulp and Paper Research Institute, Oslo;

J. GJØNNES and N. NORMAN

Central Institute for Industrial Research, Oslo, Norway

In an attempt to obtain cellulose II with a high degree of crystallinity, it was discovered that surgical cotton as well as mercerized surgical cotton after 2—4 h treatment at 20°C with 40.3 % hydrochloric acid gave X-ray diffractograms which could not be explained exclusively by the hitherto known crystalline modifications of cellulose. A microphotometer curve of such an X-ray diffractogram obtained in an evacuated Guinier camera with Cu$K\alpha$-radiation is shown in Fig. 1a. Shorter or prolonged HCl treatments gave residues containing cellulose II only, as shown by the microphotometercurve in Fig. 1b. Diffractograms like the one in Fig. 1a could also be obtained from wood pulp and ground cotton at a lower acid concentration.

To investigate whether the strong reflections corresponding to the interplanar spacings 6.31 Å, 5.70 Å, 5.13 Å and 3.99 Å in Fig. 1a may be due to sodium cellulose formed during the neutralization, or to cellulose hydrates, oxycelluloses or hemicelluloses, tests were carried out leading to the following results: Flame photometry showed only negligible (0.4 %) amounts of sodium. Drying for 18 h at 105°C gave no observable change in the diffractograms. An infrared spectrogram indicated no bonds other than those characteristic of cellulose, and a chromatogram of the sample showed glucose to be the only monosaccharide present. It should also be mentioned that viscosity measurements for the sample led to a DP-value below 100.

It must, therefore, be concluded that the sample consists of pure cellulose. The few reflections in Fig. 1a are of course insufficient for solving the structure of the unknown phase or phases, but it is reasonable to assume a unit cell and an arrangement of the glucose rings not too different from

in reflection from the surface of the bars revealed no changes during sintering.

Preliminary measurements indicated very low resistances for all samples, including PdS. Of this latter compound, only one test bar had been made, which was accidentally damaged before accurate measurements could be carried out. The preliminary experiments would put its conductivity in the same order of magnitude as that of the other palladium sulfides. For the other samples, measurements were made both with direct current and at different A. C. frequencies up to 10 kc/sec. They gave practically identical values, indicating that grain boundary resistance had no appreciable influence on the results.

The following values were recorded:

Compound	$\varrho_{20°C}$ * (Ωcm)	$d\varrho/dt$ (Ωcm/degree C)
Pd_4S	3.20×10^{-4}	$+1.05 \times 10^{-6}$
$Pd_{2.2}S$	10.00×10^{-4}	$+1.80 \times 10^{-6}$
Ag_2Pd_3S	1.93×10^{-4}	$+0.22 \times 10^{-6}$

The positive temperature coefficient as well as the low absolute values of the resistivities indicate that these compounds are metallic conductors. For the Pd sulfides, Grønvold and Røst had inferred, from magnetic susceptibility measurements, that they should be essentially nonionic, *i. e.* either metallic or covalent. The present measurements show that the former is the case. As for Ag_2Pd_3S, its high metal content suggested that this compound should be considered as an alloy phase.

I wish to thank Prof. G. Hägg for the facilities placed at my disposal. Thanks are due to Mr. S. Djurle for his assistence with the preparation of the samples, and to Telefon-AB L. M. Ericsson, Stockholm, for the loan of a conductivity bridge.

1. Grønvold, F. and Røst, E. *Acta Chem. Scand.* **10** (1956) 1620.
2. Raub, E., Wullhorst, B. and Plate, W. *Z. Metallk.* **45** (1954) 533.
3. Weibke, F. and Laar, J. *Z. anorg. Chem.* **228** (1935) 49.

* Without correction for porosity, which was of the order of 10 %.

Received April 3, 1959.

A New Crystalline Modification of Cellulose as Revealed by X-Ray Diffractograms of Hydrolyzed Cotton and Wood Pulp

Ø. ELLEFSEN

The Norwegian Pulp and Paper Research Institute, Oslo;

J. GJØNNES and N. NORMAN

Central Institute for Industrial Research, Oslo, Norway

In an attempt to obtain cellulose II with a high degree of crystallinity, it was discovered that surgical cotton as well as mercerized surgical cotton after 2—4 h treatment at 20°C with 40.3 % hydrochloric acid gave X-ray diffractograms which could not be explained exclusively by the hitherto known crystalline modifications of cellulose. A microphotometer curve of such an X-ray diffractogram obtained in an evacuated Guinier camera with Cu$K\alpha$-radiation is shown in Fig. 1a. Shorter or prolonged HCl treatments gave residues containing cellulose II only, as shown by the microphotometercurve in Fig. 1b. Diffractograms like the one in Fig. 1a could also be obtained from wood pulp and ground cotton at a lower acid concentration.

To investigate whether the strong reflections corresponding to the interplanar spacings 6.31 Å, 5.70 Å, 5.13 Å and 3.99 Å in Fig. 1a may be due to sodium cellulose formed during the neutralization, or to cellulose hydrates, oxycelluloses or hemicelluloses, tests were carried out leading to the following results: Flame photometry showed only negligible (0.4 %) amounts of sodium. Drying for 18 h at 105°C gave no observable change in the diffractograms. An infrared spectrogram indicated no bonds other than those characteristic of cellulose, and a chromatogram of the sample showed glucose to be the only monosaccharide present. It should also be mentioned that viscosity measurements for the sample led to a DP-value below 100.

It must, therefore, be concluded that the sample consists of pure cellulose. The few reflections in Fig. 1a are of course insufficient for solving the structure of the unknown phase or phases, but it is reasonable to assume a unit cell and an arrangement of the glucose rings not too different from

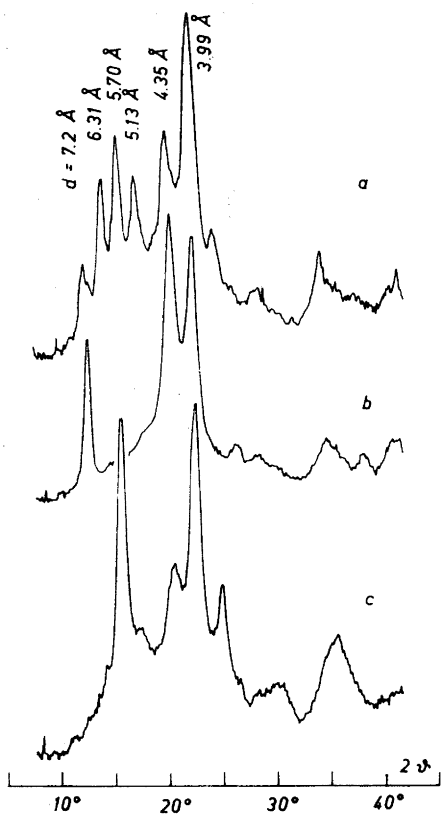


Fig. 1. Microphotometer curves: (a) surgical cotton after 3 hours treatment with HCl, (b) cellulose II, (c) cellulose IV.

cellulose I and II. Utilizing this for indexing the reflections, the three alternatives *A*, *B* and *C* in Table 1 may all be possible. In the three phase alternative *C*, the peak $d = 5.70$ Å and part of the peak $d = 3.99$ Å are ascribed to cellulose IV, a diffractogram of which is shown in Fig. 1c. Unit cell dimensions and density for this modification and for cellulose I and II based on the best crystalline samples prepared in our laboratories are also included in Table 1.

In our experiments the three lines $d = 6.31$ Å, 5.70 Å and 5.13 Å always appeared in the same reciprocal intensity ratio. This makes it likely that only one phase exists besides cellulose II. Of the two-phase alternatives *A* and *B*, the first one gives a rather long *a*-axis and a low density and must be considered the less probable one. Alternative *B* has the cell dimensions of cellulose IV and may look as an ordered form of this modification. Strong (011) and (020) reflections would require a very strict ordering of the cellulose chains with respect to each other along the chain axis in the crystalline lattice, but even then it is questionable whether reflections of the observed strength can be realized.

Further it should be mentioned that the indexing under *A* and *B* in Table 1 would make the relative intensities of the lines very sensitive to preferred orientation. No difference between transmission and reflection diffractograms could be observed, however, even after severe pressing of the samples into thin sheets. Alternative *C* thus seems to have a preference since all the strong reflections here would originate from planes containing the axis of the cellulose chain.

There are also other facts that favour the three phase alternative. Thus the proposed new monoclinic modification has a unit cell closely resembling those of cellulose I and cellulose IV, and has the same density. One would accordingly expect an arrangement of the glucose rings similar to

Table 1. Indices an dcell dimensions for possible cellulose modifications.

Modification	Indices				Cell dimensions				Density (g/cm³)
	d=6.31 Å	d=5.70 Å	d=5.13 Å	d=3.99 Å	a (Å)	b (Å)	c (Å)	(°)	
Alternative A	101	101	020	002	9.08	10.3	8.03	95.9	1.44
» B	011	101	020	002	8.12	10.3	7.99	90	1.61
» C	101		101	002	8.10	10.3	8.16	101.7	1.615
Cellulose IV		101		002	8.12	10.3	7.99	90	1.61
» I					8.20	10.3	7.90	96.7	1.625
» II					8.02	10.3	9.03	117.2	1.62

that in the lattice of cellulose I. For alternative *C* this seems to hold true since the relative intensities of the three predominant reflections (101), (10$\bar{1}$) and (002) are very near the same as for cellulose I.

Attempts have also been made to utilize the other less pronounced peaks in the X-ray diffractograms in order to discriminate between the different possibilities without this leading to any substantially new arguments. Further experimental and theoretical work are in progress. A more comprehensive description of the hydrolysis and the experimental technique will be given elsewhere.

This investigation is part of a research program on the structure of cellulose carried out at the Central Institute for Industrial Research and the Norwegian Pulp and Paper Research Institute. Financial support from the *Royal Norwegian Council for Scientific and Industrial Research* is gratefully acknowledged.

Received March 31, 1959.

Luminescence of Aqueous Solutions of Substances Irradiated with Ionizing Radiation in the Solid State

G. AHNSTROM and G. v. EHRENSTEIN

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Sweden

If we place glucose crystals irradiated with X-rays, gamma rays or with fast neutrons over the window of a sensitive photomultiplier (EMI 9514S) we observe a luminescence. This luminescence is also exhibited by some other substances irradiated in the solid state, *e. g.* sorbitol, amylopectin, saccharose and glycine. Dry seeds of *Agrostis stolonifera* also show luminescence after irradiation with X-rays.

The luminescence disappeared completely in all substances investigated during the first hour after irradiation. However, by dissolving *e. g.* irradiated glucose in water, in air and at room temperature, a new luminescence appeared which, after the lapse of one minute from the start of the dissolution, reached a comparatively constant level. During storage for one day at room temperature the luminescence decays to half of the value observed after one minute. Non-irradiated glucose showed no luminescence when dissolved in water.

Glucose which had been irradiated and stored in air at room temperature for some months, still showed luminescence after dissolving in water. The following experiments have been carried out with glucose which was irradiated three months ago in a reactor with a fast neutron dose of 200 Mrad and a contaminating gamma dose of 200 Mrad.

The water solution of this sugar had a pH of 3, 0.2 mole of acid ($pK_a = 4.2$) had been formed from one mole of glucose. The intensity of luminescence — *i. e.* the number of pulses recorded by a scaler (Ecko N530) — was a function of the pH of the solution. After alkalinization with 0.1 N NaOH the intensity of luminescence increased twentyfold. The luminescence of a solution of non-irradiated glucose was negligible on alkalinization. The absorption spectrum of the acid (pH 3) and the alkaline (pH 12) solution of irradiated glucose showed an absorption peak at 265 mμ, probably due to the presence of dihydroxyacetone [1]. The alkaline solution exhibited a stronger light absorption, thus excluding the possibility of the increase of the luminescence intensity of the alkaline solution being due to a decrease of the light absorption.

The luminescence intensity in aqueous alkaline solutions is directly proportional to the amount of irradiated glucose dissolved and also directly proportional to the radiation dose. This experiment has been performed with ^{60}Co gamma irradiated glucose at different dose levels between 60 and 170 Mrad. It should be noted here that irradiation of crystalline glucose with fast neutrons produced five to seven times more luminescence after dissolving in alkaline solution than irradiation with the same dose of gamma rays from ^{60}Co.

The addition of hydrogen peroxide, benzoylperoxide or dioxan containing hydroperoxides to the alkaline aqueous solution of irradiated glucose increases the luminescence intensity about tenfold. The luminescence of non-irradiated glucose in this system was negligible. Burnt glucose, however, also exhibited a strong luminescence in this system. On addition of trace amounts of Fe^{++} or Cu^{++} to the peroxide-irradiated glucose system at pH 3, the

that in the lattice of cellulose I. For alternative *C* this seems to hold true since the relative intensities of the three predominant reflections (101), (10$\bar{1}$) and (002) are very near the same as for cellulose I.

Attempts have also been made to utilize the other less pronounced peaks in the X-ray diffractograms in order to discriminate between the different possibilities without this leading to any substantially new arguments. Further experimental and theoretical work are in progress. A more comprehensive description of the hydrolysis and the experimental technique will be given elsewhere.

This investigation is part of a research program on the structure of cellulose carried out at the Central Institute for Industrial Research and the Norwegian Pulp and Paper Research Institute. Financial support from the *Royal Norwegian Council for Scientific and Industrial Research* is gratefully acknowledged.

Received March 31, 1959.

Luminescence of Aqueous Solutions of Substances Irradiated with Ionizing Radiation in the Solid State

G. AHNSTROM and G. v. EHRENSTEIN

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Sweden

If we place glucose crystals irradiated with X-rays, gamma rays or with fast neutrons over the window of a sensitive photomultiplier (EMI 9514S) we observe a luminescence. This luminescence is also exhibited by some other substances irradiated in the solid state, *e. g.* sorbitol, amylopectin, saccharose and glycine. Dry seeds of *Agrostis stolonifera* also show luminescence after irradiation with X-rays.

The luminescence disappeared completely in all substances investigated during the first hour after irradiation. However, by dissolving *e. g.* irradiated glucose in water, in air and at room temperature, a new luminescence appeared which, after the lapse of one minute from the start of the dissolution, reached a comparatively constant level. During storage for one day at room temperature the luminescence decays to half of the value observed after one minute. Non-irradiated glucose showed no luminescence when dissolved in water.

Glucose which had been irradiated and stored in air at room temperature for some months, still showed luminescence after dissolving in water. The following experiments have been carried out with glucose which was irradiated three months ago in a reactor with a fast neutron dose of 200 Mrad and a contaminating gamma dose of 200 Mrad.

The water solution of this sugar had a pH of 3, 0.2 mole of acid ($pK_a = 4.2$) had been formed from one mole of glucose. The intensity of luminescence — *i. e.* the number of pulses recorded by a scaler (Ecko N530) — was a function of the pH of the solution. After alkalinization with 0.1 N NaOH the intensity of luminescence increased twentyfold. The luminescence of a solution of non-irradiated glucose was negligible on alkalinization. The absorption spectrum of the acid (pH 3) and the alkaline (pH 12) solution of irradiated glucose showed an absorption peak at 265 mμ, probably due to the presence of dihydroxyacetone [1]. The alkaline solution exhibited a stronger light absorption, thus excluding the possibility of the increase of the luminescence intensity of the alkaline solution being due to a decrease of the light absorption.

The luminescence intensity in aqueous alkaline solutions is directly proportional to the amount of irradiated glucose dissolved and also directly proportional to the radiation dose. This experiment has been performed with ^{60}Co gamma irradiated glucose at different dose levels between 60 and 170 Mrad. It should be noted here that irradiation of crystalline glucose with fast neutrons produced five to seven times more luminescence after dissolving in alkaline solution than irradiation with the same dose of gamma rays from ^{60}Co.

The addition of hydrogen peroxide, benzoylperoxide or dioxan containing hydroperoxides to the alkaline aqueous solution of irradiated glucose increases the luminescence intensity about tenfold. The luminescence of non-irradiated glucose in this system was negligible. Burnt glucose, however, also exhibited a strong luminescence in this system. On addition of trace amounts of Fe^{++} or Cu^{++} to the peroxide-irradiated glucose system at pH 3, the

intensity of the chemiluminescence increases even more [2]. With our present photomultipier assembly it was not possible to obtain a time dependency curve of this reaction.

In order to elucidate this catalytic effect of irradiated glucose on the decomposition of hydrogen peroxide, and organic peroxides and hydroperoxides, we tested some compounds which possibly arise by the action of ionizing radiation on glucose [1]. The relative luminescence intensities of the non-irradiated substances investigated in the alkaline hydrogen peroxide system were: glucose 0, glyceraldehyde 100, dihydroxyacetone 7, arabinose 0, glucuronic acid 1. From these data it can be tentatively concluded that glyceraldehyde may possibly be one of the substances responsible for the luminescence of irradiated glucose. This hypothesis is strengthened by the fact that non-irradiated glyceraldehyde exhibits a strong luminescence in aqueous alkaline solution without added peroxide.

Infrared spectroscopy showed that the irradiation induced carboxyl groups already in the solid glucose, while carbonyl groups are not formed until the irradiated glucose reacts with water.

By means of electron spin resonance, it has been shown that long-lived free radicals are induced by X-rays in different substances and in living material [3-5 and quoted literature]. It has been strongly indicated — that at least part of the biological radiation effects are produced *via* free radicals. A portion of the radiation damage in dry plant seeds is latent and can be modified during storage by, *e. g.*, temperature and oxygen. However, when the water content of the seeds rises, *e. g.* during germination, the damage becomes manifest, (*cf.* discussion) [7].

The observations described in this communication may give a method by which we hope to be able to elucidate the role played by water and oxygen in radiobiological processes. The phenomena studied in this communication are also relevant in the discussion concerning the radiation preservation of foodstuffs and the possible mutagenicity of these. It is worthwhile in this connection to mention that mutations have been observed when biologic material — barley and wheat seeds — is treated with the aqueous solutions of irradiated substances, *e. g.* glucose, glycine [8], agar [9].

The investigations are being continued on a quantitative and qualitative basis, including emission spectroscopy during and after irradiation with ionizing radiation.

The authors' thanks are due to Dr. L. Ehrenberg for his promoting interest in this investigation.

1. Phillips, G. O., Mattok, G. L. and Moody, G. J. *Second U.N. Int. Conf. Peaceful Uses Atomic Energy*, Geneva 1958, P/47.
2. Mayneord, W. V., Anderson, W., Evans, H. D. and Rosen, D. *Radiation Research* **3** (1955) 379.
3. Zimmer, K. G., Ehrenberg, L. and Ehrenberg, A. *Strahlentherapie* **103** (1957) 3.
4. Ehrenberg, A. and Ehrenberg, L. *Arkiv Fysik* **14** (1958) 133.
5. Zimmer, K. G. *Radiation Research, Suppl.* **1** (1959) 519.
6. Sparrman, B., Ehrenberg, L. and Ehrenberg, A. *Acta Chem. Scand.* **13** (1959) 199.
7. Ehrenberg, L. Radiation Research, *Suppl.* **1** (1959) 102.
8. Ehrenberg, L. *et al. Unpublished.*
9. Natarajan, A. T. and Swaminathan, M. S. *Indian J. Gen. Plant Breed*, **18** (1958).

Received April 15, 1959.

Use of Butyl Acetate in Determination of Sialic Acid

T. MIETTINEN
and I. -T. TAKKI-LUUKKAINEN

Department of Medical Chemistry, University of Helsinki, Finland

Determination of sialic acid can be performed with several reactions: the Bial [1,2], direct Ehrlich [3], tryptophane-perchloric acid [4], diphenylamine [5,6] and sulphuric acid — acetic acid [7] reactions. Orcinol used in Bial's reaction has been replaced by resorcinol [8]. In all orcinol and resorcinol reactions presented in the literature the colour is extracted by amyl alcohol. This solvent extracts very effectively the developed pigments, but its disadvantages are the long procedure of purification, the necessity of centrifugation after extraction, and especially its ability to extract also the false pigments produced by other carbohydrates. Ion exchange resins and dichromatic readings are used to eliminate

intensity of the chemiluminescence increases even more [2]. With our present photomultipier assembly it was not possible to obtain a time dependency curve of this reaction.

In order to elucidate this catalytic effect of irradiated glucose on the decomposition of hydrogen peroxide, and organic peroxides and hydroperoxides, we tested some compounds which possibly arise by the action of ionizing radiation on glucose [1]. The relative luminescence intensities of the non-irradiated substances investigated in the alkaline hydrogen peroxide system were: glucose 0, glyceraldehyde 100, dihydroxyacetone 7, arabinose 0, glucuronic acid 1. From these data it can be tentatively concluded that glyceraldehyde may possibly be one of the substances responsible for the luminescence of irradiated glucose. This hypothesis is strengthened by the fact that non-irradiated glyceraldehyde exhibits a strong luminescence in aqueous alkaline solution without added peroxide.

Infrared spectroscopy showed that the irradiation induced carboxyl groups already in the solid glucose, while carbonyl groups are not formed until the irradiated glucose reacts with water.

By means of electron spin resonance, it has been shown that long-lived free radicals are induced by X-rays in different substances and in living material [3-5 and quoted literature]. It has been strongly indicated — that at least part of the biological radiation effects are produced *via* free radicals. A portion of the radiation damage in dry plant seeds is latent and can be modified during storage by, *e. g.*, temperature and oxygen. However, when the water content of the seeds rises, *e. g.* during germination, the damage becomes manifest, (*cf.* discussion) [7].

The observations described in this communication may give a method by which we hope to be able to elucidate the role played by water and oxygen in radiobiological processes. The phenomena studied in this communication are also relevant in the discussion concerning the radiation preservation of foodstuffs and the possible mutagenicity of these. It is worthwhile in this connection to mention that mutations have been observed when biologic material — barley and wheat seeds — is treated with the aqueous solutions of irradiated substances, *e. g.* glucose, glycine [8], agar [9].

The investigations are being continued on a quantitative and qualitative basis, including emission spectroscopy during and after irradiation with ionizing radiation.

The authors' thanks are due to Dr. L. Ehrenberg for his promoting interest in this investigation.

1. Phillips, G. O., Mattok, G. L. and Moody, G. J. *Second U.N. Int. Conf. Peaceful Uses Atomic Energy*, Geneva 1958, P/47.
2. Mayneord, W. V., Anderson, W., Evans, H. D. and Rosen, D. *Radiation Research* **3** (1955) 379.
3. Zimmer, K. G., Ehrenberg, L. and Ehrenberg, A. *Strahlentherapie* **103** (1957) 3.
4. Ehrenberg, A. and Ehrenberg, L. *Arkiv Fysik* **14** (1958) 133.
5. Zimmer, K. G. *Radiation Research, Suppl.* **1** (1959) 519.
6. Sparrman, B., Ehrenberg, L. and Ehrenberg, A. *Acta Chem. Scand.* **13** (1959) 199.
7. Ehrenberg, L. Radiation Research, *Suppl.* **1** (1959) 102.
8. Ehrenberg, L. *et al. Unpublished.*
9. Natarajan, A. T. and Swaminathan, M. S. *Indian J. Gen. Plant Breed*, **18** (1958).

Received April 15, 1959.

Use of Butyl Acetate in Determination of Sialic Acid

T. MIETTINEN
and I. -T. TAKKI-LUUKKAINEN

Department of Medical Chemistry, University of Helsinki, Finland

Determination of sialic acid can be performed with several reactions: the Bial [1,2], direct Ehrlich [3], tryptophane-perchloric acid [4], diphenylamine [5,6] and sulphuric acid — acetic acid [7] reactions. Orcinol used in Bial's reaction has been replaced by resorcinol [8]. In all orcinol and resorcinol reactions presented in the literature the colour is extracted by amyl alcohol. This solvent extracts very effectively the developed pigments, but its disadvantages are the long procedure of purification, the necessity of centrifugation after extraction, and especially its ability to extract also the false pigments produced by other carbohydrates. Ion exchange resins and dichromatic readings are used to eliminate

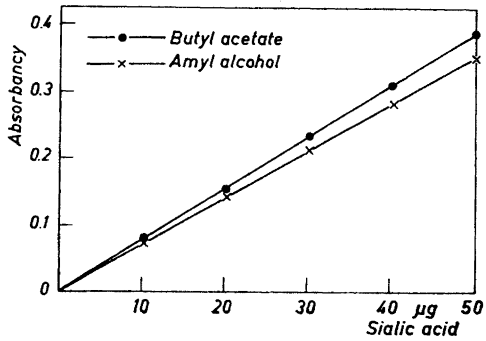


Fig. 1. Standard curves of N-acetylsialic acid read at 580 mμ against the blank. — ● — ● — Extracted with 4 ml of butyl acetate. × — × — × Extracted with 4 ml of amyl alcohol.

this last mentioned error [2,3,9]. The purpose of this work was to find an extracting solvent possessing as little of the mentioned disadvantages as possible.

The determination of sialic acid was performed by the resorcinol reaction [8], using as standard N-acetylsialic acid.* Of the many tested organic solvents, only butyl acetate, in addition to amyl alcohol, was found to be suitable. When used in 100 % concentration its ability to extract was negligible, but after addition of butyl alcohol this capacity was increased. For this reason *Butylium aceticum 85 percent normal ("Merck")* was chosen for use as such. Some minutes after extraction this solvent separates as crystal-clear layer without centrifugation also when proteins are present. The corresponding determinations were performed with both amyl alcohol and butyl acetate, using 4 ml of each solvent. The photoelectric readings were made with the Beckman Spectrophotometer model B in 10 mm cells.

It is seen from Fig. 1 that butyl acetate extracts increasing amounts of N-acetylsialic acid correctly. The two curves deviate because amyl alcohol dissolves more of the

* The N-acetylsialic acid was kindly presented to us by Dr. Lars Svennerholm, M. D., Gothenburg, Sweden.

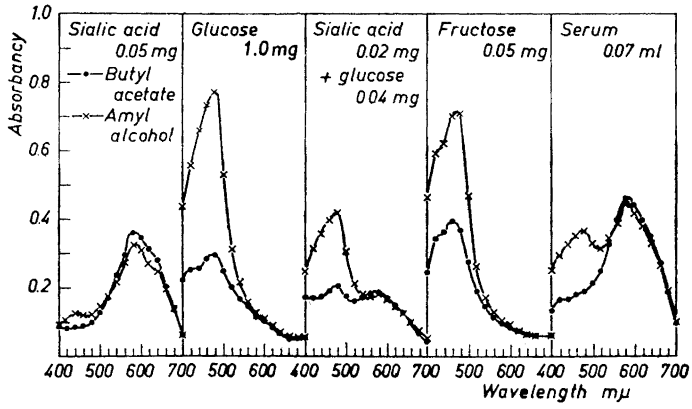


Fig 2. Absorption curves of N-acetylsialic acid, glucose, mixture of glucose and N-acetylsialic acid, fructose and serum without precipitation of proteins. Readings against reagent blank.

water layer, resulting in lower extinctions. It was observed that when the final volume in the reaction increased from 2 to 8 ml and the extraction was performed with 4 ml in each case, the absorbancy was somewhat smaller in greater volumes when butyl acetate was used. With amyl alcohol it remained constant. Thus butyl acetate seems to have a somewhat lower extracting capacity. This is, however, sufficient if the volume used in the reaction is 2—4 ml.

The shapes of the absorption curves in Fig. 2 are quite similar for both solvents, the maximum being at 580 mμ. At shorter wavelengths there is a slight difference, however. Examples of absorption spectra produced by carbohydrates (Fig. 2) are those of fructose and glucose. These substances have their maxima around 480 mμ. As is seen from the figure, butyl acetate has a remarkably lower peak at this point. Thus butyl acetate does not extract all the disturbing pigments, which is apparent already macroscopically from the strikingly yellow colour of the water layer after extraction if false pigments are present. Also at 580 mμ the absorbancy of glucose is smaller in the butyl acetate extraction. One mg of glucose corresponds to about 15 μg of sialic acid when butyl acetate is used, and to 21 μg with amyl alcohol.

From the absorption curves for a mixture of glucose and sialic acid and for serum rich in disturbing substances it is seen that butyl acetate leaves most of the disturbing pigments in the water layer.

1. Klenk, E. and Langerbeins, H. *Z. physiol. Chem. Hoppe-Seyler.* **270** (1941) 185.
2. Svennerholm, L. *Arkiv Kemi* **10** (1956) 577.
3. Werner, I. and Odin, L. *Acta Soc.Med. Upsaliensis* **57** (1952) 230.
4. Seibert, F. B., Pfaff, M. L. and Seibert, M. V. *Arch. Biochem.* **18** (1948) 279.
5. Dische, Z. *Mikrochemie* **7** (1929) 33.
6. Dische, Z. *Mikrochemie* **8** (1930) 4.
7. Hess, E. L., Coburn, A. F., Bates, R. C. and Murphy, P. *J. Clin. Invest.* **36** (1957) 449.
8. Svennerholm, L. *Biochim. et Biophys. Acta* **24** (1957) 604.
9. Svennerholm, L. *Acta Chem. Scand.* **12** (1958) 547.

Received March 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 859–863

The Reaction between Ethylidenemalonic Ester and *o*-Methoxyphenylmagnesium Iodide

GUST.-AD. HOLMBERG

Institutet för organisk kemi, Åbo Akademi, Åbo, Finland

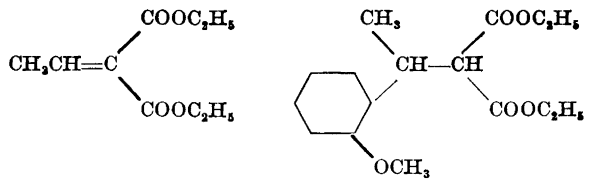
1-(*o*-Methoxyphenyl)ethylmalonic ester (II) and α-*o*-methoxybenzoyl-β-*o*-methoxyphenylbutyric ester (III) are formed in the reaction between ethylidenemalonic ester (I) and *o*-methoxyphenylmagnesium iodide. The latter reaction product has not been isolated as a pure compound, but its presence has been demonstrated by isolation of 1,3-di-*o*-methoxyphenyl-1-butanone (IV) (as its 2,4-dinitrophenylhydrazone) from the saponification products.

The identity of the isolated substances has been demonstrated by synthesis.

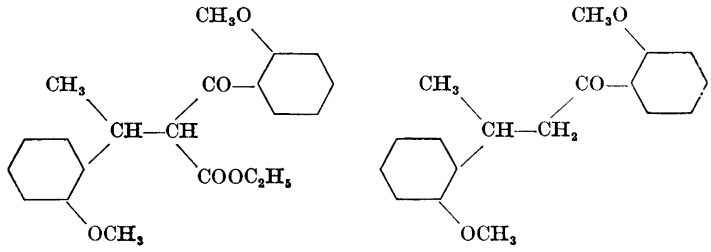
A preliminary examination of the reactions between ethylidenemalonic ester (I) and some aliphatic and aromatic Grignard reagents has shown that the main reaction seems to be 1,4-addition to the conjugated system [1].

When *o*-methoxyphenylmagnesium iodide was allowed to react with the unsaturated malonic ester, substances were found among the reaction products that could not have been formed by 1,4-addition only. The separation of the products was, however, difficult but some separation was attained by chromatography on alumina. The products were applied to the column in tetrachloromethane and the development and elution were performed successively with tetrachloromethane, ether, an ether-ethanol mixture and ethanol. The "normal" reaction product, 1-(*o*-methoxyphenyl)ethylmalonic ester (II), was eluted by tetrachloromethane. No pure product could be isolated from the ether fraction, but on saponification of the ether extract potassium carbonate and a neutral oil were obtained. The latter gave with 2,4-dinitrophenylhydrazine the 2,4-dinitrophenylhydrazone of 1,3-di-*o*-methoxyphenyl-1-butanone (IV).

This result indicates that the mixture of reaction products contains α-*o*-methoxybenzoyl-β-*o*-methoxyphenylbutyric ester (III), which is formed by 1,4-addition combined with a substitution of a methoxyphenyl group for an ethoxy group. Nothing can, of course, be concluded from the experimental results about the mechanisms leading to the above butyric ester.



I II



III IV

A similar double reaction between a Grignard reagent and an α,β-unsaturated ester has been reported by, *e.g.*, Kohler [2], who isolated α-benzhydrylpropiophenone as a by-product of the reaction between phenylmagnesium bromide and α-methylcinnamic ester.

When the ratio of the ethylidenemalonic ester to the Grignard reagent was 1:1.5, about 35 % of the ester had reacted to form 1-*o*-methoxyphenylethylmalonic ester. When the quantity of the Grignard reagent was increased so that the ratio was 1:2, about 50 % reacted in the same way. It was concluded from the quantity of potassium carbonate formed in the saponfication of the butyric ester derivative in the latter case that at least 19.6 % of the ethylidenemalonic ester had reacted twice with the Grignard reagent.

The identity of the reaction products was established by synthesis. 1-*o*-methoxyphenylethylmalonic ester was obtained in good yield by reacting methylmagnesium iodide with *o*-methoxybenzylidenemalonic ester. The same malonic and barbituric acids were obtained from this ester and the above ester. The malonic acid was decarboxylated to β-*o*-methoxyphenylbutyric acid, which was converted into the corresponding amide. This substance was dehydrated by a modification of a method of Stephens, Bianco, and Pilgrim [3] to form β-*o*-methoxyphenylbutyronitrile, which by a reaction with *o*-methoxyphenylmagnesium iodide gave 1,3-di-*o*-methoxyphenyl-1-butanone.

EXPERIMENTAL

o-*Methoxybenzylidenemalonic ester.* The yield of this substance [4] from the reaction between *o*-methoxybenzaldehyde and malonic ethyl ester in the presence of piperidine was improved and the reaction time shortened to 5 h by adding a small quantity of *o*-methoxybenzoic acid (1 g/100 g malonic ester) to the reaction mixture. The yield of material boiling at 207—209°/11 mm was 84 %. Before the ester was used in the following synthesis, it was twice recrystallized from ligroin.

1-(o-Methoxyphenyl) ethylmalonic ester. A solution of *o*-methoxybenzylidenemalonic ester (27.8 g) in dry ether (100 ml) was gradually added with cooling to a Grignard reagent prepared from magnesium (2.88 g) and methyl iodide (17.04 g) in dry ether (55 ml). During this addition two layers usually formed. Only when the cooling was less effective, the addition resulted in a homogeneous solution, which, however, separated into two layers on cooling. When the addition was complete, the reaction mixture was stirred for 20 minutes before it was poured into a mixture consisting of hydrochloric acid (70 ml), water (100 ml) and ice (about 100 g). After shaking, the organic phase was washed with water and a dilute potassium carbonate solution. The acid water solution was extracted once more with ether and this ether was also washed with water and potassium carbonate before it was combined with the main portion. After drying with sodium sulphate, the ether was evaporated and the residue distilled under reduced pressure. The yield of the ester, b.p. 183—183.5°/10 mm, was 89 % (26.19 g). (Found: C 65.10; H 7.57. Calc. for $C_{16}H_{22}O_5$: C 65.29; H 7.53.)

The same percentage yield was obtained from a larger batch (0.3 mole).

1-(o-Methoxyphenyl) ethylmalonic acid. 1-*o*-Methoxyphenylethylmalonic ester (37.66 g) was saponified with a solution of potassium hydroxide (38 g) in a mixture of water (40 ml) and ethanol (450 ml) during 5 h on a boiling water bath. The mixture was then kept in a refrigerator over night. The crystals (37.08 g) of potassium 1-*o*-methoxyphenylethylmalonate monohydrate were filtered off. (Found: H_2O 5.37. Calc. for $C_{12}H_{13}O_5K_2 \cdot H_2O$: 5.42.) This salt was dissolved in water (200 ml) and the solution acidified. The precipitated 1-*o*-methoxyphenylethylmalonic acid (25.04 g) melted at about 170° with evolution of gas. (Found: C 60.39; H 5.93. Calc. for $C_{12}H_{14}O_5$: C 60.50; H 5.92.)

The alkaline ethanolic filtrate was evaporated on a boiling water bath. The crystals that had formed on cooling were filtered and washed with ether. They were dissolved in water and the solution acidified. In this way a further quantity (3.56 g) of the malonic acid was obtained. The total yield of the acid was 93.8 %.

β-(o-Methoxyphenyl)butyric acid. 1-*o*-Methoxyphenylethylmalonic acid (28.5 g) was decarboxylated by heating in an oil bath at 170—180° for 15 min. The residue (23.0 g) was distilled under reduced pressure. In this way, pure *β-o*-methoxyphenylbutyric acid, b.p. 180—181°/15 mm, was obtained in a yield of 94.5 % (21.9 g). On standing and treatment with ligroin, the acid solidified and melted at 49—50°. The melting and boiling points correspond well with those reported by Lauer and Hansen [5], who prepared the acid by another method.

β-(o-Methoxyphenyl) butyramide. A mixture of *β-o*-methoxyphenylbutyric acid (22.8 g), thionyl chloride (20 ml) and benzene (40 ml) was heated on a boiling water bath for half an hour. The solvent and the excess of thionyl chloride were then distilled off (finally under reduced pressure). The residue, crude *β-o*-methoxyphenylbutyryl chloride, was dissolved in benzene (75 ml). This solution was gradually added to benzene (125 ml) through which dry ammonia was led at such a rate that an excess of ammonia was present during the whole addition. The flask was at the same time cooled with cold water. Next day the mixture was filtered and the solid material dissolved in boiling water. When the solution cooled, crystals of *β-o*-methoxyphenylbutyramide (16.45 g) separated. After recrystallization from water the substance melted at 127—128°. (Found: N 7.37. Calc. for $C_{11}H_{15}NO_2$: N 7.25.)

β-(o-Methoxyphenyl)butyronitrile. *β-o*-Methoxyphenylbutyramide (19.76 g) was dissolved in pyridine (90 ml), and benzenesulphonyl chloride (31.0 g) was added. After the exothermic reaction was over, the reaction flask was placed in warm water for 20 min. The mixture was then poured into water and the oil taken up in ether. The ether solution was shaken with dilute hydrochloric acid and potassium bicarbonate solution and finally dried with sodium sulphate. After evaporation of the ether, the residue was distilled under reduced pressure. The yield of *β-o*-methoxyphenylbutyronitrile, b. p. 148—149°/10.5 mm, was 81.5 % (14.60 g). (Found: N 8.03. Calc. for $C_{11}H_{13}NO$: N 7.99.)

2,4-Dinitrophenylhydrazone of 1,3-di-o-methoxyphenyl-1-butanone. A solution of *β-o*-methoxyphenylbutyronitrile (7.80 g) in dry ether (50 ml) was gradually added with cooling to a Grignard reagent prepared from magnesium (1.60 g) and *o*-iodoanisole (15.64 g) in dry ether (50 ml). During the addition, the reaction mixture separated into two layers. After all the nitrile had been added, the mixture was kept for one hour at room temperature. When it was then poured into an excess of dilute hydrochloric acid, a substance

(probably the ketimino hydrochloride) insoluble in both ether and water separated. Steam was led through the reaction mixture for one hour in order to hydrolyze the formed ketimine. The remaining oil was taken up into ether, in which it was readily soluble, and the ether solution washed with dilute sodium hydroxide. After drying with sodium sulphate, the ether was evaporated. An oil (11.06 g) remained in the flask.

A sample of this oil (1.36 g) was treated with 2,4-dinitrophenylhydrazine (0.53 g) in dilute ethanolic sulphuric acid. The quantity of hydrazine was intentionally chosen small in order to facilitate the later purification of the reaction product. On boiling yellow crystals (1.13 g) of 2,4-dinitrophenylhydrazone of 1,3-di-*o*-methoxyphenyl-1-butanone separated from the solution. After recrystallization from ethanol, the hydrazone melted at 158—159°. (Found: N 11.92. Calc. for $C_{24}H_{24}N_4O_6$: N 12.06.)

1,3-Di-*o*-methoxyphenyl-1-butanone was isolated from the above oily reaction product. It boiled at 219—221°/8.5 mm, but was not qute pure.

5-(1'-o-Methoxyphenylethyl)barbituric acid. Urea (1.44 g) and 1-*o*-methoxyphenylethylmalonic ester (5.88 g) were added to a sodium ethoxide solution prepared from sodium (0.55 g) and absolute ethanol (30 ml). The mixture was boiled for 8 h on a boiling water bath. The precipitate formed was filtered, washed with a small amount of absolute ethanol, and dissolved in water. The dihydrate of the barbituric acid (3.05 g) precipitated on adding hydrochloric acid. The ethanol was distilled off from the ethanolic filtrate, the residue treated with water and ether, and impure dihydrate of the barbituric acid (1.40 g) precipitated with acid from the aqueous phase. The crude products were repeatedly recrystallized from aqueous ethanol. The dihydrate melts at 92—94° on rapid heating and at 154—155° on slow heating. (Found: N 9.29; H_2O 12.24. Calc. for $C_{13}H_{14}N_2O_4 \cdot 2\,H_2O$: N 9.39; H_2O 12.08.) When the dihydrate is boiled with benzene or heated on a Kofler Heizbank at 70°, the anhydrous barbituric acid is formed. It melts sharply at 154—155°. (Found: N 10.60. Calc. for $C_{13}H_{14}N_2O_4$: N 10.68.) When the anhydrous barbituric acid is recrystallized from aqueous ethanol, the dihydrate is formed.

1-(o-Methoxyphenyl)ethylmalonic p-nitrobenzyl ester. 1-*o*-Methoxyphenylethylmalonic acid was characterized by preparing its *p*-nitrobenzyl ester, m. p. 144—145°, according to Reid [6]. (Found: N 5.58. Calc. for $C_{26}H_{24}N_2O_9$: N 5.51.)

Action of o-methoxyphenylmagnesium iodide on ethylidenemalonic ester. When 0.1 mole of ethylidenemalonic ester was allowed to react with 0.15 mole of *o*-methoxyphenylmagnesium iodide as previously described [1], no pure substance could be isolated by distillation under reduced pressure. Only impure 1-*o*-methoxyphenylethylmalonic ester was obtained in a yield of about 35 %. If the ratio of the ester to the Grignard reagent was changed to 1:2, a yield corresponding to about 50 % was obtained. The impurities seem to result from the pyrolysis of other reaction products than 1-*o*-methoxyphenylethylmalonic ester.

In order to obtain a sufficiently large quantity of the reaction product mixture, ethylidenemalonic ester (52.46 g) in dry ether (85 ml) was gradually added, with cooling, to a Grignard reagent prepared from magnesium (13.56 g) and *o*-iodoanisole (132 g) in dry ether (260 ml). The molar ratio of the ester to the Grignard reagent was thus 1:2. After the addition had been completed, the mixture was stirred for 20 min. The two layers formed were then poured into a mixture of hydrochloric acid, water and ice. After shaking, the organic phase was washed with water and potassium carbonate solution. After drying with sodium sulphate and evaporating the ether, an oil (105 g) was obtained.

After preliminary experiments the following separation method was considered most effective.

A sample of the oil (10.80 g) was dissolved in tetrachloromethane (50 ml). The solvent was then distilled off in order to remove any ether remaining in the oil. The residue was again dissolved in tetrachloromethane (50 ml) and this solution was transferred to a large chromatographic column consisting of standardized alumina (200 g; Riedel-de Haën A.G.) and tetrachloromethane. The chromatogram was developed with tetrachloromethane (650 ml) and the elution was performed first with ether (300 ml), then with ether and ethanol (1:1; 300 ml) and finally with ethanol (600 ml). After the solvents had been evaporated, it was found that the tetrachloromethane fraction contained 48.8 % (5.28 g) of the original oil, the ether fraction 35.4 % (3.83 g), the ether-ethanol fraction 6.6 % (0.71 g) and the ethanol fraction 1.7 % (0.18 g). The substance lost was 7.4 % (0.80 g).

When the combined tetrachloromethane fractions from two runs (22.03 g of the original oil in all) were worked up, a substance (8.61 g) boiling at 186—189°/12 mm was

obtained. The substance was in every respect identical with 1-*o*-methoxyphenylethylmalonic ester. The amount corresponds to a yield of about 49 % calculated on ethylidenemalonic ester. The forerun (1.36 g) in the distillation had a smell of anisole, but was not examined further.

Experiments conducted to separate the substances in the ether fraction chromatographically were not successful. A sample (17.81 g) of the combined residues was therefore examined in the following way. It was dissolved in ethanol (100 ml) and the solvent distilled off to drive away any remaining ether. The substance was then dissolved in ethanol (300 ml), a solution of potassium hydroxide (20 g) in water (20 ml) added, and the mixture heated on a boiling water bath. After 4 h it was cooled and kept for one night in a refrigerator. The mixture was filtered and the solid substance dissolved in water. By titrimetric analysis it was found that the substance was potassium carbonate (3.65 g in all). The ethanol was evaporated from the alkaline ethanolic filtrate and the residue treated with ether and water. After the evaporation of the solvent from the ether phase, an oil (8.51 g) was obtained. When this oil was treated with 2,4-dinitrophenylhydrazine, an impure hydrazone (8.00 g) was formed. After several recrystallizations from ethanol it melted at 158—159° and was identical with the above-mentioned 2,4-dinitrophenylhydrazone of 1,3-di-*o*-methoxyphenyl-1-butanone. On acidification of the aqueous phase, a resinous substance (5.70 g) was obtained. No pure substance has yet been isolated from it.

The ether-ethanol and the ethanol chromatographic fractions yielded no pure substance.

REFERENCES

1. Holmberg, G. A. *Acta Chem. Scand.* **12** (1958) 229.
2. Kohler, E. P. *Am. Chem. J.* **36** (1906) 529.
3. Stephens, C. R., Bianco, E. J. and Pilgrim, F. J. *J. Am. Chem.Soc.* **77** (1955) 1701.
4. Holmberg, G. A. *Acta Acad. Aboensis, Math. et Phys.* **16** (1949) No. 6.
5. Lauer, V. M. and Hansen, L. I. *J. Am. Chem. Soc.* **61** (1939) 3039.
6. Reid, E. E. *J. Am. Chem. Soc.* **39** (1917) 124.

Received February 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 864–868

The Acid Hydrolysis of Alkoxymethyl Ureas

FRODE NORDHØY and JOHN UGELSTAD

Institute of Industrial Chemistry, Norges tekniske høgskole, Trondheim, Norway

The hydrolysis of alkoxymethyl ureas in acid solution has been investigated kinetically. The hydrolysis leads to the formation of methylol urea, which in turn will decompose to give formaldehyde and urea. The hydrolysis of the ether is a very fast reaction compared to the subsequent decomposition of the methylol compound. Values for the specific catalytic constant of H_3O^+ are given for some ethers with different ether-alkyl groups. Increased branching of the alkyl group increases the rate of hydrolysis. Ethers of methylol compounds of ordinary carboxylic acid amides are much more stable to acid hydrolysis than the ethers from methylolurea.

The alkyl ethers of methylolurea decompose in acid solution, leading to the liberation of free formaldehyde. From the kinetic experiments described below evidence is obtained that the reaction proceeds as follows:

$$NH_2CONHCH_2OR + H_2O \rightarrow NH_2CONHCH_2OH + ROH$$

$$NH_2CONHCH_2OH \rightarrow NH_2CONH_2 + CH_2O$$

In the first step the ether is hydrolysed giving methylolurea and alcohol. In the next step the methylolurea decomposes into urea and formaldehyde. The latter reaction is known to be acid catalysed [1]. In the above scheme the first step, the ether hydrolysis, is a much faster reaction than the second. Formerly published results on the rate of the acid catalysed decomposition of alkoxymethyl ureas [2], where the reaction has been followed by measurement of free formaldehyde formed, have, in fact, given the rate of the decomposition of methylolurea, the second step in the above scheme.

KINETIC INVESTIGATIONS

The kinetic investigations were performed in dilute water solutions (0.01—0.03 mole/l of ether). The pH was adjusted by use of acetic acid-acetate buffers or by addition of strong acid (HNO_3). In each experiment the ionic strength was adjusted to 0.2 by the addition of KNO_3. To obtain the rate of

hydrolysis of the ether, step 1 in the above scheme, it is necessary to analyse samples from the reaction mixture for the sum of free formaldehyde and formaldehyde bound as methylol urea (= ether converted). This was achieved by transferring the samples into a solution containing excess KCN and enough NaOH to bring the pH to 13. Under these conditions the methylol urea is rapidly decomposed into urea and formaldehyde, and the latter reacts practically spontaneously with KCN. After 30 minutes at 25°C the excess KCN is titrated with $Hg(NO_3)_2$ [3].

Use of higher alkalinity to speed up the rate of decomposition of the methylol urea is not advisable as the hydrolysis of the ethers themselves then becomes more pronounced [4]. Even at pH 13 the alkoxymethyl ureas will decompose to a small degree which has to be taken into consideration in calculating the rate constants as described later.

The hydrolysis of the ethers was followed up to about 75 % conversion. The rate of the reverse reaction of step 1 could be neglected under the experimental conditions.

The reaction was found to be of 1st order with regard to ether. Values for the specific catalytic constant of H_3O^+ for different ethers are given in Table 1.

The $[H_3O^+]$ values of the acetic acid-acetate solutions were calculated from the equation:

$$[H_3O^+_{(a)}] = K_a \cdot \frac{[HA]}{[A^-]} \Big/ \frac{f_{H^+} \cdot f_{A^-}}{f_{HA}}$$

where f are activity coefficients. The value of the term $f_{H^+} \cdot f_{A^-} / f_{HA}$ was taken to be 0.60 at the given ionic strength = 0.2 [5].

Table 1. Specific catalytic constants of H_3O^+ for the hydrolysis of some alkoxymethyl ureas and methoxymethyl benzamide.

	$k_{H_3O^+}$ (l · mole^{-1}sec^{-1})	Temp. °C
$NH_2CONHCH_2OCH_3$	2.8	25
»	12.2	40
$NH_2CONHCH_2OCH_2CH_3$	3.9	25
$NH_2CONHCH_2OCH_2CH_2CH_3$	3.9	25
$NH_2CONHCH_2OCH(CH_3)_2$	7.3	25
$C_6H_5CONHCH_2OCH_3$	7.3×10^{-3}	40

As seen from the table the rate of hydrolysis increases with increased branching of the alkyl groups.

The rate constant of an ether of a methylol compound of an ordinary carboxylic amide, *i. e.* methoxymethyl benzamide is also included in the table for comparison. The rate constant of the latter is about 1 650 times lower than for the corresponding urea ether. The difference is due to the difference in activation energy, which was found to be 18.1 and 23.0 kcal/mole for methoxymethyl urea and methoxymethyl benzamide, respectively.

Any contribution of HAc to the rate observed in the acetic acid-acetate buffers could be neglected under the given experimental conditions; some results confirming this are given in Table 2.

Table 2. $[Ac^-] / [HAc] = 1$.

[HAc]	$NH_2CONHCH_2OCH_3$ Temp. 25°C $k \times 10^5$ (sec^{-1})	$C_6H_5CONHCH_2OCH_3$ Temp. 40°C $k \times 10^7$ (sec^{-1})
0.2	8.2	2.1
0.06	8.1	2.1
0.02	8.1	2.1

Increasing the buffer concentration while keeping $[H_3O^+]$ constant does not influence the rate of hydrolysis. The rate constant observed in HAc—Ac buffers can therefore be taken as solely due to the effect of H_3O^+.

Evidence for the proposed reaction scheme, *i. e.* that the monomethylol compound is initially formed by the hydrolysis, was obtained from kinetic experiments where the samples were separately analysed also for free formaldehyde. Free formaldehyde could be determined separately by reaction with KCN at pH~8. At this pH the methylol compounds are fairly stable while the KCN-formaldehyde reaction is completed [6] within 5 min.

Results of these experiments with methoxymethyl urea and methoxymethyl benzamide are shown in Tables 3 and 4 where methylol compound + formaldehyde and free formaldehyde, as found experimentally, are given as % of initial amount of ether.

Table 3. Comparison between the amount of ether converted and free formaldehyde formed during the course of the reaction. HAc = 0.02 moles/l, B/A = 1. Temp. = 25°C.

Time (10^3 sec.)	Methoxymethyl urea % Ether converted (Methylol urea + CH_2O)	Free CH_2O %
1.8		1.2
3.9	27.1	2.0
7.2	44.8	3.2
14.4	69.3	4.0

Table 4. [HAc] = 0.02 mole/l, B/A = 0.25. Temp. = 40°C.

Time (10^4 sec.)	Methoxymethyl benzamide % Ether converted (Methylol benzamide + CH_2O)	Free CH_2O %
8.28		1.6
17.37	13.0	2.6
28.71	21.0	3.1
54.72	35.5	3.8

It will be seen from Tables 3 and 4 that, even after conversion of a large percent of the ether, only very little free formaldehyde is present in the reaction mixture. This excludes the possibility that the ether is initially broken at the dotted line in

$$R-CO-NH\vdots CH_2-O-CH_3$$

The hemiacetal thus formed would rapidly decompose to give free formaldehyde. Moreover, the methylol amide found could not have been formed from a recombination of free formaldehyde and urea. The equilibrium constant for this reaction is such that, with the dilute solutions used, most of the formaldehyde should exist in the free form. Furthermore, the rate constants for the reaction of amide and formaldehyde are much too low to allow any considerable recombination during the time of hydrolysis given in the tables.

REACTION MECHANISM

In spite of the fact that HAc showed no catalytic effect a mechanism which involves a general acid catalysis seems most probable. The effect of HAc may be difficult to detect because of the much greater effect of H_3O^+ in the pH range 4—5. Thus Dunn found a catalytic effect of piperidinium ion in the pH range 10.5—11.2 where a general acid effect should be more easily detected. The H_3O^+ catalysed reaction may therefore take place according to the following mechanism.

$$1.\ RCONHCH_2OR' + H_3O^+ \rightleftharpoons RCONHCH_2\overset{H^+}{O}R' + H_2O$$

$$2.\ RCONHCH_2\overset{H^+}{O}R' + H_2O \xrightarrow{k_2} RCON{=}CH_2 + R'OH + H_3O^+$$

$$3.\ RCON{=}CH_2 + H_2O \rightarrow RCONHCH_2OH$$

In step 2, which is rate determining, alcohol is split off with the formation of an unstable Schiff's base which in step 3 adds water to give the methylol compound. The rate of the reaction catalysed by H_3O^+ will be given by:

$$v = k_2K_1[RCONHCH_2\overset{H^+}{O}R'][H_3O^+][H_2O] \quad (K_1 = [RCONHCH_2\overset{H^+}{O}R']/[RCONHCH_2OR][H_3O^+])$$

The great difference between the rate of hydrolysis of the urea and the benzamide ether is most probably explained by the effect of the group R on the value of k_2. The formation of the double bound $N{=}CH_2$ with the simultaneous liberation of alcohol in step 2 involves an electron displacement from left to right. This displacement will most probably be hampered by the electron attracting C_6H_5 group and facilitated by the electron relasing NH_2 group.

An increased branching of the alkyl group will increase the basicity of the ether oxygen, and thus the value of K_1 in the rate equation. Apparently this effect more than outweighs the small counteracting effect which might be expected on the value of k_2.

DISCUSSION

Recently Dunn [2] has published results from the acid decomposition of the methoxymethyl urea. However, he has determined the rate by measuring free formaldehyde liberated during the experiment. What really is measured therefore is the rate of reaction 2, *i. e.* the decomposition of methylolurea, which decomposes with a much slower rate than the ether hydrolysis in step 1. Thus Dunn finds a rate constant,

$$k_{H_3O} = 1 \times 10^{-3} \text{ (l} \cdot \text{sec}^{-1} \cdot \text{mole}^{-1}\text{) at } 25°C$$

which is approximately the same as formerly given by de Jong and de Jonge for the decomposition of methylolurea. The value given in this paper is 2 800 times higher than the value given by Dunn.

The ethers were prepared according to procedures given by Kadowaki [7] and Ugelstad and de Jonge [4].

As mentioned above, the alkoxymethyl urea will also decompose to a small degree during the treatment with alkaline KCN solution used for determination of methylolurea and formaldehyde formed by the acid hydrolysis. This error is serious only with measurements taken at low conversions of the ether. It could be approximately corrected for as follows:

Let E_i = moles of ether in the sample taken from the reaction mixture at the start of the kinetic run ($t = 0$),
F' = formaldehyde liberated from E_i moles of ether during the analysis,
E_1 = the actual amount of undecomposed ether in the sample taken for analysis at a given time interval t_1,
UF_1, F_1 = the corresponding values for methylolurea and free formaldehyde in the same sample,
F = formaldehyde found by analysis of the sample taken at time t_1,
Then the latter is approximately given by:

$$F = UF_1 + F_1 + F' \cdot E_1/E_i \tag{6}$$

A material balance for the ether gives:

$$E_i = E_1 + UF_1 + F_1 \tag{7}$$

Combination of eqns (6) and (7) gives the degree of conversion of ether at time t_1:

$$\frac{E_i - E_1}{E_i} = \frac{F - F'}{E_i - F'}$$

REFERENCES

1. de Jong, J. I. and de Jonge, J. *Rec. trav. chim.* **71** (1952) 643.
2. Dunn, A. S. *J. Chem. Soc.* **1957** 1446.
3. Pfeil, E. and Schroth, G. *Z. anal. Chem.* **34** (1952) 333.
4. Ugelstad, J. and de Jonge, J. *Acta Chem. Scand.* **10** (1956) 1475.
5. Larsson, E. and Adell, B. *Z. physik. Chem.* **A 156** (1931) 352, 381.
6. Ugelstad, J. and de Jonge, J. *Rec. trav. chim.* **76** (1957) 919.
7. Kadowaki, H. *Bull. Chem. Soc. Japan* **11** (1936) 248.

Received February 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 869–876

Studies on Some Metal Complexes of Catechol-3,5-disulphonic Acid

REINO NÄSÄNEN

Institute of Chemistry, University of Helsinki, Finland

The investigation of the complex formation of catechol-3,5-disulphonic acid with divalent metals has been continued. The data obtained by the pH method on the lead complexes can be explained satisfactorily by postulating the formation of the complexes $PbHL^-$ and PbL^{2-} where L is the ligand. The formation of PbL_2^{6-} could not be established by the method used. The respective equilibrium constants were calculated. The formation of copper complexes was reinvestigated. In addition to the complexes CuL^{2-} and CuL_2^{6-} previously studied by the present author, the complex $CuHL^-$ is obviously also formed at low ionic strengths, but in 1 M sodium perchlorate solution no such ion could be detected. The respective equilibrium constants have been calculated. The complexes formed between catecholdisulphonic acid and six divalent metals hitherto studied by the present author are discussed.

The complex formation of catechol-3,5-disulphonic acid with divalent metals is generally considered to involve the reactions [1,5]:

$$M^{2+} + H_2L^{2-} \rightleftharpoons MHL^- + H^+ \quad (1)$$

$$M^{2+} + H_2L^{2-} \rightleftharpoons ML^{2-} + 2\ H^+ \quad (2)$$

$$M^{2+} + 2\ H_2L^{2-} \rightleftharpoons ML_2^{6-} + 4\ H^+ \quad (3)$$

The role of the first reaction has been found to be relatively slight in all cases studied hitherto. In 1 M sodium perchlorate solution the measurements relating to the copper complexes did not even show any formation of the complex MHL^-, as will be described below. The third equilibrium is important only in the cases of zinc and copper.

Reactions (1) and (2) overlap when $c_B < 2c_M$ and the equilibrium constants defined by

$$K_H = [MHL^-] / [ML^{3-}] [H^+] \quad (4)$$

and

$$K_I = [ML^{3-}] [H^+]^2 / [M^{2+}] [H_2L^{2-}] \quad (5)$$

can be calculated from the equation

$$1/K_I = (1 + K_H [H^+]) (c_M - c') / c' + K_{II} (2c_M - c') x^2 / c', \quad (6)$$

where c_M is the total metal concentration, $c' = c - [H_2L^{2-}] (1 + K_3 / [H^+])$ and $x = [H_2L^{2-}] / [H^+]^2$. The concentration of H_2L^{2-} is obtained from

$$[H_2L^{2-}] = (2c - c_B - [H^+] - [MHL^-]) / (2 + K_3 / [H^+]) \quad (7)$$

and

$$[MHL^-] = c' [H^+] K_H / (1 + K_H [H^+] + 2 K_{II} x), \quad (8)$$

where c and c_B are the total concentrations of Na_2H_2L and sodium hydroxide, respectively. The constant K_3 is defined by

$$K_3 = [H^+] [HL^{3-}] / [H_2L^{2-}] \quad (9)$$

The second term of eqn (6) is negligible when the ionic strength is low and $c_B < 2c_M$, but in 1 M $NaClO_4$ it must be taken into account since the equilibrium constant K_{II} of the reaction

$$ML^{3-} + H_2L^{2-} \rightleftharpoons ML_2^{6-} + 2\ H^+ \quad (10)$$

increases very greatly with ionic strength. The details of the calculation of the constants K_I and K_H have been described previously [1].

The constant K_{II}

defined by

$$K_{II} = [ML_2^{6-}] [H^+]^2 / [ML^{3-}] [H_2L^{2-}] \quad (11)$$

can also be calculated by means of eqn (6), which in this case is best used in the form

$$K_{II} = \frac{(1 + K_H [H^+]) (c' - c_M)}{x (2c_M - c')} + \frac{c'}{K_I x^2 (2 c_M - c')} \quad (12)$$

The calculation of K_{II} is, of course, possible only when $2 c_M < c_B < 4 c_M$ and $c_M \leqq 2 c$. The second term of eqn (12) is negligible at low ionic strengths, but must be taken into account in 1 M $NaClO_4$. The term $K_H [H^+]$ has been found to be negligible in the calculation of K_{II}.

EXPERIMENTAL

Beckman glass electrodes, types 4990—83 and E-2 were used. As reference electrode a calomel electrode was employed. When no neutral salt had been added to the solution studied, the electrode vessel and bridge were filled with a saturated solution of potassium chloride. When the solution to be measured was 1 M in sodium perchlorate, the electrode vessel and bridge were filled with a 1 M NaCl solution. In the former case a 0.05 M solution of potassium hydrogen phthalate, pH = 4.01, and in the latter case an acetate buffer

Table 1. Determination of hydrogen ion concentration of the acetate buffer at 25°C.

Solutions of hydrochloric acid, 1 M in $NaClO_4$		Acetate buffer, 0.005 M in acetic acid, 0.005 M in sodium acetate, 1 M in $NaClO_4$	
$-\log [H^+]$	E mV	E mV	$-\log [H^+]$
2.71	203.5	90.3	4.62
2.54	214.2	90.3	4.63
2.42	221.2	90.3	4.63
2.32	227.0	90.3	4.63

1 M in $NaClO_4$, served as standard buffer solutions. The acetate buffer was 0.005 M in acetic acid and in sodium acetate. Its hydrogen ion concentration was determined by means of a series of hydrochloric acid solutions, 1 M in $NaClO_4$, to be $10^{-4.63}$. The acid dissociation constant of acetic acid should thus be $10^{-4.63}$ in 1 M $NaClO_4$. The results are given in Table 1. For this constant Ellilä [2] has obtained the value $10^{-4.61}$. When a saturated calomel electrode was used, the pH values were converted to $-\log [H^+]$ values by using the apparent pf_{H^+} values [3].

The chemicals used were of analytical grade. In potentiometric measurements a Radiometer PHM 3 m potentiometer was used.

RESULTS AND DISCUSSION

The values obtained for the constants K_H and K_I in the cases of copper and lead without addition of a neutral salt are given in Table 2. The data previously obtained for cadmium, cobalt, nickel and zinc complexes have been included. The respective values at zero ionic strength are given in Table 3.

Table 2. Equilibrium constants relating to the complex formation by some metals with catecholdisulphonic acid at 25°C. Complexes MHL^- and ML^{2-}.

$c:c_M$	$\sqrt{I}$	pK_I						$\log K_H$					
		Cd	Co	Ni	Zn	Pb	Cu	Cd	Co	Ni	Zn	Pb	Cu
1:1	0.088	11.16	10.65	10.21	9.75	6.72	5.85	6.88	—	—	5.85	—	3.54
2:1	0.108	11.19	10.70	10.25	9.80	6.74	5.87	6.84	—	—	5.82	—	3.48
3.33:1	0.129	11.19	10.72	—	9.84	6.72	5.88	6.78	—	—	5.74	—	3.40
6.67:1	0.170	11.24	10.75	10.29	9.84	—	—	6.74	—	—	5.68	—	—
mean value	(0.124)	11.20	10.71	10.25	9.81	6.72	5.87	6.81	6.48	6.00	5.79	4.46	3.47

Table 3. Equilibrium constants relating to the complex formation by some metals with catecholdisulphonic acid at 25°C and at zero ionic strength.

	pK_I°	$\log K_H^\circ$	$\log K_M^\circ$	$\log \beta_1^\circ$
Cd	11.09	7.01	4.23	10.29
Co	10.60	6.68	4.39	10.78
Ni	10.14	6.20	4.37	11.24
Zn	9.70	5.99	4.58	11.68
Pb	6.61	4.66	6.36	14.77
Cu	5.76	3.67	6.22	15.62

In extrapolating, the equation

$$pK_I = pK_I^\circ + \frac{1.018\sqrt{I}}{1+2.0\sqrt{I}} \quad (12)$$

obtained earlier for the respective copper complex was used [1]. The constant K_H was correspondingly extrapolated to zero ionic strength by means of the equation

$$\log K_H = \log K_H^\circ - \frac{2.04\sqrt{I}}{1+2\sqrt{I}} \quad (13)$$

Table 3 also contains the values of the stability constant

$$\beta_1^0 = (ML^{2-})/(M^{2+})\ (L^{4-}) \quad (14)$$

as calculated from

$$\beta_1^\circ = K_I^\circ/K_3^\circ\ K_4^\circ \quad (15)$$

For the acid dissociation constants K_3° and K_4° the values $10^{-8.31}$ and $10^{-13.07}$ (25°C), taken from a previous paper by the present author [4], were used. Further, the equilibrium constants of the reaction

$$M^{2+} + HL^{3-} \rightleftharpoons MHL^- \quad (16)$$

defined by

$$K_M^\circ = (MHL^-)/(M^{2+})\ (HL^{3-}) \quad (17)$$

are given in Table 3. This constant can be calculated from the expression

$$K_M^\circ = K_H^\circ K_I^\circ/K_3^\circ \quad (18)$$

Recently the values of β_1, K_M, K_3 and K_4 in 0.1 M KCl determined for some metals by Schwarzenbach and Willi have been published [5]. Since the value of K_4 determined by the present author differs considerably from that given by these authors, the comparison of the K_I and K_H values seemed to be more suitable than of the β_1 and K_M values. Therefore the values of K_I and K_H

Table 4. The constants K_3, K_4, K_H and K_I in 0.1 M KCl.

	pK_3	pK_4	pK_I					
			Cd	Co	Ni	Zn	Pb	Cu
Schwarzenbach and Willi [5] (20°C)	7.66	12.6	—	10.77	10.30	9.85	—	5.73
Present author (25°C)	7.64	12.2	11.29	10.80	10.34	9.90	6.81	5.96

	$\log K_H$					
	Cd	Co	Ni	Zn	Pb	Cu
Schwarzenbach and Willi (20°C)	—	6.19	5.64	5.49	—	3.55
Present author (25°C)	6.61	6.28	5.80	5.59	4.26	3.27

Table 5. Determination of the constant K_{II} for the complex CuL_4^{6-} at 25°C.

$\sqrt{I}$	$c \times 10^3$	$c_B \times 10^3$	$c_M \times 10^3$	$[H^+] \times 10^7$	$K_{II} \times 10^{11}$
0.137	4.219	2.954	1.266	4.20	0.82
0.142	4.211	3.158	1.263	3.22	0.85
0.148	4.202	3.361	1.261	2.61	0.90
0.153	4.193	3.564	1.258	2.15	0.91
0.159	4.184	3.766	1.255	1.79	0.90
0.163	4.175	3.967	1.253	1.513	0.89
0.168	4.167	4.167	1.250	1.265	0.85
0.173	4.158	4.366	1.247	1.062	0.83
				mean value	0.87

were calculated from the data of Schwarzenbach and Willi. The respective values of K_I and K_H at ionic strength 0.1 were calculated from the data of the present author by means of eqns (12) and (13). These results are given in Table 4. The pK_I values of the Co, Ni and Zn complexes are seen to agree so well that the small differences, 0.03, 0.04 and 0.05, might be attributed mainly to a temperature effect. In the case of copper, however, a greater difference exists. The log K_{II} values differ more; 0.09, 0.16 and 0.10 in the cases of Co, Ni and Zn, respectively. Here again, some of the difference may be due to the different temperatures used, but on the other hand the accuracy that can be achieved is not very high, as already mentioned above. The agreement is thus very good if one leaves out of account the constant K_4 and the constants for the copper complexes.

From the data of the second buffer range at which $2c_M < c_B < 4c_M$ the values of the constant K_{II} can be evaluated. As mentioned above, only in the case of zinc and copper [1] could a distinct formation of ML_2^{6-} be observed. The stability of CuL_2^{6-} has been reinvestigated. In Table 5 a titration is shown. The quantity K_{II} is seen to remain nearly constant, although the increase in the concentration of the CuL_2^{6-} ion during the titration causes a considerable change in ionic strength as defined by $I = \frac{1}{2}\Sigma z_i^2 c_i$. An equal change in ionic strength produced by addition of neutral salt has a considerable effect on the quantity K_{II}[1]. A similar state of affairs has previously been shown

Table 6. Determination of the constant K_I for the complex CuL^{2-} at 25°C and in 1 M $NaClO_4$.

$c \times 10^3$	$c_B \times 10^3$	$c_M \times 10^3$	$[H^+] \times 10^5$	$K_I \times 10^6$
4.329	0.433	1.299	12.02	0.95
4.320	0.648	1.296	9.90	0.98
4.310	0.862	1.293	8.51	1.07
4.301	1.075	1.290	7.08	1.03
4.292	1.288	1.288	6.03	1.05
4.283	1.499	1.285	5.13	1.05
4.274	1.709	1.282	4.27	1.01
4.264	1.919	1.279	3.55	1.00
4.256	2.128	1.277	2.95	0.99

Table 7. Determination of the constant K_I for the complex CuL^{2-} using different metal-Na_2H_2L ratios at 25°C and in 1 M $NaClO_4$.

$c:c_M$	pK_I
1:1	5.98
2:1	5.99
3.33:1	6.00
6.67:1	6.00

to prevail in the cases of the zinc [1] and aluminium [6] complexes. Regarding the effect of added electrolytes, the sum of the concentrations of the positive ions, *i.e.* the quantity $\frac{1}{2}\Sigma|z_i|c_i$, seems to be the decisive factor and not $\frac{1}{2}\Sigma z_i^2 c_i$.

It is, of course, possible that in a medium containing sodium ions species such as $Na_\nu ML_2^{(6-\nu)-}$ are formed. If so, the quantity $[ML_2^{6-}]$ should in reality be a sum $\Sigma_\nu Na_\nu ML_2^{(6-\nu)-}$ and the quantity K_{II} a function of the sodium ion concentration of the form

$$K_{II} = K_{II}^{(0)} + K_{II}^{(1)}[Na+] + K_{II}^{(2)}[Na+]^2 + \ldots\ldots \quad (19)$$

Since the quantity $K_{II}^{(\nu)}$ defined by

$$K_{II}^{(\nu)} = \frac{[Na_\nu ML_2^{(6-\nu)-}]\,[H+]^2}{[ML^{2-}]\,[H_2L^{2-}]\,[Na+]^\nu} \quad (20)$$

is also obviously a function of ionic strength, the exact treatment of this contingency is not feasible. The experimental data seem to indicate, however, that at low ionic concentration the species ML_2^{6-} is the predominant one but that at a high concentration of sodium ions the species $Na_\nu ML_2^{(6-\nu)-}$ might also be formed.

Since the complex formation under investigation, especially the formation of ML_2^{6-}, is so highly dependent on the ionic medium it seemed advisable to investigate this system in a medium containing some neutral salt. Hitherto, only copper complexes have been investigated in this way. In Table 6 a titra-

Table 8. Determination of the constant K_{II} for the complex CuL_2^{6-} in 1 M $NaClO_4$ and at 25°C.

$c \times 10^3$	$c_B \times 10^3$	$c_M \times 10^3$	$[H+] \times 10^5$	$K_{II} \times 10^8$
4.219	2.954	1.266	1.290	1.73
4.211	3.158	1.263	1.047	1.67
4.202	3.361	1.261	0.892	1.76
4.193	3.564	1.258	0.742	1.73
4.184	3.766	1.255	0.631	1.78
4.175	3.967	1.253	0.513	1.68
4.167	4.167	1.250	0.427	1.72
4.158	4.366	1.247	0.347	1.76
4.149	4.564	1.245	0.263	1.76

Table 9. Determination of the constant K_{II} for the complex CuL_2^{6-} using different metal-Na_2H_2L ratios in 1 M $NaClO_4$ and at 25°C.

$c:c_M$	pK_{II}
2:1	7.82
3.33:1	7.76
6.67:1	7.78

tion for determination of the constant K_I for the copper complex in 1 M sodium perchlorate is given. In this case, the second term in eqn. (6) must also be considered, as already mentioned above. On the other hand, the formation of the complex $CuHL^-$ is so slight that it cannot be demonstrated. This is readily understandable in view of the fact that the equilibrium constant K_H decreases with increasing ionic strength and that the concentration of $CuHL^-$ is already small at low ionic strength. Titrations for the determination of K_I were carried out for four different Na_2H_2L-metal ratios. These results are given in Table 7 and it will be seen that the accuracy is even better than that reached in the measurements without addition of neutral salts. In Table 8 a titration for the determination of the constant K_{II} is given. The accuracy reached is seen to be very good in this case too. In eqn. (12) the second term must also be considered, because reactions (2) and (3) overlap somewhat. In Table 9 the results for K_{II} have been summarized.

For the constants K_I and K_{II} in the case of copper the present author obtained the values $pK_I = 6.08$ and $pK_{II} = 7.40$ in 1 M KCl. These values thus differ in an understandable way from the values $pK_I = 6.00$ and $pK_{II} = 7.79$ now obtained in 1 M $NaClO_4$.

The stability constants β_1 and β_2 for copper complexes are given in Table 10. The constant β_1 was calculated from eqn. (15) and the constant β_2, defined by

$$\beta_2 = [CuL_2^{6-}]/[Cu^{2+}]\ [L^{4-}]^2, \tag{21}$$

from the expression

$$\beta_2 = K_I K_{II}/K_3^2 K_4^2 \tag{22}$$

The values of the acid dissociation constants K_3 and K_4 in 1 M $NaClO_4$ were determined in the same way as before, but the electrode system used was the same as in the study of complexes in 1 M $NaClO_4$. Somewhat unexpectedly, the values obtained for K_3 and K_4 in 1 M $NaClO_4$ are almost identical with those obtained for these constants in 1 M KCl.

Table 10. Stability constants of catechol-3,5-disulphonic acid chelates of copper in 1 M $NaClO_4$ and at 25°C.

$pK_3 = 7.19$	;	$pK_I = 6.00$	;	$\log \beta_1 = 12.79$
$pK_4 = 11.6$	;	$pK_{II} = 7.79$	;	$\log \beta_2 = 23.79$

REFERENCES

1. Näsänen, R. *Suomen Kemistilehti* **B 29** (1956) 91; **B 31** (1958) 19, 261; **B 32** (1959) 7.
2. Ellilä, A. *Ann. Acand. Sci. Fennicæ A II* **51** (1953).
3. Näsänen, R., Lumme, P. and Mukula, A.-L. *Acta Chem. Scand.* **5** (1951) 1199.
4. Näsänen, R. *Suomen Kemistilehti* **B 30** (1957) 61.
5. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants of Metal-ion Complexes, with Solubility Products of Inorganic Substances.* Part I: Organic Ligands, London 1957, p. 45.
6. Näsänen, R. and Veivo, J. *Suomen Kemistilehti* **B 29** (1956) 213; Näsänen, R. *Acta Chem. Scand.* **11** (1957) 1308.

Received February 17, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 877–883

Analysis of Aromatic Solvents by Gas Chromatography

BENGT SMITH

Institutionen för Organisk Kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

The utilization of stationary phases having a high degree of selectivity for different types of hydrocarbons has made possible the determination of saturated hydrocarbons in aromatic solvents over a wide range of boiling points. The application of various stationary phases to this analysis problem is discussed and examples are given of the separations obtained.

The determination of small amounts of saturated hydrocarbons in aromatic solvents such as "industrial pure benzol, toluol and xylol" has proved to be a difficult problem (*cf.* Ref.[1]). A satisfactory solution, however, can be achieved by means of gas liquid partition chromatography using stationary phases specially chosen to satisfy the specific demands put on them in this connection.

"Industrial pure benzol" and "benzene crystallizable". The separation of the benzene and saturated constituents in these solvents can be effected by means of various stationary phases. The choice of stationary phase depends on the information required. If the total amount of saturates is of major interest, then *p*-nitroaniline picrate is suitable. This stationary phase is namely rather nonselective for saturates, these compounds being eluated mainly as one peak (*cf.* Fig. 1). In order to determine the individual saturated hydrocarbons, a combination with other columns is necessary. A full analysis of "industrial pure benzol" or "benzene crystallisable" can be achieved in the following way. The saturates, eluted from the *p*-nitroaniline picrate column, are condensed and re-run on a 6 m tetra-*n*-amylsilane column. Nine major components and three trace components were identified in "industrial pure benzol" * (*cf.* Fig. 2). The same components were present in "benzene crystallizable" although in smaller amounts and other proportions. A 6 m squalane column can also be used. The chromatogram obtained with this column is very similar to the one shown in Fig. 2. The silane column was chosen here because of its better separation of the pair, *cyclo*hexane/3,3-dimethylpentane. In the actual sample, however, no 3,3-dimethylpentane was found. The pair, 2-methyl-

* The method used in the identification of the components will be described elsewhere.

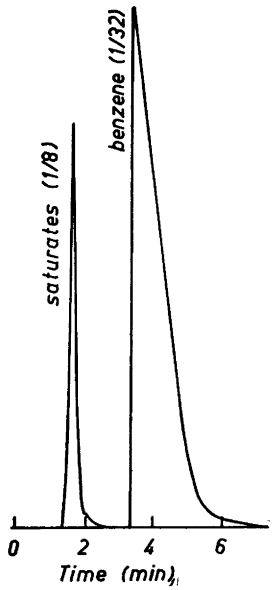


Fig. 1. "Industrial pure benzol." Column: *p*-nitroaniline picrate, 2 m. Temperature: 110°C. Flow of He: 20 ml/min. Recorder attenuation as denoted.

hexane/2,3-dimethylpentane, cannot be separated on either of these columns. If their proportions are wanted, then the peak has to be condensed and re-run on a 6 m di-*n*-octyl phthalate column.

"*Industrial pure toluol*". In addition to toluene and saturates, this solvent contains small amounts of benzene, ethylbenzene and xylenes. It is necessary to choose a stationary phase which can separate these constituents. *p*-Nitroaniline picrate has proved to be a satisfactory stationary phase. Separate peaks

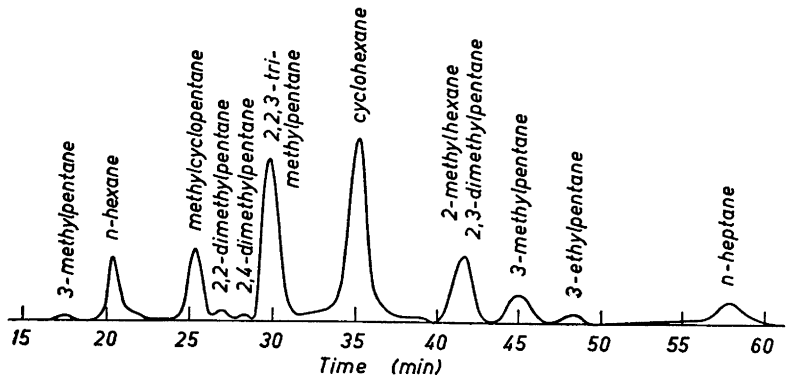


Fig. 2. Saturates in "industrial pure benzol." Column: tetra-*n*-amylsilane, 6 m. Temperature: 25°C. Flow of He: 80 ml/min.

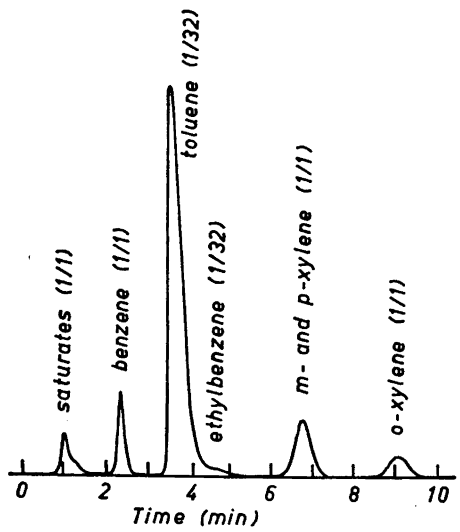


Fig. 3. "Industrial pure toluol." Column: *p*-nitroaniline picrate, 2 m. Temperature: 110°C. Flow of He: 50 ml/min. Recorder attenuation as denoted.

were obtained for saturates, benzene, toluene, ethylbenzene, *m*-plus *p*-xylene and *o*-xylene. (*cf.* Fig. 3). A full separation of *m*- and *p*-xylene by gas liquid partition chromatography has not been achieved so far *. However, if the

* Golay[2] has obtained a partial separation of *m*- and *p*-xylene using a 150 ft capillary column coated with didecyl phthalate. A partial separation has also been reported on some packed columns utilizing Apiezon[3], benzyldiphenyl[4] or tetrahalophthalates[5] as stationary phases.

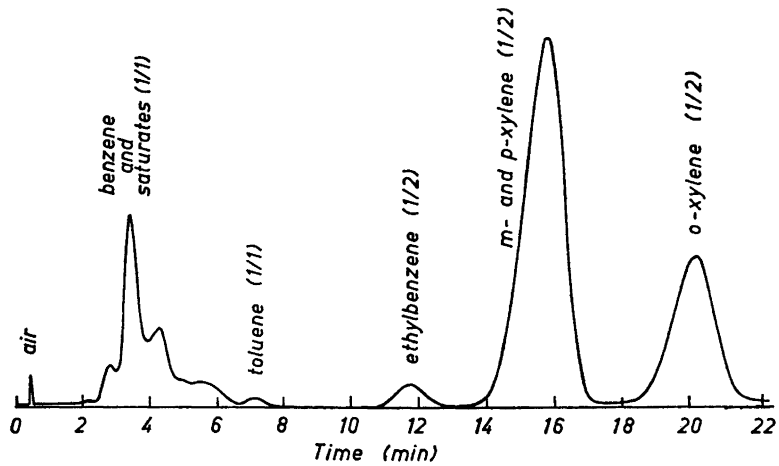


Fig. 4. "Industrial pure xylol." Column: fluorene picrate, 2 m. Temperature: 100°C. Flow of He: 80 ml/min. Recorder attenuation as denoted.

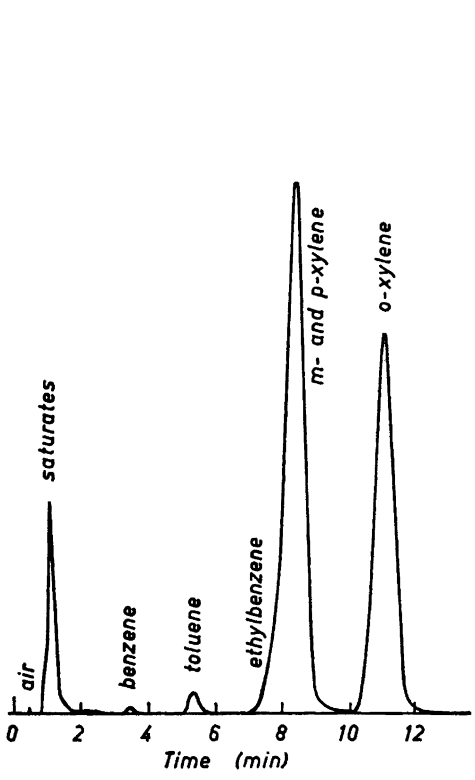


Fig. 5. "Industrial pure xylol." Column: β,β'-oxydipropionitrile, 2 m. Temperature: 100°C. Flow of He: 50 ml/min.

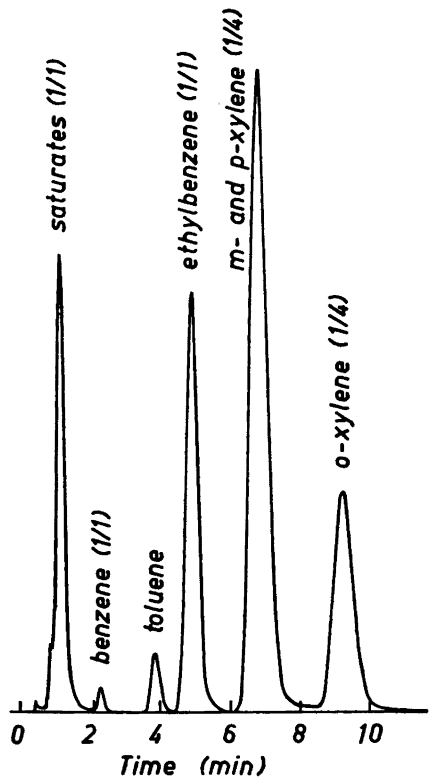


Fig. 6. "Industrial pure xylol." Column: *p*-nitroaniline picrate, 2 m. Temperature: 110°C. Flow of He: 50 ml/min. Recorder attenuation as denoted.

proportions of the two xylenes is wanted, it is possible to condense the mixture as it emerges from the column and analyze it using infrared spectrophotometry.

"Industrial pure xylol". The analysis of "industrial pure xylol" requires a stationary phase which is able to separate aromatic hydrocarbons and saturated hydrocarbons with an extremely high selectivity. To be useful it should release saturates boiling in the xylene range before releasing benzene. Fluorene picrate was reported by Keulemans, Kwantes and Zaal [6] to release *n*-octane (b.p. 125°C) and *iso*nonane (b.p. 143°C) before benzene. It is obvious, however, that a 2 meter fluorene picrate column is not selective enough to separate the saturated hydrocarbons in "industrial pure xylol" from benzene (*cf.* Fig. 4). The best selectivity is shown by β,β'-oxydipropionitrile but the separation of *m*- plus *p*-xylene from ethylbenzene is rather bad (*cf.* Fig. 5). *p*-Nitroaniline picrate leaves only *m*- and *p*-xylene unseparated and is therefore the stationary phase best suited for this analysis (*cf.* Fig. 6).

Table 1. Analysis values for "industrial pure benzol, toluol and xylol".

Sample	Saturated hydrocarbons (wt %)	Benzene (wt %)	Toluene (wt %)	Ethylbenzene (wt %)	*m*- plus *p*-Xylene (wt %)	*o*-Xylene (wt %)
Swedish "industrial pure benzol"	3.3	96.7	—	—	—	—
Swedish "industrial pure toluol"	3.4	0.3	96.2	trace	0.1	trace
Imported "industrial pure toluol"	0.2	0.3	98.7	trace	0.6	0.2
Swedish "industrial pure xylol"	11.0	0.1	0.3	4.0	57.7	26.9
Imported "industrial" pure xylol"	4.8	0.2	0.8	7.1	62.3	24.8

Quantitative analysis. Experiments with synthetic mixtures indicated that the internal normalization method, in which the percentage area is put equal to the weight per cent, was accurate enough for the purpose. The analyses obtained for some solvents are summarized in Table 1. It is seen that relatively large variations in the contents of various components exist in the case of the Swedish and imported qualities of toluol and xylol.

In order to analyze "industrial pure benzol" and "benzene crystallizable" for their individual saturated components, the method previously described

Table 2. Analysis values for "industrial pure benzol".

Compound	*wt* %
Benzene	96.7
*Cyclo*hexane	0.9
2,2,3-Trimethylbutane	0.8
Methyl*cyclo*pentane	0.3
2,3-Dimethylpentane	0.3
3-Methylhexane	0.2
2-Methylhexane	0.2
n-Hexane	0.2
n-Heptane	0.2
3-Ethylpentane	0.1
2,2-Dimethylpentane	0.02
2,4-Dimethylpentane	0.01
3-Methylpentane	0.01
Unknown compounds	0.05

was employed, *viz.* the saturated portion eluted from a *p*-nitroaniline picrate column was condensed and then analyzed on a 6 m tetra-*n*-amylsilane column or squalane column at 25°C. In both cases, the internal normalization method was used. More accurate values could be obtained by calibration using pure hydrocarbons but this was thought to be unnecessary in the present case. Typical values for "industrial pure benzol" are given in Table 2.

EXPERIMENTAL

The analyses were performed using a Perkin Elmer Vapour Fractometer Model 154.

Stationary phases

p-*Nitroaniline picrate* was prepared from *p*-nitroaniline and picric acid in chloroform solution according to Baril and Megrdichian [7]. *β,β'-Oxydipropionitrile* was prepared from acrylonitrile according to Bruson and Riener [8]. *Squalane* was obtained from May and Baker Ltd, Dagenham, England. *Tetra*-n-*amylsilane* was prepared from silicon tetrachloride and *n*-amylmagnesium bromide. Silicon tetrachloride (0.1 mole, 17.0 g) dissolved in abs. ether (25 ml) was added dropwise with stirring to a solution of *n*-amylmagnesium bromide prepared from *n*-amylbromide (1.0 mole, 151.0 g), magnesium (1.0 g.at., 24.3 g) and abs. ether (150 ml). After addition of the silicon tetrachloride, the reaction mixture was heated under reflux for 2 h and then most of the ether was distilled off. The residue was heated on a steam bath for another 2 h, cooled and hydrolyzed with dilute hydrochloric acid. The organic phase was taken up in ether, the ether solution washed with sodium carbonate solution and water and then dried. After distilling off the ether, the residue was fractionated in vacuum yielding 25.0 g (80 %) of crude tetra-*n*-amylsilane. The crude product was purified using conc. sulphuric acid and then heated over sodium and distilled. Final product: b.p. 208–210° (17 mm), $n_D^{20} = 1.4494$, $d_4^{20} = 0.8058$, $MR_D = 104.1$ (found); 104.5 (calc.[9]). % Si 8.91 (found); 8.98 (calc.).

Columns

The columns were made from glass or aluminum tubing of 4 mm internal diameter. The stationary phase was applied in solution on to the carrier, using a suitable low-boiling solvent. The solvent was evaporated and the dry powder then evenly packed in the column. Data for the columns used were:

p-*Nitroaniline picrate columns.* Two 1 m glass columns were used in series. The stationary phase (20 %) was applied on Silocel C 22 (42–60 mesh).

Fluorene picrate column. A 2 m Perkin-Elmer stainless steel column G was used. The stationary phase (about 20 %) was applied on Celite 545.

β,β'-Oxydipropionitrile column. A 2 m aluminum column was used. The stationary phase (20 %) was applied on Chromosorb (30–60 mesh).

Squalane and tetra-n-*amylsilane columns.* In both cases, two 3 m aluminum columns were used in series. The stationary phase (3 %) was applied on Chromosorb (30–60 mesh).

Di-n-*octyl phthalate columns.* Two 3 m aluminum columns were used in series. The stationary phase (10 %) was applied on Chromosorb (30–60 mesh).

Acknowledgement. The author wishes to thank the Town Council of Gothenburg for financial support to this work. The experimental assistance of Mr. M. Ervik is also gratefully acknowledged.

REFERENCES

1. Rossini, F. D., Mair, B. J. and Streiff, A. J. *Hydrocarbons from Petroleum*, ACS Monograph No. 121, Reinhold Publishing Corp., New York 1953, p. 161.
2. Golay, M. J. E. in Desty, D. H. *Gas Chromatography 1958*, Butterworths Scientific Publications, London 1958, p. **36.**
3. Scott, R. P. W. and Cheshire, J. D. *Nature* **180** (1957) 702.
4. Zlatkis, A., O'Brien, L. and Scholly, P. R. *Nature* **181** (1958) 1794.
5. Langer, S. H., Zahn, C. and Pantazoplos, G. *Chemistry & Industry* **1958** 1136.
6. Keulemans, A. J. M., Kwantes, A. and Zaal, P. *Anal. Chim. Acta* **13** (1955) 357.
7. Baril, O. L. and Megrdichian, G. A. *J. Am. Chem. Soc.* **58** (1936) 1415.
8. Bruson, H. A. and Riener, T. W. *J. Am. Chem. Soc.* **65** (1943) 23.
9. Smith, B. *Diss.* Gothenburg 1951.

Received February 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 884–887

The Influence of Some Cations on the Kinetics of Ox Liver Arylsulphatase A

S. O. ANDERSEN*

Universitetets Fysisk-Kemiske Institut, Copenhagen, Denmark

The effects of some cations on the kinetics of sulphatase A are reported. It is shown that barium ions inactivate the enzyme if substrate is present, and a possible reaction mechanism is discussed. Strontium ions have no inhibitory effect on the enzyme, and it is therefore recommended to use strontium instead of barium ions to precipitate sulphate in investigations of this enzyme.

In a previous paper investigations of the kinetics of the enzymic hydrolysis of 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) were reported [1]. As sulphate ions inhibit the reaction, the sulphate ions liberated in the course of the reaction were precipitated as barium sulphate. It was found nescessary to add barium chloride in such a manner that there would never be excess of barium ions in the reaction mixture, as barium ions have some secondary effects on the reaction. In this paper some investigations of the influence of excess barium ions on the enzymic reaction are reported together with investigations of the influence of some other cations.

EXPERIMENTAL

The progress of the reaction was followed as described previously by continued automatic titration of the liberated acid in a recording pH-stat [1]. The base which was added from a syringe in small portions consisted of a solution of equivalent amounts of KOH, substrate and $BaCl_2$, so that both pH and the substrate concentration were kept constant during the reaction, and the sulphate was precipitated as $BaSO_4$.

The cations investigated have preferably been used as their chlorides, only for Zn++ this has not been the case as pure $ZnCl_2$ was not at hand. Instead $Zn(NO_3)_2$ was used as experiments conducted with KCl and KNO_3 did not show any difference in the reaction progress. When the effect of monovalent cations was investigated the concentrations of the salts were 0.10 M and when divalent cations were used the concentrations were 0.05 M, so the concentration of chloride ions was the same in all the experiments. Only $CuCl_2$ made an exception in this respect as it is not sufficiently soluble at pH 5. A saturated solution was therefore used.

* Present address: Universitetets zoofysiologiske laboratorium B, Copenhagen, Denmark.

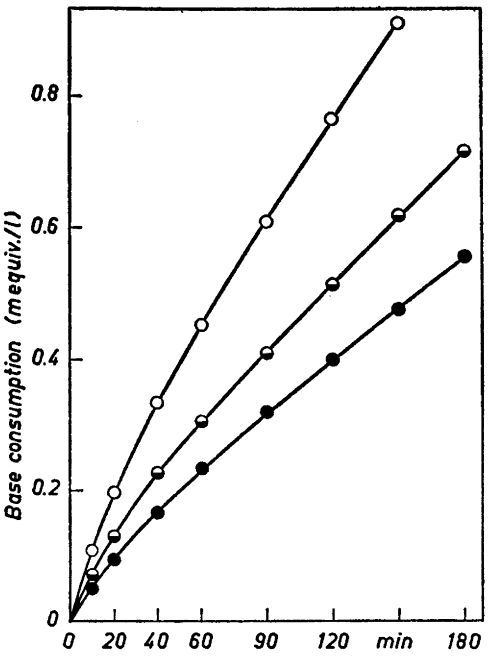


Fig. 1. Effect of cations on the activity of sulphatase A at 20°C and pH 5. The substrate concentration was 3 mM. O—O—O: 0.10 M KCl or NaCl, ◒—◒—◒: 0.05 M $CoCl_2$, ●—●—●: 0.05 M $CdCl_2$.

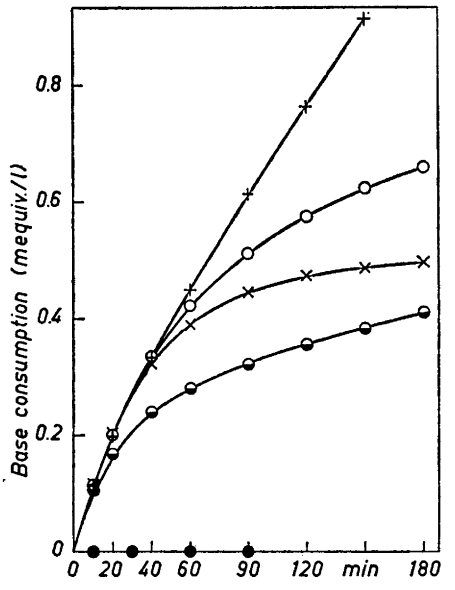


Fig. 2. Effect of cations on the activity of sulphatase A at 20°C and pH 5. The substrate concentration was 3 mM. +—+—+: 0.05 M $CaCl_2$, $MgCl_2$ or $SrCl_2$, O—O—O: 0.05 M $Zn(NO_3)_2$, ×—×—×: 0.05 M $BaCl_2$, ◒—◒—◒: 0.05 M $MnCl_2$, ●—●—●: 0.05 M $HgCl_2$ or saturated $CuCl_2$.

Mixtures of KCl and $BaCl_2$ were used in the experiments where the influence of different barium ion concentrations was investigated, so that the chloride concentration also in this experiment was kept at 0.10 M.

All the experiments were performed at pH 5.0 and 20.0°C, and the substrate concentration was 0.002 M. Enzyme and substrate were prepared as described previously[1].

RESULTS

The progress of the reaction in presence of different cations is demonstrated om Figs. 1 and 2. It is interesting to see that in the case of Ba^{++}, Zn^{++} and Mn^{++} the inhibition of the reaction first begins some time after the start of the reaction. When the enzyme is incubated for some hours together with these cations at pH 5 and the reaction is then started, then the same reaction progress is found. This indicates that the reaction between enzyme and metal ions is not simply a slow one, but that the substrate is involved in some way.

It is not surprising that Cu^{++} and Hg^{++} completely inhibit the reaction as it has been found that the enzyme contains sulfhydryl groups necessary for its activity [2].

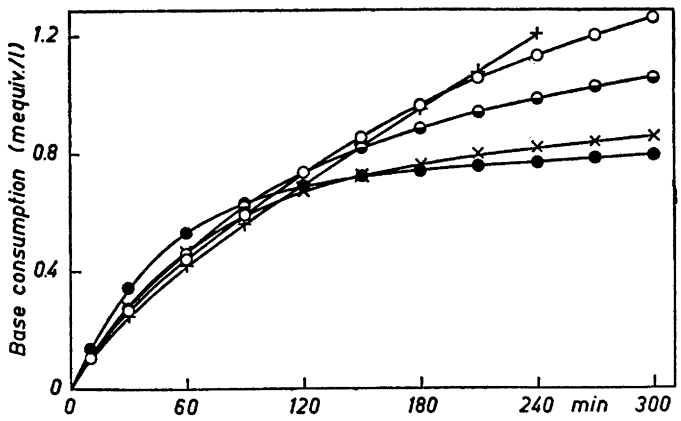


Fig. 3. Effect of $BaCl_2$ on the activity of sulphatase A at 20°C and pH 5. The substrate concentration was 3 mM. ●—●—●: 0.05 M $BaCl_2$, ×—×—×: 0.02 M $BaCl_2$, ◒—◒—◒: 0.01 M $BaCl_2$, O—O—O: 0.005 M $BaCl_2$, +—+—+: without addition of $BaCl_2$.

Co^{++} and Cd^{++} inhibit the reaction to some degree, and it is characteristic that there is a constant ratio between the activity when these ions are present and the activity of the noninhibited enzyme.

When K^+, Na^+, Mg^{++}, Ca^{++} or Sr^{++} are used no difference can be found between the progress of the reactions.

The influence of different concentrations of Ba^{++} on the reaction is shown in Fig. 3. The barium ions seem to have a double effect, first the enzyme is activated, later on inhibited. It was tried to reactivate the enzyme by removing the barium ions, but neither addition of K_2SO_4 nor dialyzing the reaction mixture against 0.1 M KCl for 24 h had any effect, so the inhibition is obviously irreversible. If the enzyme is incubated with $BaCl_2$ in the absence of substrate and the barium ions are removed by dialysis, then the enzyme shows the kinetics found in the absence of barium ions.

The reaction progress in the absence of heavy metal ions can be described by the expression:

$$\xi = At + B(1-e^{-\lambda t})$$

where ξ is the amount of substrate split at the time t, and A, B and λ depend on the enzyme and substrate concentrations[1]. The same expression with a very small value of A can describe the reaction progress in the experiment with the highest concentration of barium ions but not in the experiments with smaller concentrations.

DISCUSSION

This formal similarity between the reaction progress when no barium ions are added and when they are present in excess can be explained if it is supposed that the reaction scheme is essentially the same in both cases, but that in the presence of barium ions the enzyme is somehow modified so that the velocity constants are altered.

The scheme previously proposed for the reaction when no barium ions are present was:

$$\begin{aligned} A + X_1 &\rightleftharpoons X_2 \\ X_2 &\rightarrow X_1 + P \\ X_2 &\rightleftharpoons X_3 \end{aligned}$$

where X_1, X_2 and X_3 are different forms of the enzyme and A is the substrate and P is the reaction products. The transformation from X_2 to X_3 is assumed to be reversible and slow. If barium ions are present we should then have:

$$\begin{aligned} Ba^{++} + X_1 &\rightleftharpoons X_1' \\ A + X_1' &\rightleftharpoons X_2' \\ X_2' &\rightarrow X_1' + P \\ X_2' &\rightarrow X_3' \end{aligned}$$

X_i' represent one of the forms of the enzyme modified by barium ions. The larger initial velocity when barium ions are present can be explained if this barium modification of the enzyme is more active towards the substrate than the unmodified enzyme.

If the concentration of barium ions is so high that practically all the enzyme is in the form of the barium modification, it should be expected that the reaction progress could be described by the expression $\xi = B(1-e^{-\lambda t})$. As mentioned this is not the case, but the existence of the term At could be explained if the reaction X_2' to X_3' is not irreversible as written in the scheme, but reversible with the equilibrium far to the right. The final velocity in the experiment with 0.05 M $BaCl_2$ is about twenty times smaller than the initial velocity. In equilibrium 95 % of the enzyme must then be present as X_3', and the reaction from X_2' to X_3' must be about twenty times as fast as the opposite reaction. It should therefore be expected that a measurable reactivation could be demonstrated after 24 h dialysis. This is as mentioned not the case.

When the concentration of barium ions used is not so high that all the enzyme is in the form of the barium modification, we should expect a reaction scheme composed of both schemes here mentioned. This will give a complex reaction which cannot be simply expressed, which is in accordance with the experiments.

Barium chloride has been used to remove excess of sulphate ions in some investigations of the kinetics of sulphatases [1,3]. The results here presented show that it must be used with caution, and that it is better to use strontium chloride as this can be used in high concentrations without having any effects on the enzyme. It is peculiar that there is such a difference between the effects of barium and strontium ions. One is reminded of the fact that barium salts are toxic to the organism whereas strontium salts are not.

Acknowledgements. My thanks are due to professor J. A. Christiansen, head of this institute, for his interest and advice. Financial support from *Statens almindelige Videnskabsfond* is gratefully acknowledged.

REFERENCES

1. Andersen, S. O. *Acta Chem. Scand.* **13** (1959) 120.
2. Roy, A. B. *Biochem. J.* **59** (1955) 8.
3. Baum, H. and Dodgson, K. S. *Biochem. J.* **69** (1958) 573.

Received March 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 888–892

The Preparation of Some N-Methyl-1,2,3-Triazoles

CHRISTIAN PEDERSEN

Department of Organic Chemistry, University of Technology, Copenhagen, Denmark

The reaction between diazomethane and cyanogen bromide has been shown to give a mixture of the three N-methyl derivatives of 4-bromo-1,2,3-triazole. The structure of the three products is proved and their reaction with ammonia is studied.

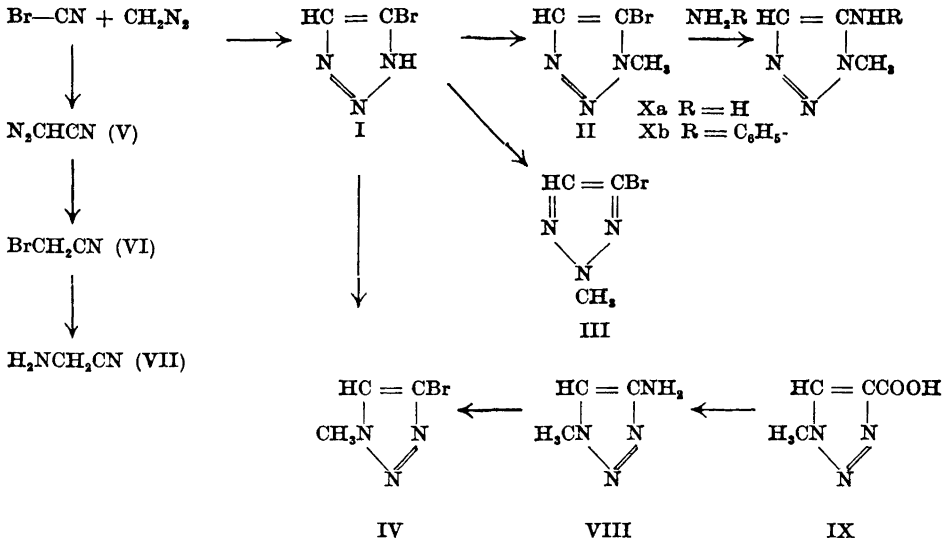
The reaction between cyanogen bromide and diazomethane has been studied by Tamburello and Milazzo [1]. According to these authors the first product of the reaction is 4-bromo-1,2,3-triazole (I) since this compound could be isolated when the reaction was carried out with excess cyanogen bromide. With excess diazomethane present in the reaction mixture I was further methylated and 2-methyl-4-bromo-1,2,3-triazole (III) was isolated.

The methylation of 1,2,3-triazole with dimethyl sulfate has been shown by Huttel and Welzel [2] to give a mixture of 1- and 2-methyl-1,2,3-triazole and in the present paper it is shown that the same two products are obtained when diazomethane is used as the methylating agent.

In view of this it would be expected that the methylation of I with diazomethane could give, besides III, two other products, namely 1-methyl-5-bromo-1,2,3-triazole (II) and 1-methyl-4-bromo-1,2,3-triazole (IV) and it was therefore decided to reinvestigate the reaction between diazomethane and cyanogen bromide.

The reaction product, on distillation, gave three fractions (1, 2 and 3, see experimental part) of which the largest and lowest boiling one (1) proved to be a mixture of α-bromoacetonitrile (VI) and 2-methyl-4-bromo-1,2,3-triazole (III). Treatment of fraction 1 with sodium hydroxide destroyed the bromoacetonitrile and gave pure III. When treated with ammonia the bromoacetonitrile of fraction 1 was converted into α-aminoacetonitrile (VII) which was isolated as the hydrochloride and identified by comparing its infrared spectrum with that of an authentic sample. Fraction 2 consisted mainly of 1-methyl-5-bromo-1,2,3-triazole (II) and fraction 3 of 1-methyl-4-bromo-1,2,3-triazole (IV).

The assignment of structure to the three isomeric methyl-bromo-triazoles is based on the following. III, in contrast to II and IV, does not form a hydrochloride when treated with hydrogen chloride in ether. A lower basic strength



of 2-substituted 1,2,3-triazoles as compared to the corresponding 1-substituted compounds seems to be a general rule. 2-Methyl-1,2,3-triazole does not form a hydrochloride whereas 1-methyl-1,2,3-triazole does [2]; the 1- and 2-methylbenzotriazoles behave in the same way [3] and so do 1- and 2-phenyl-1,2,3-triazole. This is probably analogous to the difference in basic strength between imidazoles and pyrazoles [4] since a 1-substituted 1,2,3-triazole is likely to be protonated in the 3-position giving an ion of structure XI which is analogous to a protonated imidazole, whereas a 2-substituted 1,2,3-triazole must take up the

proton in position 1- or 3- and give structure XII which is analogous to a protonated pyrazole.

IV has the same melting point as the 1-methyl-4-bromo-1,2,3-triazole described by Huttel and Welzel [2]. Besides, IV was prepared from 1-methyl-4-amino-1,2,3-triazole (VIII) by the Gattermann reaction. The synthesis of VIII is described later in this paper.

II on reaction with aniline gave 1-methyl-5-anilino-1,2,3-triazole (Xb), identical with the compound which Dimroth [5] obtained from 1-methyl-5-chloro-1,2,3-triazole.

The formation of α-bromoacetonitrile (VI) has probably taken place through diazoacetonitrile (V) which could be formed from diazomethane and cyanogen

bromide by a reaction similar to the formation of diazoketones from acyl halides and diazomethane. The conversion of V into VI by hydrogen bromide can only take place if the reaction mixture at some stage has been deficient in diazomethane since V will react much slower with hydrogen bromide than diazomethane.

The 1-methyl-5-bromo-1,2,3-triazole (II) is the most reactive of the three isomeric methyl-bromo-triazoles. With 10 % ethanolic ammonia at 100° it gives a low yield of 1-methyl-5-amino-1,2,3-triazole (Xa); III and IV do not react, under the same conditions. As shown in the present paper, II reacts with aniline and Dimroth [5] has shown that the corresponding chloro compound also reacts with aniline in contrast to 1-methyl-4-chloro-1,2,3-triazole [2].

1,2,3-Triazole was obtained by decarboxylation of 4-carboxy-1,2,3-triazole which was prepared in good yield by the reaction of propiolic acid with hydrazoic acid in boiling benzene. Since propiolic acid is easily obtained by oxidation of propargylic alcohol [6] this is probably the most convenient method for the preparation of 1,2,3-triazole. By the corresponding reaction of methyl azide with propiolic acid 1-methyl-4-carboxy-1,2,3-triazole (IX) was prepared. The isomeric 1-methyl-5-carboxy-1,2,3-triazole was not found in the reaction mixture. IX, *via* the chloride, gave the azide which on boiling in ethanol gave 1-methyl-4-carbethoxyamino-1,2,3-triazole. The latter on boiling with dilute sodium hydroxide yielded 1-methyl-4-amino-1,2,3-triazole (VIII).

EXPERIMENTAL

Melting points are uncorrected.

4-Carboxy-1,2,3-triazole. A solution of hydrazoic acid (1.5 mole) and propiolic acid (35 g, 0.5 mole) in benzene (1 200 ml) was refluxed on the steam-bath for 15 h with an effective, water-cooled condenser. Some hydrazoic acid escaped through the condenser during the heating. The product began to separate after a few hours. The mixture was cooled and the product filtered off. Recrystallization from water gave 40 g (71 %) of 4-carboxy-1,2,3-triazole as colourless crystals with m. p. 222—224° (reported [2,7]: 214—215° and 220°).

1-Methyl-4-carboxy-1,2,3-triazole. Methyl azide was generated from sodium azide (39 g, 0.6 mole) and dimethyl sulfate (37.5 ml) [8] and passed over anhydrous calcium chloride into a mixture of dry toluene (100 ml) and propiolic acid (14 g, 0.2 mole). The mixture was kept overnight at room temperature and was then heated for 2 h in a pressure-bottle in boiling water. After cooling in ice the crystalline precipitate was filtered off and dried. The product (18.4 g) was recrystallized from water giving 14.6 g (57 %) of 1-methyl-4-carboxy-1,2,3-triazole as colourless crystals with m. p. 218—219° (reported [2]: 224°).

1,2,3-Triazole. 4-Carboxy-1,2,3-triazole (40 g) was heated in an oil bath. At a bath-temperature of 220° a vigorous evolution of carbon dioxide took place. The reaction was over in a few minutes and the product was distilled giving 22.8 g (93 %) of 1,2,3-triazole with b. p. 205—206° (760 mm).

1- and *2-Methyl-1,2,3-triazole.* To an ice-cold solution of 1,2,3-triazole (10.0 g) in ether (100 ml) was added an excess of ethereal diazomethane. The mixture was kept overnight at 0°. Ether and excess diazomethane was distilled off and the residue was distilled through a column of the Podbielniak type giving 5.53 g (46 %) of 2-methyl-1,2,3-triazole with b. p. 102—103° (760 mm) and 5.02 g (42 %) of 1-methyl-1,2,3-triazol with b. p. 223—225° (760 mm).

Reaction of cyanogen bromide with diazomethane. To a solution of cyanogen bromide (31.8 g) in dry ether (100 ml) was added an ether solution of diazomethane in portions. At first there was a lively reaction and it was necessary to cool in ice now and then to prevent the solution from boiling. After *ca.* 50 % of the diazomethane had been added the reaction became slower. A total of 3 equiv. of diazomethane was added and the mix-

ture was left overnight at room temperature. Ether and excess diazomethane was distilled off and the residue was distilled through a Podbielniak type column at 16 mm pressure giving 3 fractions:

1. 28.9 g, yellow lachrymatory liquid, b. p. 45—48°.
2. 5.0 g, semi-solid mixture, b. p. 98—100°.
3. 3.9 g, yellow solid, b. p. 140°.

2-Methyl-4-bromo-1,2,3-triazole. Fraction 1 (28.9 g) was washed with four 25 ml portions of 2 N sodium hydroxide. The first portion of sodium hydroxide caused heat evolution and a decrease in the volume of the bromo compound. The compound was then dissolved in ether and washed with water and the ether solution was dried with magnesium sulfate and distilled. 2-Methyl-4-bromo-1,2,3-triazole (10.0 g, 21 % based on cyanogen bromide) was obtained as a slightly yellow solid; b. p. 161—162° (760 mm), m. p. 22°. (Found: C 22.35; H 2.46; N 26.14; Br 49.45. Calc. for $C_3H_4N_3Br$: C 22.24; H 2.49; N 25.94; Br 49.33.)

1-Methyl-5-bromo-1,2,3-triazole. Fraction 2 (5.0 g) was recrystallized from ether-light petroleum (b.p. 40—60°) giving 3.1 g (6.4 %) of colourless crystals with m. p. 37—40°. Two additional recrystallizations gave pure 1-methyl-5-bromo-1,2,3-triazole with m. p. 41—42°. (Found: C 21.95; H 2.55; N 26.23.)

1-Methyl-4-bromo-1,2,3-triazole. Fraction 3 (3.9 g) was recrystallized from benzene-light petroleum (b. p. 60—100°) to give 2.7 g (5.5 %) of colourless crystals with m. p. 95—97°. Two additional recrystallizations gave pure 1-methyl-4-bromo-1,2,3-triazole with m. p. 98—99° (reported [2]: 98.5—99°). (Found: C 22.10; H 2.68; N 25.90.)

β-Amino-acetonitrile hydrochloride. Fraction 1 (5.0 g) was dissolved in liquid ammonia (30 ml) and the mixture was kept in an open flask at room temperature until the ammonia had evaporated. The residue was treated with ether, the ammonium bromide was filtered off, and the ether solution was evaporated to remove excess of ammonia. The residue was again dissolved in ether and dry HCl was passed through the solution giving a white precipitate (1.1 g) which was recrystallized from abs. ethanol. The yield of β-amino-acetonitrile hydrochloride was 0.80 g, m. p. 165—167° (decomp.) (reported [9]: 165°). (Found: C 25.75; H 5.32; N 30.53. Calc. for $C_2H_5N_2Cl$: C 25.95; H 5.45; N 30.30.) The infrared spectrum * was identical with that of an authentic sample prepared according to Curtius [9].

The ether solution from which the amino-acetonitrile hydrochloride was precipitated gave, on evaporation, 2-methyl-4-bromo-1,2,3-triazole.

1-Methyl-4-carbethoxyamino-1,2,3-triazole. 1-Methyl-4-carboxy-1,2,3-triazole (10 g) and phosphorus pentachloride (16.5 g) were mixed and heated. The mixture melted and a vigorous reaction took place. After a few minutes the reaction was over and phosphorus oxychloride and excess phosphorus pentachloride was removed *in vacuo* at 100°. The crude chloride was dissolved in acetone (125 ml). The solution was cooled in ice and stirred and a solution of sodium azide (15 g) in water (75 ml) was added during a few minutes. The mixture was then stirred for 1 h. Water (500 ml) was added and the colourless precipitate was filtered off, washed with water and dried giving 11.2 g (93 %) of the azide of 1-methyl-4-carboxy-1,2,3-triazole. The azide decomposes at 152—154° with nitrogen evolution and forms a new, colourless compound which melts at 215—217°. This compound is probably the corresponding *iso*cyanate. The azide was not purified further.

The azide (11.2 g) in abs. ethanol (100 ml) was refluxed for 36 h. The solution was then concentrated to 30 ml and cooled yielding 9.1 g (74 %) of colourless crystals with m. p. 114—118°. Two recrystallizations from ethanol gave the pure 1-methyl-4-carbethoxyamino-1,2,3-triazole, m. p. 123—124°. (Found: C 42.60; H 5.98. Calc. for $C_6H_{10}N_4O$: C 42.35; H 5.92.)

1-Methyl-4-amino-1,2,3-triazole. The urethane (9.1 g, m. p. 114—118°) and sodium hydroxide (13.3 g, 6 equiv.) in 150 ml of water was boiled for 4 h. The solution was then acidified with conc. HCl and evaporated to dryness *in vacuo*. The residue was extracted with 3 × 50 ml of boiling ethanol, the ethanol solution was concentrated to 30 ml and cooled giving 6.35 g of crude material which was recrystallized from ethanol-ether. Yield 5.1 g (64 %), m. p. 181—184° (decomp.). Two additional recrystallizations from abs.

---

* Thanks are due to Mrs. Susanne Refn, M.Sc., for taking the IR-spectra and discussing the evidence thus obtained.

ethanol gave the pure hydrochloride of 1-methyl-4-amino-1,2,3-triazole as colourless crystals with m. p. 182—185°. (Found: C 26.50; H 5.10; N 41.35; Cl 26.45. Calc. for $C_3H_7N_4Cl$: C 26.80; H 5.24; N 41.65; Cl 26.35.)

The hydrochloride (1.0 g) was mixed with 5 ml of 40 % sodium hydroxide and extracted continuously with ether for 8 h. The ether was removed and the residue recrystallized from benzene-light petroleum. The yield of 1-methyl-4-amino-1,2,3-triazole was 0.65 g (90 %), colourless crystals, m. p. 88—90°. Recrystallized again, m. p. 88—89°. (Found: C 36.70; H 6.29; N 56.80. Calc. for $C_3H_6N_4$: C 36.72; H 6.16; N 57.11.)

1-Methyl-5-amino-1,2,3-triazole. 1-Methyl-5-bromo-1,2,3-triazole (1.0 g) was dissolved in 16 ml of 5 % ethanolic ammonia. The solution was heated to 100° in a sealed tube for 10 h. The mixture was then evaporated to dryness and the residue was extracted with ether. The ether solution was treated with dry HCl yielding 0.73 g of a colourless solid which after 2 crystallizations from abs. ethanol gave 0.18 g (22 %) of the hydrochloride of 1-methyl-5-amino-1,2,3-triazole as colourless crystals with m. p. 181—182°. (Found: C 26.90; H 5.27; N 41.90. Calc. for $C_3H_7N_4Cl$: C 26.80; H 5.24; N 41.65.)

The 1- and 2-methyl-4-bromo-1,2,3-triazoles did not react with ammonia under the same conditions as above.

1-Methyl-5-anilino-1,2,3-triazole. 1-Methyl-5-bromo-1,2,3-triazole (0.5 g) and 0.56 ml (2 equiv.) of aniline was heated on the steam bath for 6 h during which time the mixture solidified. Water was added and the crystalline product was filtered off, washed with water and dried. Yield 0.36 g. Two recrystallizations from benzene-light petroleum (b. p. 60—100°) gave 0.24 g (45 %) of colourless crystals with m. p. 169—171° (Dimroth [5] reports 172°).

1-Methyl-4-bromo-1,2,3-triazole from 1-methyl-4-amino-1,2,3-triazole. 1-Methyl-4-amino-1,2,3-triazole (0.5 g) was dissolved in 5 ml of constant boiling HBr, cooled in ice, and diazotized with 0.35 g of sodium nitrite. A small amount of copper-bronze was added causing a vigorous nitrogen evolution. After a few minutes the reaction was over and the mixture was heated to the boiling point and cooled again. The colourless crystals which separated were extracted with chloroform (3 × 10 ml). The chloroform solution was concentrated to 10 ml and 50 ml of light petroleum (b. p. 40—60°) was added. On cooling 0.34 g (41 %) of 1-methyl-4-bromo-1,2,3-triazole crystallized out; m. p. 97—99°.

Microanalyses were made by Mr. P. Hansen of The Chemical Laboratory of The University of Copenhagen.

REFERENCES

1. Tamburello, B. A. and Milazzo, A. *Gazz. chim. ital.* **38** (1908) 1, 95.
2. Huttel, R. and Welzel, G. *Ann.* **593** (1955) 207.
3. Krollpfeiffer, F., Rosenberg, A. and Muhlhausen, C. *Ann.* **515** (1935) 113.
4. Albert, A., Goldacre, R. and Phillips, J. *J. Chem. Soc.* **1948** 2240.
5. Dimroth, O. *Ann.* **364** (1908) 183.
6. Wolf, V. *Chem. Ber.* **86** (1953) 735.
7. Oliveri-Mandala, E. and Copola, A. *Gazz. chim. ital.* **40** (1910) 436.
8. Dimroth, O. and Wislicenus, W. *Ber.* **38** (1905) 1573.
9. Curtius, Th. *Ber.* **31** (1898) 2489.

Received March 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 893–899

Duodenal Glucuronide Synthesis

IV. Studies of Steroidglucuronide Conjugation by the Duodenal Mucosa in Rat

K. HARTIALA and ANITA LEHTINEN

Department of Physiology, University of Turku, Finland

Steroid hormones were incubated in the presence of rat duodena slices. Paper chromatographic analyses were then performed on the ethylacetate extracts of the incubation mixture. These extracts revealed no free steroids and also no spots indicating glucuronic acid. Treatment of the ethylacetate evaporate with β-glucuronidase resulted in the liberation of free steroids and glucuronic acid when estrone, estradiol, estriol, equiline and stilbestrol were tested. No such glucuronide conjugates were identified for progesterone, pregnandiol, androsterone or testosterone. A phenolic hydroxyl at 3 C is a common feature of the former steroid group. None of the unconjugated steroids had this characteristic.

In our previous qualitative and quantitative studies we have identified estradiol glucuronide as a conjugation product formed by rat duodenal mucosa [1,2]. In the present work a number of other steroids have been subjected to similar analyses.

MATERIAL AND METHODS

Albino rats (Wistar) were used also in these experiments.

Steroids. Estriol, estrone, equiline, stilbestrol, progesterone, pregnandiol, androsterone and testosterone propionate were used. These were dissolved in 2 ml propyleneglycol [5] in the following amounts: 5.0 mg, 4.8 mg, 4.7 mg, 4.9 mg, 5.6 mg, 4.7 mg, 5.2 mg, 6.2 mg.

Incubation. The incubation of free steroids in the Warburg apparatus was performed as in the earlier studies [3,4]. The Krebs Ringer incubation solution contained 100 mg glucose and 112 mg sodium lactate per 50 ml of the mixture. Into each Warburg flask were transferred 2.9 ml of this Ringer solution, 0.1 ml of the steroid propyleneglycol solution (0.9 μmole steroid) and after this the mucosal tissue specimens (dry weight 8–10 mg). The gas phase was 5 % carbon dioxide in oxygen.

Sampling. After incubation the contents of 2 Warburg flasks were combined to make one sample for further analyses (= 6 ml). The tissue slices were washed with 2 ml Ringer solution (6 ml + 2 ml) after being removed from the flasks.

Deproteinization. The incubation mixture was heated at 60°C for 3 min and centrifuged thereafter.

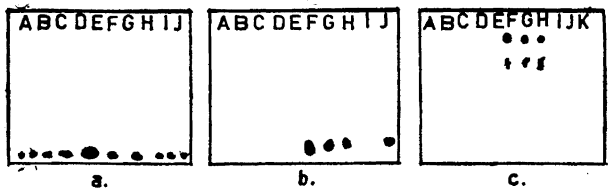


Fig. 1. Estrone. a. Unconjugated estrone. Positive spots (A, B, C, and D) for estrone present after the incubation of estrone with the tissue. The spots are, however, weaker than those obtained from the same incubation mixture without the tissue (G, H, I, and J). Control spots E (150 μg) and F (100 μg) pure estrone.

b. Estrone glucuronide acid and estrone liberated by hydrolysis. Unhydrolyzed samples show negative spots for free estrone (A, B, C, D, and E). Hydrolysis with β-glucuronidase results in liberation of free estrone (G, H, and J). Control, F, 150 μg estrone.

c. Estrone glucuronide and glucuronic acid liberated by hydrolysis. Unhydrolyzed samples give negative spots A, B, C, D, and E for free glucuronic acid. From the hydrolyzed samples (G, H, I, J, and K) G and H show positive spots for free glucuronic acid. F is obtained with pure glucuronic acid (150 μg). The spots which appear below the actual glucuronic acid spots are discussed in the text.

Extraction of free steroids with benzene, was carried out as in our earlier work [1,2].

Extraction of steroid glucuronides with ethylacetate from the acidified (2 N HCl) water phase was carried out as in the earlier work [1,2].

Hydrolysis of the conjugate was performed with β-glucuronidase (Worthington Biochem. Corp.) as in the previous papers [1,2]. Reextraction of the free steroids and glucuronic acid liberated by hydrolysis was also carried out as in the earlier work [1,2].

Paper chromatography. 1 ml of the solvent (ethylacetate/benzene) was added to each residue used for the chromatographic analyses. A mixture of butanol, glacial acetic acid and water was used as an irrigant; time 24 h, paper Whatman No. 1. Estriol, estrone, equiline, stilbestrol and pregnandiol spots were identified on the paper by a saturated aqueous solution of *p*-nitrobenzene-azodimethoxyaniline (Fast Black Salt, K. Heftman 1950 [6]). Stilbestrol gives a light brown spot on blue whereas the others give a blue red spot. Androsterone, testosterone and progesterone were identified with *m*-dinitrobenzene (1 vol. 2 % *m*-dinitrobenzene in alcohol + 1 vol. 2.5 N KOH in alcohol [7]). Glucuronic acid was identified with the $AgNO_3$-ammonia reagent (1 vol. 0.1 N $AgNO_3$, 1 vol. 5.0 N NH_3-solution + 1 vol. 2 N NaOH).

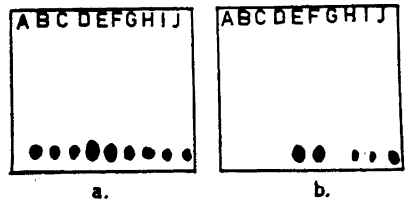


Fig. 2. Estriol. a. Unconjugated estriol. B, C, D, I, and J indicate free estriol present after incubation with the tissue slices. A is negative for some unknown reason. G and H, control runs made from the incubation mixture without the tissue. E and F pure estradiol (150 and 100 μg).

b. Estriol glucuronide acid and estriol liberated by hydrolysis. A, B, C, and D refer to the free estriol in the unhydrolyzed samples. H, I, and J, estriol liberated by hydrolysis. G is negative for some unknown reason. E and F, pure estriol (150 and 100 μg). From the hydrolyzed samples free glucuronic acid spots were never found. This is discussed in the text.

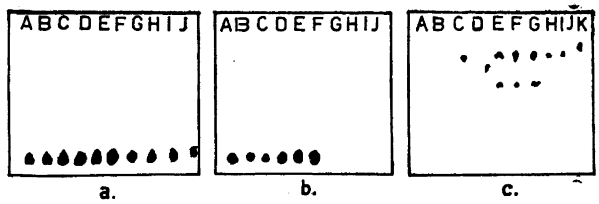


Fig. 3. Equiline. a. Unconjugated equiline. A, B, G, H, I, and J, free equiline present after incubation with the tissue slices. C and D, after incubation without the tissue. E and F, pure equiline (100 and 150 μg).

b. Unhydrolyzed equiline glucuronide and free equiline liberated by hydrolysis. G, H, I, and J refer to free equiline in the unhydrolyzed samples. A, B, C, and D free equiline after the hydrolysis. E and F pure equiline (100 and 150 μg).

c. Glucuronic acid liberated by hydrolysis. A, B, C, and E indicate the absence of free glucuronic acid before the hydrolysis. The positive spot D indicates that some liberation might take place without β-glucuronidase. H, I, J, and K, the same after enzymatic hydrolysis. F and G, pure glucuronic acid. Other spots seen in the pixture are discussed in the text.

RESULTS

In the following presentation the chromatograms of each of the steroids are given in a group. The first (left) picture of each group, a, indicates the spots due to steroids left unconjugated during the incubation. The second picture, b, indicates the results before and after hydrolysis of the conjugated steroids with β-glucuronidase. In this picture the absence of spots on the chromatograms of the unhydrolyzed steroid glucuronides or, in the cases in wich no glucuronide formation has occurred, the total absence of steroid spots is indicated. In the third picture, c, are indicated the glucuronic acid spots liberated during the hydrolysis. This picture, c, is not presented in Figs. 5—8, corresponding to experiments in which free glucuronic acid could never be demonstrated.

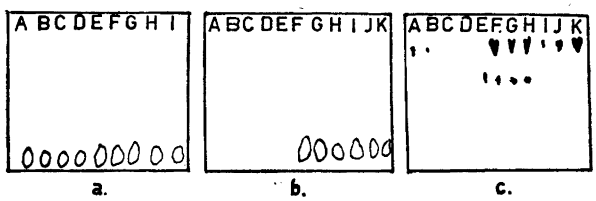


Fig. 4. Stilbestrol. a. Unconjugated stilbestrol. A, B, C, D, H, and I, free stilbestrol still present after incubation with the tissue slices. F and G, the same after incubation without the tissue. E, pure stilbestrol (150 μg).

b. Stilbestrol glucuronide and free stilbestrol liberated by hydrolysis. A, B, C, D, and E indicate negative spots for free stilbestrol before the hydrolysis. H, I, J, and K, free stilbestrol liberated by hydrolysis. F and G, pure stilbestrol (100 and 150 μg).

c. Glucuronic acid liberated by hydrolysis. A, B, C, D, and E, free glucuronic acid before the hydrolysis. Weak spots at A and B indicate some free glucuronic acid. G, H, I, J, and K the same after enzymatic hydrolysis. F, pure glucuronic acid (150 μg). The other spots present on the chromatogram are discussed in the text.

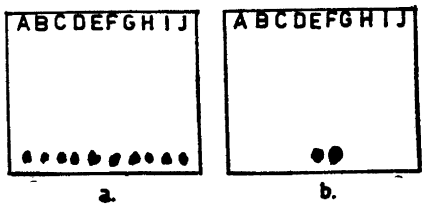


Fig. 5. Progesterone. a. Unconjugated progesterone. A, B, C, D, H, I, and J, free progesterone present after incubation with the tissue slices. E, control incubation without the tissue. F and G, pure progesterone (100 μg and 150 μg).

b. Absence of free progesterone in the unhydrolyzed and hydrolyzed samples. A, B, C, and D, before hydrolysis and G, H, I, and J, after hydrolysis with β-glucuronidase. E and F, pure progesterone (100 and 50 μg). The results indicate no glucuronide conjugation.

Positive results indicating glucuronide formation from the above listed estrogenic substances were obtained with

estrone	in	4	out	of	6	rats
estradiol	in	5	»	»	6	»
estriol	in	4	»	»	6	»
equiline	in	5	»	»	6	»
stilbestrol	in	6	»	»	6	»

Taking into consideration the size and color intensity of the different spots in the chromatogram stainings, it would appear that the degree of conjugation is not the same for the different estrogens. Using these indications as a rough estimate for the degree of conjugation, the following preliminary list can be given:

estrone	+
estradiol	+++ (reported in our previous paper [1,2])
estriol	+
equiline	++
stilbestrol	+++

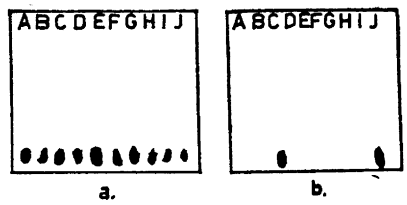


Fig. 6. Pregnandiol. a. Unconjugated pregnandiol. A, B, C, D, G, and H, free pregnandiol before incubation with the tissue slices. I and J, incubation without the tissue. E and F, pure pregnandiol (200 and 150 μg).

b. Absence of free pregnandiol in the hydrolyzed and unhydrolyzed specimens. A, B, C, before hydrolysis and E, F, G, H, and I, the hydrolyzed samples. D and J, pure pregnandiol (200 and 250 μg). The results indicate no glucuronide conjugation.

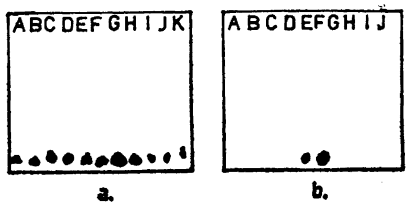


Fig. 7. Androsterone. a. Unconjugated androsterone. A, B, C, D, I, J, and K, free steroid present in the samples after incubation with the tissue slices. E and F, the same from the incubation mixture without the tissue. G and H, pure androsterone (150 and 100 μg).

b. Absence of free androsterone in the hydrolyzed and unhydrolyzed samples. A, B, C, and D, before hydrolysis and G, H, I, and J, after enzymatic hydrolysis. All spots are negative. E and F, pure androsterone (50 and 100 μg).

DISCUSSION

The chromatographic identification of the free and pure steroids used in these experiments has not met any difficulties. The presence of such spots on the chromatograms can also be taken as evidence of the reliability of the applied extraction procedures. On the other hand it occasionally appeared to be rather difficult to show free glucuronic acid on the chromatograms. In the case of estradiol, hydrolysis of the extract always resulted in the appearance of free glucuronic acid [1,2]. On the other hand glucuronic acid formation was detected only two or three times on 6 experiments with the other estrogens and never with estriol. The conjugate formation of the latter, as judged by the size and intensity of the free steroid spots of the conjugates in the different

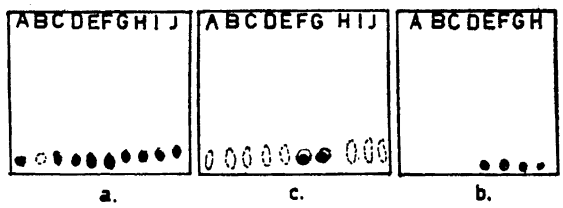


Fig. 8. Testosterone. a. Unconjugated testosterone. A, B, G, H, I, and J, free testosterone present after incubation with the tissue slices. C and D, samples incubated without the tissue. E and F, pure testosterone (150 and 100 μg). A positive spot was obtained for only one out of 6 animals.

b. Free testosterone in the hydrolyzed and unhydrolyzed specimens. A, B, C, and D negative spots for free testosterone in the unhydrolyzed samples. G and H, weak positive spots for free testosterone after hydrolysis. E and F, pure testosterone (150 and 100 μg).

c. In order to confirm the results obtained in the previous chromatograms the experiments were repeated three times with twice the amounts of solutions as used before. In the unhydrolyzed samples (A, B, C, D, and E) and in the hydrolyzed samples (H, I, and J) prolonged spots distinctly different from those given by pure testosterone (F and G, 150 and 100 μg) were found. These are discussed in the text. The results would indicate no glucuronide conjugation for the androgens.

chromatograms, is very small. It could be that the amount of glucuronic acid present is too small to give a positive spot on the paper. This possibility is enhanced by the fact that the experimental conditions do not eliminate the possibility of bacterial decomposition. This could reduce the amount of glucuronic acid present in the final steps of the analyses. This point was proved in our own experiments when it was found that free glucuronic acid added to the solutions under the same conditions as in the hydrolysis experiments disappeared completely in few days.

Extra spots are occasionally seen together with the glucuronic acid spots which arise from the apparent sensitivity of the $AgNO_3$ reagent towards various reducing substances. The small spots seen in several pictures beneath the glucuronic acid spots are identified as the lactone formed from the acid during the chromatographic procedure. These spots are also present when pure glucuronic acid alone is used.

The large, longish spots obtained with large amounts of the hydrolyzed and unhydrolyzed samples in connection with the repeated experiments with testosterone could not be more exactly identified due to lack of available pure testosterone conjugates. Since these spots stained with the same reagents as testosterone they might have an androgen molecular structure. The form of these spots would imply some kind of a salt compound. The possibility of a sulphate conjugate must also be considered. It may be pointed out that the tissue itself never interfered with the chromatographic analyses. Analyses of incubation mixture which contained tissue slices but not steroids never gave positive staining for spots which could erroneously be taken as steroid or glucuronic acid spots.

Finally, one interesting point can be stressed. If the interpretation of our results is correct, it would appear that glucuronide conjugation of substances possessing sexhormone activity is of a rather selective nature. Whereas the estrogens are conjugated, neither progesterone nor its metabolic product pregnandiol, nor the androgens are conjugated in this form. Examination of the molecular structure of these different substances reveals one interesting similarity of the glucuronide conjugated steroids which separates them from the others. They all have a phenolic hydroxyl group in the 3 C position. All of the compounds found to be unconjugated to a glucuronide lack this characteristic. It may be pointed out that in our previous studies indications of phenolphthalein and cinchophen glucuronide conjugation by the intestinal mucosa were found. The same molecular specificity also holds for these substances. The interpretation of this observation becomes however, rather complicated and difficult since recently a similar molecular characteristic, namely a phenolic hydroxyl at 3 C, has been found to belong to those steroids undergoing a sulphate conjugation[8].

This study has been supported by grants from the *State Council for Natural Sciences (Valtion Luonnontieteellinen Toimikunta)* and from the *Sigfrid Jusélius Stiftelse.*

REFERENCES

1. Lehtinen, A., Nurmikko, V. and Hartiala, K. *Acta Chem. Scand.* **12** (1958) 1585.
2. Lehtinen, A., Hartiala, K. and Nurmikko, V. *Acta Chem. Scand.* **12** (1958) 1589.
3. Hartiala, K. J. W. *Acta Physiol. Scand.* **31** (1954) Suppl. 114, 20.

4. Hartiala, K. J. W. and Rontu, S. *Ann. Med. Exptl. et Biol. Fenniae Helsinki* **33** (1955) 213.
5. Sie, H. G. and Fishman, W. H. *J. Biol. Chem.* **225** (1957) 451.
6. Heftman, E. *Science* **111** (1950) 571.
7. Kritchevsky, P. H. *Science* **114** (1951) 299.
8. Cohen, H. and Bates, R. W. *Endocrinology* **45** (1949) 86.

Received December 22, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 900–909

Kinetics of the Hydrolysis of Phenylphosphate Catalyzed by Potato Phosphatase

O. B. JØRGENSEN

Department of Technical Biochemistry, Royal Danish Technical University. Copenhagen, Denmark

The hydrolysis of phenylphosphate catalyzed by potato phosphatase was investigated to a high degree of reaction, and the liberation of phenol was followed colorimetrically. The reaction was studied with different initial concentrations of enzyme and substrate and with and without phosphate added. It was found that the following chronometric integral can describe the reaction:

$$E \cdot t = [k_a + k_b\ (p + a)] \ln \frac{a}{a-x} - k_c \cdot x$$

where t is time, a initial substrate concentration, x phosphate concentration at the time t, p the concentration of phosphate added and k_a, k_b and k_c are constants. A reaction mechanism based on the chronometric integral found is suggested.

Potato phosphatase is a group-specific acid phosphatase present in potato juice. Pfankuch [1] found the optimum pH 5.8, and he showed that Mg^{++} had little or no inhibiting effect on the activity of the enzyme, whereas F^- was a strong inhibitor. The Michaelis constant of β-glycerophosphate was $K_s = 18$ mM, and strong inhibition was found on addition of phosphate. The dissociation constant, K_p, of the enzyme-phosphate complex was found to be 1.8 mM. Helferich and Stetter [2] found the optimum pH for the substrate phenylphosphate to be 5.2—5.3. At a low degree of reaction it was found that the reaction followed first-order kinetics. Sripathi *et al.*[3] found optimum pH 5.3 and $K_s = 10$ mM for β-glycerophosphate. For an enzyme preparation from sweet potato Kondo *et al.*[4,5] found optimum pH 5.3—6.0 and $K_s = 1.7$—2.0 mM for β-glycerophosphate, $K_s = 12$ mM for glucose-1-phosphate and $K_s = 0.6$ mM for phenylphosphate. Very active preparations of the potato phosphatase have also been prepared by Schramm and Flammersfeld [6].

The reaction is by all the investigations quoted above only examined to low degrees of reaction. In this investigation the reaction was studied to high degrees of hydrolysis of the substrate, and the influence of the inhibition of phosphate was examined. The reaction was followed by determining the phenol liberated during the hydrolysis.

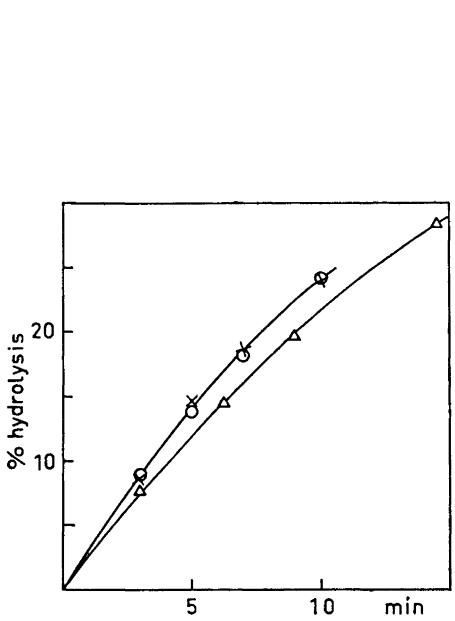


Fig. 1. Effect of Mg++ and phenol upon the rate of hydrolysis. ⊙——⊙ phenylphosphate concentration 4 mM, ×——× phenylphosphate concentration 4 mM and phenol concentration 6 mM, Δ——Δ phenylphosphate concentration 4 mM and concentration of Mg++ = 15 mM.

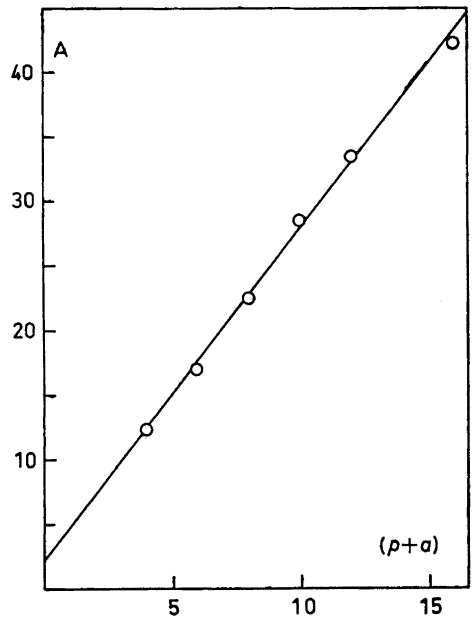


Fig. 2. Relation between the values of ($p_{\perp}$ + a) (abscissa) and the values of A (ordinate).

EXPERIMENTAL

Enzyme. Potato juice was fractionated by means of acetone and tannin as described by Helferich and Stetter [2]. Aqueous solutions of the most active fractions were used for the experiments. A slight activity of phenoloxydase in the enzyme solutions was completely inhibited by 0.5 p.p.m. sodiumsulfide. This reagent had no influence on the phosphatase activity. The enzyme solutions were stored at 0°C with some toluene added.

Substrate. The substrates were composed of 5 ml freshly prepared disodiumphenylphosphate solutions (Merck) and 4 ml 0.2 M citrate buffer (pH = 5.3). Investigating the phosphate inhibition the quantity of phosphate wanted was added to the phenylphosphate solution as KH_2PO_4.

Phenol determination. The phenol liberated during the reaction was determined colorimetrically with 4-aminoantipyrene as described by Hockenhull *et al.*[7]. The extinction was measured at 510 mμ in a Beckman spectrophotometer in 1 cm cells. A 0.1 mM solution of crystalline phenol (Merck) in 0.1 M HCl was used as a phenol standard. This solution was checked by iodometric determination. By neutralisation with 0.1 M NaOH and dilution of this stock solution different solutions with known contents of phenol were prepared. With a content of between 5×10^{-5} and 27×10^{-5} millimole phenol a linear relation between extinction and phenol concentration was found. The relative error of the phenol determination of the digest is 1.5 %.

Table 1. $a = 4$ mM, E = 1, A = 12.28 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	3.176	2.05	0.05	0.06
3	2.830	3.14	0.14	0.09
4	2.480	4.43	0.43	0.13
6	2.045	6.38	0.38	0.20
8	1.728	8.15	0.15	0.27
11	1.334	10.95	−0.05	0.41
14	1.048	13.65	−0.35	0.57
17	0.787	16.92	−0.08	0.80
21	0.544	21.22	0.22	1.22
25	0.421	24.25	−0.75	1.62
30	0.267	29.69	−0.31	2.63
35	0.178	34.59	−0.41	4.01
40	0.141	37.41	−2.59	5.10
46	0.075	45.10	−0.90	9.70
52	0.056	48.67	−3.33	13.04

Procedure. The substrate (9 ml) and the enzyme solution (1 ml) were preheated in a water thermostat at 20.0°C. The reaction was started by adding 1 ml of enzyme solution. The moment of half emptied pipette was defined as time zero. At different times 0.5 ml of the digest was withdrawn with a constriction pipette and poured into 2 % K_2CO_3 (1.5—4.5 ml) by means of which the enzyme reaction was stopped. The moment of half emptied pipette was defined as the time of the withdrawal. The phenol determination was performed on samples of this solution either directly or after suitable dilution. In all the experiments the concentrations of phenylphosphate and of added phosphate are given as millimoles of the substance concerned per litre of digest.

RESULTS

Optimum pH. Different digests with phenylphosphate concentration $a = 4$ mM and the same amount of enzyme but with varying pH (4.75—5.60) were examined. After the same time of reaction ($\alpha < 5$ %) a sample of the digest was withdrawn, and the degree of reaction at this time was chosen as a measure of the enzyme activity. Optimum was found at pH 5.3.

Effects of Mg^{++} and phenol. Fig. 1 shows the results of an experiment with $a = 4$ mM and the concentration of $Mg^{++} = 15$ mM. It is seen that Mg^{++} inhibits the reaction slightly. Fig. 1 shows too that addition of phenol (6 mM) had no effect on the rate of hydrolysis. These results are in agreement with the examinations by Pfankuch [1] and Helferich and Stetter [2].

The influence of varying substrate, phosphate and enzyme concentrations

The influence of the substrate concentration on the rate of reaction. Tables 1—4 show the results of four experiments performed at different substrate concentrations, a, but with the same enzyme concentration. The reactions did not follow zero-order, first-order or second-order kinetics, but the experi-

Table 2. $a = 6$ mM, E = 1, A = 17.00 min, B = −0.90 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	5.076	2.01	0.01	0.06
3	4.691	3.00	0.00	0.09
4	4.320	4.08	0.08	0.12
6	3.718	6.09	0.09	0.19
8	3.300	7.73	−0.27	0.25
11	2.630	10.99	−0.01	0.37
14	2.251	13.29	−0.71	0.47
17	1.757	17.06	0.06	0.67
21	1.452	20.03	−0.97	0.86
25	1.051	25.16	0.16	1.27
30	0.828	29.01	−0.99	1.66
35	0.540	36.02	1.02	2.65
41	0.425	39.98	−1.02	3.42
47	0.228	50.40	3.40	6.53
54	0.202	52.43	−1.57	7.40
62	0.086	66.84	4.84	17.61
80	0.029	85.27	5.27	52.61

mental results were fitted by the following rate equation consisting of a sum of a first-order and a zero-order term:

$$t = \mathrm{A} \cdot \ln \frac{a}{a - x} + \mathrm{B} \cdot x \qquad (1)$$

where t is time, a initial substrate concentration, x phosphate concentration at the time t and A and B are constants.

Table 3. $a = 8$ mM, E = 1, A = 22.55 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	6.987	2.09	0.09	0.06
3	6.602	3.01	0.01	0.09
4	6.228	3.97	−0.03	0.12
6	5.482	6.13	0.13	0.19
8	4.906	8.09	0.09	0.26
11	4.259	10.66	−0.34	0.35
14	3.597	13.84	−0.16	0.48
17	3.136	16.50	−0.50	0.59
21	2.512	20.91	−0.09	0.82
25	2.058	24.98	−0.02	1.06
30	1.574	30.56	0.56	1.47
35	1.222	35.92	0.92	1.97
41	0.842	43.97	−2.97	2.98
47	0.704	47.86	0.86	3.61
54	0.461	57.18	3.18	5.64
62	0.358	62.80	0.80	7.33
70	0.211	74.56	4.56	12.59
100	0.147	82.66	−17.34	18.21

Table 4. a = 10 mM, E = 1, A = 28.50 min, B = −0.90 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	8.962	2.19	0.19	0.06
3	8.593	3.06	0.06	0.09
4	8.177	4.10	0.10	0.12
5	7.812	5.06	0.06	0.15
6	7.332	6.45	0.45	0.19
8	6.764	8.23	0.23	0.25
10	6.196	10.22	0.22	0.31
12	5.824	11.65	−0.35	0.36
15	5.064	14.96	−0.04	0.48
18	4.592	17.32	−0.68	0.58
21	3.880	21.47	0.47	0.76
24	3.532	23.83	−0.17	0.87
28	2.968	28.28	0.28	1.11
33	2.488	32.88	−0.12	1.39
39	2.056	37.93	−1.07	1.76
46	1.508	46.27	0.27	2.52
55	1.188	52.78	−3.22	3.29
70	0.624	70.63	0.63	6.55

The values of A and B were determined graphically by the method described by Schønheyder and Volqvartz [8]. The values of A and B at different substrate concentrations are shown in Table 9. The enzyme concentration in experiments 1—4 is arbitrarily fixed as 1. In Tables 1—4 corresponding values of experimental t values and t values calculated by eqn. (1) are shown.

By differentiation of eqn. (1) is found:

Table 5. a = 8 mM, p = 4 mM, E = 1, A = 33.50 min, B = −0.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.307	2.38	0.38	0.06
3	7.036	3.38	0.38	0.08
4	6.792	4.34	0.34	0.11
6	6.328	6.26	0.26	0.16
8	5.869	8.36	0.36	0.21
11	5.328	11.09	0.09	0.29
14	4.746	14.41	0.41	0.39
17	4.259	17.56	0.56	0.49
21	3.792	21.02	0.02	0.62
25	3.318	25.03	0.03	0.78
30	2.842	29.77	−0.23	0.99
35	2.445	34.43	−0.57	1.22
41	1.990	40.90	−0.10	1.60
47.5	1.645	46.95	−0.55	2.03
54	1.344	53.44	−0.56	2.58
62	1.005	62.85	0.85	3.60
70	0.838	68.79	−1.21	4.40
80	0.669	76.17	−3.83	5.61
100	0.454	88.95	−11.05	8.46

Table 6. $a = 8$ mM, $p = 8$ mM, E = 1, A = 42.3 min, B = −1.1 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.468	2.31	0.31	0.05
3	7.248	3.36	0.36	0.08
4	7.040	4.34	0.34	0.10
6	6.681	6.16	0.16	0.15
8	6.333	8.05	0.05	0.19
11	5.856	10.84	−0.16	0.27
14	5.299	14.46	0.46	0.37
17	4.893	17.37	0.37	0.45
21	4.470	20.74	−0.26	0.56
25	3.971	25.21	0.21	0.71
30	3.462	30.44	0.44	0.91
35	3.040	35.48	0.48	1.12
41	2.554	42.29	1.29	1.44
47	2.198	48.27	1.27	1.77
54	1.789	56.53	2.53	2.31
62	1.504	63.54	1.54	2.85
80	0.880	85.54	5.54	5.25
100	0.586	102.40	2.40	8.15

$\Delta t = (A/(a-x) + B) \cdot \Delta x$ where Δx is the deviation of x; this deviation varies during the reaction. The relative error of the phenol determination $\Delta x/x = 0.015$, which gives $\Delta t_{calc} = (|A|/|a-x| + |B|) \cdot 0.015 \cdot x$. Δt_{calc} of all the experimental times are calculated and shown together with $\Delta t_{expt} = (t_{calc} - t_{expt})$ in the tables. The deviation of t_{expt} is not taken into account as it is only of importance for small values of t.

Table 7. $a = 8$ mM, E = 0.5, A = 47.5 min, B = −1.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
2	7.470	2.23	0.23	0.07
3	7.239	3.27	0.27	0.10
4	7.030	4.25	0.25	0.13
6	6.646	6.18	0.18	0.18
8	6.322	7.90	−0.10	0.24
11.5	5.680	11.73	0.23	0.36
14	5.312	14.21	0.21	0.44
18	4.816	17.90	−0.10	0.56
21	4.506	20.45	−0.55	0.65
25	4.086	24.28	−0.72	0.80
30	3.635	28.96	−1.04	0.98
35	3.251	33.51	−1.49	1.18
41	2.806	39.63	−0.37	1.47
47	2.355	47.08	0.08	1.87
54.5	2.026	53.59	−0.91	2.28
62	1.680	61.81	−0.19	2.86
70	1.504	66.71	−3.29	3.27
81.5	1.120	79.97	−1.53	4.58
100	0.861	91.96	−8.04	6.12

Table 8. a = 8 mM, E = 0.25, A = 93.00 min, B = −3.95 min/mM.

t_{expt} min	$a - x$ mM	t_{calc} min	Δt_{expt} min	Δt_{calc} min
3	7.558	3.54	0.54	0.11
4	7.449	4.46	0.46	0.14
6	7.204	6.56	0.56	0.20
8	7.013	8.35	0.35	0.25
11	6.715	11.15	0.15	0.34
14	6.476	13.62	−0.38	0.42
17.5	6.168	16.96	−0.54	0.52
21	5.898	20.03	−0.97	0.62
25	5.501	24.95	−0.05	0.78
30	5.149	29.75	−0.25	0.94
35	4.816	34.60	−0.40	1.11
41	4.400	41.37	0.37	1.35
47	4.051	47.70	0.70	1.59
54	3.725	54.20	0.20	1.85
62.5	3.354	62.49	0.01	2.22
70.5	2.973	72.20	1.70	2.66
80	2.723	79.40	−0.60	3.02
100	2.202	97.08	−2.92	4.02

The influence of phosphate concentration on the rate of reaction. Experiment 3 with phenylphosphate concentration a = 8 mM was repeated with phosphate of a concentration p = 4 mM and p = 8 mM added to the digest. Enzyme concentration as in experiment 3, E = 1. The results are shown in Tables 5 and 6. t_{calc} has been calculated by eqn. (1). The experiments show that phosphate has a strong inhibiting effect on the hydrolysis.

The influence of enzyme concentration on the rate of reaction. With constant substrate concentration, a = 8 mM, the enzyme concentration was varied. Tables 7 and 8 show the experimental results with E = 0.5 and E = 0.25. t_{calc} has been calculated by eqn. (1).

The variation of A and B with varying a, p and E. Table 9 shows that B is a constant, when a and p vary. Experiments 1—6 give an average value $B_{av} = -k_c = -0.96$ min/mM (E = 1). The A values change when a and p vary. In experiments 1—6 the values of A are found to fit a linear function of $(p + a)$, see Fig. 2. By the method of the least squares is found when E = 1:

Table 9.

Expt. No.	a mM	p mM	E	A min	B min/mM
1	4	0	1	12.28	−0.95
2	6	0	1	17.00	−0.90
3	8	0	1	22.55	−0.95
4	10	0	1	28.50	−0.90
5	8	4	1	33.50	−0.95
6	8	8	1	42.30	−1.10
7	8	0	0.5	47.50	−1.95
8	8	0	0.25	93.00	−3.95

$$A = 2.18 + 2.56\ (p + a) \tag{2}$$

Experiments 3, 7 and 8 show that A and B are inversely proportional to the enzyme concentration: $A = A'/E$ and $B = B'/E$. The empirical equation (1) then becomes:

$$E \cdot t = [k_a + k_b\ (p + a)] \cdot \ln \frac{a}{a - x} - k_c \cdot x \tag{3}$$

where $k_a = 2.18$, $k_b = 2.56$ and $k_c = 0.96$ when $E = 1$.

REACTION MECHANISM

The simplest reaction schemes that might describe the experimental results will be the following four where X_{1-4} are different enzyme forms, S phenylphosphate, P phosphate and F phenol:

1)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_1 + P \rightleftharpoons X_3 \qquad (\pm 3)$$

2)

$$X_1 + S \rightleftharpoons X_2 + P \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + F \qquad (+ 2)$$

3)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_2 + P \rightleftharpoons X_3 \qquad (\pm 3)$$

4)

$$X_1 + S \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P + F \qquad (+ 2)$$
$$X_2 + P \rightleftharpoons X_3 \qquad (\pm 3)$$
$$X_1 + P \rightleftharpoons X_4 \qquad (\pm 4)$$

The reaction with one molecule water, which is necessary in the hydrolysis, is omitted in the schemes, as it cannot be detected in the reaction kinetics, perhaps this reaction is involved in the step (+ 2). Scheme 1) represents competitive inhibition, scheme 3) uncompetitive and scheme 4) noncompetitive inhibition.

The reaction schemes were then treated mathematically using the steady state method as described by Christiansen [9,10]. Calling the steady state rate v and the reaction probabilities for the steps indicated w_i, we have from reaction scheme 1):

$$v = w_1 \cdot x_1 - w_{-1} \cdot x_2$$
$$v = w_2 \cdot x_2$$
$$w_3 \cdot x_1 = w_{-3} \cdot x_3$$

(x_{1-3} are the concentrations of the enzyme forms indicated). Solutions of the equations yield:

$$x_1 = v(w_2 + w_{-1})/w_1 \cdot w_2$$
$$x_2 = v/w_2$$
$$x_3 = v \cdot w_3(w_2 + w_{-1})/w_1 \cdot w_2 \cdot w_{-3}$$

The total enzyme concentration is $E = x_1 + x_2 + x_3$. Introducing the values of x_1, x_2, x_3 and $v = \frac{dx}{dt}$ and the values of w_i: $w_1 = k_1 \cdot (a—x)$, $w_{-1} = k_{-1}$, $w_2 = k_2$, $w_3 = k_3 \cdot (p + x)$ and $w_{-3} = k_{-3}$ where a is the initial substrate concentration, x the phosphate concentration at the time t and p the initial phosphate concentration, we find:

$$E \cdot dt/dx = (k_2 + k_{-1})/k_1 \cdot k_2 \cdot (a—x) + 1/k_2 + k_3 \cdot (k_2 + k_{-1})\,(p + x)/k_1 \cdot k_2 \cdot k_{-3} \cdot (a-x)$$

which by integration yields

1)

$$E \cdot t = \frac{k_2 + k_{-1}}{k_1 \cdot k_2} \cdot \left[1 + \frac{k_3}{k_{-3}}(p + a)\right] \cdot \ln \frac{a}{a—x} + \frac{k_2 + k_{-1}}{k_1 \cdot k_2}\left[\frac{k_1}{k_{-1} + k_2} - \frac{k_3}{k_{-3}}\right] \cdot x \quad (4)$$

which the has same form as the empirically found chronometric integral, eqn. (3).

When reaction scheme 2) is treated in the same manner, we find the following chronometric integral:

2)

$$E \cdot t = \frac{1}{k_1} \cdot \left[1 + \frac{k_{-1}}{k_2}(p + a)\right] \cdot \ln \frac{a}{a—x} + \frac{1}{k_2} \cdot \left[1— \frac{k_{-1}}{k_1}\right] \cdot x \quad (5)$$

which has also the same form as the empirically found chronometric integral, eqn. (3).

Scheme 3) gives the following chronometric integral:

3)

$$E \cdot t = \frac{k_2 + k_{-1}}{k_1 \cdot k_2} \ln \frac{a}{a—x} + \frac{1}{k_2}\left[1 + \frac{k_3}{k_{-3}} \cdot p\right] \cdot x + \frac{k_3}{2 \cdot k_2 \cdot k_{-3}} \cdot x^2$$

and 4) a similar integral. As both of these integrals are different from eqn. (3), the experimental facts cannot be satisfied by the schemes 3) and 4).

Comparing the chronometric integral of reaction scheme 2), eqn. (5), and the empirically found eqn. (3), it is found that:

$$k_{-1}/k_2 = k_b/k_a = 1.167$$

From this equation and reaction scheme 2) we then find, if the rate of reaction (— 1) is v_{-1} and the rate of reaction (+ 2) is v_2:

$$v_{-1}/v_2 = k_{-1} \cdot x/k_2 = 1.167 \cdot x \quad (6)$$

where x is the concentration of phosphate at the time considered. Eqn. (6) shows how many times X_2 will fall back after reaction (—1) compared with reaction (+ 2). By adding radioactive phosphate to the digest it should be

expected that the phosphate of the unhydrolyzed phenylphosphate and the radioactive phosphate were exchanged. Axelrod [11] has shown that citrus phosphatase cannot catalyze the transfer of inorganic phosphate to alcohols.

It was now investigated whether potato phosphatase would catalyze the exchange of phosphate. Radioactive phosphate, $NaH_2{}^{32}PO_4$, representing 10^9 counts per min per mM total phosphate, was added to 50 ml 20 mM phenylphosphate with buffer. Enzyme was added. The reaction was stopped, when 50 % of the substrate was hydrolyzed, and not hydrolyzed phenylphosphate was isolated from the digest by extraction with chloroform and several precipitations as described by Axelrod [11]. The isolated phenylphosphate showed 10^4 counts per min per mM phosphate. From eqn. (6) and the rate of hydrolysis is found that the isolated phenylphosphate should have shown about 10^8 counts per min per mM phosphate. This shows that reaction scheme 2) is not valid. Of the four reaction schemes proposed only scheme 1) can explain all the experimental results.

Introducing Michaelis constant, $K_s = \frac{k_2 + k_{-1}}{k_1}$, and the dissociation constant of the enzyme-phosphate complex, $K_p = k_{-3}/k_3$, in eqn. (4), it is found that:

$$E \cdot t = \frac{1}{k_2}\left[K_s + \frac{K_s}{K_p}(p + a)\right] \cdot \ln \frac{a}{a-x} + \frac{1}{k_2}\left[1 - \frac{K_s}{K_p}\right] \cdot x$$

Comparing this equation with the experimental eqn. (3), it is seen that with the enzyme and substrate here examined the values of K_s and K_p are found to be:

$$K_s = 1.36 \text{ mM and } K_p = 0.85 \text{ mM}.$$

Acknowledgements. I am much indebted to Professor Holger Jørgensen for excellent working conditions and for his interest in this work. I wish to thank Professor J. A. Christiansen for his helpful reading of the manuscript of this paper.

REFERENCES

1. Pfankuch, E. *Z. physiol. Chem. Hoppe-Seyler* **241** (1936) 34.
2. Helferich, B. and Stetter, H. *Ann.* **558** (1947) 234.
3. Sripathi, C. E., Nagana, B., Raman, A. and Venugopal, B. *Biochem. J.* **60** (1955) 215.
4. Watanabe, S., Ito, E., Takeuchi, I. and Kondo, S. *J. Chem. Soc. Japan* **74** (1953) 361; *Chem. Abstracts* **1953** 10569 b.
5. Ito, E. and Kondo, S. *Symposia on Enzyme Chem.* **9** (1954) 113; *Chem. Abstracts* **1955** 13317 f.
6. Schramm, G. and Flammersfeld, H. *Naturwiss.* **34** (1947) 216.
7. Hockenhull, D. J. D., Ashton, G. C., Fantes, K. H. and Whitehead, B. K. *Biochem. J.* **57** (1954) 93.
8. Schønheyder, F. and Volqvarts, K. *Biochim. et Biophys. Acta* **6** (1950) 147.
9. Christiansen, J. A. *Acta Chem. Scand.* **3** (1949) 493.
10. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.
11. Axelrod, B. *J. Biol. Chem.* **176** (1948) 295.

Received February 27, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 910–924

Crystal and Molecular Structure of Cytidylic Acid b

EYVIND ALVER and SVEN FURBERG

Chemical Institute, University of Bergen, Bergen, Norway

Cytidylic acid *b*, $C_9H_{14}N_3O_8P$, forms crystals of symmetry $P2_12_12$ with unit cell dimensions $a = 8.80$ Å, $b = 21.7$ Å, $c = 6.85$ Å. The structure was derived from electron density and difference syntheses along the *a* and *c* axes.

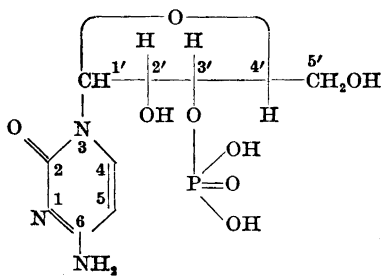
The molecule contains no unusual bond lengths, but some of the bond angles in the ribose ring differ significantly from "normal" values. The phosphate group is attached to the 3′-position of the sugar. The puckering of the ribose ring is different from what was found in crystals of cytidine, different ring atoms being out of the plane of the four other atoms in the two cases. The ribose and the phosphate group are arranged so as to make the molecule as extended as possible, the $P-O_3'$ bond having the same direction as the N_3-C_1' bond. This structure differs considerably from the structure hitherto assumed for nucleotides when building models of nucleic acids.

As constituents of the nucleic acids, the nucleosides and nucleotides are compounds of great biological interest, and it is important to have a detailed knowledge of their stereochemistry. The crystal structure of a nucleoside, cytidine, has been determined by one of us [1], and since then a number of heavy atom derivatives of nucleosides have been studied by X-ray methods [2,3]. As for the nucleotides, a note on the determination of a projection of adenylic acid has appeared in the literature [4]. Furthermore, a nucleotide-like component forms part of the molecule of vitamine B_{12}, the crystal structure of which has been determined by Hodgkin *et al*[5]. In the present paper the determination of the complete crystal structure of a nucleotide, cytidylic acid *b*, the 3′ phosphate ester of cytidine, is described. A preliminary note on this work has been published [6].

EXPERIMENTAL. CRYSTAL DATA

A specimen of cytidylic acid *b* was kindly supplied by Dr. Waldo E. Cohn, of the Oak Ridge National Laboratory, Tennessee. Large, well-developed crystals were obtained by evaporation of an aqueous solution, generally with (010) dominating and elongated along *c*. Forms {011}, {001}, {100} and {110} were also observed. (100) is a cleavage plane.

An examination by the polarising microscope showed the crystals to be biaxial positive, with (001) as the optic axial plane, and with $\alpha \| a$, $\gamma \| b$ and $\beta \| c$. The optic axial angle is great.



Structural formula, cytidylic acid b.

Oscillation and Weissenberg photographs were taken about the a and c axes, using filtered copper radiation (λ = 1.542 Å). The crystals are orthorhombic, with unit cell dimensions a = 8.80 Å, b = 21.7 Å and c = 6.85 Å (± 0.5 %). The only systematic absences occur in the $h00$ reflections for h odd and in the $0k0$ reflections for k odd, and the space group is therefore $P2_12_12$. Some of the odd-order $0k0$ reflections appear with low intensity on long-exposure photographs, but their form indicate that they are Renninger reflections. The density, measured by flotation, was found to be 1.66 g/cm³. There are four molecules $C_9H_{14}N_3O_8P$ in the unit cell, which gives a calculated density of 1.64 g/cm³.

For the structure determination, Weissenberg photographs of the zones $hk0-hk4$ and $0kl$-$5kl$ were taken, and about 1 300 independent reflections were recorded. The intensities were estimated visually and corrected for Lorentz-polarization effects [7] and for the variation of spot shape on equi-inclination photographs [8]. Small crystals were used and no correction for absorption was applied. The $hk0$ reflections were recorded from two different crystals and the mean value of the two sets of measurements was used. The calculations were carried out on desk machines and Beevers-Lipson strips were employed for the Fourier summations.

DETERMINATION OF THE STRUCTURE

Projections of the structure along the a- and c-axes were determined. The a-axis projection was first solved, as the strongest reflections are found in this zone. Approximate positions for most of the 21 atoms were derived by Patterson methods, inequalities and "trial and error", use being made of the Patterson function to determine phases. The refinement was carried out mainly by means of (F_o-F_c) syntheses.

Location of the phosphorus atom. Patterson projections along the a and c axes were first calculated (Fig. 1). The highest maxima were supposed to represent P—P vectors, and it can be seen from Fig. 1b that the phosphorus atom probably has the approximate coordinates $y_P = 0.25$, $z_P = 0.10$. The maxima in Fig. 1a are too diffuse to give reliable coordinates, but indicate that the phosphorus atom is situated near $x = 0$ or $x = 0.25$.

The Harker sections $(u, v, 0)$, $(1/2, v, w)$ and $(u, 1/2, w)$ were calcu'ated in the hope that they might give more reliable coordinates for the phosphorus atom. The section $u = 1/2$ has its highest maximum at (0, 0.23), corresponding to coordinates $y_P = 0.25$ and $z_P = 0.115$. This section was "sharpened" by dividing F^2 by $\hat{f}^2$. In the Harker section $w = 0$ the P—P vector $(2x, 2y)$ should then be found at $(2x, 0.5)$. However, two maxima of equal heights appeared at $v = 1/2$, leaving the two possibilities $x_P = 0.06$ and $x_P = 0.25$. This difficulty was overcome by an application of the "vector convergence"

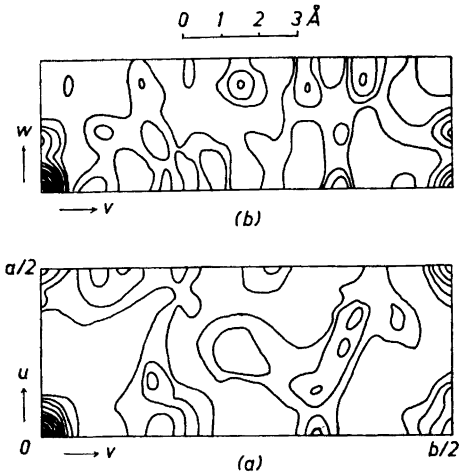


Fig. 1. Patterson projections on the (001) plane (a) and the (100) plane (b).

method [9]. The vector convergence map obtained by overlapping four transcriptions of the Patterson *c*-projection was derived for the two possible *x*-coordinates. In the case of $x = 0.25$, the map showed strong overlapping of peaks in the region near the axis of rotation, where no atoms can be situated. In fact, no atomic centre should be placed at a shorter distance from this axis than its van der Waals radius. The vector convergence map corresponding to $x = 0.06$ had a very low value in the "forbidden" region. Consequently, the phosphorus atom probably has the position (0.06, 0.25, 0.115). The agreement with the coordinates determined later on by Fourier methods is excellent. The "correct" vector convergence map also showed that the molecule probably should lie roughly diagonally in the *c*-projection.

Very little information on the positions of the lighter atoms could be derived from the Harker sections because of strong masking by non-Harker maxima.

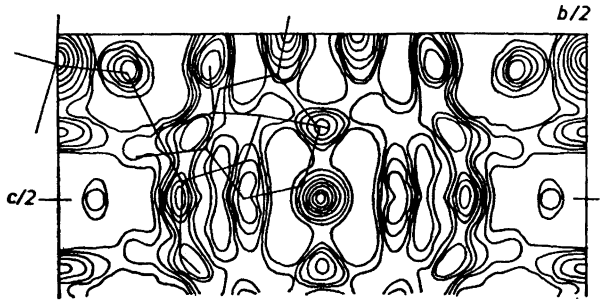


Fig. 2. The *a*-projection vector convergence map.

The a-projection. Attempts to solve the structure by the standard heavy atom technique were not successful. The phosphorus atom carries less than 10 % of the electrons in the molecule and is hardly heavy enough to determine the phases. Furthermore, its contribution to many structure factors is zero, as $y_P = 0.25$. Some information on the position of the lighter atoms was obtained from the Patterson functions in two ways. Firstly, a vector convergence map (Fig. 2) was derived by adding four transcriptions of the Patterson *a*-projection with their origins transferred to (0,0), (0.5, 0), (0, 0.23) and (0.5, 0.23). The map did not show sharp maxima which could be interpreted as individual atoms. Its symmetry is also too high. However, it indicated roughly the distribution of the atoms in the unit cell, and was used in conjunction with Bragg-Lipson charts to determine the probable signs of a number of structure factors. The second application of the Patterson function was based on the assumption that the lines $w = 0$ in the Patterson *a*-projection and $u = 1/2$ in the Patterson *c*-projection probably to a large extent represent vectors between equivalent atoms. By combining these two curves the vectors between non-equivalent atoms can be partly eliminated, and the curves may be expected to indicate the *y*-coordinates of the atoms. The probable signs of the four strongest $0k0$-reflections were derived in this way. The procedure may possibly be improved by sharpening the Patterson function.

The structure factors were put on an approximately absolute scale by Wilson's method [10]. The unitary structure factors (U) all had values below 0.30, except for $U_{0,14,1} = 0.40$ and $U_{005} = 0.34$. The signs of the strong reflections 005, 060, 080, 0,14,0, 0,16,0, 0,14,1 and 0,25,2 were derived from the vector convergence map and the distribution curve described above. As the unitary structure factors are small, the usual inequality $(U_H \pm U_K)^2 \leqslant (1 \pm U_{H+K})\ (1 \pm U_{H-K})$ could give no more signs. However, it was used to determine seven more probable signs after multiplying the U values by exp $(1.8 \sin^2\Theta/\lambda^2)$ [11]. The probable signs of eight additional reflections were indicated by the "multiplication rule" [12]. The F_o map calculated from these 22 important reflections was unclear, but indicated the positions of the oxygen atoms in the phosphate group. This was confirmed by calculating the three-dimensional Patterson function in this region and by the Harker sections. A number of other terms could now be included in the F_o synthesis, but in spite of this, the rest of the molecule had to be placed mainly by trial and error procedures. This was rather difficult, as freedom of rotation about a number of single bonds and the flexibility of the sugar ring make a great variety of molecular shapes possible. Extensive use was made of Bragg-Lipson charts and evidence from the Patterson, vector convergence, and F_o maps considered. The vector convergence map was particularly useful at this stage, as it seemed improbable that any atom should be lying in a strongly negative area. In effect, it represents the correct structure remarkably well, as will be seen from Fig. 2. An important clue is furthermore provided by the optical properties of the crystal, which indicate that the plane of the pyrimidine ring is roughly perpendicular to the *a*-axis. Reasonable qualitative agreement between observed and calculated structure factors for reflections of lower orders was finally obtained with a structure, in which the sugar and pyrimidine parts of different molecules overlapped very badly. By F_o refinement the reliability factor

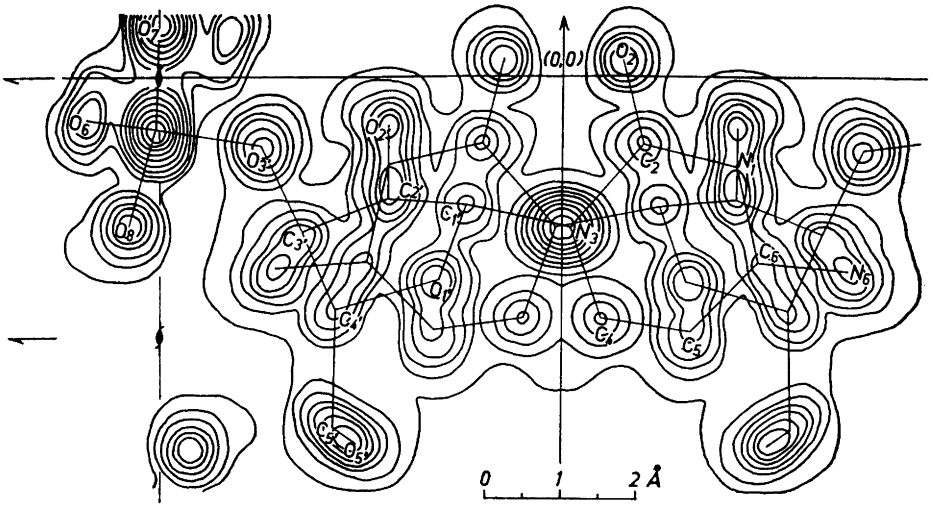


Fig. 3. Electron density projection in direction of *a* axis. Contours at 2, 4, 6, ... e . Å^{-2}.

$R = \Sigma\ (||F_o| - |F_c||)/\Sigma|F_o|$ was brought down to about 0.45 (ignoring reflections with sin Θ >0.75), but the lack of resolution made further improvement by this method difficult. The refinement was continued by successive (F_o—F_c) syntheses, about twenty of these being calculated. In the beginning only terms for which $|F_c|$ was considerably greater than $|F_o|$ were included. The refinement process did not seem to be automatic at the earliest stages and a certain election amongst the shifts indicated by the difference maps had to be made. The positions of most of the fourteen hydrogen atoms were indicated by the final maps. The others were placed by stereochemical considerations. Their inclusion did not improve the agreement significantly. Furthermore, the eight strongest reflections were corrected for extinction effects, following the procedure described by Lipson et *al.*[19] The final value for R for all $0kl$ reflections observable by copper radiation is 0.11. In calculating R the non-observed reflections are only taken into account when F_c exceeds the observable limit of intensity. In the temperature factor $\exp(-B\sin^2\Theta/\lambda^2)$ the value of B is 2.2—0.5$\sin^2\varphi$ for the phosphorus atom and 2.7—0.5$\sin^2\varphi$ for all the other atoms, φ being the angle between the *c*-axis and the normal to the reflecting plane. The atomic scattering curves of McWeeny [13] (for C, N, O and H) and Viervoll and Øgrim [14] (for P) were used. The final electron density map is reproduced in Fig. 3.

The c-projection. Approximate x-coordinates were derived by means of the *a*-projection and approximately known interatomic distances, assuming that x_P was correctly determined from the Harker section and that there were hydrogen bonds between the NH_2-group and the neighbouring PO_4-group. The postulated structure gave a factor R of 0.50, which was brought down to 0.11 by about ten successive (F_o—F_c) refinements. Some very radical shifts in the

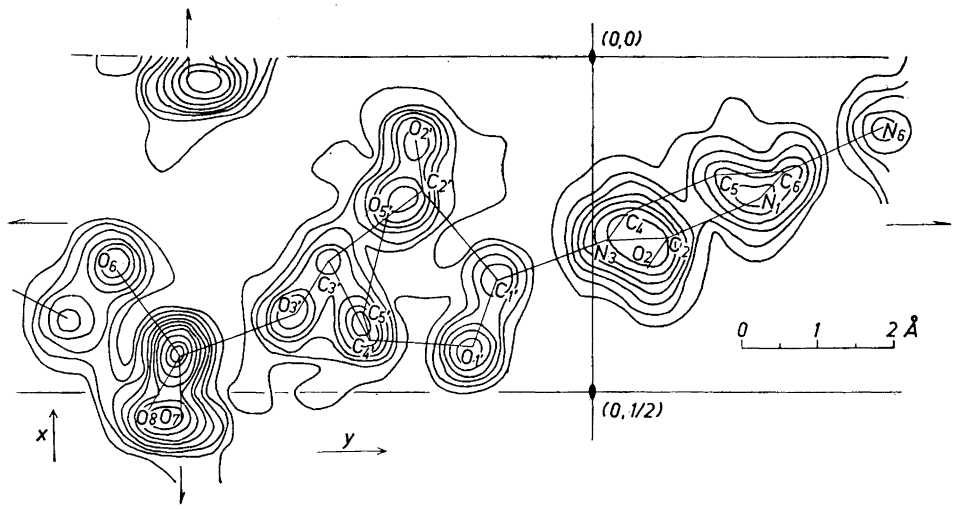


Fig. 4. Electron density projection in direction of c axis. Contours at 2, 4, 6, ... e . Å^{-2}.

rotation about single bonds were clearly indicated by the difference maps in the course of this process. The hydrogen atoms were located in the same way as in the *a*-projection and their inclusion made *R* drop to 0.10. Correction for extinction led to a final value of 0.09 for *R*. The final value chosen for *B* was 1.7 for all the atoms, but a comparison of F_o and F_c indicated that the vibrations of the phosphorus atom and the keto oxygen atom were anisotropic. This was taken into account by applying a factor *B* of 1.7—0.5 $\sin^2 \varphi$ to the phosphorus atom and of 1.7 + 1.0 $\cos^2\varphi$ to the keto oxygen atom, φ being the angle between the *a*-axis and the normal to the reflecting plane. Further refinements of the thermal parameters were not carried out. The final electron density map is given in Fig. 4. In Table 4 observed and calculated structure factors are compared for the *hk*0 reflections. A corresponding list for the 0*kl* reflections can be supplied by the authors on request.

In Table 1 the atomic coordinates derived from the final difference maps in the two projections are given. The two sets of *y*-coordinates were in good agreement and the mean value was taken. In Table 2 the coordinates employed in the calculation of the contribution from the hydrogen atoms are given.

DESCRIPTION AND DISCUSSION OF THE STRUCTURE

The bond lengths and bond angles calculated from the coordinates in Table 1 are given in Table 3 and Fig. 5. A rough estimate of the accuracy of the structure was obtained by comparing the two sets of independently determined *y*-coordinates. For several atoms, inclusive the phosphorus atom, the two values are identical. The greatest deviation, 0.04 Å, occur for the atom C_6, and the

Table 1. Atomic coordinates as fractions of the corresponding cell edge.

Atom	x	y	z
P	0.060	0.2466	0.114
O_6	0.195	0.206	0.066
O_7	−0.042	0.2495	−0.069
O_8	−0.025	0.2278	0.291
O_3'	0.119	0.3162	0.136
O_2'	0.382	0.392	0.086
O_1'	0.064	0.426	0.397
O_5'	0.279	0.3755	0.711
O_2	0.187	0.5342	−0.030
C_3'	0.195	0.337	0.309
C_2'	0.300	0.395	0.260
C_1'	0.168	0.443	0.255
C_4'	0.076	0.362	0.459
C_5'	0.120	0.358	0.673
C_4	0.266	0.520	0.474
C_5	0.320	0.579	0.511
C_6	0.330	0.622	0.366
C_2	0.235	0.546	0.135
N_3	0.223	0.505	0.289
N_1	0.280	0.603	0.181
N_6	0.388	0.679	0.387

standard deviation of the y-coordinates from their means, all atoms taken together, is 0.012 Å. It is assumed that the accuracy of the x- and z-coordinates is about the same as that of the y-coordinates from one projection only, corresponding to a standard deviation of about 0.017 Å. This gives a standard deviation of 0.015—0.025 Å in the bond lengths and of 1—2° in the bond angles. The errors within the phosphate group probably correspond to the lower limit, whereas somewhat greater errors may occur where the atoms overlap most badly.

Table 2. Coordinates assumed for the hydrogen atoms.

Atom	x	y	z
HO_6	0.170	0.168	0.934
HO_7	0.840	0.267	0.930
H_3'	0.237	0.302	0.435
H_2'	0.366	0.420	0.350
HO_3'	0.330	0.384	0.943
H_1'	0.107	0.442	0.110
H_4'	0.970	0.334	0.430
H_5'	0.055	0.395	0.755
H''_5'	0.086	0.326	0.750
HO_2'	0.346	0.340	0.735
H_4	0.280	0.500	0.560
H_5	0.350	0.584	0.610
H_{N_6}	0.450	0.683	0.510
H'_{N_6}	0.430	0.692	0.286

Table 3. Bond lengths (in Å units) and bond angles.

Bond lengths.

$P-O_6$	1.52	$C_{1'}-O_{1'}$	1.39	C_5-C_6	1.36
$P-O_7$	1.54	$O_{1'}-C_{4'}$	1.46	C_6-N_1	1.40
$P-O_8$	1.48	$C_{4'}-C_{3'}$	1.56	N_1-C_2	1.34
$P-O_{3'}$	1.60	$C_{4'}-C_{5'}$	1.52	C_2-N_3	1.38
$O_{3'}-C_{3'}$	1.43	$C_{5'}-O_{5'}$	1.47	C_2-O_2	1.23
$C_{3'}-C_{2'}$	1.60	$C_{1'}-N_3$	1.45	C_6-N_6	1.35
$C_{2'}-O_{2'}$	1.40	N_3-C_4	1.36		
$C_{2'}-C_{1'}$	1.56	C_4-C_5	1.39		

Bond angles.

O_8-P-O_6	115°	$C_{2'}-C_{1'}-O_{1'}$	107°	$C_{1'}-N_3-C_2$	120°
O_8-P-O_7	112°	$C_{1'}-O_{1'}-C_{4'}$	114°	$N_3-C_4-C_5$	119°
$O_8-P-O_{3'}$	110°	$C_{4'}-C_{3'}-C_{2'}$	105°	$C_4-C_5-C_6$	122°
O_6-P-O_7	108°	$O_{1'}-C_{4'}-C_{3'}$	101°	$C_5-C_6-N_1$	116°
$O_6-P-O_{3'}$	108°	$C_{4'}-C_{3'}-O_{3'}$	110°	$C_6-N_1-C_2$	125°
$O_7-P-O_{3'}$	103°	$O_{1'}-C_{4'}-C_{5'}$	111°	$N_1-C_2-N_3$	116°
$P-O_{3'}-C_{3'}$	121°	$C_{3'}-C_{4'}-C_{5'}$	116°	$C_2-N_3-C_4$	122°
$O_{3'}-C_{3'}-C_{2'}$	110°	$C_{4'}-C_{5'}-O_{5'}$	113°	$N_1-C_2-O_2$	121°
$C_{3'}-C_{2'}-O_{2'}$	116°	$O_{1'}-C_{1'}-N_3$	111°	$N_3-C_2-O_2$	123°
$O_{2'}-C_{2'}-C_{1'}$	113°	$C_{2'}-C_{1'}-N_3$	112°	$C_5-C_6-N_6$	125°
$C_{3'}-C_{2'}-C_{1'}$	96°	$C_{1'}-N_3-C_4$	118°	$N_1-C_6-N_6$	119°

The molecular structure. It is seen from the projections of the structure that the N-glycosidic linkage is of the β-type and that the phosphate group is bound to ribose in the 3'-position. Cytidylic acid *b* is consequently the 3'-phosphate ester of cytidine, in agreement with the conclusions drawn from more indirect physical studies [15].

The dimensions of the phosphate group agree well with those found in dibenzylphosphate[17] and in crystalline phosphoric acid [16]. Values between 1.48 Å and 1.60 Å are derived for the P—O bond lengths and all of them evidently have a large amount of double bond character. Correspondingly the angle $P—O_{3'}—C_{3'}$ is 121°, close to the value usually found for angles between double and single bonds. The longest P—O bond (1.60 Å) is the $P—O_{3'}$ ester bond. It runs roughly in the direction of the *y*-axis and should thus be expected to be more accurately determined than the other bonds. The lengthening of this bond relative to $P—O_6$ and $P—O_7$ is therefore probably significant. No such effect was, however, observed in the case of dibenzylphosphate. The shortest P—O distance ($P—O_8$) is 1.48 Å, indicating that O_8 is the "lone" oxygen atom. Correspondingly, all the angles involving $P—O_8$ are somewhat greater than the tetrahedral angle and the angles between other P—O bonds somewhat smaller, the mean values being 112° and 106°, respectively, as was also found in phosphoric acid.

Four of the atoms of the ribose ring lie nearly in one plane, namely $C_{1'}$, $O_{1'}$, $C_{3'}$ and $C_{4'}$, but the fifth member of the ring, $C_{2'}$, is out of this plane by about 0.5 Å in such a direction that its oxygen atom $O_{2'}$ is brought close to the plane. In cytidine a different atom, $C_{3'}$, was displaced from the plane of the other atoms by about 0.5 Å. The bond lengths are normal within the limits

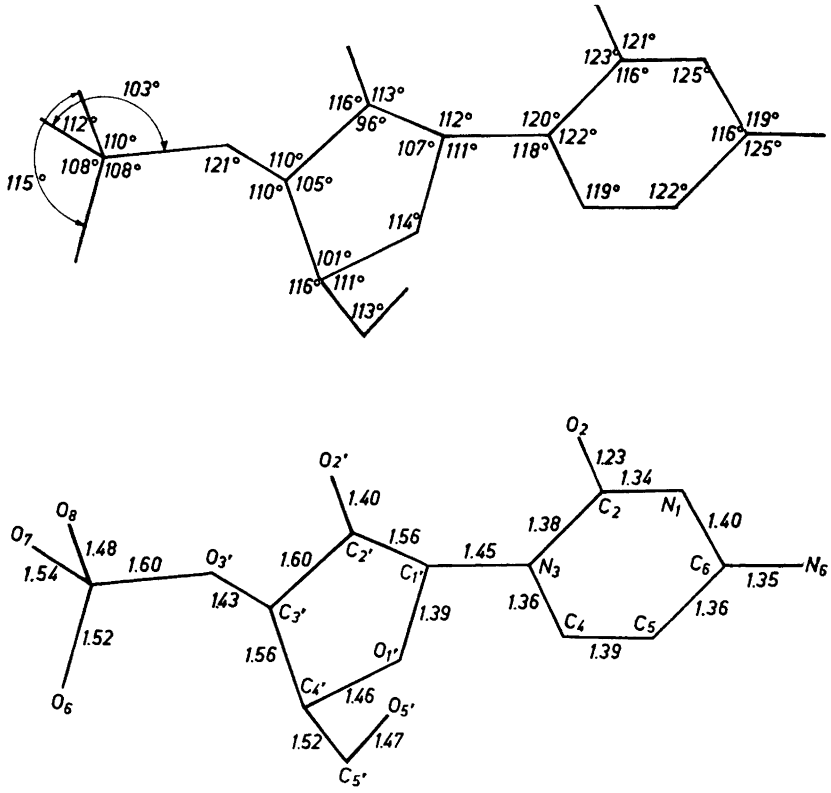


Fig. 5. Bond lengths and bond angles in cytidylic acid *b*.

of error, but some of the bond angles differ significantly from the expected values. Thus the ring angles at $C_{4'}$ and $C_{2'}$ are found as small as 101° and 96°, respectively. By a deformation of the furanose ring from a hypothetical planar structure the sum of the ring angles must become smaller. If it is deformed so as to bring base and phosphate into the relative orientation found in this work, it is seen by means of models that the most reasonable way of obtaining this is to make the angles at $C_{2'}$ and $C_{4'}$ smaller. Unfortunately the coordinates of $C_{2'}$ are very difficult to determine by two-dimensional methods because of overlapping with other atoms in both projections. The hydrogen and oxygen atoms attached to the ring are in a nearly staggered position with respect to the C—C ring bonds, corresponding to a stable conformation of the sugar.

The bond N_3—$C_{1'}$ is found to be 1.45 Å and is thus essentially a single bond. It lies in the plane of the pyrimidine ring and forms angles of 111° and 112° with the adjacent ring bonds in the ribose. The bond angles and bond lengths in the pyrimidine part of the molecule are all normal, being in close agreement with those derived by Parry for uracil [18]. Thus the ring angles at the nitrogen

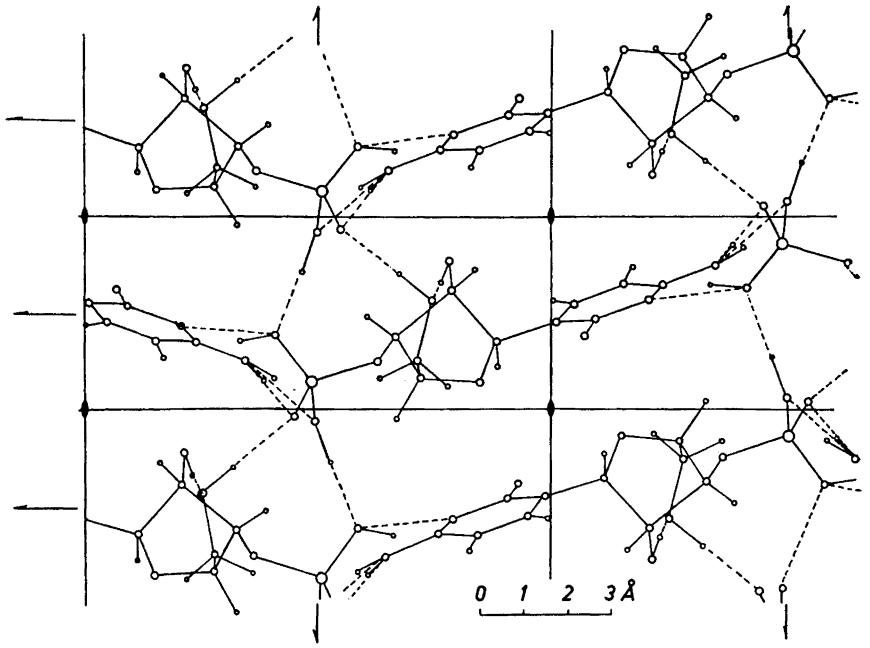


Fig. 6. The *c*-projection of the structure. Broken lines indicate hydrogen bonds. The hydrogen atoms are shown as small circles.

atoms are greater than 120°, the values 124° and 125° being found in uracil and 122° and 125° in the present structure. The C=O distance of 1.23 Å agrees with the values found for keto groups in similar compounds. All the ring atoms and substituents lie in the same plane within the limits of error. The C_6—N_6 distance of 1.35 Å indicates a considerable amount of double bond character. The peaks corresponding to the two hydrogen atoms of the amino group are well defined in both projections, and show that the three bonds from N_6 form a flattened pyramid, as in other amines. This is also in agreement with the directions of the two hydrogen bonds from N_6.

The mutual orientation of the pyrimidine and ribose rings is very nearly as in crystals of cytidine, the two ring "planes" being roughly perpendicular to each other. The midpoints of the bonds $C_{3'}$—$C_{4'}$ and $C_{5'}$—$O_{5'}$ lie nearly in the pyrimidine plane. The distance C_4—$O_{5'}$ is 3.53 Å, corresponding to normal van der Waals separation between H_4 and $O_{5'}$. In cytidine this distance was found to be as short as 3.24 Å*.

Intermolecular forces. The chemical formula of the compound shows that six of the hydrogen atoms in the molecule would be expected to engage in the

* *Note added in proof.* In a recent paper [22] Spencer has discussed most of the available information on the structure of nucleic acid components and related compounds. The present work seems to support his views regarding the conformations of the ribose ring.

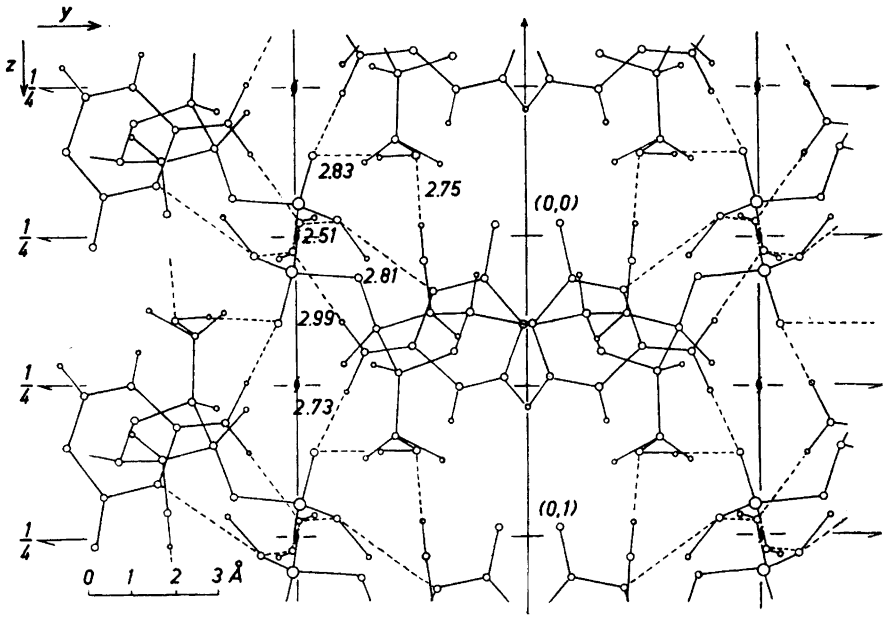


Fig. 7. The *a*-projection of the structure. The lengths of the hydrogen bonds are indicated (Ångström units).

formation of hydrogen bonds as there are two P—OH groups, one NH_2 group and two sugar hydroxyl groups in the molecule. Correspondingly only six intermolecular distances are found to be shorter than 3.1 Å and it appears therefore reasonable to interpret these interactions as hydrogen bonds.

Two hydrogen bonds of lengths 2.73 Å and 2.99 Å form linkages from the amino group to O_8 and O_7 in different phosphate groups. They are situated symmetrically with respect to C_6—N_6 and lie in a plane which forms an angle of about 30° with the plane of the pyrimidine ring.

The oxygen atom O_6 of the phosphate group is linked by a hydrogen bond (length 2.81 Å) to the nearest ring nitrogen atom. This bond lies nearly in the ring plane. Furthermore, the atom O_6 is engaged in a very short hydrogen bond (length 2.51 Å) to O_7 in a neighbouring phosphate group. The two hydroxyl groups in the ribose, $(OH)_{2'}$ and $(OH)_{5'}$, are connected by an O—H...O bond of length 2.75 Å running approximately in the *z* direction and $(OH)_{5'}$ takes also part in a similar bond (2.83 Å) to O_8.

The amino derivatives of purines and pyrimidines are known to occur in crystals in the amino form. Thus there are two hydrogen atoms at N_6 and none at N_1. Taking this into account, a consideration of the hydrogen bonding scheme shows that there are hydrogen atoms attached to O_6 and O_7, but not to O_8. Consequently O_8 is the "lone" oxygen atom of the phosphate group, as was also deduced above. The hydrogen bonds conform remarkably well to

the general stereochemistry of the molecule, and the angles between the hydrogen bonds and the adjacent intramolecular bonds do not deviate much from normal valency angles.

Neither the C=O group nor the ring oxygen atom of the ribose take part in hydrogen bonds. The number of "active" hydrogen atoms is not sufficient for hydrogen bond formation between all the electronegative atoms in the molecule.

None of the other intermolecular distances are shorter than normal van der Waals separations. There are "holes" in the structure around the twofold rotation axes. Every alternate one of these axes is surrounded by pairs of equivalent oxygen atoms at distances 3.4 Å ($O_{1'}$—$O_{1'}$) and 3.6 Å (O_2—O_2) whereas the packing around the others is even less close, the shortest distance across the axis being 3.9 Å (H_4—H_4).

The general arrangement of the molecules and the hydrogen bonds are shown in Figs. 6 and 7.

Because of the great amount of overlap in both projections the structure can hardly be determined very accurately by two-dimensional methods, and probably not much more accurately than in this work. It is planned to carry out further refinements in three dimensions.

Relation to nucleic acid structure. The structure of nucleotides and its relation to nucleic acid structure has been discussed by one of us on the basis of the crystal structure analysis of cytidine [21]. It was concluded that base and sugar were likely to be nearly perpendicular to one another and that in the most probable orientation of the 3′ phosphate group the bond P—$O_{3'}$ was roughly perpendicular to the plane of the base. The distance from this plane to the phosphorus atom was about 3.4 Å, and it was shown how nucleotides of this structure could be combined to macromolecules of helical and other shapes. Roughly the same nucleotide structure has been maintained in later models of nucleic acids, such as the Crick-Watson double helix model for DNA [20], and it would therefore appear to be of interest to compare it with the structure derived for cytidylic acid in the present work.

Firstly, some of the main features of the nucleotide model used are confirmed. Thus the relative orientation of pyrimidine and ribose is almost exactly as in crystals of cytidine, the two "planes" being roughly perpendicular to one another. Furthermore, the length of the glycosidic linkage N_3—$C_{1'}$, as well as the angles between this bond and the adjacent bonds in the ribose are also the same in the two cases within the limits of error. At present there would appear to be no reason to revise the nucleotide model in this respect.

As both base and phosphate are linked directly to the sugar, the structure of the latter is very important for the shape of the whole molecule. In the models hithertho constructed for nucleic acids the sugar ring is supposed to be puckered in the same way as in crystals of cytidine. As shown above, the ring has a different shape in crystals of cytidylic acid. In both conformations there is a nearly staggered arrangement of the substituent hydrogen and oxygen atoms. They would appear to be about equally favourable and should therefore both be considered when building models of nucleic acids.

As for the position of the phosphate group relative to the rest of the molecule, two directions of $C_{3'}$—$O_{3'}$ differing by about 60° should be considered,

Table 4. Observed (F_o) and calculated (F_c) structure factors for the *hk*0 zone (* Values corrected for extinction).

*hk*0	F_o	F_c	*hk*0	F_o	F_c	*hk*0	F_o	F_c
0 2 0	41 *	−41	2 15 0	13	+ 8	4 16 0	13	−10
0 4 0	4	− 4	2 16 0	28	+27	4 17 0	30	−29
0 6 0	59 *	−65	2 17 0	45	−45	4 18 0	7	+ 6
0 8 0	65 *	+69	2 18 0	33	−32	4 19 0	22	+20
0 10 0	18	−19	2 19 0	8	+ 7	4 20 0	8	+ 6
0 12 0	22	+20	2 20 0	<4	+ 4	4 21 0	<4	+ 4
0 14 0	55	−55	2 21 0	<4	+ 0	4 22 0	11	− 7
0 16 0	52	+46	2 22 0	6	− 4	4 23 0	4	+ 5
0 18 0	18	−18	2 23 0	6	+ 4	4 24 0	12	+12
0 20 0	18	+14	2 24 0	<3	− 1	4 25 0	5	− 3
0 22 0	20	−18	2 25 0	13	−13	5 1 0	19	+15
0 24 0	14	+14	2 26 0	9	− 9	5 2 0	6	− 7
0 26 0	14	−13	2 27 0	11	+11	5 3 0	42	−47
1 1 0	21	−22	3 1 0	17	−17	5 4 0	<4	− 3
1 2 0	34	+32	3 2 0	59 *	+68	5 5 0	22	−23
1 3 0	28	−31	3 3 0	43	+43	5 6 0	12	−12
1 4 0	36	−38	3 4 0	42	+45	5 7 0	16	−16
1 5 0	5	− 1	3 5 0	74 *	+80	5 8 0	20	−16
1 6 0	98 *	−99	3 6 0	29	+32	5 9 0	33	+35
1 7 0	27	−31	3 7 0	8	+ 9	5 10 0	7	− 2
1 8 0	<3	− 4	3 8 0	3	− 3	5 11 0	8	− 8
1 9 0	78 *	+79	3 9 0	47	−48	5 12 0	15	−16
1 10 0	32	−28	3 10 0	15	+13	5 13 0	7	+ 1
1 11 0	<3	− 2	3 11 0	35	−35	5 14 0	9	− 7
1 12 0	30	−32	3 12 0	11	+10	5 15 0	23	+23
1 13 0	6	− 6	3 13 0	27	+25	5 16 0	17	+17
1 14 0	18	−18	3 14 0	5	+ 3	5 17 0	13	−11
1 15 0	37	−38	3 15 0	18	−19	5 18 0	6	+ 8
1 16 0	11	−12	3 16 0	26	+25	5 19 0	13	−13
1 17 0	21	+23	3 17 0	12	+ 8	5 20 0	<4	− 1
1 18 0	9	+10	3 18 0	9	−11	5 21 0	6	− 4
1 19 0	<4	+ 3	3 19 0	<4	− 5	5 22 0	25	−29
1 20 0	8	+ 7	3 20 0	7	+ 3	5 23 0	<3	− 2
1 21 0	<4	+ 0	3 21 0	25	+21	5 24 0	<3	− 4
1 22 0	7	+ 7	3 22 0	<4	+ 5	5 25 0	7	− 7
1 23 0	12	−11	3 23 0	4	+ 2	6 0 0	22	−23
1 24 0	5	+ 5	3 24 0	10	+ 9	6 1 0	<4	+ 3
1 25 0	12	+10	3 25 0	<2	− 2	6 2 0	5	+ 4
1 26 0	15	−12	3 26 0	16	−16	6 3 0	6	− 6
1 27 0	8	− 8	4 0 0	14	+14	6 4 0	11	− 7
2 0 0	78 *	−85	4 1 0	<3	− 1	6 5 0	16	−16
2 1 0	114 *	−114	4 2 0	17	+19	6 6 0	3	+ 1
2 2 0	154 *	−148	4 3 0	16	−17	6 7 0	28	+27
2 3 0	8	+ 7	4 4 0	58	+60	6 8 0	25	−25
2 4 0	66 *	+65	4 5 0	19	−14	6 9 0	11	+13
2 5 0	24	+26	4 6 0	21	−25	6 10 0	9	+ 9
2 6 0	75 *	−76	4 7 0	9	+ 6	6 11 0	11	+10
2 7 0	42	−42	4 8 0	44	+44	6 12 0	22	−20
2 8 0	17	+18	4 9 0	37	−40	6 13 0	12	− 9
2 9 0	30	+29	4 10 0	<4	− 5	6 14 0	13	+11
2 10 0	68	−67	4 11 0	20	+17	6 15 0	9	−10
2 11 0	50	+54	4 12 0	4	− 1	6 16 0	18	−19
2 12 0	62	+60	4 13 0	20	−18	6 17 0	<4	− 1
2 13 0	<4	+ 4	4 14 0	7	+ 7	6 18 0	18	+17
2 14 0	7	− 6	4 15 0	26	+22	6 19 0	8	+ 6

*hk*0	F_o	F_c	*hk*0	F_o	F_c	*hk*0	F_o	F_c
6 20 0	8	−10	7 21 0	6	− 4	9 5 0	18	−17
6 21 0	21	−25	8 0 0	29	−31	9 6 0	12	+13
6 22 0	<3	+ 1	8 1 0	14	+13	9 7 0	<7	− 6
6 23 0	20	+21	8 2 0	20	+14	9 8 0	14	−14
7 1 0	5	+ 5	8 3 0	25	−25	9 9 0	<4	+ 2
7 2 0	16	−14	8 4 0	20	−18	9 10 0	7	+ 7
7 3 0	18	+16	8 5 0	<4	+ 1	9 11 0	4	+ 3
7 4 0	8	+ 5	8 6 0	36	+38	9 12 0	<3	− 1
7 5 0	<4	+ 4	8 7 0	<4	+ 4	9 13 0	9	−11
7 6 0	16	+16	8 8 0	15	−13	9 14 0	<3	− 0
7 7 0	7	+ 4	8 9 0	<4	− 1	10 0 0	<3	+ 1
7 8 0	11	+ 9	8 10 0	25	+24	10 1 0	21	+21
7 9 0	17	−16	8 11 0	7	− 6	10 2 0	6	+ 5
7 10 0	9	−10	8 12 0	16	−16	10 3 0	<3	+ 2
7 11 0	5	+ 1	8 13 0	10	+12	10 4 0	21	−23
7 12 0	9	− 8	8 14 0	<4	+ 1	10 5 0	18	+18
7 13 0	14	+14	8 15 0	8	+10	10 6 0	19	+20
7 14 0	15	+12	8 16 0	12	−10	10 7 0	21	−25
7 15 0	10	+ 6	8 17 0	<3	+ 0	10 8 0	11	−12
7 16 0	9	−12	8 18 0	15	+17	10 9 0	13	+11
7 17 0	<4	− 0	9 1 0	10	+12	10 10 0	18	+22
7 18 0	<3	+ 1	9 2 0	<4	− 1	10 11 0	9	−11
7 19 0	<3	+ 1	9 3 0	<4	− 2	10 12 0	11	−12
7 20 0	11	+10	9 4 0	7	− 8			

corresponding to the two conformations of the sugar. Furthermore, the angle $C_{3'}$—$O_{3'}$—P is 121°, in agreement with the results of the structure analysis of dibenzylphosphate [17], whereas the tetrahedral angle has been assumed in the nucleic acid models. The bond $O_{3'}$—P is in a *trans* position with respect to the ribose ring and is almost parallel to the N_3—$C_{1'}$ bond at a distance of about 0.6 Å, whereas these bonds are nearly perpendicular to one another in current nucleic acid models. In fact, the puckering of the ribose ring and the large angle at $O_{3'}$ make the molecule a very extended one.

The question then arises if nucleotides with the sugar conformation found in the present work can be combined to form macromolecules by 3′—5′ diester phosphate linkages, making the assumption that the bases are perpendicular to the molecular axis and separated by a distance of 3.4 Å corresponding to the dominating X-ray reflection. We have tried to study this problem by means of models.

In the helical structures proposed for DNA, such as our model I [21] and the Crick-Watson model [20], the phosphate groups of the external sugar-phosphate spiral lie roughly in the plane of the bases, which form a central column. In order to build these structures using the sugar conformation found in the present work, we have found it necessary to place the phosphate group roughly midway between successive bases, a position which may possibly not be consistent with the strength of the 3.4 Å reflection. Even with this position of the phosphate group, some of the van der Waals separations are too short. This is also the case in the existing helical models, but on the whole it appears more promising to maintain the ribose conformation found in crystals of cytidine

as a basis for helical models. The possibility that different ribose residues within the same macromolecule may have different conformations should also be borne in mind.

Turning now to other possible structures for the macromolecules, such as the zig-zag model II described by one of us [21], no difficulties are encountered when combining nucleotides of the shape found in the present work to models of this type. Evidently the two sugar conformations may both be used in this case to construct models which contain no unfavourably short van der Waals separations. However, there is considerable evidence that DNA has a double helix structure and other types of structures may therefore serve as models only for RNA. Very little is known about the space structure of this macromolecule, and so it does not seem worth while to discuss the models for it in more detail at the present stage.

We wish to express our thanks to *Norges Almenvitenskapelige Forskningsråd*, whose financial support made this work possible. We should also like to thank Dr. Waldo E. Cohn for providing us with a sample of cytidylic acid *b*, and the Medical Research Council Unit for Molecular Biology, Cavendish Laboratory, Cambridge, in particular Mrs. L. Barnett, for checking our calculations of bond lengths and bond angles.

REFERENCES

1. Furberg, S. *Acta Cryst.* **3** (1950) 325.
2. Zussmann, J. *Acta Cryst.* **6** (1953) 504.
3. Huber, M. *Acta Cryst.* **10** (1957) 129.
4. Brown, D. M., Fasman, G. D., Magarth, D. I., Todd, A. R., Cochran, W. and Woolfson, M. M. *Nature* **172** (1953) 1184.
5. Hodgkin, D. C., Kamper, J., Lindsey, J., MacKay, M., Pickworth, J., Robertson, J. H., Shoemaker, C. B., White, J.G., Prosen, R. J. and Trueblood, K. N. *Proc. Roy. Soc. London* **A 242** (1957) 228.
6. Alver, E. and Furberg, S. *Acta Chem. Scand.* **11** (1957) 188.
7. Lu, C. S. *Rev. Sci. Instr.* **14** (1943) 331.
8. Philips, D. C. *Acta Cryst.* **7** (1954) 746.
9. Beevers, C. A. and Robertson, J. H. *Acta Cryst.* **3** (1950) 164; **4** (1951) 63.
10. Wilson, A. J. C. *Acta Cryst.* **2** (1949) 318.
11. Burbank, R. D. *Acta Cryst.* **5** (1952) 236.
12. Zachariassen, W. H. *Acta Cryst.* **5** (1952) 68.
13. McWeeny, R. *Acta Cryst.* **4** (1951) 513.
14. Viervoll, H. and Øgrim, O. *Acta Cryst.* **2** (1949) 277.
15. Fox, J. J., Cavalieri, L. F. and Chang, N. *J. Am. Chem. Soc.* **75** (1953) 4315.
16. Furberg, S. *Acta Chem. Scand.* **9** (1955) 1557.
17. Dunitz, J. D. and Rollet, J. S. *Acta Cryst.* **9** (1956) 327.
18. Parry, G. S. *Acta Cryst.* **7** (1954) 313.
19. Pinnock, P. R., Taylor, C. A. and Lipson, H. *Acta Cryst.* **9** (1956) 173.
20. Crick, F. H. C. and Watson, J. D. *Proc. Roy. Soc. London* **A 223** (1954) 80.
21. Furberg, S. *Acta Chem. Scand.* **6** (1952) 634.
22. Spencer, M. *Acta Cryst.* **12** (1959) 59.

Received February 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 925–944

The Crystal Structure of $TlOHSO_4(H_2O)_{2.5}$

GEORG JOHANSSON

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The structure of $TlOHSO_4(H_2O)_{2.5}$ has been determined by means of X-ray crystallographic methods. Each thallium atom is octahedrally surrounded by six oxygen atoms at an average distance of 2.22 Å. The thallium atoms form chains in the structure with the shortest distance between two thallium atoms equal to 4.01 Å. The positions of the light atoms have been determined from reflections to which the thallium atoms give no contribution.

In the course of a study of the structures of basic salts of some trivalent metals a basic thallium(III) sulfate has also been prepared. It has been described in the literature by Meyer and Wilk [1], who found that it crystallized from solutions of Tl_2O_3 in sulfuric acid containing between about 10 and 40 % H_2SO_4. They ascribed to it the formula $TlOHSO_4(H_2O)_2$.

PREPARATION AND ANALYSIS

Dilute sulfuric acid containing about 20 % H_2SO_4 was saturated with Tl_2O_3 and the solution was placed in a vacuum desiccator. After a couple of days, colourless, rod-shaped crystals of the basic sulfate had formed and were large enough for single crystal measurements. They were filtered off on a glass filter plate and drained by being pressed between filter papers. They could not be washed with water as this immediately resulted in a hydrolysis to Tl_2O_3.

Thallium was determined by precipitation with ammonia and weighing as Tl_2O_3. The sulfate was determined gravimetrically as $BaSO_4$. The water was determined by Hartwig-Bendig's [2] modification of Brush and Penfield's method as described by Kolthoff and Sandell [3].

The density of the crystals was obtained from the apparent loss of weight in benzene.

	Found	Calculated for $TlOHSO_4(H_2O)_2$	Calculated for $TlOHSO_4(H_2O)_{2.5}$
% Tl_2O_3	63.2	64.61	63.00
% SO_3	22.4	22.65	22.09
% H_2O	15.1	12.74	14.91
Density	3.94	3.85	3.94

The analysis data show a better agreement with the formula $TlOHSO_4(H_2O)_{2.5}$ than with $TlOHSO_4(H_2O)_2$ which was assumed by Meyer and Wilk [1]. As will be shown later, only the first formula is consistent with the results of the X-ray analysis.

Table 1. Powder photograph of $TlOHSO_4(H_2O)_{2.5}$. Cu*Ka* radiation.

hkl	$10^4 \sin^2\Theta$ calc.	$10^4 \sin^2\Theta$ obs.	*I* obs.	*hkl*	$10^4 \sin^2\Theta$ calc.	$10^4 \sin^2\Theta$ obs.	I obs.
101	78	77	w+	225	1515	1516	vw+
200	153	152	m	602	1535	1536	vw+
002	159	158	s	504	1591	1592	vvw
201	193	192	s	405	1604	1605	vw
210	245	245	vvw	042	1635	1636	m
211	285	284	vvw	241	1669	1671	w
112	289	289	vvw	142	1673		
202	312	310	m	603	1733	1734	vvw
020	369	369	m	620	1745	1745	vw+
103	396	395	w	306	1773	1773	vvw
121	447	447	vvw	621	1785	1787	m
311	476	476	vw	242	1788		
302	503	502	w	143	1872	1873	vw
203	510	509	m	622	1904	1904	w
220	522	522	s	425	1973	1974	vw
022	528	528	s	107	1984	1986	w
221	562	562	s	243	1986		
122	566	566	m	604	2011	2011	vvw
004	635	635	w−	207	2098	2102	vw
401	651	651	m	623	2102		
222	681	681	m	441	2127	2130	w+
303	701	702	vw−	416	2133		
411	744	744	vw	244	2264	2263	vw
123	765	765	vw	127	2353	2352	w−
114	766			506	2385	2387	vw
204	788	787	w−	443	2445	2445	w+
322	872	873	vvw	800	2447		
223	879	880	w+	145	2507	2509	vvw
214	880			704	2508		
403	969	968	m	723	2599		
304	979			714	2601	2602	vvw
420	981	980	vw	336	2604		
230	983			802	2605		
024	1004	1004	vw	245	2622	2624	vvw
421	1020	1021	m	625	2738	2741	vvw
105	1031	1030	vw	731	2743		
124	1042	1044	vvw	606	2805		
502	1115	1115	vvw	352	2809		
205	1146	1146	vvw	345	2813	2811	vw
224	1157	1158	w	137	2814		
305	1337			820	2816		
423	1338	1340	m	821	2855	2858	vvw
414	1339			732	2862		
324	1348	1349	vw	641	2892		
600	1376	1376	vvw	813	2896	2897	vw−
125	1400	1400	w−	616	2898		
601	1416	1417	vw	507	2901		
006	1429	1429	vvw	822	2974	2973	vw
040	1476	1478	m	318	2977		

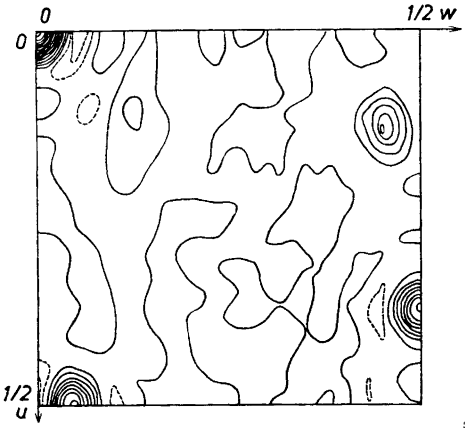


Fig. 1. Patterson projection $P(upw)$ calculated from the intensities of the $h0l$-reflections.

UNIT CELL AND SPACE GROUP

The crystals have orthorhombic symmetry which was shown by rotation and Weissenberg photographs taken with CuK-radiation for two of the axes ($0kl$—$4kl$ and $h0l$—$h4l$). The dimensions of the unit cell were calculated to be $a = 12.5$ Å $b = 8.0$ Å and $c = 12.1$ Å. From powder photographs in a Guinier focusing camera (Table 1) the following more accurate values for the cell dimensions were obtained (Cu$K\alpha$ radiation. $\lambda_{CuK\alpha_1} = 1.54050$ Å)

$$a = 12.453 \pm 0.005 \text{ Å}$$
$$b = 8.019 \pm 0.005 \text{ Å}$$
$$c = 12.224 \pm 0.005 \text{ Å}$$
$$V = 1220.7 \text{ Å}^3$$

According to the observed density, the unit cell must contain 8 formula units of $TlOHSO_4(H_2O)_{2.5}$.

The reflections systematically absent were such that

$0kl$ was observed only for $k + l = 2n$
$hk0$ was observed only for $h = 2n$

This is characteristic for the space groups No 62: $Pnma$ and No 33: $Pn2_1a$.

Intensities were estimated visually and were corrected for Lorentz and polarization factors. No absorption corrections were applied but only very small crystals were used (about 0.1 mm in length).

POSITIONS OF THE THALLIUM ATOMS

On the rotation photograph around the b-axis the odd layer lines were very weak, and the intensities did not seem to increase for the higher layer lines. Therefore the positions of the Tl-atoms may be described by a unit cell with a b-axis of only half that of the structure as a whole.

For the determination of the x and z coordinates the $h0l$-intensities were used to calculate the Patterson projection $P(upw)$. $P(upw)$ shows three sharp maxima which can be ascribed to the Tl-atoms (Fig. 1). For the space group *Pnma* this can be explained by the point position 8(d) which gives the vectors:

$(2x,\ 2z)$	weight 1
$(2x\text{-}\frac{1}{2},\ \frac{1}{2})$	» 2
$(\frac{1}{2},\ 2z\text{-}\frac{1}{2})$	» 2

By comparison with the maxima in $P(upw)$ the following coordinates were obtained:

$$x = 0.064 \qquad z = 0.226$$

All other coordinate possibilities can be transferred to these by shifting the coordinate system half a unit along one of the axes.

As it was possible to place the Tl-atoms in *Pnma* it was first tried to solve the structure by means of this space group.

The Tl-atoms give no or at least only a very small contribution to the reflections $h1l$, $h3l$, . . . and the y-coordinates must therefore be very close to $y = 0$ and $y = \frac{1}{2}$.

POSITIONS OF THE SULFATE GROUPS

The unit cell of the space group *Pnma* has mirror planes in $y = \pm\ ¼$. As the distance between them is only 4.01 Å the SO_4-groups cannot be situated between the planes, which would have required an interplanar distance not less than about 4.3 Å. The SO_4-groups must then be in the mirror planes and the S-atoms and two of the four O-atoms of the SO_4-group must be in point positions 4(c). The other two O-atoms must be outside the mirror planes and therefore have the same x and z parameters. In an electron density projection on this plane they should therefore give a maximum about 0.85 Å from the peak of the sulfur atom and of approximately the same height.

From the structure factors for the Tl-atoms, calculated with the above coordinates, all the signs of the intensities of the $h0l$-reflections were determined and the projection of the electron density on the xz-plane, with the contribution from the Tl-atoms subtracted, was calculated.

$$\varrho'(xpz) = \sum_h \sum_l [F_{obs}(h0l) - F_{Tl}(h0l)] \cos 2\pi(hx + lz)$$

F_{Tl} are the calculated structure factors for the Tl-atoms multiplied by the factor $\Sigma F_{obs}/\Sigma|f_{Tl}A_{Tl}|$. (The temperature factor obtained by means of the expression $F_{obs} = c\,.\,F_{calc} \exp(-B \sin^2 \Theta/\lambda^2)$ was negligible. Probably the temperature factor is cancelled by the absorption effects).

Fig. 2 shows $\varrho'(xpz)$. There are two double maxima in the projection:

A	$x = 0.236$, $z = 0.343$	$x = 0.162$, $z = 0.367$	(Distance 0.97 Å)
B	$x = 0.374$, $z = 0.382$	$x = 0.440$, $z = 0.397$	(Distance 0.84 Å)

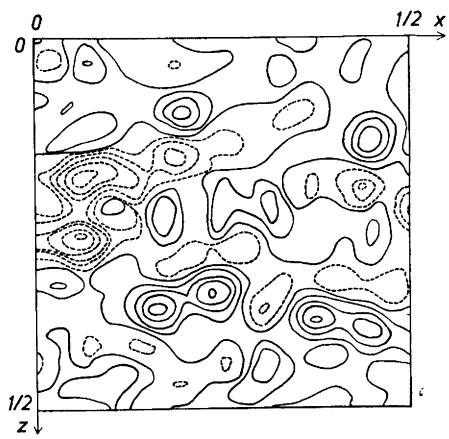


Fig. 2. The electron density projection $\varrho'(xpz)$ calculated from the structure factors of the $h0l$-reflections with the contribution from the thallium atoms subtracted. Dashed lines indicate negative regions.

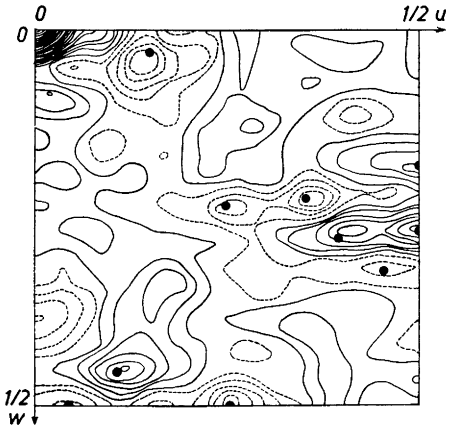


Fig. 3. The generalized Patterson projection $P_1(uw)$ calculated from the intensities of the $h1l$-reflections. The dots indicate the positions of S—S-vectors.

The heights of the maxima are approximately the same and are also the same as might be expected for one sulfur or two oxygen atoms.

From purely geometrical reasons it is difficult to decide which of the maxima comes from the sulfur atom and which from the oxygen atoms, particularly as the coordinates obtained could be expected to be only approximate because of the influence of the heavy thallium atoms. It should, however, be possible to reduce the contribution from the two oxygen atoms relative to the sulfur atom by calculating a generalized Patterson projection [4] $P_1(uw)$ from the intensities of the $h1l$-reflections to which the Tl-atoms do not contribute:

$$P_1(uw) = \frac{1}{A} \sum_h \sum_l |F(h1l)|^2 \cos 2\pi(hu + lw)$$

The vectors in $P_1(uw)$ are equal to the vectors in the $(h0l)$-projection multiplied by the factor $\cos 2\pi(y_1 - y_2)$. For vectors between atoms in the planes $y = \pm \frac{1}{4}$, *i.e.* the sulfur atoms, this will not reduce the size of the maxima but the signs for vectors between atoms in different planes will be negative. The maxima from vectors between atoms in the planes $y = \pm \frac{1}{4}$ and those oxygen atoms from the SO_4-groups which are in the position $8(d)$ will be reduced to about half the magnitude they would have had in the $h0l$-projection. Atoms in the planes $y = 0$ and $y = \frac{1}{2}$ will not contribute at all.

The vectors between the sulfur atoms must therefore have the largest relative contribution to $P_1(uw)$ and the correspinding maxima should be easily recognized.

Within the point position $4(c)$ the following two vectors occur with only one variable:

$u = 2x - \frac{1}{2}$, $w = \frac{1}{2}$ (with negative sign)
$u = \frac{1}{2}$, $w = 2z - \frac{1}{2}$ (with positive sign)

From Fig. 3 it can be seen that for both $u = \frac{1}{2}$ and $w = \frac{1}{2}$ there are two maxima which probably come from the S-atoms and from which the following parameters can be calculated:

$x = 0.377$ $\quad z = 0.383$
$x = 0.228$ $\quad z = 0.340$

A comparison with the coordinates obtained from $\varrho'(xpz)$ shows that the parameters of the sulfur atoms must be:

A $x = 0.228$, $z = 0.340$ $\quad$ B $x = 0.377$, $z = 0.383$

By means of these values the other vectors between the sulfur atoms were calculated and Fig. 3 shows that they also occur in the projection with their correct signs. Because of the great number of vectors in the projection it cannot be used for determining the coordinates of other atomic positions.

The positions of the sulfate groups are now approximately determined as having the following parameters (the coordinates for the O-atoms in 4(c) have been obtained by assuming the dimensions of the SO_4-group to be O—O = 2.4 Å and S—O = 1.5 Å):

SO_4 (A)	4 S in 4(c)	$x = 0.228$	$y = 1/4$	$z = 0.340$
	8 O in 8(d)	$x = 0.162$	$y = 0.10$	$z = 0.367$
	4 O in 4(c)	$x = 0.26$	$y = 1/4$	$z = 0.22$
	4 O in 4(c)	$x = 0.33$	$y = 1/4$	$z = 0.41$
SO_4 (B)	4 S in 4(c)	$x = 0.377$	$y = 3/4$	$z = 0.383$
	8 O in 8(d)	$x = 0.440$	$y = 0.60$	$z = 0.397$
	4 O in 4(c)	$x = 0.33$	$y = 3/4$	$z = 0.27$
	4 O in 4(c)	$x = 0.29$	$y = 3/4$	$z = 0.47$

ELECTRON DENSITY PROJECTIONS OF THE LIGHT ATOMS

From the coordinates of the SO_4-groups the signs of most of the $h1l$-reflections were determined and the generalized electron density projection, $\varrho_1(xz)$, was calculated:

$$\varrho_1(xz) = -\int_0^1 \varrho(xyz) \sin 2\pi y dy = \sum_h \sum_l F(h1l) \sin 2\pi(hx + lz)$$

In this projection the electron density at each point has been multiplied by $-\sin 2\pi y$ which means that the atoms in the planes $y = \pm 1/4$ will give the same contribution as in a $h0l$-projection, with the exception of the signs, but atoms outside these planes will give smaller contributions and atoms in the planes $y = 0$ and $y = \frac{1}{2}$ will not be seen at all.

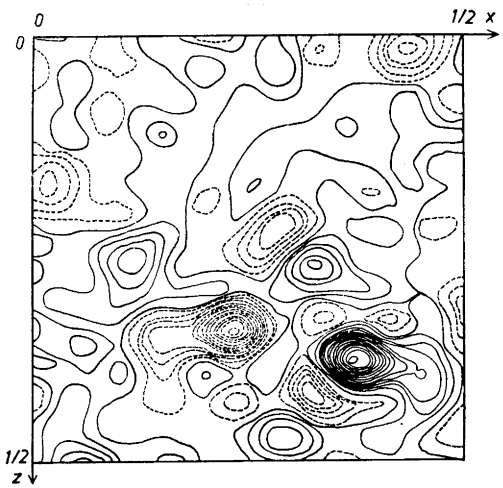


Fig. 4. The generalized electron density projection $\varrho_1(xz)$. The two large peaks come from the sulfur atoms; the negative peak (dashed lines) from S_A and the positive peak from S_B.

Fig. 4 shows the final result with all intensities from the $h1l$-level included. The two largest maxima can be ascribed to the sulfur atoms and give the coordinates:

A: $x = 0.232$ $z = 0.347$ $(y = 1/4)$
B: $x = 0.377$ $z = 0.382$ $(y = 3/4)$

Among the other maxima that can be seen in Fig. 4, the six maxima from the oxygen atoms of the sulfate groups can be identified in positions with coordinates which are in fair agreement with those calculated above.

A new projection $\varrho_1'(xz)$ with the S-atoms substracted was now calculated:

$$\varrho_1'(xz) = \sum_h \sum_l [F_{obs}(h1l) - F_S(h1l)] \cdot \sin 2\pi(hx+lz)$$

This projection, which is shown in Fig. 5, was used for the final determination of the parameters of the oxygen atoms in the SO_4-group:

SO_4 (A): 8 O_1 in 8(d) : $x = 0.167$ $y = 0.10$ $z = 0.353$
4 O_2 in 4(c) : $x = 0.324$ $y = 1/4$ $z = 0.424$
4 O_3 in 4(c) : $x = 0.278$ $y = 1/4$ $z = 0.232$

SO_4 (B): 8 O_4 in 8(d) : $x = 0.439$ $y = 0.60$ $z = 0.390$
4 O_5 in 4(c) : $x = 0.331$ $y = 3/4$ $z = 0.272$
4 O_6 in 4(c) : $x = 0.299$ $y = 3/4$ $z = 0.473$

The y-coordinates of O_1 and O_4 have been calculated from the dimensions assumed for a sulfate group. An approximate value can, however, be obtained from the electron density projection by a comparison between the heights of

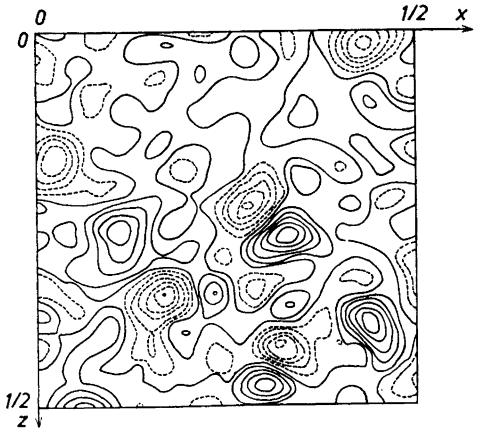


Fig. 5. The generalized electron density projection $\varrho'_1(xz)$ calculated from the structure factors of the $h1l$-reflections with the contribution from the sulfur atoms substracted.

the peaks of O_1 and O_4 and the heights of the peaks of the oxygen atoms in the planes $y = \pm 1/4$, which gives the following values for the y-coordinates of O_1 and O_4:

$$y = 0.09_8 \text{ for } O_1$$
$$y = 0.59_8 \text{ for } O_4$$

This leads to a distance between these oxygen atoms of 2.4_4 Å for both SO_4(A) and SO_4(B), which is the expected distance between the O-atoms of a sulfate group. This method of determination, however, is rather approximate. Some information about the accuracy can be obtained from the scatter of the heights of the 4-fold O-positions around the calculated mean. This gives a standard deviation of 0.013 in the y-coordinates of O_1 and O_4.

In the electron density projection in Fig. 5 there are three further peaks of about the same height as for an oxygen atom in one of the planes $y = \pm 1/4$. The two maxima with the coordinates

$$x = 0.024,\ z = 0.170 \quad \text{and} \quad x = 0.109,\ z = 0.267$$

cannot be ascribed to oxygen atoms outside the mirror planes as this would lead to a Tl—O distance less than about 1.2 Å. Their y-coordinates must therefore be 1/4 and 3/4, respectively, which leads to the Tl—O distances 2.17 and 2.14 Å. Each of these two oxygens (O_7 and O_8) is therefore in contact with two thallium atoms.

Of the thallium atoms no trace can be seen in Fig. 5 and their y-parameters must therefore be exactly or very nearly $y = 0$ and $y = \frac{1}{2}$.

The result of the analysis shows that, in addition to the 40 oxygen atoms that have already been found, there are at least 16 and probably 20 more

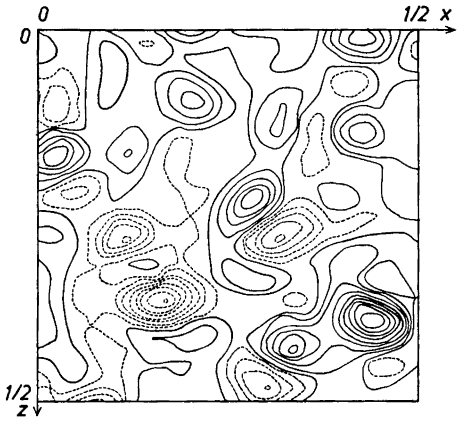


Fig. 6. The generalized electron density projection $\varrho_3'(xz)$ calculated from the structure factors of the $h3l$-reflections with the contribution from the sulfur atoms substracted.

oxygen atoms in the unit cell. In the electron density projection in Fig. 2 there are, besides the maxima from the sulfate groups, two other maxima of about the same height. These might therefore correspond to two sets of oxygens (O_9 and O_{10}) in position 8(d).

Their coordinates are:

$$x = 0.198 \text{ and } x = 0.447$$
$$z = 0.099 \quad » \quad z = 0.142$$

In the electron density projection from the $h1l$-reflections, Fig. 5, there are, however, no marked maxima corresponding to these positions, and the y-coordinates must therefore be close to $y = 0$ and $y = \frac{1}{2}$. This would give a Tl—O distance of about 2.25 Å for O_9 and the rather short distance of 2.05 Å for O_{10}.

In order to obtain a more accurate value for the y-parameters the following electron density projection was calculated:

$$\varrho_3'(xz) = \sum_h \sum_l [F_{obs}(h3l) - F_S(h3l)] \sin 2\pi(hx + lz)$$

This will give the same result as the corresponding projection from the $h1l$-reflections with the exception that the electron density is now multiplied by $-\sin 6\pi y$ instead of $-\sin 2\pi y$. This will change only the signs of the maxima of the atoms in the planes $y = \pm 1/4$, but will greatly increase the maxima from atoms near — but not in — the planes $y = 0$ and $y = \frac{1}{2}$. The result is shown in Fig. 6. The projection is in the main the same as $\varrho_1'(xz)$ in Fig. 5, but the maxima from the 8(d) atoms of the sulfate groups have increased by about the same factor as could be calculated from the relationship $\sin 6\pi y / \sin 2\pi y \approx 1.6$, and in the assumed positions for O_9 and O_{10} there are now marked maxima. From the heights of these maxima in relation to the heights of the maxima of the other oxygen atoms the following y-parameters can be calculated:

$$O_9 : y = -0.020 \qquad O_{10} : y = -0.022$$

(An estimation of the accuracy of these parameter values, analogous to that for O_1 and O_4, gives an approximate value for the standard deviation of about 0.003). The positions of the maxima in the xz-plane are:

O_9: $x = 0.203$ $z = 0.097$ (From the $h3l$-projection)
$x = 0.198$ $z = 0.099$ (From the $h0l$-projection)

O_{10}: $x = 0.425$ $z = 0.137$ (From the $h3l$-projection)
$x = 0.447$ $z = 0.142$ (From the $h0l$-projection)

In both the $h1l$- and $h3l$-projections (Figs. 5 and 6) there is also another maximum with the coordinates:

$x = 0.433$ $z = 0.017$ (From the $h1l$-projection)
$x = 0.427$ $z = 0.012$ (From the $h3l$-projection)

This can be explained by assuming four oxygen atoms in the position 4(c). From space considerations this also seems very probable.

FINAL PARAMETERS

As the structure seems to be satisfactorily explained by means of the space group *Pnma* no further investigation has been made with the space group $Pn2_1a$, which would also have been in accordance with the systematically absent reflections.

The positional parameters of the light atoms were refined by the method of least squares from the 82 observed $h1l$-reflections, and independently from the 78 $h3l$-reflections. The average values of the differences between the final parameters as calculated from the $h1l$- and the $h3l$-reflections, respectively, was 0.002 for the x-parameters and 0.001 for the z-parameters. The standard deviations in atomic coordinates calculated from the least squares residuals are 0.002 for S, 0.004 for O in 4(c), 0.003 for O_1 and O_4, 0.007 for O_9, and 0.005 for O_{10}. For the y-parameters of O_9 and O_{10} the standard deviation is 0.004 which is close to the estimation made above by comparing the heights of the maxima in the Fourier-projections. These values lead to standard deviations of about 0.06 Å for S—O and 0.07 Å for O—O distances except those involving O_9 and O_{10} for which the values are somewhat higher.

Assuming the SO_4-group to be a regular tetrahedron a standard deviation of 0.04 Å in the S—O-distances can be calculated from the scatter around the average value of the S_A—O_2, S_A—O_3, S_B—O_5, and S_B—O_6 distances, which is close to the value calculated above. This would indicate that the estimation of the standard deviations from the least squares residuals is essentially correct in spite of the high absorption from the heavy Tl-atoms. However, the presence of these heavy atoms may introduce systematic errors that are not included in the calculations of the standard deviations and the accuracy may therefore be lower than the values indicate.

The final parameters are shown in Table 2. For the light atoms the average value of the parameters obtained from the *h*1*l*- and the *h*3*l*-reflections has been used. The *y*-parameters of O_1 and O_4 have been calculated assuming the O_1—O_1 and O_4—O_4 distances to be the same as the average value of the O_2—O_3 and O_5—O_6 distances.

Table 2. Final parameters.

		x	*y*	*z*
Tl:	8 Tl in 8(d):	0.064	0	0.226
SO_4(A):	4 S_A in 4(c):	0.235	1/4	0.346
	8 O_1 in 8(d):	0.164	0.09_9	0.361
	4 O_2 in 4(c):	0.323	1/4	0.428
	4 O_3 in 4(c):	0.286	1/4	0.232
SO_4(B):	4 S_B in 4(c):	0.376	3/4	0.382
	8 O_4 in 8(d):	0.441	0.59_9	0.393
	4 O_5 in 4(c):	0.328	3/4	0.273
	4 O_6 in 4(c):	0.299	3/4	0.469
OH^-:	4 O_7 in 4(c):	0.021	1/4	0.170
	4 O_8 in 4(c):	0.112	3/4	0.271
H_2O:	8 O_9 in 8(d):	0.193	0.982	0.096
	8 O_{10} in 8(d):	0.441	0.974	0.144
	4 O_{11} in 4(c):	0.432	1/4	0.018

From these parameters the structure factors which are listed in Table 4 were calculated assuming the temperature factor $B = 0$ for thallium and $B = 0.1$ Å^2 for the light atoms. For the calculation of the reliability index

$$R = \frac{\Sigma||F_o| - |F_c||}{\Sigma|F_o|}$$

shown below for different classes of reflections, only observed reflections have been included:

*h*0*l* : $R = 0.16$
*h*1*l* : $R = 0.13$ (no contribution from the Tl-atoms)
*h*3*l* : $R = 0.11$ (» » » » »
0*kl* : $R = 0.20$
1*kl* : $R = 0.12$

DESCRIPTION OF THE STRUCTURE

In the structure proposed each thallium atom is octahedrally surrounded by six oxygen atoms. There seems to be no significant difference between the six Tl-O distances (Table 3). The average value is 2.22 Å which is close to the value 2.26 Å found by Zachariasen [5] for the Tl—O distances in Tl_2O_3. Of the oxygen atoms in the TlO_6-octahedron O_1 and O_4 also belong to a sulfate group and each of them is in contact with only one thallium atom. O_7 and O_8 are both in contact with two thallium atoms (Fig. 7) and Pauling's rule for

Table 3. Interatomic distances.

Within a TlO_6-group:			
$Tl-O_1$ = 2.21 Å	O_1-O_7 = 3.18 Å	O_4-O_9 = 3.21 Å	
O_4 = 2.26	O_1-O_8 = 3.08	O_4-O_{10} = 3.10	
O_7 = 2.19	O_1-O_9 = 3.39	O_7-O_9 = 3.17	
O_8 = 2.16	O_1-O_{10} = 3.00	O_7-O_{10} = 3.33	
O_9 = 2.26	O_4-O_7 = 3.07	O_8-O_9 = 3.01	
O_{10} = 2.22	O_4-O_8 = 3.17	O_8-O_{10} = 2.97	
Within the SO_4-groups:			
SO_4(A):	S_A-O_1 = 1.51 Å	O_1-O_2 = 2.46 Å	
	$-O_2$ = 1.49	O_1-O_3 = 2.50	
	$-O_3$ = 1.53	O_2-O_3 = 2.44	
		(O_1-O_1 = 2.43)	
SO_4(B):	S_B-O_4 = 1.46 Å	O_4-O_5 = 2.37 Å	
	$-O_5$ = 1.46	O_4-O_6 = 2.34	
	$-O_6$ = 1.43	O_5-O_6 = 2.42	
		(O_4-O_4 = 2.43)	
Short O—O distances:			
	O_7-O_2 = 2.74 Å	O_8-O_5 = 2.69 Å	
	O_9-O_2 = 2.78 Å	$O_{10}-O_5$ = 2.77	
	O_9-O_6 = 2.65 Å	$O_{10}-O_{11}$ = 2.70	
Other O—O distances less than 3.5 Å:			
	O_1-O_9 = 3.44 Å	O_4-O_9 = 3.13 Å	
	O_2-O_4 = 3.19	O_5-O_9 = 3.31	
	O_3-O_6 = 3.40	O_6-O_7 = 3.33	
	O_3-O_7 = 3.39, 3.16	O_6-O_8 = 3.35	
	O_3-O_9 = 2.95	O_6-O_{11} = 2.94	
	O_3-O_{10} = 3.13	O_8-O_{11} = 3.07	
	O_3-O_{11} = 3.19	O_9-O_{10} = 3.14	
	O_4-O_4 = 3.39	$O_{10}-O_{11}$ = 3.11	

the sum of the bond strengths [6] therefore suggests that one hydrogen atom is probably bound to each of O_7 and O_8. The remaining two oxygen atoms in the TlO_6-group, O_9 and O_{10}, are in contact with only one thallium atom and it seems reasonable to assume that each of O_9 and O_{10} is associated with two hydrogen atoms. The oxygen atom, O_{11}, which is not in contact with any of the thallium atoms, must be a water of crystallization. These results of the structure determination show that the formula should probably be written $Tl_2(OH)_2(H_2O)_4(SO_4)_2 \cdot H_2O$

Chains of TlO_6-octahedra running parallel to the *b*-axis can be distinguished in the structure; the thallium atoms within such a chain being joined together by means of O_7, O_8 and the oxygens of the sulfate groups, O_1 and O_4 (Fig. 7). The shortest distance between two thallium atoms linked together in this way is 4.01 Å. The different chains have no oxygens or sulfate groups in common. The packing of the atoms is shown in Fig. 8, which is a projection of half the unit cell on the *xz*-plane.

Water molecules in contact with cations with a high charge usually tend to form hydrogen bonds to surrounding oxygen atoms and it therefore seems reasonable to assume that in this structure the forces that hold the different

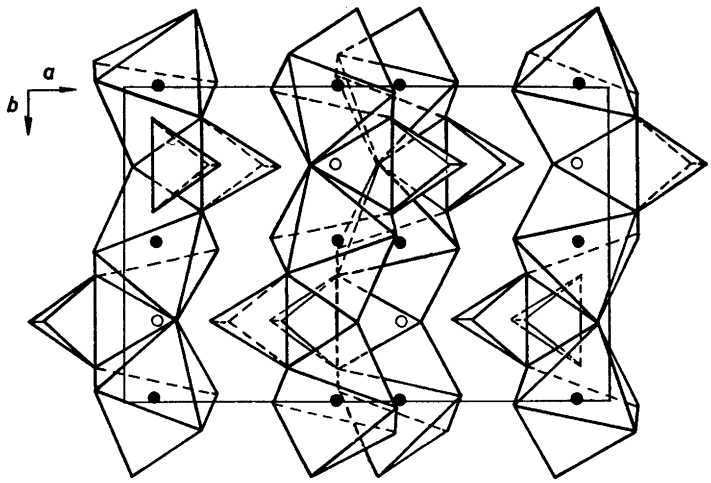


Fig. 7. Projection of the structure along the *c*-axis showing the chains of TlO_6-octahedra running parallel to the *b*-axes. Filled circles indicate Tl-atoms; open circles indicate O_{11}-atoms.

chains together are hydroxyl bonds from the water molecules with O_9 and O_{10} and perhaps also from the oxygen atoms O_7 and O_8. Among the interatomic distances listed in Table 3 there are also some short O—O-distances of about the length expected for a hydroxyl bond. The accuracy of the parameter values is probably not high enough definitely to distinguish these as being significantly shorter than a normal O—O-distance. However, each of O_7 and O_8 is involved in one and each of O_9 and O_{10} in two such short bonds, which is the same as

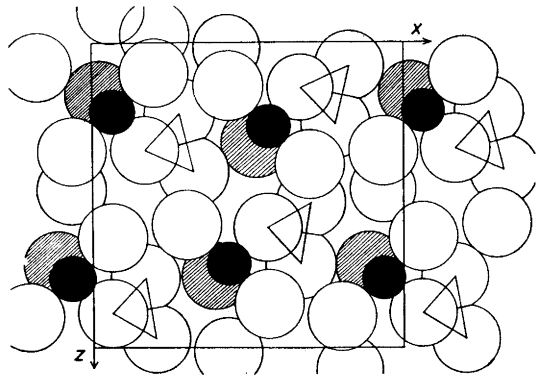


Fig. 8. Projection of half the unit cell on the *xz*-plane. Small circles represent thallium atoms and large circles oxygen atoms. Large shaded circles represent O_7 or O_8.

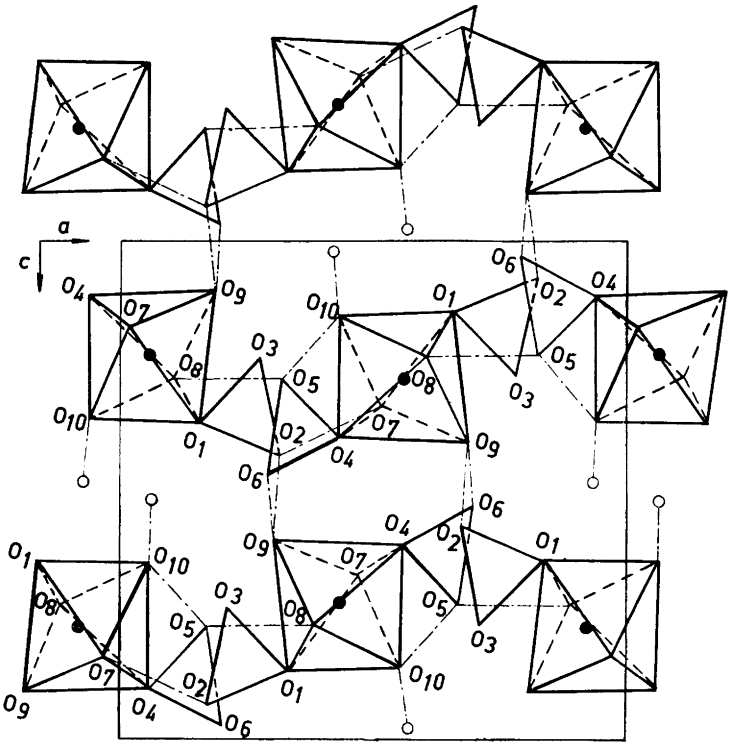


Fig. 9. Projection of the unit cell along the *b*-axis. The projection shows how the sulfate groups (represented as triangles) and the TlO_6-octahedra are linked together. Lines marked —.—.—, between oxygen atoms represent short O—O distances which may indicate the presence of hydrogen bonds. Filled circles indicate Tl-atoms; open circles indicate O_{11}-atoms.

the number of hydrogen atoms that can be assumed to be associated with these oxygens. If hydroxyl bonds were present the arrangement around these oxygen atoms could be expected to be approximately tetrahedral. Except for O_{10} the deviations from an approximate tetrahedral arrangement do not seem so large as to make the assumption of hydrogen bonds unlikely and even if small parameter changes are assumed no other way of forming the hydrogen bonds seems to give a more plausible arrangement. The water molecule O_{11} is in contact with two O_{10} atoms at a distance of 2.70 Å. The angle O_{11}—O_{10}—O_{11} is 110°, which is close to the tetrahedral angle 109° and therefore supports the assumption of a hydrogen bond between O_{10} and O_{11}. The other short distance in which O_{10} is involved is O_5—O_{10} but the angle O_{11}—O_{10}—O_5 is as large as 147°.

With the possible exception of O_5—O_{10} it therefore seems reasonable to assume that the short O—O distances listed in Table 3 and shown in the projection in Fig. 9

Table 4. Observed and calculated structure factors. The F_c and F_o-values are listed in groups in the following order: *h0l*, *0kl*, *1kl*, *h1l*, *h3l*. Structure factors belonging to more than one group are listed only once.

h 0 l	F_o	F_c	*h 0 l*	F_o	F_c
0 0 2	167	−312	3 0 13	82	66
4	133	148	14	162	−146
6	172	−159	15	47	− 43
8	169	134			
10	42	− 26	4 0 0	127	−173
12	51	− 43	1	203	−241
14	81	88	2	—	6
			3	230	310
			4	12	− 5
1 0 1	37	65	5	213	−242
2	21	24	6	31	31
3	93	−123	7	94	89
4	41	48	8	35	− 25
5	117	127	9	50	− 29
6	97	− 91	10	30	− 15
7	229	−216	11	—	− 7
8	129	105	12	45	25
9	242	207	13	71	67
10	82	− 61			
11	179	−152	5 0 1	20	6
12	64	50	2	96	− 99
13	140	122	3	72	69
14	65	− 60	4	123	141
15	119	−130	5	59	− 57
			6	143	−164
2 0 0	147	179	7	55	57
1	144	−198	8	179	168
2	130	−190	9	101	− 87
3	148	214	10	204	−194
4	96	149	11	102	88
5	104	−125	12	185	166
6	88	− 90	13	61	− 57
7	82	73	14	107	−120
8	83	57			
9	36	− 22	6 0 0	184	−187
10	30	6	1	133	−156
11	—	9	2	135	177
12	50	− 36	3	119	127
13	51	47	4	129	−145
14	58	49	5	78	− 83
15	78	− 87	6	83	84
			7	78	75
3 0 1	22	− 14	8	69	− 65
2	74	− 79	9	34	− 13
3	85	− 76	10	—	14
4	117	166	11	—	− 11
5	78	83	12	30	27
6	129	−190	13	41	37
7	55	− 51	14	53	− 61
8	145	126			
9	63	57	7 0 1	37	− 30
10	187	−168	2	51	− 46
11	94	− 74	3	70	77
12	209	181	4	63	58

Table 4. (Continued)

h 0	F_o	F_c	*h* 0 *l*	F_o	F_c
7 0 5	126	−121	11 0 7	36	40
6	39	− 36	8	138	−144
7	172	187	9	60	− 62
8	45	53	10	139	173
9	181	−181	11	39	43
10	75	− 56			
11	164	148	12 0 0	—	12
12	57	50	1	163	167
13	128	−129	2	34	− 23
			3	160	−167
8 0 0	225	−265	4	29	20
1	28	19	5	135	123
2	172	190	6	—	3
3	—	− 14	7	64	− 66
4	177	−174	8	—	− 4
5	22	14	9	36	39
6	157	141			
7	—	− 14	13 0 1	23	− 20
8	73	− 69	2	40	49
9	—	− 6	3	—	− 19
10	—	1	4	76	− 95
11	—	− 6	5	65	70
12	38	35	6	86	120
			7	72	− 91
9 0 1	27	− 20	8	108	−124
2	50	55			
3	71	79	14 0 0	92	128
4	52	− 54	1	63	70
5	142	−128	2	90	−120
6	51	46	3	86	−113
7	163	180	4	75	112
8	85	− 81	5	71	108
9	155	−155	6	53	− 74
10	89	87			
11	140	140	15 0 1	53	43
12	51	− 73	2	—	0
			3	65	− 88
10 0 0	138	−143	0 *k l*		
1	138	143			
2	129	127	0 2 0	232	217
3	132	−130	2	293	−252
4	76	− 75	4	141	168
5	102	105	6	145	−159
6	51	51	8	73	71
7	88	− 90	10	—	4
8	57	− 51	12	46	− 41
9	37	27	14	67	88
10	33	20			
11	37	32	0 4 0	376	359
			2	347	−247
11 0 1	—	2	4	190	193
2	45	39	6	99	−133
3	—	15	8	73	85
4	84	− 93	10	34	− 25
5	34	− 26	12	47	− 31
6	144	141	14	50	86

Table 4. (Continued)

0 *k l*	F_o	F_c
0 5 1	—	9
3	—	10
5	—	6
7	24	— 31
9	—	4
11	47	30
13	—	— 8
0 6 0	177	131
2	211	—188
4	201	178
6	98	—125
8	41	40
10	—	2
12	33	— 33
0 7 1	—	17
3	—	7
5	24	— 25
7	—	24
9	—	10
11	35	— 37
0 8 0	220	248
2	178	—174
4	88	125
6	68	—103
8	51	78
0 10 0	68	132
1 *k l*		
1 2 1	41	22
2	137	—107
3	61	— 69
4	47	47
5	138	169
6	55	— 60
7	174	—205
8	76	81
9	195	197
10	87	— 81
11	162	—158
12	81	72
13	166	147
14	71	— 66
15	106	—125
1 4 1	54	36
2	30	— 25
3	119	—105
4	58	46
5	131	134
6	60	— 56
7	129	—171
8	79	86
9	164	168

1 *k l*	F_o	F_c
1 4 10	73	— 68
11	168	—156
12	64	54
13	131	132
1 5 1	—	3
2	—	0
3	—	— 14
4	—	15
5	—	— 15
6	—	4
7	—	12
8	—	— 7
9	32	— 30
1 6 1	—	15
2	90	— 80
3	74	— 65
4	51	43
5	133	141
6	39	— 36
7	134	—152
8	53	60
9	144	151
10	76	— 76
11	134	—153
12	60	70
1 7 1	—	10
2	—	— 27
3	—	8
4	—	— 3
5	—	— 3
6	—	22
7	—	— 16
8	—	— 5
9	33	33
10	26	— 24
1 8 1	33	33
2	—	3
3	77	— 79
4	31	33
5	79	94
6	59	— 59
7	113	—135
8	62	71
9	114	150
h 1 *l*		
0 1 1	—	— 5
3	—	4
5	24	27
7	48	— 43
9	—	— 5
11	55	42

Table 4. (Continued)

h l l	F_o	F_c	*h l l*	F_o	F_c
13	—	— 2	5 1 1	14	— 12
15	31	— 26	2	—	— 5
			3	20	— 21
1 1 1	—	— 4	4	16	— 17
2	22	29	5	—	2
3	18	— 23	6	19	20
4	17	20	7	12	8
5	17	— 19	8	—	3
6	25	— 20	9	—	0
7	24	23	10	—	11
8	—	— 5	11	13	— 13
9	44	— 42	12	24	— 20
10	27	27	13	—	5
11	—	8	14	21	17
12	—	14			
13	24	21	6 1 0	—	— 8
			1	—	— 2
2 1 0	40	— 41	2	10	8
1	30	32	3	18	— 17
2	—	4	4	—	— 4
3	12	12	5	—	5
4	51	54	6	17	— 17
5	17	— 23	7	—	11
6	—	— 11	8	14	13
7	20	— 19	9	14	12
8	25	— 23	10	14	13
9	—	7	11	—	— 1
10	—	— 1	12	15	— 11
11	27	— 18			
12	24	18	7 1 1	—	3
13	—	— 10	2	—	— 10
14	—	9	3	—	3
			4	—	1
3 1 1	51	— 46	5	23	— 22
2	38	— 42	6	17	— 19
3	24	33	7	—	— 8
4	22	28	8	13	— 11
5	—	14	9	17	16
6	9	13	10	—	3
7	22	— 21	11	—	— 4
8	15	— 13	12	—	0
9	—	— 7	13	15	— 10
10	26	— 20			
			8 1 0	16	— 15
4 1 0	9	10	1	—	— 2
1	58	— 56	2	21	21
2	—	4	3	—	5
3	10	9	4	—	— 11
4	19	24	5	—	11
5	19	19	6	13	9
6	22	26	7	15	— 16
7	12	12	8	—	— 9
8	9	11	9	—	0
9	15	— 14	10	—	— 3
10	13	— 11	11	17	15

Table 4. (Continued)

h l l	F_o	F_c
9 1 1	—	3
2	13	10
3	—	7
4	—	3
5	14	17
6	11	— 11
7	—	7
8	—	9
9	—	— 5
10	11	— 6
10 1 0	25	— 21
1	—	6
2	14	— 17
3	—	4
4	28	25
5	—	— 4
6	16	— 17
7	—	11
8	—	1
9	11	13
11 1 1	16	— 17
2	26	— 24
3	25	24
4	—	1
5	—	2
6	14	13
12 1 0	16	12
1	14	— 16
2	—	— 5
3	—28	26
4	17	— 19

h 3 l	F_o	F_c
0 3 1	19	16
3	30	24
5	17	— 13
7	16	15
9	19	— 14
11	32	— 26
13	25	21
1 3 1	23	24
2	15	15
3	—	— 6
4	—	— 2
5	—	8
6	—	— 4
7	—	— 1
8	—	— 12
9	38	35
10	21	— 16
11	22	— 20
1 3 12	—	— 1
13	18	— 12
2 3 0	22	— 24
1	—	6
2	—	1
3	—	15
4	—	— 1
5	—	— 4
6	—	0
7	—	3
8	—	— 2
9	—	6
10	—	9
11	25	22
12	—	— 8
13	—	4
14	17	— 15
3 3 1	20	19
2	—	4
3	28	— 27
4	11	— 11
5	11	11
6	—	6
7	36	29
8	—	— 5
9	14	— 12
10	14	12
11	14	— 12
12	15	12
13	15	14
4 3 0	36	— 34
1	55	62
2	13	— 14
3	—	5
4	—	— 2
5	32	— 31
6	—	— 1
7	17	— 15
8	17	— 14
9	21	22
10	16	— 11
5 3 1	23	— 23
2	20	18
3	45	40
4	10	12
5	22	26
6	24	— 25
7	13	— 17
8	—	2
9	19	— 17
10	—	— 8

Table 4. (Continued)

h 3 *l*	F_o	F_c	*h* 3 *l*	F_o	F_c
5 3 11	16	13	8 3 4	25	31
12	16	13	5	—	— 7
			6	—	— 14
6 3 0	18	— 14	7	31	33
1	27	— 30	8	—	— 4
2	—	— 8	9	—	6
3	—	— 9	10	—	10
4	22	20	11	28	— 24
5	25	24			
6	18	18	9 3 1	—	— 3
7	—	1	2	32	— 38
8	21	— 22	3	—	— 8
9	29	— 29	4	—	— 7
10	19	— 16	5	14	— 15
11	—	— 1	6	25	30
12	16	14			
			10 3 0	43	43
7 3 1	—	7	1	—	— 9
2	—	7	2	—	13
3	17	— 18	3	—	— 9
4	17	— 19	4	43	— 44
5	18	20	5	—	4
6	18	16	6	19	20
7	21	22	7	—	— 4
8	27	33	8	13	12
9	28	— 21			
10	—	0	11 3 1	16	16
11	—	— 8	2	25	26
12	19	— 21	3	16	— 13
			4	—	— 6
8 3 0	12	— 13	5	—	— 10
1	—	— 14	6	—	— 14
2	13	— 16	7	—	— 6
3	31	— 27	8	13	11

indicate hydrogen bonds. However, the standard deviations of the parameters are too large for a definite conclusion in this respect.

Acknowledgements. I am deeply indebted to Professor Lars Gunnar Sillén for his help and advice. I also wish to thank Dr Georg Lundgren for many valuable discussions.

REFERENCES

1. Meyer, J. and Wilk, H. *Z. anorg. Chem.* **132** (1924) 239.
2. Hartwig-Bendig, M. *Z. angew. Mineral.* **3** (1941) 195.
3. Kolthoff, I. M. and Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*, New York, p. 753.
4. Lipson, H. and Cochran, W. *The Determination of Crystal Structures*, London 1953, p. 175.
5. Zachariasen, W. *Strukturbericht*, Vol. 2, p. 39, 310.
6. Pauling, L. *The Nature of the Chemical Bond*, New York 1948.

Received February 17, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 945–953

Studies on the Heat Inactivation of Intestinal Invertase, Maltase and Trehalase

ARNE DAHLQVIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The heat inactivation of the invertase, maltase, and trehalase activities in a solubilized glycosidase preparation from swine intestinal mucosa has been studied.

The trehalase activity was completely separated from the invertase and maltase activities, which is in accordance with earlier findings.

Two different maltase activities were recognized, one *heat-labile* (inactivated at 50°C) and one more *heat-stable* (stable at 70°C when heated for 1 h at pH 6.0). These activities were chromatographically separable, and must therefore be caused by different enzymes.

The invertase activity was inactivated parallel with the heat-labile maltase activity. These two activities are, however, not quite identical, since a heat-labile maltase with low invertase activity was isolated chromatographically. The heat-labile maltase activity is possibly caused by two different enzymes, one of which is combined with the invertase activity, while the other one is not.

Particle-bound glycosidases in microsomes show the same behaviour as the solubilized enzymes when exposed to heat, which demonstrates that all the activities observed have their origin in the intestinal mucosa and that the properties of the enzymes are not altered during solubilization by trypsin digestion.

The hydrolysis of different α-D-glucopyranosides, including sucrose, by intestinal mucosa has been proposed to be caused by one single α-D-glucopyranosidase [1,2]. Recently has, however, been found that intestinal trehalase activity in preparations from swine intestine could be separated from the invertase and maltase activities by anion exchange chromatography [3]. The invertase and maltase activities formed one common peak, but this peak was not homogeneous, since the maltase/invertase activity quotient was lower in the beginning of the peak than in its end. It was therefore apparent, that the intestinal mucosa contains several different enzymes capable of hydrolyzing α-D-glucopyranosides, but for further studies a more complete separation is necessary.

Different enzymes show great variations in their sensitivity to heat inactivation during standardized conditions. This fact may be used for distinguishing

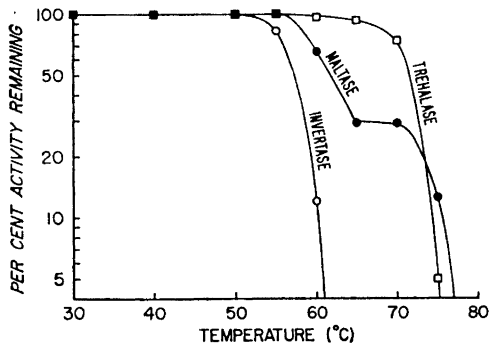


Fig. 1. Heat inactivation of intestinal glycosidases at continuously increasing temperature (gradient 1°C per min). The enzyme solution used was purified by ethanol fractionation and contained 90 U of invertase, 180 U of maltase, 20 U of trehalase and 6.1 mg of protein per ml in 0.01 M phosphate buffer pH 6.0.

different enzymes in mixture. This method has been used for the demonstration of two different bromo-naphthyl-β-D-galactopyranosidase activities in calf intestinal mucosa, only one of which was combined with lactase activity [4]. Invertase from human jejunum is known to be more sensitive to heat than yeast invertase [5], but a comparison between the heat sensitivities of the different α-D-glucopyranosidase activities of intestinal mucosa seems never to have been performed.

In the present paper is described a study of the heat inactivation of the invertase, maltase, and trehalase activities of a solubilized glycosidase preparation from swine intestinal mucosa. The heat inactivation of different fractions from an anionic exchange chromatogram of this preparation also was studied. In addition the heat inactivation of these activities in their non-soluble form in a preparation of microsomes from swine intestinal mucosa was studied.

RESULTS AND DISCUSSION

A. Heat inactivation of solubilized glycosidases

The enzyme preparation used was a solubilized preparation of glycosidases from swine intestinal mucosa, purified by ethyl alcohol fractionation [3]. It contained 90 U of invertase, 180 U of maltase, 20 U of trehalase, and 6.1 mg of protein per ml. Before use the solution was dialyzed against 0.01 M phosphate buffer pH 6.0.

Inactivation at increasing temperature. In a preliminary experiment, 5 ml of the enzyme solution was heated in a waterbath at increasing temperature (increasing 1°C per min.). At intervals 0.5 ml samples were taken, and immediately blown down into test-tubes chilled with crushed ice, in which they were stored until analysed.

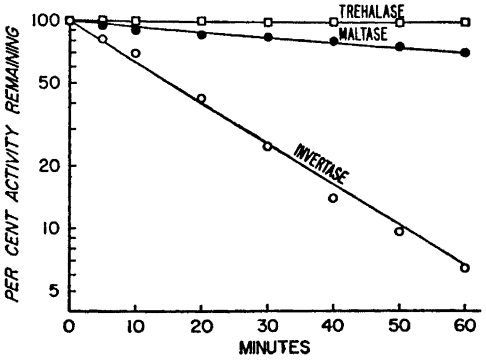


Fig. 2. Heat inactivation of intestinal glycosidases at 50°C. Enzyme solution the same as in Fig. 1.

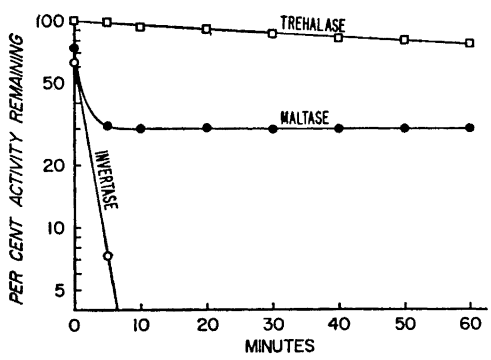


Fig. 3. Heat inactivation of intestinal glycosidases at 60°C. Enzyme solution the same as in Fig. 1.

The different activities investigated were found to differ widely in their sensitivity to heat. The invertase actvity was abolished between 50 and 60°C while the trehalase activity was not influenced below 65°C (Fig. 1). These activities must therefore be caused by two different enzymes, which has also been revealed by ion exchange chromatography [3]. The maltase was inactivated in two steps. 70 % of the maltase activity disappeared in the same temperature interval as the invertase activity, while the remaining 30 % of the total maltase activity were inactivated first when the temperature exceeded 70°C.

Inactivation at constant temperature. At constant temperature the heat inactivation of enzymes usually follows the kinetics of a first order reaction, *i.e.* if the logarithm of the remaining enzymatic activity is plotted against time, a straight line is obtained [6].

To reach the desired temperature within reasonable time, it was necessary to heat the enzyme solution, in a thin-walled test tube, in a preheating bath which has a temperature 8°C higher than the desired inactivation temperature. When the desired temperature was reached (within 45 sec.) the tube was immediately transferred to the inactivation bath, and in the same moment a zero-time sample was taken and a stop-watch was started. At time intervals samples were taken as described above. The glycosidase activities in the samples are expressed as per cent of the original activity (before exposure to heat).

At 50°C (Fig. 2) the invertase activity diminished to 50 % of its original value in 18 min. The maltase was inactivated more slowly, and the trehalase activity was not affected at all.

At 60°C (Fig. 3) 99 % of the invertase activity was destroyed in about 10 min. The maltase activity rapidly diminished to 30 % of its original value, but even after heating for 1 hour at 60°C the maltase activity was not further affected. The trehalase activity decreased only very slightly.

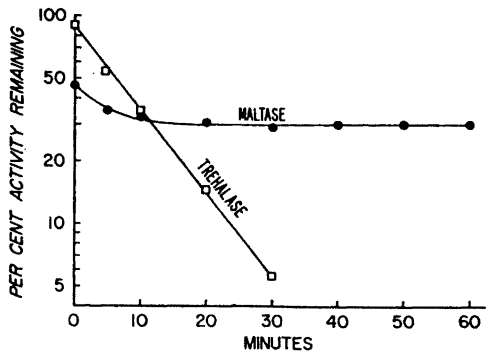


Fig. 4. Heat inactivation of intestinal glycosidases at 70°C. Enzyme solution the same as in Fig. 1.

At 70°C (Fig. 4) the invertase activity was practically immediately destroyed, and the trehalase activity was rapidly diminishing, following first order kinetics. The maltase activity decreased immediately to 30 % of its original value, but was then not measurably affected. At higher temperature also the maltase activity was completely destroyed.

The maltase activity apparently consists of two components, one more *heat-labile*, constituting 70 % of the total activity, and one more *heat-stable*, constituting 30 % of the total maltase activity. The maltase activity must therefore be caused either by two different enzymes or by one enzyme which in inactivated in two steps. As will be seen below, the heat-labile and the heat-stable maltase activities are chromatographically separable, and must therefore be caused by two different enzymes.

From the inactivation experiment at 70°C (Fig. 4) it is clearly seen that the trehalase activity is independent of both the invertase and heat-labile maltase activities (which are practically immediately destroyed) and the heat-stable maltase activity (which is not affected at all). This is in accordence with the earlier reported isolation of a trehalase fraction from intestinal mucosa which had no maltase or invertase action.

The relations between the heat-stable and heat-labile maltase activities and the invertase activity were further investigated by analysis of fractions obtained by chromatography of these activities upon DEAE-cellulose.

B. Heat inactivation of fractions from DEAE-cellulose chromatography

In chromatography upon DEAE-cellulose [3] the invertase and maltase activities form one common peak, which, however, is inhomogeneous. The maltase/invertase activity quotient is 0.6 in the beginning of the peak, while it rises to over 4 in its end.

Chromatography on DEAE-cellulose. A solubilized glycosidase preparation, containing 24 000 U of invertase, 48 000 U of maltase, and 400 mg of protein in 0.05 M phosphate buffer pH 6.0 was applied to a column containing 10 g (dry weight) of DEAE-cellulose. The column was first eluted with 2 liters of 0.05 M phosphate buffer pH 6.0. This fraction contained no maltase or invertase activity and was discarded. The column was then eluted with phosphate buffer of the same pH with step-wise increasing molarity (Table 1).

Table 1. Chromatography of intestinal maltase and invertase on DEAE-cellulose with step-wise elution.

Fraction No.	Molarity of buffer	Volume	Mg of protein	U of invertase	U of maltase
I	0.065	300 ml	24	3 300	2 000
II	0.150	300 »	229	20 000	42 000
III	0.200	200 »	7.6	400	1 700

Concentration of the fractions. After dilution of the fractions with distilled water to give a final concentration of 0.05 M buffer, the fractions were applied to small DEAE-cellulose columns (dry weight of DEAE-cellulose 1 g for fraction I and III and 5 g for fraction II) and thereafter eluted with 50 ml of 0.2 M phosphate buffer pH 6.0. Before heat inactivation the solutions were dialyzed against 0.01 M phosphate buffer pH 6.0.

Heat inactivation of the fractions obtained. Fraction I contained after concentration 80 U of invertase, 50 U of maltase, and 0.6 mg of protein per ml. This means that the maltase/invertase activity quotient is 0.6. When heated at 48°C both the invertase and the maltase activity diminished, following first order kinetics, and closely following each other (Fig. 5). The maltase activity in this fraction is apparently all heat-labile. The question arises if the maltase

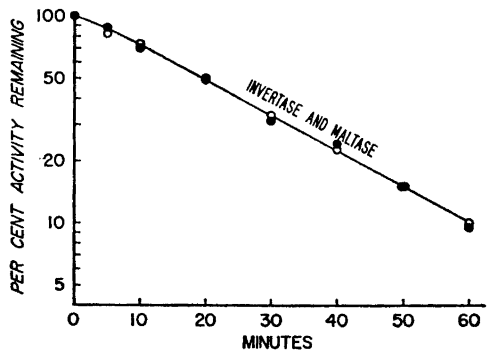


Fig. 5. Heat inactivation at 48°C of fraction I from DEAE-cellulose chromatography. The solution contained 80 U of invertase, 50 U of maltase, and 0.6 mg of protein per ml in 0.01 M phosphate buffer pH 6.0. The maltase activity is all heat-labile, and invertase and maltase are inactivated parallel.

and invertase activities in this preparation are caused by the same enzyme. When a sample of this fraction was rechromatographed on DEAE-cellulose, using gradient elution, the invertase and maltase activities appeared as one peak, which was quite homogeneous. Heat inactivation was performed in buffers of varying pH in order to find any differences in behaviour of the maltase and the invertase activities, but no difference was found (Table 2).

Table 2. Heat inactivation of fraction I at 45°C in varying buffers. Maltase/invertase activity quoient 0.6.

Buffer	Half-time for the invertase activity, min	Half-time for the maltase activity min
0.01 M acetate pH 5.2	10	10
0.01 M phosphate pH 6.0	90	90
0.01 M phosphate pH 7.0	80	80
0.01 M veronal pH 8.0	3	3

Fraction III contained after concentration 10 U of invertase, 41 U of maltase, and 0.2 mg of protein per ml. When the solution was heated at 60°C, the invertase activity rapidly disappeared together with 20 % of the maltase activity (Fig. 6). The remaining 80 % of the maltase activity were however not affected even after heating for 1 h. This part therefore consists of heat-stable maltase. That the heat-labile and the heat-stable maltase activities are chromatographically separable, demonstrates that they are caused by two different enzymes.

It is apparent, that the heat-stable maltase has no invertase action. On the other side the invertase fraction obtained (fraction I) had (heat-labile) maltase activity, with a maltase/invertase activity quotient of 0.6. This maltase activity could not be separated from the invertase activity, and there

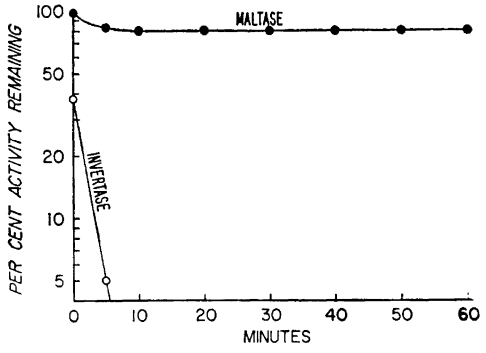


Fig. 6. Heat inactivation at 60°C of fraction III from DEAE-cellulose chromatography. The solution contained 10 U of invertase, 41 U of maltase, and 0.3 mg of protein per ml in 0.01 M phosphate buffer pH 6.0. The maltase activity is mainly heat-stable.

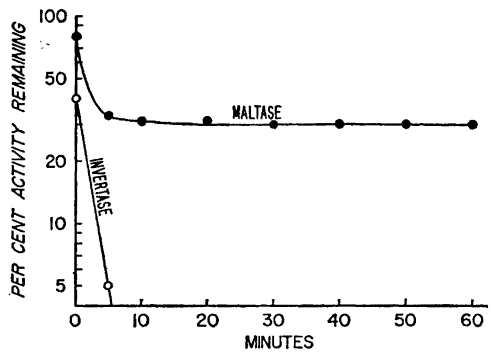


Fig. 7. Heat inactivation at 60°C of a preparation obtained by repeated rechromatography of the middle fraction from DEAE-cellulose chromatography. The solution contained 3.9 U of invertase, and 67.5 U of maltase per ml. In spite of the low invertase activity, the major part of the maltase activity is heat-labile.

are reasons to believe that these two activities are caused by the same enzyme. The original enzyme preparation had, however, a maltase/invertase activity quotient of 2.0. Since the heat-labile maltase activity amounted to 70 % of the total maltase activty, this preparation had a heat-labile maltase/invertase activity quotient of 1.4. It stands therefore clear, that even if the invertase has maltase activity, it cannot cause more than 50 % of the heat-labile maltase activity in the original preparation.

When fraction II, which contained the main part of the original enzyme activity, was rechromatographed on DEAE-cellulose, again a first fraction with a maltase/invertase activity quotient of 0.6, and a last fraction mainly consisting of heat-stable maltase were obtained. By repeated collection and rechromatography of the middle fraction, a preparation was obtained which contained all three activities, but in which the heat-labile maltase/invertase quotient was gradually increased. After four times repeated chromatography, this fraction had a heat-labile maltase/invertase quotient of 12.1 (Fig. 7). There exists therefore one heat-labile maltase activty which has no (or very low) invertase action. On DEAE-cellulose it is only possible to purify this activity partially, wherefore more suitable separation methods are under investigation. The relation between this invertase-free heat-labile maltase activity and the maltase activity which accompanies the invertase activity will be further studied.

If it is supposed that there exist *two* different *heat-labile* maltases, the following intestinal maltases will be recognized:

Maltase I: heat-labile maltase activity accompanying the invertase activity and amounting to 30 % of the total maltase activity.

Maltase II: heat-labile maltase activity without invertase action, amounting to 40 % of the total maltase activity.

Maltase III: heat-stable maltase activity, amounting to 30 % of the total maltase activity.

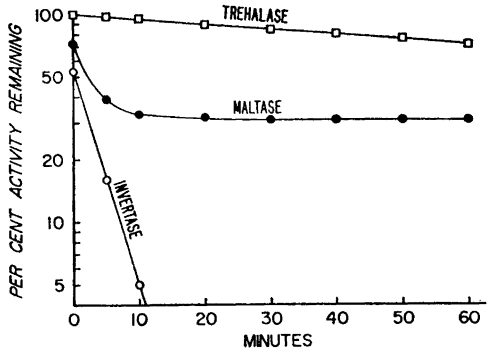


Fig. 8. Heat inactivation at 60°C of particle-bound intestinal glycosidases. The sample contained 35 U of invertase, 70 U of maltase, 10 U of trehalase and 7 mg of protein per ml in 0.01 M phosphate buffer pH 6.0.

C. Heat inactivation of particulate glycosidases

In the method used for preparation of soluble glycosidases, the intestinal contents are not removed from the mucosa [3]. This offers the possibility for contamination with enzymes secreted from other organs or bacterial enzymes. It has, however, been found that the invertase obtained in this way has the same properties as the invertase in washed microsomes from the intestinal mucosa, and that the relation of the maltase and trehalase activities to the invertase activity is unaltered [3]. It must also be considered, that the solubilization by trypsine digestion may alter the properties of the enzymes. This seems, however, not probable, because the enzymatic activity is unaltered [3].

The behaviour of particle-bound intestinal glycosidases during heat inactivation was studied on a desoxycholate-extract from washed intestinal microsomes [3]. This extract is known to contain only particle-bound glycosidases, which can be sedimented in a centrifuge if the desoxycholate is removed [3]. It contained 35 U of invertase, 70 U of maltase, 10 U of trehalase, and 7 mg of protein per ml. The extract was dialyzed against 0.01 M phosphate buffer pH 6.0 before use.

At 60°C (Fig. 8) the invertase activity disappeared within 10 min together with 68 % of the total maltase activity. The remaining 32 % of the maltase activity was not further affected. The trehalase activity was only very slowly diminishing. This behaviour is quite identical with that which was found with solubilized glycosidases (Fig. 3). This shows that all the activities observed have their origin in the microsomes of the intestinal mucosa, and that the properties of the enzymes are not altered during their solubilization by trypsin digestion.

MATERIALS AND METHODS

Enzymes. Solubilized glycosidases were prepared from swine intestinal mucosa by trypsin digestion and ethyl alcohol fractionation as described earlier [3]. *Particle-bound glycosidases* were prepared from washed microsomes from swine intestinal mucosa by extraction with sodium desoxycholate as described earlier [3].

Substrates. Sucrose was obtained from J. T. Baker Co., maltose (monohydrate) from Merck A. G. (Germany), and trehalose (dihydrate) from Pfanstiehl Chemical Co.

Determination of glycosidase activity was performed at 37°C. The reaction mixture had 0.1388 M substrate concentration and contained 0.05 M maleate buffer of pH 6.5 for invertase and maltase and 6.0 for trehalase. The degree of hydrolysis was determined with the 3,5-dinitrosalicylic acid reagent of Sumner [7] for sucrose and trehalose, and with the acid copper reagent of Tauber and Kleiner [8] for maltose. The degree of hydrolysis was not allowed to exceed 10 % in one hour. During these conditions the amount of monosaccharides produced was proportional to the glycosidase activity (zero order reaction). One U of glycosidase activity means the activity producing 1 mg of monosaccharides in one hour during the above conditions.

Protein determination was made with the method of Lowry *et al.*[9] A standard curve was made with human serum albumin (kindly supplied by A. B. Kabi). *Spectrophotometric readings* were made with a Beckman B spectrophotometer.

Diethyl-aminoethyl-cellulose (DEAE-cellulose) was prepared by the method of Peterson and Sober [10]. It contained 0.78 mequiv. titrable hydroxyl groups per g dry weight.

Acknowledgment. The author's thanks are due to laborator Bengt Borgström for his interest in my work and for valuable discussions.

This work has been supported by grants from the *Medical Faculty of the University of Lund.*

Mrs. Kerstin Hagebris is acknowledged for skilful technical assistance.

REFERENCES

1. Weidenhagen, R. *Ergeb. Enzymforsch.* **1** (1932) 168.
2. Neuberg, C. and Mandl, I. in Sumner, J. B. and Myrbäck, K. *The Enzymes* Vol. I. Part 1., New York 1950.
3. Borgström, B. and Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 1997.
4. Heilskov, N. S. C. *Studies on Animal Lactase,* Dissertation, Copenhagen 1956.
5. von Euler, H. and Myrbäck, K. *Z. physiol. Chem. Hoppe-Seyler* **115** (1921) 68.
6. Moelwyn-Hughes, E. A. in Nord F. F. und Weidenhagen, R. *Handbuch der Enzymolgie,* Leipzig 1940.
7. Hostettler, F., Borel, E. und Deuel, H. *Helv. Chim. Acta* **34** (1951) 2132.
8. Tauber, H. and Kleiner, I. S. *J. Biol. Chem.* **99** (1932) 249.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
10. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.

Received March 18, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 954–962

Studies on Molybdenum Oxides *

LARS KIHLBORG

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The MoO_2–MoO_3 region of the molybdenum-oxygen system has been investigated within the temperature range 500°–870°C, by means of X-ray diffraction techniques. Temperature regions are given for the formation of the seven intermediate phases found, among which are two previously undescribed oxides designated ϑ- and $\varkappa$-oxides.

Unit cell dimensions and X-ray diffraction data are given and formulae are suggested for the ζ-oxide (Mo_9O_{26}, triclinic), the $\varkappa$-oxide ($Mo_{17}O_{47}$, orthorhombic) and the η-oxide (Mo_4O_{11}, monoclinic). The unit cell dimensions have also been determined for the ϑ-oxide ($MoO_{\sim 2.80}$, tetragonal), which is probably metastable.

The electrical resistivity at room temperature has been determined for the various intermediate phases excluding the ϑ-phase.

The oxidation of the oxides in air at 230°–350°C has been studied. An intermediate stage, called λ-oxide, has been observed in the oxidation of the two Mo_4O_{11} phases to MoO_3.

The first phase analysis by means of X-ray methods on the molybdenum-oxygen system was published in 1944 by Hägg and Magnéli [1]. They reported the existence of four oxides with compositions between MoO_2 and MoO_3, called β-, β'-, γ- and ε-oxide. By determination of the crystal structures Magnéli was later able to establish the exact formula Mo_8O_{23} (monoclinic) for the β-oxide [2], Mo_9O_{26} (monoclinic) for the β'-oxide [2] and Mo_4O_{11} (orthorhombic) for the γ-oxide [3]. These and other structural studies are reviewed and further discussed in Ref.[4].

In 1950 Glemser and Lutz published a paper on molybdenum oxides studied by X-ray powder methods, electrical resistivity measurements and chemical and microscopical techniques [5]. Their phase analysis in general confirmed the results of Hägg and Magnéli, but as has already been pointed out [6], the notations of the phases used in the two articles are inconsistent (*v. infra*).

In connection with studies of the thermal decomposition of MoO_3 *in vacuo*, two other oxide phases were observed, which were provisionally called ζ- and

* A survey of part of the work reported in this article was presented to the XVIth International Congress for Pure and Applied Chemistry, Paris 1957, and has been published in *Mémoire présenté à la Section de Chimie Minérale*, Sedes, Paris 1958, p. 59–61.

η-oxide [7]. There were indications that the composition of ζ-oxide is near $MoO_{2.88}$, but at that time nothing could be said about the composition of the η-oxide.

A review of the structural investigations of oxygen compounds of molybdenum and tungsten carried out at this Institute up to 1954 is given in Ref.[8]

As the investigations by Hägg and Magnéli were limited mainly to a restricted region of temperature around 650—750°C, it was thought desirable to extend the phase analysis studies to a wider range of temperature and to try to establish the formulae of the new oxides.

The results obtained to date are presented in this article. Crystal structure determinations of several of the new oxides are in progress.

EXPERIMENTAL

The starting materials for the preparations were molybdenum trioxide ("Baker's Analyzed", 99.9 % dried at 200°C), and molybdenum dioxide prepared by reducing the trioxide in a stream of dry hydrogen gas at 500°C, the degree of reduction being controlled by weighing. In a few cases molybdenum powder ("Kahlbaum", heated *in vacuo* at about 400°C) was used instead of the dioxide.

The samples, prepared by thoroughly mixing weighed amounts of MoO_2 (or Mo) and MoO_3, were heated in evacuated, sealed silica tubes, which had been cleaned and preheated in air at about 800°C. As had been observed previously by Hägg and Magnéli, MoO_3 attacks the silica to some extent, especially above 700°C, giving a white, crystalline coating inside the tubes. This coating showed the powder lines of low-cristobalite, often together with those of MoO_3. To avoid contamination, those samples which did not contain molybdenum metal were often packed into platinum foil when heated at the higher temperatures.

The high volatility of MoO_3 made it necessary to minimize any temperature gradient within the tubes which were therefore kept in appropriate borings in heavy steel cylinders placed in vertical electrical furnaces [6]. In spite of these precautions a slight escape of MoO_3 from the main sample could not be avoided, especially during the heat treatments above 700°C. This gave rise to a small drift of the composition towards lower oxygen content ($\Delta x < 0.02$ in MoO_x) which had to be taken into account.

A calibrated platinum-platinum rhodium thermocouple was used, inserted into a boring of the steel cylinder. The cold junction was kept at 0°C. The temperature was measured with a potentiometer bridge at the beginning and the end of each heat treatment, and was kept constant by a Wheelco regulator which controlled only about 5 % of the stabilized voltage of the furnace [6]. In this way the short-time variations of the temperature were reduced to within ± 2°C, but a long-time drift of the temperature level was sometimes observed. The total variations were, however, always within ± 4°C.

The studies have been concerned with the temperature interval 500° — 870°C, the lower limit being set by the rapidly decreasing reaction rate. At the lower temperatures heating times of several weeks and repeated powdering of the samples were necessary in order to obtain equilibrium. At the higher temperatures heating for some days was usually sufficient.

The silica tubes were quenched in water from the reaction temperatures. No chemical analysis of the products was considered necessary.

The samples were examined microscopically and X-ray powder photographs were taken in Guinier focusing cameras of 80 mm diameter using monochromatized Cu$K\alpha$ or Cr$K\alpha$ radiation. These cameras, covering the angular region $1.5° < \Theta < 45°$, give a high resolution and very low background which greatly facilitated the identification of the patterns and the detection of phases present in small amounts. The powder patterns were evaluated according to the method given by Hägg, which introduces an automatic correction for film shrinkage [9]. Potassium chloride (Analar, BDH, ground and recrystallized) was added to the powder specimens as an internal standard, and its lattice parameter was taken to be $a = 6.29215$ Å (21°C) according to Hambling [10]. The use of Guinier

Table 1. X-Ray diffraction patterns of molybdenum oxides; Cu*Ka* radiation, $\lambda = 1.5418$ Å.

a Triclinic Mo_9O_{26} (ζ-molybdenum oxide)

I	$\sin^2\Theta_{obs}$	*hkl*	$\sin^2\Theta_{calc}$
w	0.01139	$10\bar{1}$	0.01138
vw−	0.01565	$10\bar{2}$	0.01571
w	0.03657	113	0.03647
vw−	0.04187	$20\bar{1}$	0.04152
		$\bar{1}31$	0.04197
vw−	0.04551	$20\bar{2}$	0.04552
st	0.04641	015	0.04653
vw	0.04802	$\bar{1}32$	0.04800
		114	0.04827
st	0.05235	$03\bar{3}$	0.05218
vw−	0.05254	$20\bar{3}$	0.05262
vw	0.05498	$\bar{2}30$	0.05510
m	0.05549	$2\bar{3}1$	0.05561
vw	0.06135	$03\bar{4}$	0.06136
vw	0.06373	212	0.06374
vw	0.07732	$2\bar{2}5$	0.07718
vw	0.08082	041	0.08080
w	0.08749	$\bar{1}43$	0.08772
		042	0.08774
vw	0.09029	$1\bar{2}7$	0.09036
vw+	0.09355	$\bar{3}31$	0.09368
		$2\bar{2}6$	0.09381
vw−	0.09671	$13\bar{5}$	0.09656
vw−	0.10602	$3\bar{2}4$	0.10616
		140	0.10635
vw	0.11007	$02\bar{8}$	0.10968
		$\bar{2}44$	0.11047
vw+	0.11234	$30\bar{4}$	0.11230
vw	0.11372	311	0.11376
w	0.15029	128	0.15028
vw	0.15494	$23\bar{6}$	0.15452
		$24\bar{1}$	0.15469
		$\bar{4}32$	0.15512
m	0.17009	$2\bar{6}4$	0.17008

b $Mo_{17}O_{47}$ ($\varkappa$-molybdenum oxide)

I	$\sin^2\Theta_{obs}$	*hkl*	$\sin^2\Theta_{calc}$
m	0.00280	110	0.00281
w	0.00507	200	0.00509
w	0.00616	020	0.00616
w	0.00661	210	0.00663
w	0.00742	120	0.00744
w	0.01124	220	0.01126
w−	0.01893	230	0.01897
w−	0.02979	240	0.02976
m	0.03331	510	0.03334
w	0.03612	340	0.03612
st	0.03804	520	0.03797
		001	0.03806
st	0.04507	440	0.04502
w+	0.05199	031	0.05194
		620	0.05196
vw	0.05553	060	0.05551
vw	0.05648	540	0.05647
w−	0.05686	160	0.05678
w−	0.05699	231	0.05703
m	0.05843	401	0.05841
st	0.05966	630	0.05967
m	0.06272	041	0.06273
vw	0.06388	710	0.06387
vw	0.07032	550	0.07035
w	0.07139	511	0.07140
w	0.07587	460	0.07586
m	0.07683	170	0.07683
w	0.08063	270	0.08065
m	0.08138	800	0.08141
vst	0.08307	441	0.08308
w−	0.08754	820	0.08758
vw	0.09353	061	0.09357
vw	0.09459	541	0.09453
m	0.09764	631	0.09773

c ϑ-molybdenum oxide

I	$\sin^2\Theta_{obs}$	*hkl*	$\sin^2\Theta_{calc}$
w	0.00448	400	0.00449
w	0.00563	420	0.00562
w	0.01126	620	0.01124
vw−	0.01460	640	0.01461
vw	0.01800	800	0.01798
w	0.02028	660	0.02023
vw−	0.02248	840	0.02247
vw−	0.03262	10,4,0	0.03259
st	0.03829	001	0.03834
m	0.04045	12,0,0	0.04045
w	0.04156	12,2,0	0.04158
vw	0.04494	12,4,0	0.04495
m	0.04607	10,8,0	0.04607
w	0.05619	10,10,0	0.05618
w	0.05845	12,8,0	0.05843
w	0.05954	14,4,0	0.05956
vw−	0.07086	10,4,1	0.07093
vw	0.07192	16,0,0	0.07192
m	0.07305	14,8,0	0.07304
		16,2,0	

d Monoclinic Mo_4O_{11} (η-molybdenum oxide)

I	$\sin^2\Theta_{obs}$	*hkl*	$\sin^2\Theta_{calc}$
w	0.00393	200	0.00397
w	0.01332	001	0.01331
w	0.01618	$20\bar{1}$	0.01619
vw	0.01836	201	0.01836
w+	0.02697	$40\bar{1}$	0.02702
m−	0.03336	011	0.03340
vw	0.03629	$21\bar{1}$	0.03628
vst+	0.03844	211	0.03845
vw	0.04393	311	0.04396

vst	0.04571	$60\bar{1}$	0.04578
st	0.05145	411	0.05145
vw	0.05574	610	0.05582
vw	0.07235	611	0.07238
		$80\bar{1}$	0.07248
vw+	0.07352	012	0.07333
		402	0.07346
m	0.07522	$21\bar{2}$	0.07513
		112	0.07541
m	0.08031	020	0.08036
m	0.08345	502	0.08348
		810	0.08360
m	0.08490	$41\bar{2}$	0.08487
w+	0.09927	10,0,0	0.09924
vw	0.10262	$61\bar{2}$	0.10254
vw	0.10805	$80\bar{2}$	0.10807
w−	0.11911	$10\bar{3}$	0.11915
		10,1,0	0.11933
w	0.12008	003	0.11979
w	0.12606	$62\bar{1}$	0.12614
vw	0.13975	222	0.13974
		013	0.13988
w−	0.14148	$10,0,\bar{2}$	0.14163
w	0.15381	313	0.15370
		422	0.15382
vw	0.16245	413	0.16227
		12,0,1	0.16273
vw	0.16383	522	0.16384
m	0.17590	622	0.17584
w	0.18282	12,1,1	0.18282
m	0.19051	813	0.19037

e λ-molybdenum oxide

I	$\sin^2\Theta_{obs}$
st	0.0398
w	0.0437
m	0.0469
vw	0.0527
vw	0.0865
vw	0.0890
vw	0.0907
vw	0.1594
vw	0.1689
vw	0.1878

vst = very strong
st = strong
m = medium
w = weak
vw = very weak

cameras for accurate determination of lattice dimensions has recently been demonstrated in this journal [11]. The powder patterns of several of the molybdenum oxide phases are given in Table 1.

Single crystal photographs were taken in Weissenberg and precession cameras using Cu*K* and Mo*K* radiation.

The electrical resistivity of the oxides was determined in a simple cell, permitting the measurements to be carried out on a powder specimen under considerable pressure, applied by means of a screw press. The data thus obtained are given in Table 2.

RESULTS OF THE PHASE ANALYSIS

The results of the phase analysis within the 500°—800°C region are summarized in Fig. 1, where the formation temperatures *versus* composition are indicated for the intermediate phases found. As far as can be determined from the powder photographs, none of the phases has an extended homogeneity range. It must be emphasized that the temperature ranges indicated do not necessarily represent the true stability regions. These may be very difficult to establish because of an observed disposition of several phases to remain for a long time in a metastable state.

Preparations — the starting mixtures as well as the pure oxides prepared — heated above 800°C and quenched in water always contained at least three phases, *viz.* MoO_2, MoO_3 and orthorhombic Mo_4O_{11} (γ-oxide), and thus did not represent a state of equilibrium. When the cooling rate was slower, the lines of MoO_2 generally disappeared. This seems to indicate that MoO_2 and MoO_3 are the only phases stable above 800°C, but that orthorhombic Mo_4O_{11}

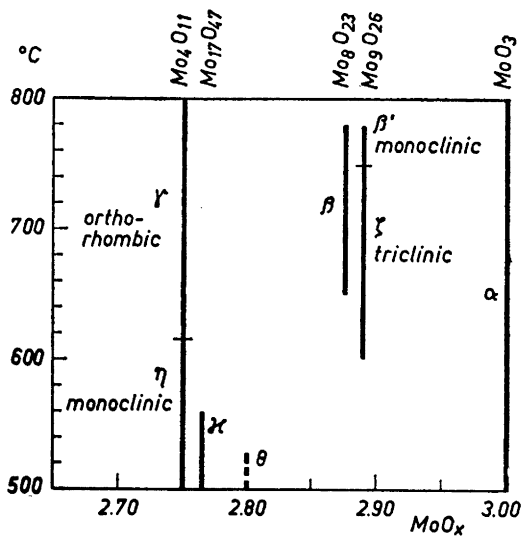


Fig. 1. Formation temperature *v.* composition diagram for molybdenum oxides.

forms fairly rapidly when the temperature is lowered. The triclinic Mo_9O_{26} (ζ-oxide) likewise was sometimes found in samples slowly cooled from above 800°C. According to Fig. 1, the formation of this phase should occur below 750°C.

Hägg and Magnéli reported that the β'-oxide (monoclinic Mo_9O_{26}) and the γ-oxide (orthorhombic Mo_4O_{11}) decompose when heated to 850°C, giving MoO_2, MoO_3 and a new phase which they designated ε-oxide[1]. Upon reexamination of samples prepared by these authors and reported to contain ε-oxide, it became evident that this phase was identical with the ζ-oxide (triclinic Mo_9O_{26}). The cooling rate was, however, not very high in these early experiments and may explain the occurrence of this phase.

Description of the phases observed

Triclinic Mo_9O_{26} (ζ-oxide). The ζ-phase has been obtained between 600° and 750°C *, and is the purest in samples of compositions close to $MoO_{2.89}$. It is transformed into monoclinic Mo_9O_{26} (β'-oxide) when heated at 760°—780°C (within 2 h at 780°C), and is slowly decomposed into MoO_3 and monoclinic Mo_4O_{11} (η-oxide) at 550°C. It forms almost-black, irregular, but rather flaky crystals, readily distinguishable from the other molybdenum oxides. With the aid of single crystal data, its powder pattern (see Table 1a) has been indexed and the following triclinic unit cell has been derived by least squares refinement

* The accuracy of the formation temperature limits given in the following are, in general, about ± 10°C.

$a = 8.145$ Å	$b = 11.89$ Å	$c = 19.66$ Å	$V = 1779.5$ Å^3
$\alpha = 95.47°$	$\beta = 90.39°$	$\gamma = 109.97°$	

As is the case with all the oxides below MoO_3 the electrical conductivity makes impossible the use of piezo-electrical methods for detection of any non-centrosymmetry.

The density was found to be 4.74, which corresponds to 35.7 $MoO_{2.89}$ per unit cell and suggests a cell content of 4 formula units Mo_9O_{26}.

Mo_8O_{23} (β-oxide) and monoclinic Mo_9O_{26} (β'-oxide). These two phases, first described by Hägg and Magnéli [1], have been obtained within the temperature ranges 650°—780°C for Mo_8O_{23} (by decomposition of $Mo_{17}O_{47}$ even at 630°C; see below) and 750°—780° for monoclinic Mo_9O_{26}. Both decompose into MoO_3 and orthorhombic Mo_4O_{11} when heated at 785° and 800°C. At temperatures below their formation regions they are, however, markedly stable; no change was actually observed after tempering Mo_8O_{23} for three days at 643°C or three weeks at 550°C and monoclinic Mo_9O_{26} three days at 700°C or one week at 648°C.

The structures of the two oxides have been determined by Magnéli [2]. They constitute the first two members of a series of structurally interrelated oxides of the general formula Me_nO_{3n-1} [12]. By substituting tungsten for part of the molybdenum, higher homologues in this series have been prepared, *e.g.* $(Mo, W)_{10}O_{29}$ and $(Mo, W)_{12}O_{35}$ [6]. The introduction of a small proportion of tungsten has furthermore been found to extend the formation range for the β'-phase down to 650°C [6].

The *d*-values of the powder patterns of Mo_8O_{23} and monoclinic Mo_9O_{26} are listed in Ref.[13], and the unit cell dimensions are given in Table 2 of Ref.[6]

It has been pointed out earlier [6] that, in view of its chemical properties, the "β-molybdenum oxide" of Glemser and Lutz [5] is probably identical with the triclinic Mo_9O_{26} (ζ-oxide), while their "β'-oxide" is Mo_8O_{23}, monoclinic Mo_9O_{26} or a mixture of these two phases. The resistivity measurements made (*cf.* Table 2) strongly support this view: the triclinic Mo_9O_{26} is seen to have by far the highest resistivity of all the intermediate phases, as has the "β-oxide" according to Glemser and Lutz.

$Mo_{17}O_{47}$ ($\varkappa$-oxide). In samples heated below about 560°C, a new phase appeared with a composition close to $MoO_{2.78}$. It decomposes into orthorhombic Mo_4O_{11} (γ-oxide) and Mo_8O_{23} (β-oxide) at 630°C, but does not change when kept at 590°C for one week.

Table 2. Specific electrical resistivity of molybdenum oxides (at room temperature).

Phase	Resistivity ohm . cm
Mo_9O_{26} triclinic	~ 250
Mo_9O_{26} monoclinic	~ 3.7
Mo_8O_{23}	~ 1.2
$Mo_{17}O_{47}$	< 0.05
Mo_4O_{11} orthorhombic	~ 0.25
Mo_4O_{11} monoclinic	~ 0.2

The $\varkappa$-oxide forms long, flat, extremely thin, black needles and looks dark grey when powdered. Its powder pattern, given in Table 1b has revealed an orthorhombic unit cell with the dimensions

$$a = 21.61_5 \text{ Å} \qquad b = 19.63_2 \text{ Å}, \qquad c = 3.951_5 \text{ Å}.$$

The reflection absences found in the single crystal photographs are in accordance with the space groups No. 32, *Pba*2 and No. 55, *Pbam*.

The density was measured to be 4.72, which corresponds to 34.0 $MoO_{2.78}$ units per cell and suggests the formula $Mo_{17}O_{47}$, with 2 formula units per cell.

The low electrical resistivity of this phase is noteworthy (see Table 2).

ϑ-Oxide. A second phase not previously reported has been observed in preparations heated for a relatively short time below 530°C. The composition of this phase, called ϑ-oxide, seems to be in the neighbourhood of $MoO_{2.80}$, but it has not been possible to obtain it in a very pure state. With prolonged heating, it slowly decomposes into $Mo_{17}O_{47}$ ($\varkappa$-oxide) and MoO_3. It seems, therefore, probable that the ϑ-oxide is not stable within the temperature region investigated, but is formed in a metastable state.

Its powder pattern is given in Table 1c. The unit cell is tetragonal with the dimensions

$$a = 45.99 \text{ Å}, \qquad c = 3.937 \text{ Å}.$$

The reflections *hkl* with h or k odd are absent for $l = 0$; they are revealed in the layer line $l = 1$ as very weak, diffuse spots growing stronger in the higher layer lines. This phenomenon evidently indicates the presence of a subcell of the dimensions

$$a' = \tfrac{1}{2}a = 23.00 \text{ Å}, \qquad c' = c = 3.937 \text{ Å}.$$

The most probable space-group of the large cell is No. 129, *P*4/*nmm*.

As it has not been possible to prepare the oxide in an amount sufficient for density determinations, no proposal of its formula can be given at present.

Orthorhombic Mo_4O_{11} (γ-oxide). The orthorhombic Mo_4O_{11} (γ-oxide) forms between 615° and 800°C approximately. As was mentioned above, it is often found also in samples heated above this upper limit and furthermore sometimes in preparations treated at about 500°C. In these cases equilibrium had, however, never been reached. It is transformed into the monoclinic form, η-oxide (see below), by heating for three weeks at 550° but remains unchanged after one week at 590°C.

The crystal structure has been determined by Magnéli [3] and the X-ray powder pattern is listed in Ref.[13]. The unit cell dimensions are *

$$a = 24.49 \text{ Å}, \qquad b = 5.457 \text{ Å}, \qquad c = 6.752 \text{ Å}, \qquad V = 902.3 \text{ Å}^3.$$

Monoclinic Mo_4O_{11} (η-oxide). Below about 615°C another phase of composition $MoO_{2.75}$ is formed. This phase, η-oxide, has previously been observed

* The present values are somewhat more exact than those previously given [13].

as a product of thermal decomposition of MoO_3 *in vacuo* at 549° and 608°C [7]. It is probably identical with the phase designated γ' by Glemser and Lutz which was rarely obtained at 600°C [5]. It remains unchanged after heating for two days at 634°, but is transformed into the orthorhombic, high-temperature form (γ-oxide) within two weeks at 655°C.

This phase forms wine-red, rather irregular crystals, which are similar to those of the orthorhombic form (γ-oxide). The powder pattern given in Table 1d has been evaluated with the aid of single crystal photographs, and the following unit cell has been derived

$a = 24.54$ Å, $b = 5.439$ Å, $c = 6.701$ Å, $\beta = 94.28°$ $V = 891.8$ Å^3.

The observed density is 4.17, which is in good agreement with the value 4.168 calculated for Mo_4O_{11} with 4 formula units in the unit cell. The space group in probably No. 14, $P2_1/a$.

The close similarity of the unit cell dimensions of the two Mo_4O_{11}-modifications is striking; the slight monoclinic deformation of the η-oxide is the main difference. In addition there are many common features in the intensity distribution of the diffraction pattern indicating that the structures of the two oxides are probably interrelated. In view of this the sluggishness of the transformation between the two forms is remarkable.

By means of differential thermal analysis no polymorphic transformation has been observed[14], which is not surprising in the light of the sluggishness of the transformation.

OXIDATION IN AIR OF MOLYBDENUM OXIDES

A few experiments have been carried out on the oxidation of molybdenum oxides in air at elevated temperatures. Finely ground specimens of the various oxides (except the ϑ-oxide) were heated in a muffle furnace at a few temperatures within the region 230°—350°C.

It was found that MoO_2 and triclinic Mo_9O_{26} (ζ-oxide) were the phases most resistant to oxidation (hardly affected by heating for two days at about 270°C), while orthorhombic Mo_4O_{11} (γ-oxide) was the most sensitive phase (completely transformed to MoO_3 within 18 h at about 300°C). The high-temperature forms of Mo_4O_{11} and Mo_9O_{26}, in a metastable state at the oxidation temperature, were much more rapidly oxidized than the low-temperature forms.

Indications from the powder photographs of intermediate stages in the course of oxidation were found only in the cases of monoclinic and orthorhombic Mo_4O_{11} heated at about 230° and 270°C, at which temperatures a quite new, somewhat diffuse pattern appeared in the photographs of partially oxidized products. In the samples treated at about 300° and 350°C, this intermediate stage, here designated λ-oxide, was not observed. When a mixture of λ-oxide and MoO_3 (the mixture obtained by oxidizing orthorhombic Mo_4O_{11} for 13 days at about 230°C) was heated *in vacuo* at 390° for 20 h, the lines of the λ-oxide disappeared: the sample consisted of MoO_3 and monoclinic Mo_4O_{11}. It is thus evident that the λ-oxide is unstable, at least at higher temperatures, and that it has a composition lying somewhere within the range

$MoO_{2.75} \leq MoO_x < MoO_3$, but is probably not very close to the latter limit. The main lines of its powder pattern are listed in Table 1e.

Acknowledgements. I wish to express my sincere gratitude to Professor G. Hägg and Dr. A. Magnéli for their encouraging interest in this investigation and for putting their early preparations as well as many facilities at my disposal. I am also indebted to Mr. A. Rönnquist for valuable assistance in part of the experimental work.

A scholarship from "Th. Nordströms testamentsfond" of the *Royal Swedish Academy of Sciences* is gratefully acknowledged.

The research reported in this article has been sponsored in part by the *Swedish Natural Science Research Council* and in part by the *Office, Chief of Research and Development, U.S. Department of Army*, through its *European Office*.

REFERENCES

1. Hägg, G. and Magnéli, A. *Arkiv Kemi, Mineral. Geol.* **A 19** (1944) No. 2.
2. Magnéli, A. *Acta Chem. Scand.* **2** (1948) 501.
3. Magnéli, A. *Acta Chem. Scand.* **2** (1948) 861.
4. Magnéli, A. *Nova Acta Regiae Soc. Sci. Upsaliensis* [IV] **14** (1950) No. 8.
5. Glemser, O. and Lutz, G. *Z. anorg. u. allgem. Chem.* **263** (1950) 2.
6. Magnéli, A., Blomberg-Hansson, B., Kihlborg, L. and Sundkvist, G. *Acta Chem. Scand.* **9** (1955) 1382.
7. Kihlborg, L. and Magnéli, A. *Acta Chem. Scand.* **9** (1955) 471.
8. Hägg, G. and Magnéli, A. *Revs. Pure and Appl. Chem. (Australia)* **4** (1954) 235.
9. Hägg, G. *Rev. Sci. Instr.* **18** (1947) 371.
10. Hambling, P. G. *Acta Cryst.* **6** (1953) 98.
11. Westman, S. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1587.
12. Magnéli, A. *Acta Cryst.* **6** (1953) 495.
13. Magnéli, A., Andersson, G., Blomberg, B. and Kihlborg, L. *Anal. Chem.* **24** (1952) 1998.
14. Hegedüs, A. J., Sasvári, K. and Neugebauer, J. *Z. anorg. u. allgem. Chem.* **293** (1957) 56.

Received March 5, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 963–969

On the Reaction of *tert*-Butyl Chloride with Ethylene

ARNE BRÄNDSTRÖM

Research Laboratory, Pharmacia Ltd., Uppsala, Sweden

The reaction between *tert*-butyl chloride and ethylene has been studied. The following four chlorides have been isolated.
1-chloro-3,3-dimethylbutane
1-chloro-3,3-dimethylpentane
1-chloro-3,3,4-trimethylpentane
1-chloro-3,3,6,6-tetramethylheptane
A mechanism which explains these products is given for the reaction.

The condensation of some saturated halides with ethylene has been studied by Schmerling [1]. With *tert*-butyl chloride the main reaction is

$$(CH_3)_3C{-}Cl + CH_2{=}CH_2 \xrightarrow{AlCl_3} (CH_3)_3C{-}CH_2{-}CH_2{-}Cl$$

This reaction has now been studied in greater detail. The byproducts obtained in the reaction have been examined and the structure has been proved for three of them.

The reaction was carried out by bubbling pure ethylene at —15° to —20° into pure *tert*-butyl chloride containing about 1.5 % by weight of $AlCl_3$ [2]. By careful fractionation of the crude chloride mixture obtained in the reaction, the following chlorides have been isolated from the reaction of 132 kg of *tert*-butyl chloride and 47.5 kg of ethylene:

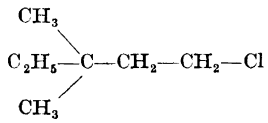
3.6 kg (2.7 %) of *tert*-butyl chloride
146.9 kg (85.4 %) of the main product $C_6H_{13}Cl$, b.p. 115—122°
1.35 kg (0.7 %) of a chloride $C_7H_{15}Cl$, b.p. 145—150°
12.00 kg (5.7 %) of a chloride $C_8H_{17}Cl$, b.p. 45—50°/8 mm Hg and
1.65 kg (1.2 %) of a chloride $C_{11}H_{23}Cl$, b.p. 82—84°/8 mm Hg

The yield of the chloride $C_{11}H_{23}Cl$ was calculated on the assumption that two moles of *tert*-butyl chloride is consumed to produce one mole of the chloride $C_{11}H_{23}Cl$.

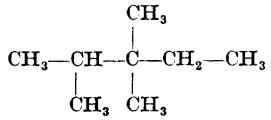
The chlorides mentioned above correspond to 95.7 % of the *tert*-butyl chloride. There were also obtained a residue boiling above 85°/8 mm weighing 5 kg and several small intermediate fractions having a total weight of 1.5 kg. Gas chromatographic analysis of these intermediate fractions showed that they

consisted of mixtures of the chlorides mentioned above together with a small quantity of a fraction boiling at 72—74°/8 mm Hg, probably a chloride $C_{10}H_{21}Cl$, which could not be obtained in a pure state. The weight of this chloride is about 0.1 % of the total weight of the chlorides.

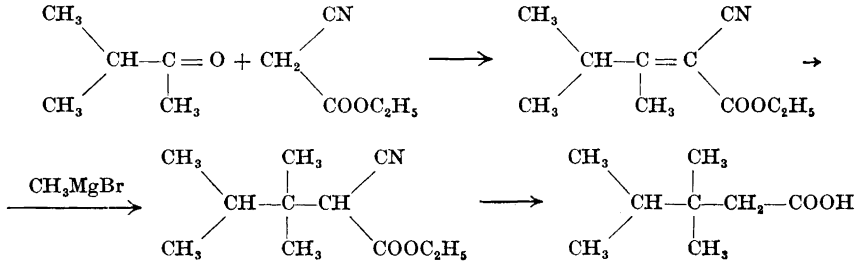
The main product $C_6H_{13}Cl$ has the formula $(CH_3)_3C—CH_2—CH_2—Cl$ [1] and the chloride $C_7H_{15}Cl$ is identical with the main product obtained from *tert*-amyl chloride and ethylene [1] and has the structure



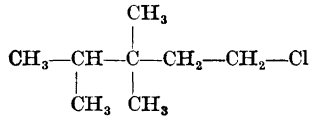
The chloride $C_8H_{17}Cl$ reacts with magnesium to form a Grignard reagent which on treatment with water gives the hydrocarbon



The chloride $C_8H_{17}Cl$ can further be converted to an alcohol $C_8H_{17}OH$ which can be oxidized to an acid $C_7H_{15}COOH$ [3]. This acid is identical with that obtained by the reactions



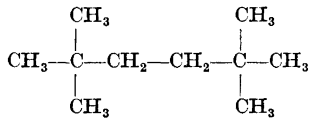
The chloride $C_8H_{17}Cl$ has thus the following structure



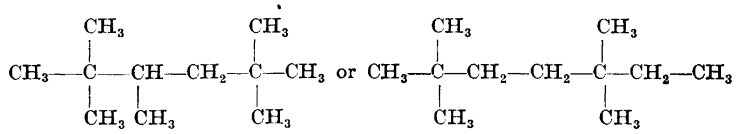
The chloride $C_{11}H_{23}Cl$ reacts with magnesium to form a Grignard reagent which on treatment with water gives a hydrocarbon $C_{11}H_{24}$ which seems not to have been described before. The chloride can be converted to a hydrocarbon $C_{10}H_{22}$ by the following reactions.

$$C_{11}H_{23}Cl \rightarrow C_{11}H_{23}OH \rightarrow C_{10}H_{21}COOH \rightarrow C_{10}H_{21}COOAg \rightarrow C_{10}H_{21}Br \rightarrow C_{10}H_{22}$$

This hydrocarbon has the same infrared absorption and boiling point as

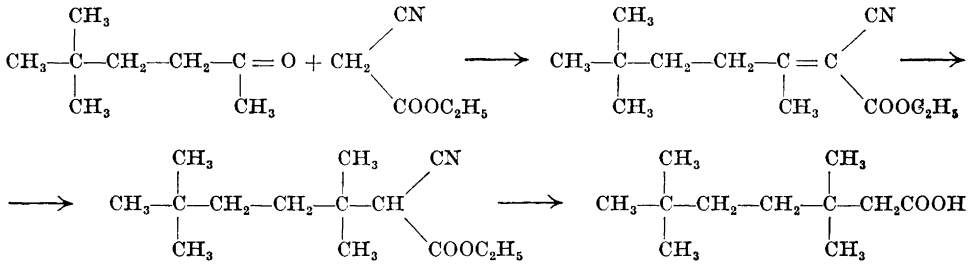


The hydrocarbon $C_{11}H_{24}$ must then be

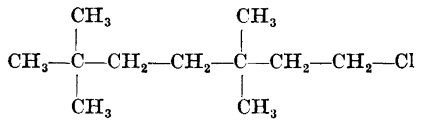


The left hand of these has been described by Moersch and Whitmore [3] and has a boiling point, a refractive index and a density which is different from that of the hydrocarbon $C_{11}H_{24}$ obtained from the chloride $C_{11}H_{23}Cl$. This hydrocarbon must therefore have the right hand of the two structures given.

The acid $C_{10}H_{21}COOH$ obtained from the chloride $C_{11}H_{23}Cl$ is identical with the acid obtained by the reactions:



The chloride $C_{11}H_{23}Cl$ has thus the following structure



The fact that the yield of the main product is high, together with the limited number of byproducts obtained in the reaction suggests that a very simple reaction mechanism is most probable. Such a mechanism which explains all the byproducts obtained can in fact be conceived. It is based on the following considerations:

The reaction conditions are such that a carbonium ion mechanism is probable. The reaction has further a definite period of initiation during which it is very slow, followed by a rapid increase in velocity so that the absorption of ethylene becomes as rapid as the rate of addition and the capacity of the cooling bath permits. This indicates that a chain reaction is operating. This chain is probably

$$(CH_3)_3C{-}Cl + AlCl_3 \xrightarrow{\text{slow}} (CH_3)_3\overset{+}{C} + (AlCl_4)^- \qquad \text{I}$$

$$(CH_3)_3\overset{+}{C} + CH_2 = CH_2 \xrightarrow{\text{rapid}} (CH_3)_3C{-}CH_2{-}\overset{+}{C}H_2 \qquad \text{II}$$

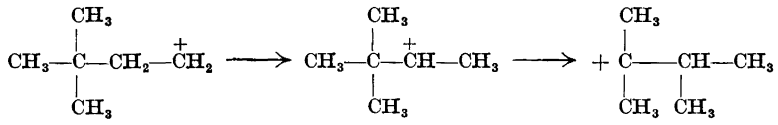
$$(CH_3)_3C{-}CH_2{-}\overset{+}{C}H_2 + Cl{-}C(CH_3)_3 \xrightarrow{\text{rapid}} (CH_3)_3C{-}CH_2{-}CH_2{-}Cl + \overset{+}{C}(CH_3)_3 \qquad \text{III}$$

In step I, which is a slow heterogeneous reaction, the reactive component, a carbonium ion, is formed. This is rapidly used up in step II, and regenerated in step III. The mechanism up to this point is just a slight modification of that generally accepted [4], which however, does not explain the period of initiation.

The very important point in the mechanism given above is the existence of the carbonium ion $(CH_3)_3C\text{-}CH_2\text{-}\overset{+}{C}H_2$. This is a primary carbonium ion which will undergo rearrangements or other reactions unless the step III is extremely rapid.

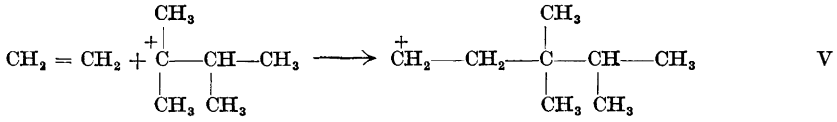
The fact that $(CH_3)_3C\text{-}CH_2\text{-}CH_2\text{-}Cl$ is obtained in a 85.4 % yield indicates that step III is in fact extremely rapid. All byproducts obtained can, however, be explained as results of subsequent reactions of the ion $(CH_3)_3C\text{-}CH_2\text{-}\overset{+}{C}H_2$.

The ion $(CH_3)_3C\text{-}CH_2\text{-}\overset{+}{C}H_2$ may undergo a succession of 1,2-shifts to give a more stable carbonium ion. The most likely succession is:

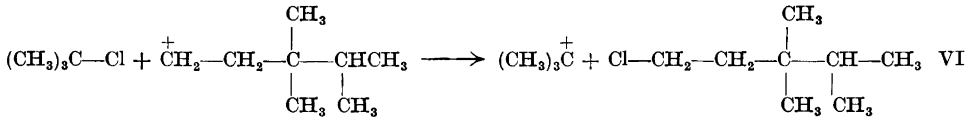


IV

The carbonium ion thus formed can then undergo reactions similar to steps II and III.



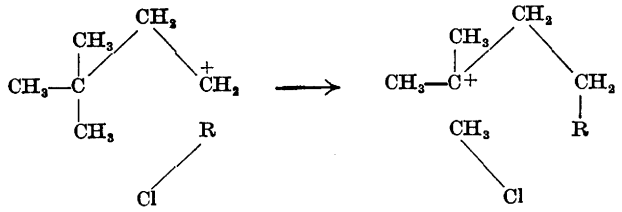
V



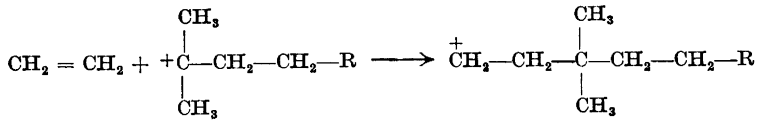
VI

By the reactions IV, V and VI the formation of the main byproduct, the chloride $C_8H_{17}Cl$ is explained.

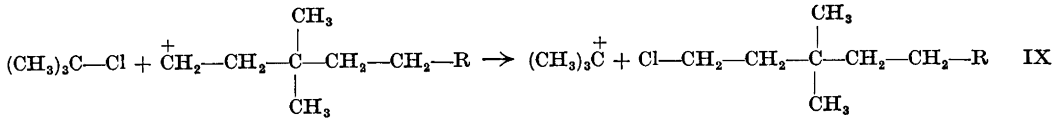
The formation of the chlorides $C_7H_{15}Cl$ and $C_{11}H_{23}Cl$ means that an uneven amount of carbon atoms are added or lost. Only two chlorides containing an uneven number of carbon atoms could be isolated from the reaction mixture. The reaction is further probably ionic and the chlorides $C_7H_{11}Cl$ and $C_{11}H_{23}Cl$ differ by 4 carbon atoms. All this suggests that a methyl group is lost in the $(CH_3)_3C\text{-}CH_2\text{-}CH_2$ ion and replaced by an hydrogen atom or a *tert*-butyl group. Such a mechanism may be



VII



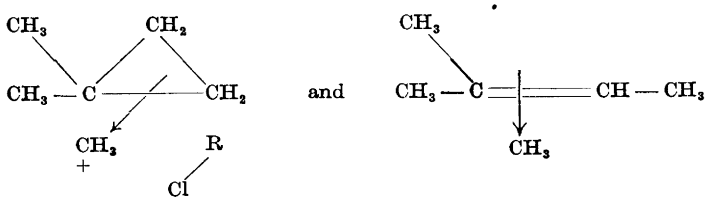
VIII



IX

When R represents H the chloride $C_7H_{15}Cl$ is formed and when it represents $(CH_3)_3C$- the chloride $C_{11}H_{23}Cl$ is formed.

The weakness in the scheme of step VII is that the wrong end of the R-Cl molecule appears to attack the positive charge of the carbonium ion. The point of attack is the significant difference between step III and step VII. The charge distribution in the carbonium ion $(CH_3)_3C\text{-}CH_2\text{-}\overset{+}{C}H_2$ is, however, not so absolutely certain as that given by the conventional formula. Dewar [5] has *e.g.*, explained the tendency of carbonium ion to rearrange itself by the formation of a π-complex between the migrating group and the rest of the molecule. Whether Dewar's explanation of the carbonium ion rearrangement is correct or not, it indicates the presence of a positive charge on the methyl group and some similarity between this type of reaction and reaction VII, which can be seen from the following formulas:



These represent the hypothetical structures in the steps VII and IV, respectively.

A still closer similarity exists between the mechanism given in step VII and the mechanism for a 1,3-rearrangement [6]. Step VII is in fact an exact intermolecular analogue to the intramolecular 1,3-shift, and can be expressed as a 1,3-substitution.

The halides R-Cl which can be expected to be reactive in step VII and which are present in the reaction mixture are HCl and $(CH_3)_3C$-Cl, which exactly correspond to the two halides $C_7H_{15}Cl$ and $C_{11}H_{23}Cl$ which have been found. Whether the chloride $C_6H_{13}Cl$ which is obtained as the main product in the reaction is capable of functioning as R-Cl in step VII is impossible to say. If it can, a chloride $C_{13}H_{27}Cl$ should be present in the high boiling residue. The residue was, however, not examined for this product.

From the formulations given for step VII it follows that CH_3Cl can be expected as a byproduct. No attempts have been made to isolate it from the reaction mixture due to experimental difficulties and the fact that the presence of CH_3Cl can have many explanations and its absence can be explained by subsequent reactions.

EXPERIMENTAL

The reaction between *tert*-butyl chloride and ethylene was performed as described by Brändström [7] but on a larger scale using 12 kg of *tert*-butyl chloride in each run. 11 such runs were collected and distilled through a 2 m packed column.

The conversion of the chloride obtained into the acid with the same number of carbon atoms has been described by Brändström [2].

Preparation of 3,3,4-trimethylpentanoic acid. 115 g of ethyl cyanoacetate, 96.5 g of 3-methylbutanon-2, 15.6 g of ammonium acetate, 50 g of glacial acetic acid, and 170 ml of benzene are mixed and refluxed in a 1-litre flask fitted with a water separator. When no more water is obtained, the mixture is cooled and treated in the usual way [8]. The yield is 134 g or ethyl 2-cyano-3,4-dimethylpent-2-enoate, b. p. 115–120°/8 mm Hg.

A Grignard reagent is prepared from 0.3 moles of magnesium, 0.3 moles of methyl iodide and 120 ml of ether. This reagent is added to a mixture of 50 ml of ether, 0.25 moles of ethyl 2-cyano-3,4-dimethylpent-2-enoate, and 0.5 g of Cu_2Cl_2 during 30 min. The temperature is kept below +5° during this operation by cooling. The reaction mixture is then treated in the usual way [9]. The yield is 84 % of ethyl 2-cyano-3,3,4-trimethylpentanoate, b. p. 118–122°/8 mm.

This ester is saponified by refluxing for 1 h with a solution of 21 g of potassium hydroxide in 60 ml of water. The resulting solution is acidified and extracted with benzene. The benzene extract is distilled at ordinary pressure during which operation decarboxylation occurs. The resulting distillate is fractionated under reduced pressure giving a 67 % yield of 1-cyano-2,2,3-trimethylbutane, b. p. 64–65°/10 mm Hg.

A portion of this cyanide is saponified [9] by refluxing with a solution of potassium hydroxide in ethylene glycol. When the resulting solution is worked up in the usual way, 3,3,4-trimethyl-pentanoic acid, b. p. 112–113°/8 mm Hg is obtained. This acid is in all respects identical with the acid obtained from the chloride $C_8H_{17}Cl$.

Preparation of 3,3,6,6-tetramethylheptanoic acid. 5,5-Dimethylhexanone-2 is prepared from 4,4-dimethylpentanoyl chloride by acylating diethyl malonate and hydrolysing the resulting diethyl acylmalonate [10].

The diethyl 4,4-dimethylpentanoylmalonate, b. p. 145–155°/10 mm Hg, is obtained in a yield of 89 % and the treatment of this ester with propionic acid and sulphuric acid gives 5,5-dimethylhexanone-2, b. p. 145–157°, in a yield of 76 %.

5,5-Dimethylhexanone-2 is condensed with ethyl cyanoacetate by the method given above [8], giving the ethyl 2-cyano-3,6,6-trimethylhept-2-enoate, b. p. 145–160°/10 mm Hg, in a yield of 68 %.

This ester is treated with an ether solution of methylmagnesium bromide by the method given above [9] giving ethyl 2-cyano-3,3,6,6-tetramethylheptanoate, b. p. 145–155°/10 mm Hg, in a yield of 70 %.

This ester is hydrolysed and decarboxylated by the method given above [9], giving 1-cyano-2,2,5,5-tetramethyl hexane, b. p. 205–215°, in a yield of 64 %.

A portion of this cyanide is hydrolysed by the method given above [9], giving 3,3,6,6-tetramethylheptanoic acid. This acid is in all respects identical with the acid obtained from the chloride $C_{11}H_{23}Cl$.

Preparation of 2,3,3-trimethylpentane from the chloride $C_8H_{17}Cl$. A Grignard reagent is prepared from 1 mole of the chloride $C_8H_{17}Cl$, 1 mole of magnesium and 200 ml of ether. This reagent is decomposed by cautious addition of water and hydrochloric acid. The layers are separated and distilled giving a 80 % yield of 2,3,3-trimethylpentane, b. p. 112–115°. The structure was established by a comparison of the infrared spectra of the product with that given for 2,3,3-trimethylpentane.

Preparation of 2,2,5,5-tetramethyl hexane. 50.2 g of the dry powdered silver salt of 3,3,6,6-tetramethylheptanoic acid are placed in a dry 250 ml three-necked flask fitted with a stirrer, a reflux condenser and a dropping funnel. The flask is cooled in ice-water and a solution of 9.0 ml of bromine in 40 ml of dry carbon tetrachloride is added during 20 min. The flask is then heated on a water-bath for 3 h.

The reaction mixture is cooled and filtered. The solid is washed with some ether. The combined filtrates are washed with a sodium bisulphite solution and then with a sodium carbonate solution. The ether and carbon tetrachloride are distilled off and the residue fractionated under reduced pressure.

The yield of 1-bromo-2,2,5,5-tetramethylhexane, b. p. 76–78/10 mm Hg, is 60 %.

A Grignard reagent is prepared from 10 g of the bromide above and magnesium which is then treated with hydrochloric acid giving 2,2,5,5-tetramethylhexane, b. p. 134–138°. The structure of this hydrocarbon was established by comparing the infrared spectra with that given for 2,2,5,5-tetramethylhexane.

Preparation of 2,2,5,5-tetramethylheptane from the chloride $C_{11}H_{23}Cl$. A Grignard reagent, prepared from the chloride $C_{11}H_{23}Cl$ and magnesium, is treated with hydrochloric acid giving a 50 % yield of 2,2,5,5-tetramethylheptane, b. p. 161.5–162.5°, $d_4^{20} = 0.7452$, $n_D^{20} = 1.4186$.

The author is indebted to Lennart Storgärds and Ingvar Eriksson for the large scale preparation of the chlorides, to Wolfgang Kirsten and Mats Carlsson for the gas chromatographic analyses, to Bengt Nygård for the infrared analyses and to Ragnar Lindblom for good help with the manual work.

REFERENCES

1. Schmerling, L. *J. Am. Chem. Soc.* **67** (1945) 1152.
2. Brändström, A. *Acta Chem. Scand.* **13** (1959) 610.
3. Moersch, G. W. and Whitmore, F. C. *J. Am. Chem. Soc.* **71** (1949) 819.
4. Alexander, E. R. *Principles of ionic organic reactions*, New York 1950, p. 146.
5. Dewar, M. J. S. *The electronic theory of organic chemistry*, Oxford 1949, p. 211.
6. Bartlett, P. D. in Gilman, H. *Organic Chemistry* III, New York 1953, p. 69.
7. Brändström, A. *Acta Chem. Scand.* **13** (1959) 611.
8. *Organic Syntheses* **III** (1955) 399.
9. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1954) 561.
10. Walker, H. G. and Hauser, C. R. *J. Am. Chem. Soc.* **68** (1946) 1386.

Received March 3, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 970–975

On the Metabolism of Ursodeoxycholic Acid in the Rat

Bile Acids and Steroids 84

BENGT SAMUELSSON

Department of Physiological Chemistry, University of Lund, Lund and the Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

The metabolism of intraperitoneally administered tritium labelled ursodeoxycholic acid has been studied in bile fistula rats. This acid is partly converted into 3α,6β,7β-trihydroxycholanic acid (Acid I), chenodeoxycholic acid and one unidentified metabolite.

Ursodeoxycholic acid (3α,7β-dihydroxycholanic acid) has been isolated from certain bear species, the coypu [1] and recently also from the rat [2] and man [3]. In the two last-mentioned species it occurs in very small amounts. Studies of the metabolism of chenodeoxycholic acid in the rat have shown that together with the two principal metabolites [4,5], 3α,6β,7β-(Acid I) and 3α,6β,7α- (Acid II) trihydroxycholanic acid, small amounts of 7-ketolithocholic acid [6] and ursodeoxycholic acid [2,6] are formed. Furthermore it has been shown that 7-ketolithocholic acid is mainly transformed into ursodeoxycholic acid and Acid I in the rat liver [2,7]. In order to get more information about the course of the metabolism of chenodeoxycholic acid, tritium labelled ursodeoxycholic acid has been injected intraperitoneally into bile fistula rats, and the labelled products excreted in the bile have been separated by chromatography.

EXPERIMENTAL

Tritium labelled ursodeoxycholic acid. Ursodeoxycholic acid was prepared by the procedure of Samuelsson [6], (M. p. 201–202°). 10 mg of this acid was exposed to tritium gas (2C, 200 mm Hg, 95.5 % pure) for 6 days at room temperature according to the method of Wilzbach [8]. The tritium labelled product was diluted with 25 mg of inactive ursodeoxycholic acid and chromatographed with phase system F[9]. The titration and activity peaks coincided.

Crystallization from ethylacetate/light petroleum yielded 24 mg of ursodeoxycholic acid (M. p. 201–202°), specific activity; 40 × 10^6 c.p.m/mg when counted in a Tracerlab flow counter or approximately 70 μC/mg. A sample of this acid was diluted with inactive ursodeoxycholic acid and recrystallized from aqueous acetic acid, ethylacetate/light petroleum and aqueous methanol. The specific activity remained constant.

3α,6β,7β-Trihydroxycholanic acid (Acid I) was prepared according to the method of Doisy *et al.*[5] (M. p. 226–227°).

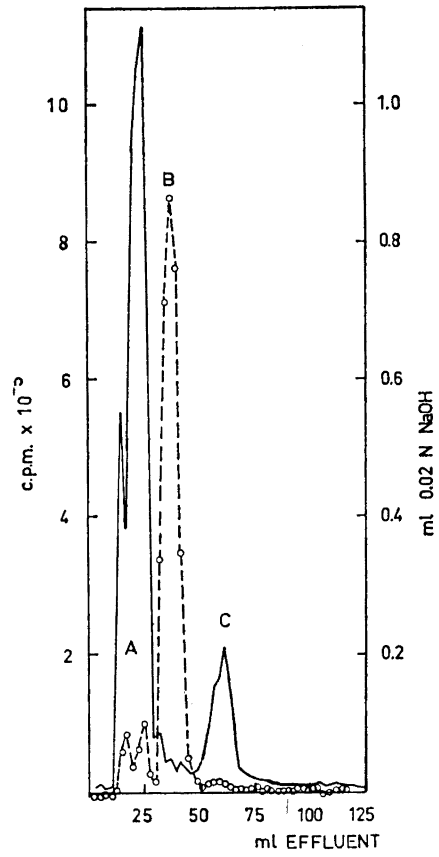


Fig. 1. Chromatographic separation of acids from hydrolyzed bile, excreted during 24 h following intraperitoneal administration of 0.3 mg of tritium labelled ursodeoxycholic acid. Column: 4.5 g hydrophobic Supercel. Phase system: Type F, see page 971. Solid line: Titration values. Broken line: Radioactivity.

Animal experiments. Bile fistulas were made on white male rats of the institute stock weighing about 200 g. The rats had free access to white bread and oats and 0.9 % sodium chloride solution. The bile was collected in ethanol. 0.3 mg of the tritium labelled ursodeoxycholic acid was neutralized with sodium hydroxide and injected intraperitoneally in 0.9 % sodium chloride solution into each of three rats 12 h after the bile fistula had been made. The bile was collected for 24 h. The conjugated bile acids were hydrolyzed in 1.5 N NaOH for 6 h at 120° in a sealed tube. The free bile acids were extracted with ether after acidification with hydrochloric acid.

Chromatographic separations. The free bile acids were chromatographed on hydrophobic Supercel as described by Bergström, Sjövall and Norman [9,10]. The following solvent systems were used.

System	Moving phase	ml	Stationary phase	ml
F[9]	Methanol-water	165:35	Chloroform-heptane	45:5
C[10]	Methanol-water	150:150	Chloroform-*iso*octanol	15:15

4 ml of the stationary phase was supported on 4.5 g of hydrophobic Supercel. Each fraction from the chromatography was titrated with 0.02 N NaOH and an aliquot plated in an "infinitely thin" layer on an aluminium planchet which was counted in a Tracerlab flow counter.

Table 1. Recrystallizations of radioactive bands isolated by chromatography from the bile after administration of tritium labelled ursodeoxycholic acid to bile fistula rats.

Sample	Inactive bile acid added	Crystallizing solvent	Weight mg	*c.p.m.* / mg
Chenodeoxycholic acid band. Fig. 1.	Chenodeoxycholic acid		80	2 100
		Ethylacetate-light petroleum	62	1 980
		Acetic acid-water	43	1 920
		Ethanol-water	28	2 020
		Ethylacetate-light petroleum	17	1 930
3*α*, 6*β*, 7*β*-Trihydroxycholanic acid (Acid I) band. (Fig. 2)	3*α*, 6*β*, 7*β*-Trihydroxycholanic acid		60	9 600
		Ethylacetate	53	9 850
		Acetone-water	42	10 050
		Acetone-light petroleum	34	9 900
		Methanol-water	21	9 750

RESULTS

Tritium labelled ursodeoxycholic acid was administered intraperitoneally into three bile fistula rats. 70—82 % of the administered ^3H was recovered in the bile collected during 24 h following the injection. The saponified bile from each animal was chromatographed with phase system F. With this system ursodeoxycholic acid (peak at 40 ml effluent) chenodeoxycholic acid (60 ml) and more hydrophobic bile acids separate from cholic acid and other more polar bile acids which appear with the front. In Fig. 1 is shown a chromatogram of the hydrolyzed bile from one rat (R I). Most of the activity is eluted at the place of ursodeoxycholic acid (B), but about 10 % of the activity appears almost with the front as an incompletely separated double peak (A). A small but significant amount of the activity was found to coincide with the titration peak, caused by chenodeoxycholic acid present in the bile (C). Less than one per cent of the activity remained in the stationary phase. The material in the first band (A) was rechromatographed with phase system C, suitable for the separation of trihydroxycholanic acids (Fig. 2). The activity then appears as two separate peaks, one at 40—50 ml and one at the place of Acid I (120—160 ml), immediately after the titration peak of inactive cholic acid.

Identification of the labelled products separated by chromatography. Unchanged ursodeoxycholic acid (Fig. 1, peak B) was identified by recrystallizations from four different solvent systems after rechromatography with unlabelled ursodeoxycholic acid with phase system F. The labelled material eluted at the place of chenodeoxycholic acid was rechromatographed with phase system F and identified by isotope dilution (Table 1). The radioactive material eluted immediately after cholic acid (Fig. 2) was diluted with inactive Acid I and identified by isotope dilution after chromatography with phase system C.

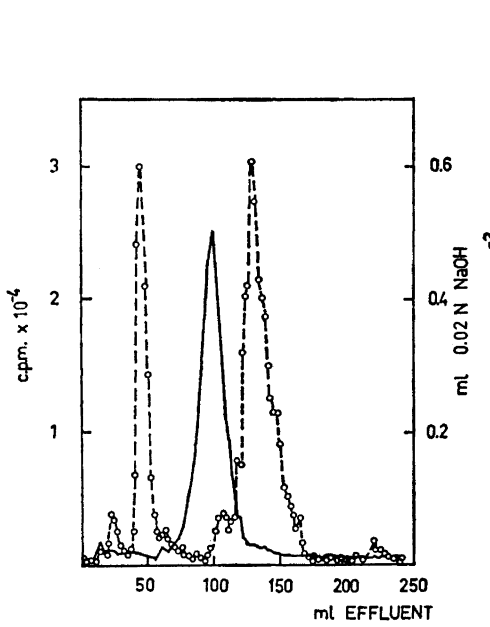


Fig. 2. Chromatographic separation of peak A in the chromatogram shown in Fig. 1. Column: 4.5 g hydrophobic Supercel. Phase system: Type C, see page 971. Solid line: Titration values. Broken line: Radioactivity.

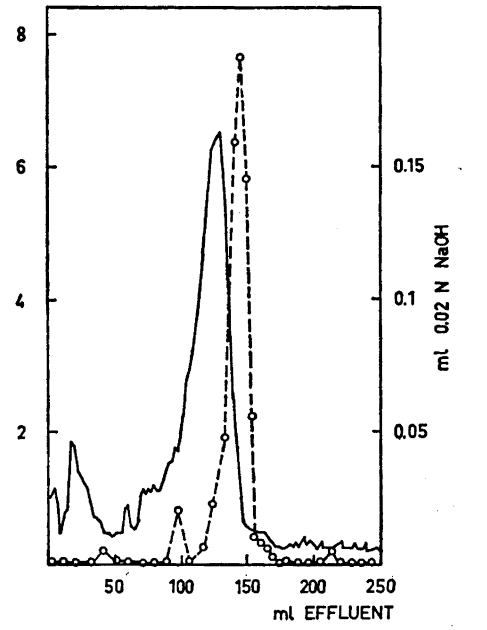


Fig. 3. Chromatographic separation of the reaction product after chromic acid oxidation of Metabolite III and 25 mg of inactive cholic acid. Column: 4.5 g hydrophobic Supercel. Phase system: Type C, see page 971. Solid line: Titration values. Broken line: Radioactivity.

The appearence of the radioactive band at about 50 ml (metabolite III) with phase system C (Fig. 2) is characteristic of a trihydroxycholanic acid or a dihydroxymonoketocholanic acid. As it was chromatographically identical with another 7β-hydroxylated bile acid (3α,7β,12α-trihydroxycholanic acid) with this phase system [6], it was, together with inactive cholic acid, oxidized with chromic acid under conditions [11] known to give dehydrocholic acid from 3,7,12-trihydroxycholanic acids. The chromatography of the reaction product with phase system C is shown in Fig. 3. The radioactivity is eluted immediately after the titration peak of inactive dehydrocholic acid. This result also excludes that metabolite III is a 3,6,7-trihydroxycholanic acid as chromic acid oxidation of these acids under the conditions used results in the formation of the more hydrophilic 3-keto-6,7-secocholanic acid-6,7-dioic acid [12,13], which appears with the front with phase system C. No further attempts were made to identify metabolite III. The percentage composition of the labelled products excreted in the bile is given in Table 2.

Table 2. Percentage composition of the labelled products excreted in the bile during 24 h following intraperitoneal injection of tritium labelled ursodeoxycholic acid into three rats with bile fistulas.

Compound	Per cent		
	R I	R II	R III
3*α*, 6*β*, 7*β*-Trihydroxycholanic acid	7.4	3.9	6.1
Chenodeoxycholic acid	1.5	0.6	1.4
Metabolite III	2.7	1.9	3.1
Ursodeoxycholic acid	79.7	91.5	85.3
Total	91.3	97.9	95.9

DISCUSSION

The results show that ursodeoxycholic acid is only slightly transformed in the rat liver. Its principal metabolite consists of 3*α*,6*β*,7*β*-trihydroxycholanic acid (Acid I), *i.e.* a 6*β*-hydroxyl group is introduced.

However, ursodeoxycholic acid seems only to be a minor intermediate in the formation of Acid I from chenodeoxycholic acid, as it is formed in very small amounts from chenodeoxycholic acid [2,6]. The demonstration of the direct transformation of Acid II into Acid I also points in this direction [6]. Another more polar bile acid was also isolated, but the structure of this metabolite has not been determined. A metabolite with the same elution rate as this acid was also found [7] in the bile after intraperitoneal administration of 7-ketolithocholic acid-24-^{14}C[7].

A small amount (0.6—1.5 %) of the recovered avtivity was identified as chenodeoxycholic acid. This acid is probably formed by dehydrogenation of ursodeoxycholic acid to 7-ketolithocholic acid which is then reduced to chenodeoxycholic acid.

Ursodeoxycholic acid present in normal rat bile may probably be formed by two routes. The direct formation of ursodeoxycholic acid from chenodeoxycholic acid in the liver has been demonstrated [2,6], and furthermore *in vitro* experiments have shown that chenodeoxycholic acid is dehydrogenated by different strains of *E. coli* to 7-ketolithocholic acid [14], which is readily reduced in the liver to ursodeoxycholic acid.

The technical assistence of Miss H. Rytz is gratefully acknowledged. This work is part of an investigation supported by the *Medical Faculty of the University of Lund* and by a research grant (H 2842) to Prof. S. Bergström from the *National Institutes of Health, United States Public Health Service*, Bethesda, Maryland.

REFERENCES

1. Haslewood, G. A. D. *Physiol. Rev.* **35** (1955) 178.
2. Mahowald, T. A., Yin, M. W., Matschiner, J. T., Hsia, S. L., Doisy, E. A., Jr., Elliott, W. H. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 581.
3. Sjövall, J. *Acta Chem. Scand.* **13** (1959) 711.
4. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **226** (1957) 667.

5. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 573.
6. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 976.
7. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 236.
8. Wilzbach, K. E. *J. Am. Chem. Soc.* **79** (1957) 1013.
9. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
10. Bergström, S. and Norman, A. *Proc. Soc. Exptl. Biol. Med.* **83** (1953) 71.
11. Hammarsten, O. *Ber.* **14** (1881) 71.
12. Ziegler, P. *Can. J. Chem.* **34** (1956) 523.
13. Haslewood, G. A. D. *Biochem. J.* **62** (1956) 637.
14. Bergman, S. and Norman, A. *To be published.*

Received March 21, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 976–983

On the Metabolism of Chenodeoxycholic Acid in the Rat

Bile Acids and Steroids 85

BENGT SAMUELSSON

Department of Physiological Chemistry, University of Lund, Lund and the Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

Chenodeoxycholic acid-7β-^{3}H and randomly tritium labelled 3α, 6β, 7α-(Acid II) and 3α, 6β, 7β- (Acid I) trihydroxycholanic acids have been prepared. Acid II is converted into Acid I when injected into a bile fistula rat, whereas the reverse reaction does not occur. The inversion of the 7α-hydroxyl group has been studied with 7β-tritio, 24-^{14}C labelled chenodeoxycholic acid. The tritium label is completely lost in this reaction. The mechanism for the inversion and the course of the metabolism of chenodeoxycholic acid are discussed.

Chenodeoxycholic acid constitutes a minor component of the bile acids normally present in rat bile[1,2]. In 1954 Bergström and Sjövall showed that chenodeoxycholic acid is transformed in the rat liver into two more polar acids which are not identical with cholic acid [2]. The same metabolites had been observed earlier by Bergström and Norman after administration of cholesterol-4-^{14}C to bile fistula rats [3]. These metabolites have recently been identified by Doisy *et al.* as 3α,6β-7β- (Acid I) and 3α,6β,7α-trihydroxycholanic acid (Acid II) [4,5].

The aim of the present investigation was to study the mechanism of the conversion of chenodeoxycholic acid into Acid I and II with chenodeoxycholic acid 7β-^{3}H, 24-^{14}C and randomly tritium labelled Acid I and Acid II.

EXPERIMENTAL

Syntheses

Chenodeoxycholic acid-7-^{3}H. 60 mg of 7-ketolithocholic acid (M. p. 200–202°) prepared through oxidation of methyl 3α-cathyloxy-7α-hydroxycholanate with sodium dichromate,[6] were dissolved in 10 ml of "diglyme" (Diethyleneglycoldimethylether, Fluka A. G., Buchs/S.G., Switzerland, refluxed over calciumhydride and distilled from lithium-aluminium hydride) and 40 mg of sodium borohydride were added. The latter compound had been labelled through exposure to an athmosphere of tritiumgas [7] (2C, 95 % pure, 200 mm Hg) for ten days at room temperature. After 12 h at room temperature the reaction mixture was diluted with water, acidified with 2 N hydrochloric acid and extrac-

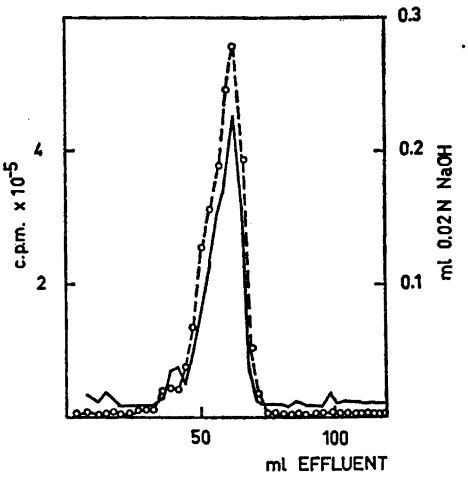


Fig. 1. Chromatographic separation of the reaction product after NaB^3H_4 reduction of 7-ketolithocholic acid. Column: 4.5 mg hydrophobic Supercel. Phase system: Type F. Solid lines: Titration values. Broken line: Radioactivity.

ted with ether. After evaporation *in vacuo* the residue was chromatographed with phase system F[10]. The single peak (Fig. 1) appeared at the place of chenodeoxycholic acid, which with this system separates clearly from the 7β-isomer, ursodeoxycholic acid. The radioactive material was crystallized from ethylacetate/light petroleum yielding 47 mg of chenodeoxycholic acid-7β-^{3}H, m. p. 141—143°. Mixed melting point with authentic chenodeoxycholic acid showed no depression. Specific activity: ~0.5 μC/mg (1 μC ^{3}H is equivalent to approximately 6×10^5 c.p.m. when counted in an infinetely thin layer in a Tracerlab gas flow counter). Dilution of a sample of this acid with inactive chenodeoxycholic acid and extensive recrystallizations gave no depression of the specific activity. 25 mg of this diluted acid was oxidized with chromic acid in aqueous acetic acid to 3,7-diketocholanic acid (m. p. 152—154°). The diketoacid contained 2.5 % of the amount of tritium label present in the acid before the oxidation.

A stock solution of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was made by dissolving 10 mg of chenodeoxycholic acid-7β-^{3}H (0.5 μC/mg) and 0.25 mg of chenodeoxycholic acid-24-^{14}C (10 μC/mg) in 10 ml of acetone.

Acid I and Acid II were prepared by the methods of Doisy *et al.*[4,5] They were labelled with tritium by exposing 5 mg of the acid to tritium gas (2 C, 200 mm Hg, 95 % pure) for 6 days at room temperature [7]. The tritium labelled products were diluted with 20 mg of the corresponding inactive acid and chromatographed with phase system C[3]. Acid I was crystallized from ethylacetate yielding 17 mg, m. p. 226—227°. Specific activity ~50 μC/mg.

Acid II was crystallized from acetone/light petroleum. Yield 13 mg, m. p. 201—202°. Specific activity: ~35 μC/mg.

Ursodeoxycholic acid was prepared according to Samuelsson [8]. M. p. 201—202°.

Chenodeoxycholic acid-24-^{14}C was prepared according to the method of Bergström *et al.*[9] Specific activity: 10 μC/mg.

Animal experiments

The labelled bile acids were injected intraperitoneally as the sodium salt in 0.9 % sodium chloride solution into 200—250 g white male rats of the institute stock. The bile duct was cannulated 12 h before the administration. The rats had free access to white bread and oats and 0.9 % sodium chloride during the experimental period. The bile was collected daily in ethanol and hydrolyzed with 1.5 N NaOH in a sealed tube for 6 h at 120°. The free acids were extracted from the acidified solution with ether.

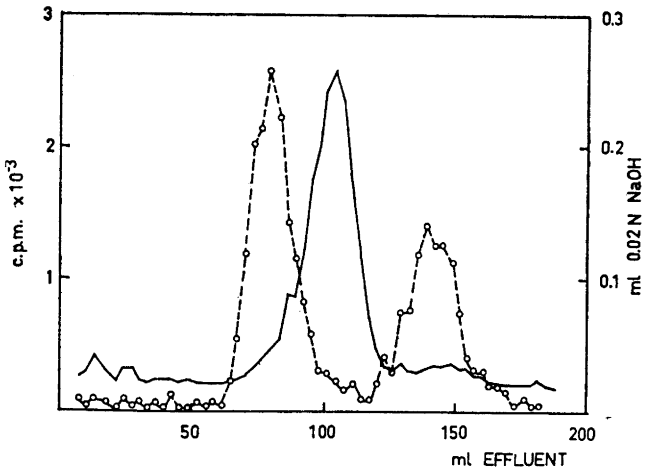


Fig. 2. Chromatographic separation of acids from hydrolyzed bile, excreted during 24 hours following intraperitoneal administration of 2 mg of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C. Column: 4.5 g hydrophobic Supercel. Phase system: Type C. Solid line: Titration. Broken line: Radioactivity.

Chromatographic separations

The free acids were chromatographed on hydrophobic Supercel as described by Bergström, Norman and Sjövall[3,10]. The following solvent systems were used.

System	Moving phase	ml	Stationary phase	ml
F[10]	Methanol: water	165:135	Chloroform: heptane	45:5
C[3]	Methanol: water	150:150	Chloroform: isooctanol	15:15

4 ml of the stationary phase were used per 4.5 g of hydrophobic Supercel. All the chromatograms were run at a constant temperature of +23°.

Isotope determinations

Suitable aliquots of the titrated fractions of the chromatographies were plated on aluminium planchets and counted in a Tracerlab gas flow counter, when tritium labelled compounds were chromatographed.

The radioactivity of the products obtained after injection of chenodeoxycholic acid-7β-^{3}H,24-^{14}C was determined in a Tracerlab end window counter, in which only the ^{14}C activity is recorded. In this way it was possible to get a quantitative picture in spite of the fact that some of the metabolites had lost their tritium label. The ^{3}H and ^{14}C activity in the administered and isolated acids were determined by gas phase counting after combustion to $^{14}CO_2$ and ^3H_2O and conversion of the latter to tritio butane by the methods of Glascock [11].

RESULTS

Metabolism of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C. 2 mg of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was injected into each of two bile fistula rats. The bile excreted during the first 24 h following the injection was hydrolyzed

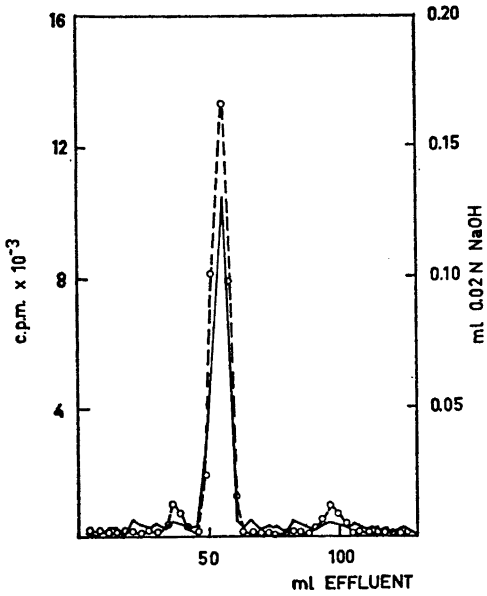


Fig. 3. Chromatographic separation of acids remaining in the stationary phase of the chromatogram shown in Figure 2. Column: 4.5 g hydrophobic Supercel. Phase system Type F. Solid line: Titration values. Broken line: Radioactivity.

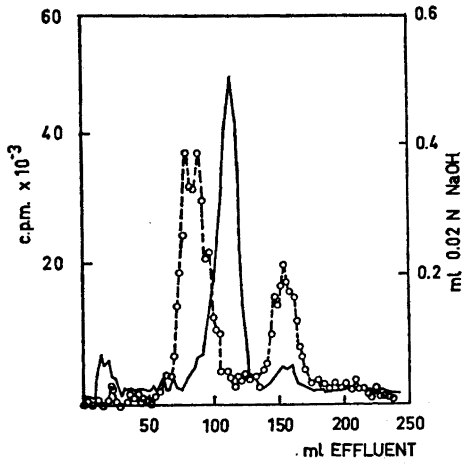


Fig. 4. Chromatographic separation of acids from hydrolyzed bile, excreted during 24 hours following intraperitoneal administration of 0.2 mg of Acid II. Column: 4.5 g hydrophobic Supercel. Phase system: Type C. Solid line: Titration values. Broken line: Radioactivity.

and chromatographed with phase system C (Fig. 2). The material remaining in the stationary phase was separated by chromatography with phase system F. The chromatogram is shown in Fig. 3.

As found earlier [2] the two main metabolites are eluted with phase system C (Fig. 2). One of them (Acid II) appears before the inactive cholic acid (100 ml) and the other (Acid I) after this acid. Acid I and II were diluted, with the corresponding inactive acid to suitable specific activity for the $^{3}H/^{14}C$ determinations and crystallized from ethylacetate and acetone/light petroleum respectively.

In the chromatogram shown in Fig. 3, most of the activity is eluted at the place of chenodeoxycholic acid, but two small radioactive bands also occur, one before (ursodeoxycholic acid) and one after (7-ketolithocholic acid) chenodeoxycholic acid. The radioactive material in these two peaks were rechromatographed with the corresponding inactive acid and were finally identified by isotope dilution.

The percentage composition of the recovered ^{14}C is shown in Table 1. The ^{3}H and ^{14}C content was determined in the administered doubly labelled chenodeoxycholic acid and in the isolated metabolites (Table 2).

Table 1. Percentage composition of the ^{14}C recovered in the bile during 24 h following intraperitoneal injection of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C.

Compound	Per cent	
	Rat I	Rat II
Chenodeoxycholic acid	48.3	53.1
7-Ketolithocholic acid	5.3	4.9
Ursodeoxycholic acid	4.0	3.7
Acid II (3α, 6β, 7α)	17.3	15.5
Acid I (3α, 6β, 7β)	24.0	21.0
Total	98.9	98.2

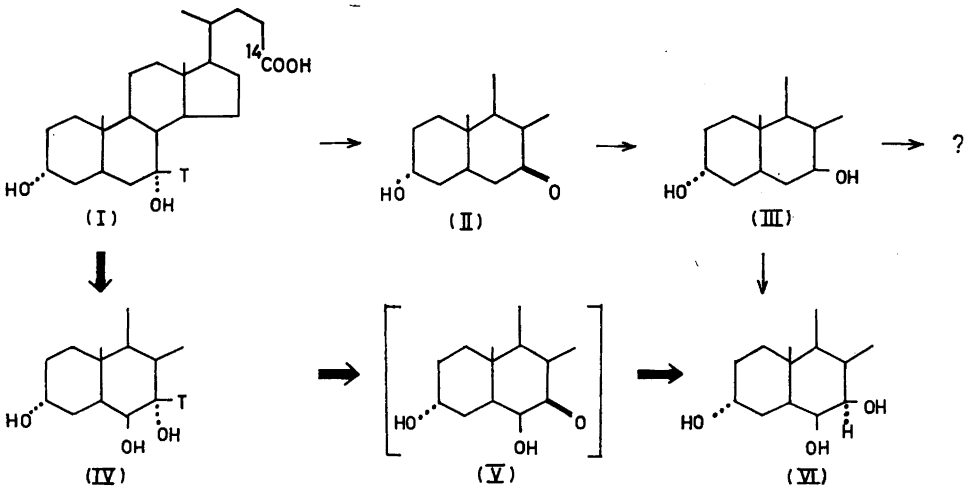
7-Ketolithocholic acid, ursodeoxycholic acid and Acid I only contained traces of ^{3}H whereas this isotope was retained in Acid II.

Metabolism of ^{3}H-labelled Acid II. 0.2 mg of randomly tritium labelled Acid II was injected intraperitoneally into a bile fistula rat and the bile excreted during 24 h following the injection was chromatographed with phase system C after hydrolysis. (Fig. 4). About 35 % of the chromatographed activity was eluted as Acid I, which was identified by isotope dilution.

Metabolism of ^{3}H labelled Acid I. Chromatography of the hydrolyzed bile excreted during 24 h following administration of 0.2 mg of tritium labelled Acid I to a bile fistula rat showed only unchanged Acid I.

Table 2.

Compound	^{3}H c.p.m./mg	^{14}C c.p.m./mg	$^{3}H/^{14}C$	Per cent ^{3}H retained
Administered chenodeoxycholic acid-7β-^{3}H, 24-^{14}C	253	356	0.71	
7-Ketolithocholic acid Rat I and II	3	319	0.01	1
Ursodeoxycholic acid Rat I and II	8	382	0.02	3
Acid II (3α, 6β, 7α)				
Rat I	154	237	0.65	92
Rat II	202	298	0.68	96
Acid I (3α, 6β, 7β)				
Rat I	3	244	0.01	2
Rat II	4	242	0.02	2
Acid I (after administration of acid II-7β-^{3}H, 24-^{14}C)	5	209	0.02	3



Metabolism of biosynthetically formed doubly labelled Acid II. Acid II, which had been isolated from the bile after administration of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was injected into a bile fistula rat and the excreted bile separated with phase system C after hydrolysis. About 8 % of the injected Acid II had been transformed into Acid I. Determination of ^{3}H and ^{14}C in Acid I showed that the tritium label is completely lost during this transformation (Table 2).

DISCUSSION

Based on results obtained in this and previous investigations [2,4,5,12] the course of the metabolism of chenodeoxycholic acid in the rat liver may be formulated as in scheme 1. The main pathway leads from chenodeoxycholic acid to Acid I (VI) *via* Acid II (IV). Acid II is formed from chenodeoxycholic acid in good yield through direct 6β-hydroxylation, and as was shown with tritium labelled Acid II this acid is readily transformed into Acid I when a tracerdose is injected. The proportion between Acid II and Acid I after injection of chenodeoxycholic acid is about 1:0.7 and after injection of Acid II 1:0.6.

The experiments with 7β-tritiochenodeoxycholic acid showed that Acid I is formed with loss of the tritium label and it was further established with biosynthetic 7β-tritio Acid II, that the tritium loss occurs in the reaction Acid II $\rightarrow$ Acid I, *i.e.* the inversion of the hydroxyl at C7 from α to β position.

Concerning the mechanism of the inversion it is most likely that the 7α-ol is dehydrogenated to a ketone, which is reduced stereospecifically to the equatorial 7β-ol in Acid I. The steroid alcohol dehydrogenases show a very high degree of stereospecificity for the substrate [13], and there are presumably two separate enzymes which carry out the inversion. The following equilibrium might then be obtained:

$$7\alpha\text{-ol} \rightleftharpoons \text{7-ketone} \rightleftharpoons 7\beta\text{-ol}$$

The presence of both 7α- and 7β-hydroxydehydrogenases active in the bile acid series has been demonstrated in the rat liver through metabolic studies of 7-ketohydroxycholanic acids. As mentioned above 7-ketolithocholic acid is formed in small amounts from chenodeoxycholic acid and the former acid is mainly reduced to ursodeoxycholic acid in the liver [12,14]. About equal parts of cholic acid (7α-ol) and 3α,7β,12α-trihydroxycholanic acid are obtained when 7-ketodeoxycholic acid is administered [15]. A mechanism consisting of the formation of a double bond through dehydration of the 7α-ol and a rehydration to the opposite steric position can be excluded by the isotope experiments. The above-mentioned oxidation-reduction reactions which were suggested for the inversion are usually linked to DPN or TPN[13]. The requirement of DPN for the lactate racemase described by Kaufman *et al.*[16] has recently also been found for the β-hydroxybuturyl CoA racemase by Wakil[17]. The latter author also shows that the racemization is effected by two different dehydrogenases (D(—) and L(+) β-hydroxy buturyl-CoA-dehydrogenase) with acetoacetyl CoA as an intermediate. A problem similar to the transformation of Acid II into Acid I is the epimerization of the hydroxyl at C4 in the conversion of uridinediphosphogalactose to uridinediphosphoglucose. The mechanism of this reaction has been studied extensively with ^3H_2O and $H_2{}^{18}O$ in the medium and also with tritium labelled DPN or DPNH [18]. Of the originally proposed mechanisms essentially only one involving a dehydrogenation remains. This reaction, however, is considered to be carried out by one enzyme bearing two specific groups, the epimerization consisting of a shift of a hemiacetal between the 4-carbonyl group and these groups under the influence of a certain catalyst [18].

A minor pathway (I→II→III→VI) for the metabolism of chenodeoxycholic acid consists of the formation of 7-ketolithocholic acid, reduction of the 7-carbonyl group to ursodeoxycholic acid and a 6β-hydroxylation of this acid to Acid I.[19] Another metabolite of ursodeoxycholic acid was also isolated but the structure of this acid has not been determined.[19] It is probable that a direct 6β-hydroxylation of 7-ketolithocholic acid also can take place, as the proportion between ursodeoxycholic acid, Acid I and the unidentified metabolite after administration of essentially the same amount of a tracerdose of 7-ketolithocholic acid and ursodeoxycholic acid, respectively, are 1:0.75:0.05 and 1:0.07:0.03 [12].

The technical assistence of Miss I. Lindell, Miss H. Rytz and Mr. S. Jönsson is gratefully acknowledged. This work is part of an investigation supported by *Karolinska Institutets Reservationsanslag* and by a research grant (H 2842) to Prof. S. Bergström from the *National Institutes of Health, United States Public Health Service*, Bethesda, Maryland.

REFERENCES

1. Bergström, S. and Borgström, B. *Ann. Rev. Biochem.* **25** (1956) 177.
2. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **8** (1954) 611.
3. Bergström, S. and Norman, A. *Proc. Soc. Exptl. Med.* **83** (1953) 71.
4. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **226** (1957) 667.
5. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A. Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 573.

6. Fieser, L. F., Herz, J. E., Klohs, M. W., Romero, M. A. and Utne, T. *J. Am. Chem. Soc.* **74** (1952) 3309.
7. Wilzbach, K. E. *J. Am. Chem. Soc.* **79** (1957) 1013.
8. Samuelsson, B. *To be published.*
9. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
10. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
11. Glascock, R. *Isotopic Gas Analysis for Biochemists*, Academic Press N.Y. 1954.
12. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 236.
13. Talalay, P. *Physiol. Rev.* **37** (1957) 362.
14. Mahowald, T. A., Mao Wu Yin, Matschiner, J. T., Hsia, S. L., Doisy, E. A., Jr., Elliott, W. H. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 581.
15. Norman, A and Sjövall, J. *To be published.*
16. Kaufman, S., Korkes, S. and del Campillo, A. *J. Biol. Chem.* **192** (1951) 301.
17. Wakil, S. J. *Biochim. et Biophys. Acta* **18** (1955) 314.
18. Kalckar, H., *Advances in Enzymol.* **20** (1958) 111.
19. Samuelson, B. *Acta Chem. Scand.* **13** (1959) 970.

Received March 21, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 984–988

The Thermal Expansion of the Crystalline B- and C-Forms of Stearic Acid

GUNNAR DEGERMAN and ERIK von SYDOW

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The changes with temperature of the cell dimensions and the density of the B- and C-forms of stearic acid have been determined. The influence on the c-axis is very slight. The effect on the subcell axes is similar to the disturbing effect of branching groups, *i. e.* the a_s-axis, ~ 4.95 Å, is almost unchanged but the b_s-axis, ~ 7.40 Å, is appreciably increased.

In connection with studies on polymorphism of normal fatty acids and transitions between the different polymorphs [1-3] it was found necessary to determine the variation of the cell dimensions of two polymorphs of an acid for temperatures between 0°C and the transition and melting points, respectively. The B- and C-forms of stearic acid were chosen. The structure of the B-form of stearic acid has been described by von Sydow [4]. The C-form of stearic acid has a structure analogous with the C-form of lauric acid, which has been described by Vand *et al.*[5] The cell dimensions of the C-forms of the whole homologous series have been determined by Abrahamsson and von Sydow [6] at one temperature (+ 23°C). Some of these data are given in Table 1.

Table 1. Dimensions of the B- and C-form of stearic acid and the orthorhombic subcell (O ⊥) at room temperature (+23°C) [3,4,6].

	a Å	b Å	β	Space group	a_s, $a \sin \beta$ and b, respectively	b_s, b and $a \sin \beta$, respectively
O ⊥	4.95	7.40	90°	$P\ b\ n\ m$	4.95	7.40
B-form	5.59	7.40	117°22′	$P2_1/a$	4.96	7.40
C-form	9.36	4.96	128°14′	$P2_1/a$	4.96	7.35

These crystal forms have both the same chain packing: the common orthorhombic type [3] with every second chain plane perpendicular to the others ($O_{\perp}$). The main difference between the structures is that in the case of the B-form the structure is tilted over the long subcell axis (7.40 Å) giving an increase in the short axis (4.95 Å to 5.59 Å) while in the case of the C-form the structure is tilted over the short subcell axis (4.95 Å) increasing the long axis from 7.40 Å to 9.36 Å (Table 1). These tilts are necessary to give space for the carboxylic groups, which are held together two and two by hydrogen bonds.

The B-form, which is obtained by crystallization from various solvents [2], is the stable form at room temperature but it is irreversibly transformed into the C-form by raising the temperature above + 46°C[1]. The C-form is stable up to the melting point + 69°.7C [1]. Solid phase in equilibrium with melt is always of the C-form.

By X-ray diffraction spectrometry the variations with temperature of the cell-dimensions and the densities of the B- and C-forms of stearic acid have been determined by measuring the variations with temperature of the diffraction angles of four reflexions of each crystal form.

TECHNIQUE

The X-ray spectrometer equipped with a scintillation counter and the heating arrangement used are constructed by Prof. G. Hägg. A shallow teflon beaker was used as specimen holder for the finely chopped crystal specimens.

The determination of the diffraction angle for a reflexion at a particular temperature was carried out in the following manner. After stabilising the specimen at the correct temperature for half an hour, the diffraction peak was located within ± 5′. The peak was scanned back and forth 4 times and exactly for each ½′ an artificial extra pulse was given to the rate meter. In this way the peak could be located within 0.1′, the standard deviation for the eight measurements generally being 0.07—0.09′. During these manipulations the temperature was recorded five times. The mean temperature is correct within ± 0.4°C.

Owing to serious orientation effects and comparatively low intensities, the best position of the specimen holder for registration of one particular reflexion

Table 2. The cell dimensions and the calculated density of the B-form of stearic acid at temperatures between 0°C and its transition point.

Temp. °C	a Å	b Å	c Å	β	ϱ_{calc} gcm $^{-3}$
0	5.591	7.368	49.439	117° 15′.2	1.0444
10	5.592	7.381	49.412	117° 18′.8	1.0432
20	5.594	7.400	49.388	117° 21′.9	1.0414
30	5.595	7.420	49.365	117° 24′.5	1.0391
40	5.595	7.443	49.343	117° 25′.3	1.0365
45	5.595	7.457	49.337	117° 26′.4	1.0349

was found empirically. This, of course, implies that no absolutely correct diffraction angles were obtained, but the changes with temperature were very accurately determined. The curves Θ *vs.* temperature for every reflexion were therefore put on an absolute scale by comparison with the data from the previous structure investigations [4,6], which were of course, also used for indexing.

The following reflexions were used:

B-form (005), (020), (201) and (2, 1, $\bar{1}9$)
C-form (005), (110), (200) and (2, 1, $\bar{2}0$)

The cell dimensions and the density were calculated for each 10°C in both cases.

It was not possible to measure (2, 1, $\bar{2}0$) at temperatures over 50°C with any accuracy, owing to the lowering of intensity and broadening of the peak. Thus some of the data above this temperature are obtained by extrapolation.

RESULTS AND DISCUSSION

The cell dimensions and densities of the crystalline B- and C-forms of stearic acid at different temperatures are given in Tables 2 and 3. The accuracy is the same as in the earlier papers [4,6], but as regards changes in dimensions and densities, which are given in Figs. 1 and 2, the accuracy is much higher (< 0.001 Å for a- and b-axes, < 0.005 Å for c-axes, < 0.1 for β and < 0.0002 gcm^{-3} for ϱ_{calc}).

As can be seen the changes in the c-dimensions are comparatively very small. As the molecules are very nearly parallel to the c-axis [3-5], this means that the length of the molecule of stearic acid is almost independent of temperature in the interval investigated. Of the other axes, the shortest in both cases are hardly at all influenced by temperature changes, but the medium ones are very sensitive. As the changes in β are very small, these conclusions are directly transferable to the subcell-dimensions, *i.e.* the shortest subcell

Table 3. The cell dimensions and the calculated density of the C-form of stearic acid at temperatures between 0°C and the melting point.

Temp. °C	a Å	b Å	c Å	β	ϱ calc gcm^{-3}
0	9.268	4.961	50.747	128° 7′.4	1.0293
10	9.312	4.960	50.762	128° 13′.8	1.0259
20	9.351	4.958	50.750	128° 16′.3	1.0226
30	9.399	4.955	50.761	128° 17′.1	1.0183
40	9.452	4.950	50.753	128° 16′.2	1.0135
50	9.508	4.942	50.721	128° 12′.1	1.0087
60	9.562 *	4.935	50.69 *	128° 7′ *	1.0040
70	9.617 *	4.923	50.66 *	128° 0′ *	0.9995

* extrapolated

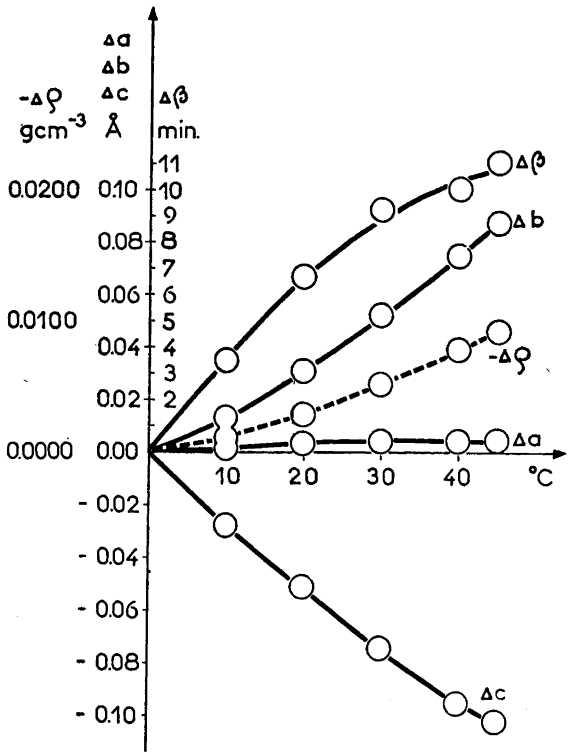


Fig. 1. The changes of the cell dimensions and the calculated density of the B-form of stearic acid.

dimension (~ 4.95 Å) is almost unchanged but the largest one (~ 7.40 Å) If very variable with temperature. This is also observed for *n*-hydrocarbons [7]. is the packing of the hydrocarbon chains is disturbed in other ways, *e.g.* by branching groups, the effect on the subcell dimensions is the same. In the case of 14-heptacosanol [8] the dimensions are 4.95 and 7.8 Å, respectively, and for *trans*-DL-9,10-methyleneoctadecanoic acid [9] they are 4.98 and 8.12 Å. As that of branching [9] the effect of temperature is certainly a trend to achieve a hexagonal closepacking of cylindrical rods (*e.g.* rotating hydrocarbon chains) which happens when the b_s-axis is increased from 7.40 Å to 8.6 Å.

The decrease in the density of the C-form is larger than for the B-form, which indicates that the C-structure is more flexible than the B-structure. It might have been expected that the density curve of the B-form would intersect the curve of the C-form at the transition temperature (B → C, 46°C) but this is not the case (Tables 2 and 3). This calls for caution in discussing molecular interactions and its connection with density. It is obvious from this investigation that not only chain packing and end group packing [3], but also the arrangement of the carboxylic groups are important factors in this connection.

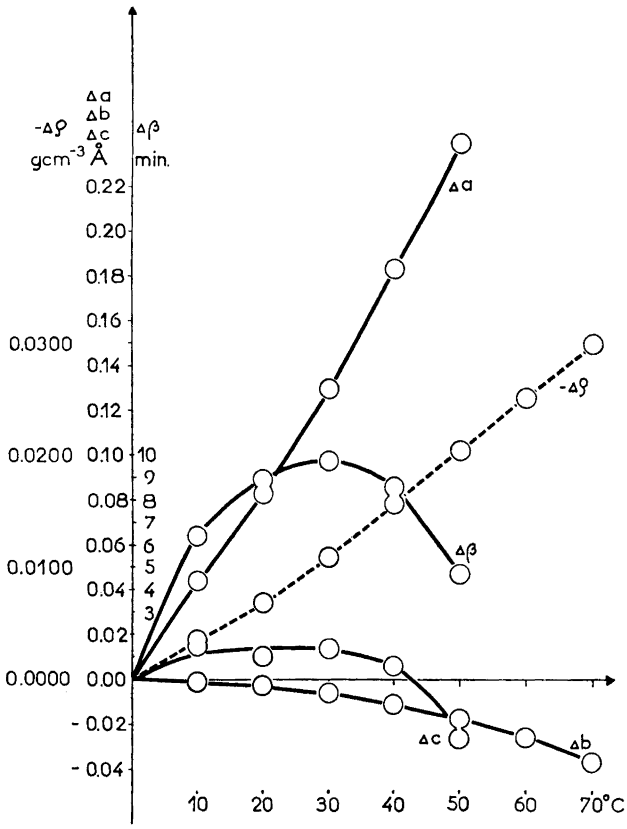


Fig. 2. The changes of the cell dimensions and the calculated density of the C-form of stearic acid.

We are indebted to Professors G. Hägg and E. Stenhagen for the facilities put at our disposal and to Dr. S. Abrahamsson for interesting discussions. The expenses involved have been defrayed by grants from the *Swedish Natural Science Research Council.*

REFERENCES

1. Stenhagen, E. and von Sydow, E. *Arkiv Kemi* **6** (1953) 309.
2. von Sydow, E. *Acta Chem. Scand.* **9** (1955) 1685.
3. von Sydow, E. *Arkiv Kemi* **9** (1956) 231.
4. von Sydow, E. *Acta Cryst.* **8** (1955) 557.
5. Vand, V., Morley, W. M. and Lomer, T. R. *Acta Cryst.* **4** (1951) 324.
6. Abrahamsson, S. and von Sydow, E. *Acta Cryst.* **7** (1954) 591.
7. Müller, A. *Proc. Roy. Soc. London.* **A 138** (1932) 514.
8. Welsh, H. K. *Acta Cryst.* **9** (1956) 89.
9. Brotherton, T., Craven, B. and Jeffrey, G. A. *Acta Cryst.* **11** (1958) 546.

Received March 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 989–997

A Homologous Series of Mixed Titanium Chromium Oxides $Ti_{n-2}Cr_2O_{2n-1}$ Isomorphous with the Series Ti_nO_{2n-1} and V_nO_{2n-1}

STEN ANDERSSON, ANITA SUNDHOLM and ARNE MAGNÉLI

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

By heating mixtures of titanium(IV)oxide and chromium(III)oxide at 1 300°—1 400°C in an inert atmosphere, mixed oxides $Ti_{n-2}Cr_2O_{2n-1}$ (n = 6, 7, 8, and 9) are obtained. These phases are isomorphous with the members of the homologous series Ti_nO_{2n-1} and V_nO_{2n-1}. Some structural data are given for the mixed oxide homologues. When heated at still higher temperatures, the structures seem to undergo a partial disordering.

From a consideration of an X-ray phase analysis of the vanadium-oxygen system carried out by Georg Andersson, it was suggested by one of the present authors (A.M.) that the phases occurring in the composition region $VO_{1.75-1.87}$ form a "homologous series" based structurally on the rutile-type structure [1]. The evidence constituting the grounds for this suggestion was the following:

1) The compositions of the phases, none of which shows an extended range of homogeneity, are all in good agreement with the general formula V_nO_{2n-1}, n being an integer. The phases prepared by Andersson correspond to values of n equal to 4, 5, 6, 7 and 8.

2) The powder photographs of the various phases are of a closely related appearance and are similar to the pattern of a structure of rutile type. Thus, all the strong reflexions of the former occur as doublets or triplets close to the positions of the rutile type lines. With increasing n, the distribution of the reflexions converges towards that of the rutile-type pattern.

Recent studies on the titanium-oxygen system have revealed the existence of an analogous family of oxides with the general formula Ti_nO_{2n-1} [2-4]. The members of this series ($4 \leq n \leq 10$) should, as indicated by the analogous appearance of the powder diagrams, in every case be isomorphous with the corresponding vanadium oxides.

An alternative formulation of a series M_nO_{2n-1} is $(n—2)MO_2 \cdot M_2O_3$. The latter formula suggests the possibility of preparing an oxide series from appropriate mixtures of oxides of tetravalent and trivalent metals. This article will give results obtained in this way using titanium(IV) and chromium(III) oxides.

The TiO_2-Cr_2O_3 system has previously been studied by Hamelin [5] who found a wide region of solid solubility in preparations rich in titanium. A phase transition was observed for this region at temperatures rising from about 1 440° to 1 500°C with increasing contents of chromium. It was pointed out that the X-ray powder patterns of the low temperature form were very similar to those given by products obtained by reducing rutile. The existence of a phase with the stoichiometric composition $2TiO_2 \cdot Cr_2O_3$ was also stated.

A different picture resulted from the studies on this system by Dietzel and Flörke [6,7] who found that the rutile-type structure broke down when the chromium content was raised above 3—5 %, giving rise to a series of new phases structurally related to the rutile type.

EXPERIMENTAL

The starting materials for preparing the mixed titanium chromium oxides were titanium dioxide (Baker analyzed, anatase modification, about 99.97 % pure) and dichromium trioxide (Matheson Coleman and Bell, reagent grade). Intimate mixtures of weighed amounts of the oxides were pressed into tablets and then heated at temperatures between 1 200° and 1 450°C for periods of time ranging from a few hours to ten days. In several cases, the samples had to be refired after grinding. The heating was performed in a glow-bar furnace provided with a horizontal heating tube made of pythagoras mass. The tablets were always placed on a thin sheet of platinum which prevented the sample from reacting with the corundum boat carrying the specimen. The heating was performed in air or a stream of oxygen or argon. After completion of the period of heating, the boat was quickly moved out of the hot zone in order to ensure a fairly rapid cooling of the sample. In a few instances, the mixed oxide tablets were melted in an electric arc furnace under argon atmosphere.

X-Ray powder photographs were taken of all the samples using a Guinier set-up with monochromatized $CuK\alpha_1$ radiation. An aluminium foil (35 μ) covering the film served to reduce the back-ground fogging caused by the fluorescent radiation from the samples. Potassium chloride (a = 6.2919 Å at 20°C [8]) was always added to the specimens as an internal standard. Details about the techniques employed in the X-ray work have been given in a previous article [9].

Table 1 gives a survey of several of the experiments performed during this investigation. The colour of the samples listed was always graphite grey after the heat-treatment but, upon grinding, it turned to a dark brown. Density data for several preparations are given in Table 5.

PHASE ANALYSIS

Most of the synthesis experiments were carried out in an argon atomsphere. It is obvious from Table 1 that the attainment of a state of equilibrium requires a more intense heat-treatment of the reaction mixture at low titanium contents than at higher ones. Fig. 1 gives a schematic representation of the powder patterns of several equilibrium samples.

The non-existence of a compound $TiO_2 \cdot Cr_2O_3$ and the existence of the phase $2TiO_2 \cdot Cr_2O_3$ ($Ti_2Cr_2O_7$) reported by Hamelin [5] are clearly demonstrated by our results. For higher contents of titanium dioxide, the powder patterns

Table 1. Survey of some preparations and results of phase analyses.

Starting composition of reacted mixture	Heating conditions			X-ray evidence
	Temperature °C	Time	Atmosphere	
1—6 $TiO_2 \cdot Cr_2O_3$	1 200	4 + 2 days	air or oxygen or argon	non-equilibrium
1—4 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 »	—»—	incomplete equilibrium
5 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 »	argon	M_7O_{13}
5 $TiO_2 \cdot Cr_2O_3$	1 300	2 days	air	$M_7O_{13} + M_8O_{15}$
5 $TiO_2 \cdot Cr_2O_3$	1 300	2 + 5 days	»	M_8O_{15}
5.5 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 days	argon	$M_7O_{13} + M_8O_{15}$
5.5 $TiO_2 \cdot Cr_2O_3$	1 300	1 day	air	M_8O_{15}
6 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 days	argon	M_8O_{15}
7 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 »	»	M_9O_{17}
8—15 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 »	»	M_nO_{2n-1}-type
16 $TiO_2 \cdot Cr_2O_3$	1 300	4 + 2 »	»	M_nO_{2n-1}-type + rutile
$TiO_2 \cdot Cr_2O_3$	1 450	4 + 2 »	»	$Ti_2Cr_2O_7 + Cr_2O_3$
2 $TiO_2 \cdot Cr_2O_3$	1 450	4 + 2 »	»	$Ti_2Cr_2O_7$
3 $TiO_2 \cdot Cr_2O_3$	1 450	4 + 2 »	»	$Ti_2Cr_2O_7 + M_6O_{11}$
4 $TiO_2 \cdot Cr_2O_3$	1 450	4 + 2 »	»	M_6O_{11}
5— 7 $TiO_2 \cdot Cr_2O_3$	1 450	4 + 2 »	»	M_nO_{2n-1}-type pattern with several lines absent.

are of a mutually related appearance and the whole of this region could easily be mistaken for being one sigle phase of a composition varying within wide limits. A careful study, however, reveals the existence, at least up to the composition $7TiO_2 \cdot Cr_2O_3$, of a regular alternation of one-phase and two-phase regions. The compositions of the former are underlined in Fig. 1. The ranges of homogeneity should be very narrow since the positions of the X-ray reflexions of the various phases do not change appreciably with the composition of the samples. The discontinuous character thus demonstrated for this region of the titanium chromium oxide system is in agreement with the opinion put forward independently by Dietzel and Flörke [6,7] but conflicts with the results reported by Hamelin [5].

For titanium dioxide contents exceeding $7TiO_2 \cdot Cr_2O_3$, the powder patterns still maintained the same general appearance. It was not, however, possible to observe the existence of any two-phase regions or to demonstrate a discontinuous character in this range of composition. This may indicate either that there is actually a continuos series of solid solutions or that the series of discrete oxide phases observed at lower titanium dioxide contents is continued up into this range of composition. In the latter case, the failure to demonstrate the existence of two-phase regions would be due to an inadequate resolving power of the powder camera. The upper limit of this range of composition is at about $15TiO_2 \cdot Cr_2O_3$, where the first indications of another two-phase region show up, rutile being the additional phase.

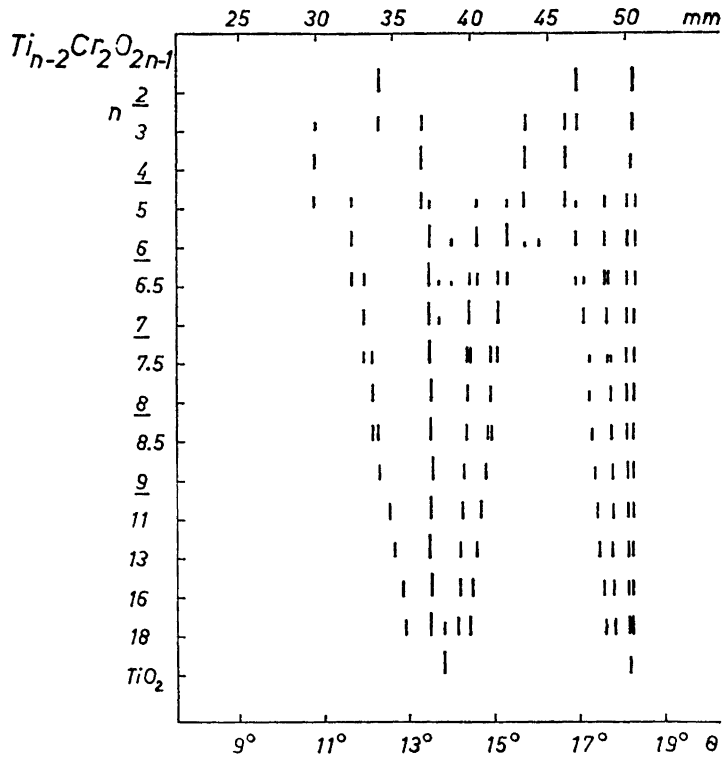


Fig. 1. Powder patterns of samples $Ti_{n-2}Cr_2O_{2n-1}$.

The phase analysis evidently demonstrates the existence of at least four discrete, related phases of compositions $xTiO_2 \cdot Cr_2O_3$, x having values close to the integers 4, 5, 6, and 7. An alternative way of expressing these compositions is $Ti_{n-2}Cr_2O_{2n-1}$, where n stands for $x + 2$. The powder patterns of the four phases show pronounced similarities and approach that of a rutile-type pattern with increasing values of n. The results obtained for the series of mixed titanium chromium oxides thus suggest that a far-reaching analogy exists with the homologous series Ti_nO_{2n-1} and V_nO_{2n-1}. Actually, a comparison of the powder photographs of all these phases reveals that the pattern of a member of the mixed oxide series may generally be described as something intermediate between the patterns of the titanium and vanadium oxide phases with the same value for n. The isomorphism of the three series has been further elucidated by the detailed interpretation of the powder photographs (*v. infra*).

The identification of the phases of the series $Ti_{n-2}Cr_2O_{2n-1}$ made possible the phase analysis of preparations obtained by heating the mixed oxide samples in air or in oxygen (*cf.* Table 1). Preparations heat-treated in this way give X-ray powder patterns showing phases of higher n values than those corresponding to the compositions of the starting mixtures. These starting mixtures

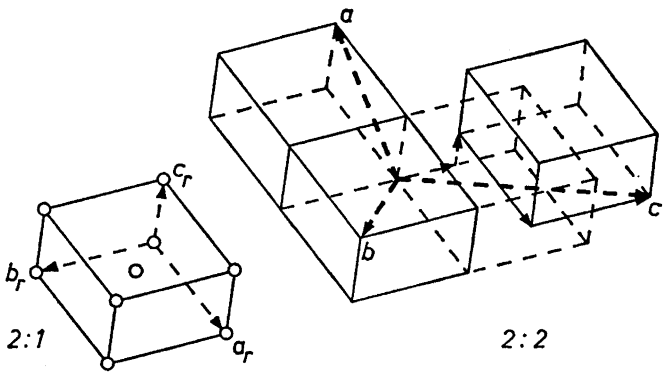


Fig. 2. 1) Unit cell of rutile with positions of metal atoms indicated. 2) Interrelation of basic rutile-type structure of adjacent slabs of Ti_5O_9. Unit cell axes of the latter indicated by dashed heavily-drawn arrows.

have therefore evidently been partly oxidized during the firing. This process is accompanied by a slight loss of weight of the sample. This phenomenon has so far not been further investigated. An oxidation of chromium(III)oxide when it was heated together with titanium(IV)oxide in air at 700°C was observed by Dietzel and Flörke. They also found that chromium(III)oxide when heated in air at 1 000°C is slightly volatile, forming a higher oxide [6,7].

Upon heating members of the series $Ti_{n-2}Cr_2O_{2n-1}$ at sufficiently high temperatures (*cf.* Table 1), preparations are obtained which give powder patterns that differ in a characteristic way from those shown in Fig. 1. Thus, while part of the reflexions are unchanged, several appear diffuse and with reduced intensities while others are not even visible after the high-temperature firing. This phenomenon, which is probably associated with the phase-transition reported by Hamelin [5], may indicate a partial disordering of the structures of the homologues. Further studies on this matter are in progress.

STRUCTURAL DATA OF THE SERIES $Ti_{n-2}Cr_2O_{2n-1}$

Crystals of the members of the homologous titanium chromium oxide series grow only very slowly and this of course made X-ray studies of these compounds with single-crystal methods impracticable. The deduction of detailed structural data of the homologues under such circumstances had to be based on information now available for the Ti_nO_{2n-1} series and especially for its second member, *i.e.* Ti_5O_9.

The crystal structure of Ti_5O_9 has recently been determined by one of the present authors (S.A.)[10] and is now being refined. (A detailed account of this investigation will, in due time, appear elsewhere.) The structure may be described as being built up of slabs of a rutile-structure type, *i.e.* TiO_6 octahedra joined by edges and corners as in the rutile structure. The slabs are of infinite extension in two dimensions and have a characteristic finite width correspond-

ing to five TiO_6 octahedra in a third direction. The mutual connection of the slabs takes place by means of TiO_6 octahedra sharing faces. If the axes of the rutile-type subunit cell ($\boldsymbol{a}_r$, $\boldsymbol{b}_r$ ($\boldsymbol{b}_r = \boldsymbol{a}_r$) and $\boldsymbol{c}_r$) are used to define the coordinate system, then the axes of the "ideal" Ti_5O_9 unit cell will be $\boldsymbol{a} = \boldsymbol{c}_r - \boldsymbol{a}_r$, $\boldsymbol{b} = \boldsymbol{a}_r + \boldsymbol{b}_r + \boldsymbol{c}_r$ and $\boldsymbol{c} = \boldsymbol{a}_r - \frac{3}{2}\boldsymbol{b}_r + \frac{1}{2}\boldsymbol{c}_r$. (The term, "ideal" cell, is used here to imply that no deformation of the geometry of the rutile subunit takes place.) The infinite extension of the slabs is parallel to the *ab* plane. The rutile arrangements of adjacent slabs are out of step by $\frac{1}{2}(\boldsymbol{b}_r + \boldsymbol{c}_r)$ (*cf.* Fig. 2).

Assuming the structural principles found for Ti_5O_9 to be valid also for the other members of the series, general expressions may be derived for the characteristics of the "ideal" structures of all the homologues. This is done in a way similar to that applied when deducing general structural data for the homologous series of molybdenum wolfram oxides M_nO_{3n-1} [11]. A detailed account of this investigation will be presented elsewhere. For the present discussion, the following vectorial expressions for the unit cell parameters will suffice.

$$\boldsymbol{a} = \boldsymbol{c}_r - \boldsymbol{a}_r$$

$$\boldsymbol{b} = \boldsymbol{a}_r + \boldsymbol{b}_r + \boldsymbol{c}_r$$

$$\boldsymbol{c} = \frac{n-3}{2}\boldsymbol{a}_r - \frac{n-2}{2}\boldsymbol{b}_r - \frac{n-6}{2}\boldsymbol{c}_r$$

The fundamental role of the rutile-type subcell in the structures of the various homologues shows itself in the character of the X-ray patterns. Thus, the latter only show strong reflexions when the reciprocal lattice points are situated close to those of the strong rutile reflexions. The indices of the strong reflexions of a homologue M_nO_{2n-1} may thus be found by means of the following matrix giving the relations between the homologue and rutile.

	→ rutile		
M_nO_{2n-1}	$\bar{1}$	0	1
	1	1	1
	$\frac{n-3}{2}$	$\frac{2-n}{2}$	$\frac{6-n}{2}$

Thus the reciprocal point of the strong rutile reflexion 110 corresponds, for all the homologues, to the point $1\bar{2}\frac{1}{2}$, which implies that the reflexions of the adjacent points $1\bar{2}0$ and $1\bar{2}1$ should always be strong. On the other hand, the rutile point $1\bar{1}0$ corresponds, for the various homologues, to $1\ 0\ \frac{5-2n}{2}$. The reflexions generated by the strong $1\bar{1}0$ reflexion of rutile should thus be $10\bar{3}$ and $10\bar{4}$ for M_6O_{11}, $10\bar{4}$ and $10\bar{5}$ for M_7O_{13} *etc.*

Such considerations should in principle make it possible to index the powder pattern of any member of a homologous series M_nO_{2n-1} possessing the structural characteristics given above. Actually all well resolved patterns of members

Table 2. Powder patterns of the phases $Ti_{n-2}Cr_2O_{2n-1}$. Guinier camera. Cu$K\alpha_1$ radiation.

Compound	I	$\sin^2\Theta_{obs}$	$h\,k\,l$	$\sin^2\Theta_{calc}$
$Ti_4Cr_2O_{11}$	w	0.02468	$10\bar{2}$	0.02467
	m	0.04017	$10\bar{3}$	0.04011
	st	0.05347	$1\bar{2}0$	0.05343
	w	0.05771	$\bar{1}21$	0.05769
	m	0.06232	$1\bar{2}1$	0.06237
	m	0.06870	$10\bar{4}$	0.06875
	w	0.07517	$02\bar{3}$	0.07518
	m	0.08325	021	0.08326
	w	0.08758	$20\bar{3}$	0.08760
	m	0.08968	$20\bar{2}$	0.08972
	m	0.09494	102	0.09491
	m	0.09707	$12\bar{2}$	0.09707
$Ti_5Cr_2O_{13}$	m	0.04214	$10\bar{4}$	0.04214
	st	0.05347	$1\bar{2}0$	0.05346
	w	0.05535	$\bar{1}21$	0.05527
	m	0.06115	$1\bar{2}1$	0.06115
	m	0.06656	$10\bar{5}$	0.06659
	m	0.08491	021	0.08491
	m	0.09029	$20\bar{3}$	0.09031
	m	0.09494	102	0.09494
	m	0.09656	$12\bar{3}$	0.09659
$Ti_6Cr_2O_{15}$	m	0.04376	$10\bar{5}$	0.04375
	st	0.05386	$1\bar{2}0$	0.05383
	m	0.06055	$1\bar{2}1$	0.06057
	m	0.06516	$10\bar{6}$	0.06507
	m	0.08631	021	0.08632
	m	0.09104	$20\bar{4}$	0.09100
	m	0.09501	102	0.09499
	m	0.09648	$12\bar{4}$	0.09639
$Ti_7Cr_2O_{17}$	m	0.04510	$10\bar{6}$	0.04509
	st	0.05395	$1\bar{2}0$	0.05395
	m	0.06001	$1\bar{2}1$	0.06000
	m	0.06399	$10\bar{7}$	0.06404
	m	0.08723	021	0.08724
	m	0.09148	$20\bar{5}$	0.09146
	m	0.09508	102	0.09509
	m	0.09630	$12\bar{5}$	0.09626

of the series Ti_nO_{2n-1}, V_nO_{2n-1} and $Ti_{n-2}Cr_2O_{2n-1}$, have been indexed in this way *. Parts of the powder diagrams of the latter homologues are given in Table 2. The good agreement between the calculated and the observed diffraction data may be taken as a confirmation of the structural character discussed above for these phases.

* According to a private communication Mr. Jørgen Villadsen of F:a Haldor Topsøe, Hillerup, Denmark, has indexed the powder patterns of Ti_4O_7 and V_4O_7 by applying a procedure according to De Wolff [12] and has found parameter values in perfect agreement with those derived in this investigation.

Table 3. Observed and calculated unit cell parameters and densities of the members of the $Ti_{n-2}Cr_2O_{2n-1}$ series.

Phase	Unit cell parameters						Density
	a_{obs} a_{calc}	b_{obs} b_{calc}	c_{obs} c_{calc}	α_{obs} α_{calc}	β_{obs} β_{calc}	γ_{obs} γ_{calc}	obs calc
$Ti_4Cr_2O_{11}$	5.50 Å	7.07 Å	11.89 Å	98.6°	120.6°	107.4°	4.42
	5.46	7.14	11.48	97.4°	120.3°	108.5°	4.47
$Ti_5Cr_2O_{13}$	5.50	7.09	15.24	99.1°	125.3°	108.2°	4.36
	5.46	7.14	14.78	98.1°	125.2°	108.5°	4.42
$Ti_6Cr_2O_{15}$	5.49	7.08	18.64	99.5°	128.2°	108.1°	4.33
	5.46	7.14	18.18	98.6°	128.3°	108.5°	4.40
$Ti_7Cr_2O_{17}$	5.49	7.09	22.13	99.5°	130.3°	107.3°	4.24
	5.46	7.14	21.40	98.9°	130.8°	108.5°	4.38

Table 3 gives a comparison between the unit cell parameters of the members of the $Ti_{n-2}Cr_2O_{2n-1}$ series derived from the experimentally obtained X-ray data and parameter values calculated from the expressions given above. For simplicity, the parameters a_r and c_r are taken here to be equal to those of rutile, *viz.* 4.59 and 2.96 Å, respectively. The agreement between the observed and calculated data is satisfactory when considering the simplifying assumptions involved in the derivation of the latter. Thus, for the a and b axes, which should be constant for all the homologues, the deviation does not exceed 1 %. The observed length of the c axis, which increases with increasing n, is throughout larger than the calculated value by about 4 %. This difference is, however, easily accounted for by the repulsion between the metal atoms of octahedra sharing faces. The resulting expansion of the structure, which was not considered when deriving the ideal lattice parameters, has its largest component in the c direction. The agreement is satisfactory also for the angle parameters.

Further developments of the expressions giving the structural characteristics of »ideal» and eventually also real members of a series M_nO_{2n-1} should make it possible to calculate rather accurate positions for the metal and oxygen atoms of the various phases. The distribution of the titanium and chromium atoms on the metal atom sites might possibly be derived from comparisons of detailed structural data, including interatomic distances, obtained from single-crystal studies of titanium chromium and titanium oxides.

This investigation has been sponsored in part by the *Office, Chief of Research and Development, U.S. Department of Army* through its *European Research Office* and in part by the *Swedish Natural Science Research Council.*

REFERENCES

1. Andersson, G. *Acta Chem. Scand.* **8** (1954) 1599.
2. Andersson, S. and Magnéli, A. *Naturwiss.* **43** (1956) 495.
3. Andersson, S., Collén, B., Kuylenstierna, U. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1641.
4. Andersson, S. *XVI*e *Congrès International de Chimie Pure et Appliquée, Paris 1957. Mémoires présentés à la Section de Chimie Minérale* 53.
5. Hamelin, M. *Bull. soc. chim. France* (1957) 1421.
6. Dietzel, A. and Flörke, O. W. *Mitteilungen des Vereins Deutscher Emailfachleute* **5** (1957) 99.
7. Ditzel, A. and Flörke, O. W. *Fortschr. Min.* **36** (1958) 72.
8. Hambling, P. G. *Acta Cryst.* **6** (1953) 98.
9. Westman, S. and Magnéli, A. *Acta Chem. Scand.* **11** (1957) 1587.
10. Magnéli, A., Andersson, S., Westman, S., Åsbrink, S. and Holmberg, B. *Final Technical Report* (1958) DA-91-508-EUC-245.
11. Magnéli, A. *Acta Cryst.* **6** (1953) 495.
12. De Wolff, P. M. *Acta Cryst.* **10** (1957) 590.

Received March 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 998–1010

On the Complex Chemistry of the Tervalent Rare-Earth Ions

III. The Glycolate Systems

ARTUR SONESSON

Department of Inorganic and Physical Chemistry, Chemical Institute, University of Lund, Lund, Sweden

The complexity constants of the glycolate systems of ten rare earths have been determined by potentiometric measurements similar to those used previously in the investigation of the acetate systems [1,2]. The results are given in Tables 5 and 6 and Fig. 5.

The glycolate systems show a considerably higher complexity than the corresponding acetate systems. This is probably due to the formation of chelates in the glycolate systems. The value of the first complexity constant, $\beta_1 = [MA] \cdot [M]^{-1} \cdot [A]^{-1}$, increases from La to Sm. For the Gd-system a somewhat lower value of β_1 is found than for the Sm-system. From Gd to Yb, however, β_1 increases again, contrary to the variation of β_1 for the corresponding acetate systems.

The potentiometric measurements show that the formation of anionic glycolate complexes must be assumed.

The neodymium glycolate system has also been investigated extinctiometrically. The complexity constants obtained in this way are in good agreement with those obtained potentiometrically.

The glycolate ion, $HO \cdot CH_2 \cdot COO^-$, contains a hydroxide group but has in other respects the same constitution as the acetate ion, $CH_3 \cdot COO^-$. On account of the OH-group the glycolate ion has a more or less pronounced tendency to form chelates. A five-membered ring can be formed. In those cases where chelates are not formed, the glycolate solutions ought to show a lower degree of complexity than the corresponding acetate solutions, in as much as glycolic acid is stronger than acetic acid (*cf.* Sundén [3] and Ahrland [4]). If on the other hand chelates are formed, we would expect the first complexity constants (the very first one at least) to be greater than the constants of the corresponding acetate complexes.

Investigations indicating that the glycolate systems of the rare earths probably contain chelates are to be found in the literature (Jantsch and Grünkraut [5]), but no attempts to compute the complexity onstants have been made. In order to make possible a comparison between the complexity of the

corresponding acetate and glycolate systems, the investigation of the glycolate systems described in this paper has been performed in the same way as the investigation of the acetate systems reported in papers I [1] and II [2]. Thus all measurements have been made at 20°C and with an ionic strength of 2.0 M. The main method of investigation has been the potentiometric determination of the hydrogen-ion concentration in solutions with known total concentrations of rare-earth ions, glycolate ions and glycolic acid. As a check on the potentiometric investigations the neodymium glycolate system has been examined extinctiometrically in the way described in II for the erbium acetate system.

Concerning the symbols and the formulae used in the calculations, the reader is referred to I and II.

CHEMICALS USED

Glycolic acid ((Fluca puriss. and Kahlbaum puriss.) was dried *in vacuo* over concentrated H_2SO_4. Both preparations gave after drying the equivalent weight 76.1 (calc. 76.0).

A stock solution of glycolic acid * HAcOH and sodium glycolate NaAcOH in the proportion 2 M HAcOH: 2 M NaAcOH was prepared by partial neutralization of calculated amounts of dried acid by carbonate-free sodium hydroxide. Afterwards the concentrations of HAcOH and NaAcOH were checked.

Buffer solutions with δ = 1.0, 2.5 and 4.0 were prepared from calculated amounts of the stock solution and dried glycolic acid. Sodium perchlorate from a stock solution was added to make I = 2.0 M. Besides, a buffer solution with the composition 0.387 M HAcOH, 1.107 M NaAcOH and $NaClO_4$ to make I = 2.0 M was used.

A. POTENTIOMETRIC MEASUREMENTS

The cells in which the electromotive forces were measured were of the following composition (Me^{3+} = the rare earth ion):

Au	Quinhydrone 10.2 mM $HClO_4$ $NaClO_4$ to I = 2.0 M	2.0 M $NaClO_4$	Quinhydrone C_M mM $Me(ClO_4)_3$ $C_H = \nu \cdot C_M$ mM $HClO_4$ C'_A mM NaAcOH $C'_{HA} = \delta \cdot C'_A$ mM HAcOH $NaClO_4$ to I = 2.0 M	Au	(1)

The solution of the right cell was prepared from a known volume of the rare-earth perchlorate solution S_1 and increasing volumes of the buffer solution S_2:

	S_1	S_2		
$Me(ClO_4)_3$	C°_M	0	mM	
$HClO_4$	$C^\circ_H = \nu \cdot C^\circ_M$	0	mM	
NaAcOH	0	C°_A	mM	(2)
HAcOH	0	$C^\circ_{HA} = \delta \cdot C^\circ_A$	mM	
$NaClO_4$	$2\,000 - 6\,C^\circ_M - C^\circ_H$	$2\,000 - C^\circ_A$	mM	

The relation between C_M and C'_A is given by

$$C_M = C^\circ_M(1 - C'_A \times 10^{-3}) \text{ mM} \qquad (3)$$

except in the case where C°_A = 1 107 mM.

* $AcOH^-$ = the glycolate ion.

Table 1. Determination of E' as a function of C'_A by the addition of glycolate buffers with different values of δ to a 2 M solution of sodium perchlorate.

$\delta = 0.350$				$\delta = 1.0$		$\delta = 4.0$	
C'_A mM	E' mV	$K_c \times 10^{+4}$	C'_A mM	E' mV	$K_c \times 10^{+4}$	E' mV	$K_c \times 10^{+4}$
10.96	129.2	1.79	9.90	102.9	1.77	68.4	1.82
21.71	128.8	1.80	19.61	102.1	1.80	67.0	1.85
			29.1	101.8	1.81	66.5	1.86
42.6	128.5	1.81	38.5	101.7	1.81	66.1	1.88
			56.6	101.6	1.81	65.6	1.90
72.4	128.4	1.81	74.1	101.5	1.82	65.2	1.93
109.7	128.2	1.83	99.1	101.3	1.83	64.7	1.96
			122.8	101.2	1.84	64.2	2.00
168.9	128.1	1.83	152.5	101.1	1.84	63.7	2.03
			200.0	100.9	1.86	62.9	2.09
287.0	128.0	1.84	285.7	100.6	1.88	61.6	2.2
			375	100.2	1.91	60.4	2.3
492	127.9	1.84	500	99.7	1.94	58.6	2.5
604	127.8	1.85	546	99.6	1.95	58.1	2.6

The emf of the element in (1) is denoted by E when $C_M \neq 0$, and by E' when $C_M = 0$, that is, when the right cell contains only the buffer solution. During the experiments the solutions were kept in a thermostat in which the temperature was 20.0° ± 0.1°C.

Every titration series was repeated at least once. With the exception of the cases where the rare-earth glycolate was precipitated, the potential was steady and could with few exceptions be reproduced within 0.2 mV.

The variation of the stoichiometric acid constant K_c with C'_A and C'_{HA} is shown in Table 1. It is evident that the addition of glycolic acid and the exchange of perchlorate ions for glycolate ions have a marked influence on the ionic medium. The variation of E' may to some degree depend on the salt error of the quinhydrone electrode. As only $E_A = E'—E$ is used for the computation of [A] (except for the determination of some correction terms), the salt error caused by the addition of the buffer solution is reduced to a minimum.

However, the medium changes probably also influence the activity factors of other ion species in the complex solution. Then it is possible that the complexity constants β_n vary with C'_A and C'_{HA}. As the variation of E' is especially significant at high values of δ, some difference might be expected between the $\bar{n}$/[A]-values obtained with buffer solutions having great differences in δ. Therefore buffer solutions with $\delta = 0.350$ and $\delta = 4.0$ were used in investigating the complexity systems. The results of the measurements on the gadolinium glycolate system are shown in Tables 2a and 2b and Fig. 1. The agreement between the $\bar{n}$/[A]-values obtained with the two buffers is rather good. The differences fall generally within the limits of the random errors. Thus it appears that a high concentration of glycolic acid does not have any great influence on the complexity constants.

Table 2 a. Corresponding values of $\bar{n}$/[A] and [A] for the gadolinium glycolate system determined by means of a glycolate buffer with $\delta = 0.350$ (*cf.* Fig. 1).

	$C_M = C_M^o\ (1-0.903 \cdot C'_A \cdot 10^{-3})$ mM					
	$C_M^o = 23.65$ mM $C_H = 8.30 \cdot 10^{-2} \cdot C_M$ mM Buffer 0.387:1.107 Symbol: O			$C_M^o = 47.3$ mM $C_H = 3 \cdot 10^{-5} \cdot C_M$ mM Buffer 0.387:1.107 Symbol: □		
C'_A mM	$E_A - \Delta E_A$ mV	[A] mM	$\frac{\bar{n}}{[A]}$ M^{-1}	$E_A - \Delta E_A$ mV	[A] mM	$\frac{\bar{n}}{[A]}$ M^{-1}
10.96	60.6—0.1	1.365	261	60.4—0.1	0.847	272
21.71	52.0—0.1	3.35	217	61.5—0.2	1.756	254
32.25	45.6—0.2	6.13	175	60.1—0.2	2.85	230
42.6	40.6—0.2	9.58	144	58.1—0.3	4.16	206
52.7	35.9—0.2	14.03	117	55.7—0.3	5.73	184
72.4	28.8—0.3	25.05	82.5	50.3—0.4	9.90	144
91.4	23.3—0.3	38.8	60.6	44.6—0.5	15.81	110.5
109.7	19.5—0.3	53.6	47.6	39.1—0.5	23.67	85.5
136.0	15.6—0.3	76.9	36.0	32.3—0.5	38.5	61.1
168.9	12.3—0.3	108.0	27.4	25.6—0.5	62.4	42.6
221.4	8.9—0.3	160.7	19.5	18.5—0.5	108.5	27.5
287.0	6.5—0.2	226.9	14.8	13.2—0.5	173.5	18.7
369	4.8—0.2	310.7	11.6	9.3—0.5	260.4	13.3
492	3.0—0.2	443	8.2	6.0—0.4	394	9.4
604	2.0—0.2	565	6.3	4.2—0.3	517	7.8

As the glycolic acid has a OH-group, it is possible that the unprotolyzed acid molecules form complexes with the rare-earth ions. We have therefore examined the question as to whether the addition of glycolic acid to an acidified solution of a rare-earth perchlorate has any effect on the extinction curve of the rare earth. It was found that the extinction of the holmium ions within the wave length range 320—700 mμ was unchanged, except for the peak at 450 mμ, when C_{HA} was increased from 0 mM to 750 mM ($C_M = 50$ mM, $[H^+] = 125$ mM, $NaClO_4$ to $I = 2.0$ M). The molar extinction coefficient of the peak at 450 mμ increased from 4.0 to 4.8. This increase, however, can be attributed to a complex formation between holmium ions and glycolate ions, as $[A] \approx 1.1$ mM in a solution having $C_{HA} = 750$ mM ($K_c = 1.8 \times 10^{-4}$) and $[H^+] = 125$ mM. This assumption was confirmed by an examination of the extinction of holmium perchlorate solutions ($C_H^o \approx 0.5$ mM), to which different amounts of glycolate buffer ($\delta = 1$) were added. It was found that the molar extinction coefficient of the peak at 450 mμ was about 4.8 when $[HA] \approx 18$ mM and $[A] \approx 1.0$ mM ($\bar{n} \approx 0.3$ according to the potentiometric measurements) *. Thus, complex formation between unprotolyzed glycolic

* The formation of holmium glycolate complexes is accompanied by a rather strong increase of the extinction band at 450 mμ, whereas the increase of the band at 360 mμ is considerably lower. The band at 450 mμ has two peaks at a distance of about 1 mμ. It is the peak with the shorter wave length that increases when glycolate complexes are formed.

Table 2 b. Corresponding values of $n/[A]$ and [A] obtained for the gadolinium glycolate system by means of a buffer solution with $\delta = 4.0$ (*cf.* Fig. 1).

	$C_M = C_M^o\ (1 - C'_A \cdot 10^{-3})$ mM					
	$C_M^o = 23.65$ mM $C_H = 8.30 \cdot 10^{-2} \cdot C_M$ mM Buffer 4:1 Symbol: ●			$C_M^o = 47.3$ mM $C_H = 3.10^{-5} \cdot C_M$ mM Buffer 4:1 Symbol: ■		
C'_A mM	$E_A - \Delta E_A$ mV	[A] mM	$\frac{\bar{n}}{[A]}$ M^{-1}	$E_A - \Delta E_A$ mV	[A] mM	$\frac{\bar{n}}{[A]}$ M^{-1}
9.90	44.3—0.1	1.776	248	52.8—0.1	1.147	271
19.61	42.5—0.1	3.73	208	55.0—0.2	2.152	241
29.13	39.8—0.2	6.18	174	55.0—0.2	3.24	219
38.5	36.7—0.2	9.21	146	53.8—0.3	4.55	196
56.6	30.8—0.2	17.09	106	50.5—0.4	7.73	159
74.1	25.8—0.3	27.3	78.7	46.3—0.4	12.00	127.5
82.6	23.6—0.3	33.2	68.8	44.0—0.4	14.70	114
99.1	20.2—0.3	45.5	55.3	39.8—0.5	20.93	92.0
122.8	16.2—0.3	66.0	41.3	33.8—0.5	33.0	68.0
152.5	12.8—0.3	93.7	31.2	27.7—0.5	52.1	49.3
200.0	9.4—0.3	140.3	22.4	20.5—0.5	90.9	32.3
285.7	5.9—0.2	229.0	14.6	13.2—0.5	173.3	19.4
375	4.0—0.2	323.5	10.7	8.9—0.4	268.5	13.6
500	2.4—0.2	459	7.5	5.5—0.3	408	9.7
546	1.9—0.1	509	6.7	4.5—0.3	463	8.4

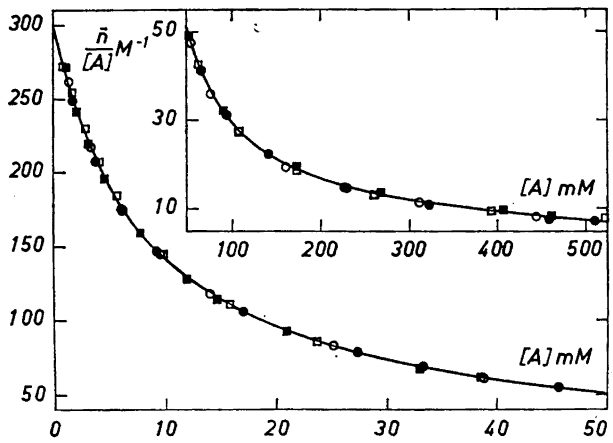


Fig. 1. The relation between $\bar{n}/[A]$ and [A] for the gadolinium glycolate system according to the titration series in Tables 2a and 2b.

acid and holmium ions (and other rare-earth ions) may probably be excluded.

Any significant signs of polynuclear complexes have not been found, as the $\bar{n}$/[A]-functions of series with different C_M coincide at low [A] within the limits of the random errors.

The rare-earth glycolates are rather slightly soluble. The solubilities of the glycolates of the lighter rare earths have been determined by Jantsch and Grünkraut [5]. They found that the solubility at 18°C increases from 9.1 mmole/l at La to 12.5 mmole/l at Nd and 17.0 mmole/l at Sm. These glycolates have the composition $Me(AcOH)_3$ when dried in air at room temperature. The gadolinium glycolate showed a considerably higher solubility, 33.8 mmole/l, and contained 2 H_2O after drying.

The solubilities of the higher rare-earth glycolates seem to decrease from Gd to Yb. No determinations of the solubilities have been performed, but Jantsch and Grünkraut found that if a solution containing a mixture of rare-earth nitrates was partially precipitated with sodium glycolate, the higher rare earths were concentrated in the precipitate. It was also found by the present author in the investigation of the complexity of the glycolate systems that in order to avoid precipitation solutions with considerably lower rare-earth concentrations must be used for the higher rare earths than for gadolinium. The precipitate obtained in the Ho^{3+}—$AcOH^-$-system was analyzed after drying in air at room temperature. Its composition was $Ho(AcOH)_3 \cdot 2H_2O$ (Found: Ho 38.3; H_2O 8.4. Calc. Ho 38.7; H_2O 8.45).

The precipitates of the rare-earth glycolates were formed rather slowly, except at $\bar{n} \approx 3$. Therefore it was also possible to investigate the glycolate systems at high ligand concentrations and relatively high rare-earth concentrations.

According to the potentiometric measurements anionic glycolate complexes are formed. Then it was expected that the precipitate of the rare-earth glycolate should dissolve at least partially when the concentration of glycolate ions was increased. However, such a dissolution could not be proved directly.

As the complex solutions have pH < 4, the degree of hydrolysis of the rare-earth ions must be so slight that it can be neglected (*cf.* I[p. 171] and II[p. 1940]).

As the ionic strength of the complex solutions decreases by the complex formation, the E_A-values must be corrected. By using the method indicated in Paper I the relation between the correction term, ΔE_A, and the depression of the ionic strength, ΔI, has been determined. The correction term should be subtracted from E_A to give the true E_A at $I = 2.0$ M. For the glycolate systems the following function was obtained:

$$\Delta E_A = 2.3 \cdot \Delta I \tag{4}$$

Thus the E_A-values of the glycolate systems are not so sensitive to changes of the ionic strength as the E_A-values of the acetate systems.

For the calculation of ΔI the following formula was used:

$$\Delta I = 0.5 \cdot C_M \cdot \bar{n}(7-\bar{n}) \tag{5}$$

where $\bar{n}$ was obtained by means of the uncorrected E_A-values (*cf.* I[p. 171]).

Table 3. The corresponding values of $\bar{n}/[A]$ and [A], given at some round values of [A] (in mM), for the rare-earth glycolate systems investigated potentiometrically.

	$\bar{n}/[A]$ for [A] =									
	0	2	5	10	15	20	30	40	50	70
La	153	132	112	90	76.5	67	54.5	46	40.5	32.3
Ce	226	180	142	109	88.5	75.5	60	49.5	42.5	34.0
Pr	270	208	161	121	98.5	83	65	54	46	36.2
Nd	320	238	177	130	103.5	86.5	66.5	55	47	36.6
Sm	364	270	202	145	114	95	72.5	59	50	38.3
Gd	297	244	190	142	113.5	95	73	59.5	50.5	39.1
Dy	330	259	197	146	116	96.5	74.5	60.5	51	39.3
Ho	350	262	199	146	116	96.5	74	60	51	39.5
Er	402	288	212	150	118	98	74	60	51	39.1
Yb	520	348	246	167	129	106	78.5	63	53	40.2
	$\bar{n}/[A]$ for [A] =									
	100	150	200	250	300	350	400	450	500	550
La	25.3	18.3	14.6	12.4	10.6	9.4	8.5	7.7	7.2	6.8
Ce	26.1	19.3	15.5	13.0	11.2	9.8	8.7	7.8	7.1	—
Pr	28.0	20.3	16.1	13.4	11.6	10.2	9.1	8.1	7.6	7.1
Nd	28.0	20.0	15.8	13.2	11.3	10.0	8.8	8.0	7.4	—
Sm	29.2	20.9	16.5	13.6	11.7	10.3	9.1	8.3	7.6	7.0
Gd	29.5	21.3	16.6	13.8	11.9	10.6	9.3	8.3	7.4	6.8
Dy	29.7	21.5	17.0	14.0	12.0	10.4	9.2	8.3	7.5	7.0
Ho	29.8	21.5	17.0	14.2	12.2	10.7	9.5	8.6	7.8	7.3
Er	29.6	21.0	16.7	13.9	12.0	10.7	9.6	8.7	7.8	7.1
Yb	30.2	21.2	16.7	14.0	12.2	11.0	9.8	8.9	8.0	7.3

Table 3 gives $\bar{n}/[A]$ for various values of [A] for all the rare-earth glycolate systems examined. [A] and $\bar{n}/[A]$ have been calculated according to eqns. II(2), II(3) and II(4). Fig. 2 shows the complex formation curve $\bar{n} = f(\log[A])$ for the glycolate systems of La^{3+}, Sm^{3+}, Dy^{3+}, and Yb^{3+}.

The complexity constants $\beta_n = [MA_n]\cdot[M]^{-1}\cdot[A]^{-n}$ have been computed according to eqns. II(5), II(7) and II(8) after a graphical integration of the function $\bar{n}/[A] = f([A]))$. The complexity constants are given in Table 5.

B. THE EXTINCTIOMETRIC INVESTIGATION

The neodymium glycolate system was examined extinctiometrically in the same way as the erbium acetate system described in II[p. 1947]. As the potentiometric investigation showed that polynuclear complexes can be formed only to a very limited extent, the terms depending on polynuclear complexes are neglected in the extinctiometric equations.

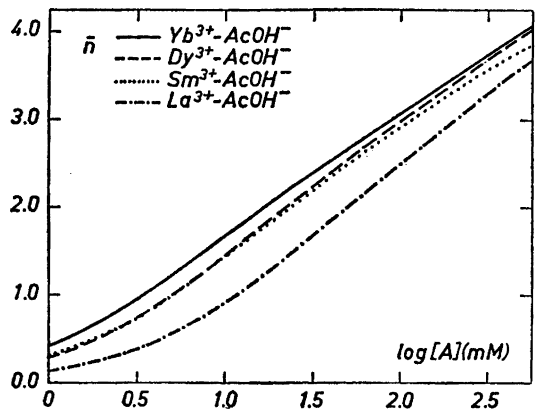


Fig. 2. The complex formation curves $\bar{n} = f(\log[A])$ for four rare-earth glycolate systems.

The measurements were performed at 20°C by means of a Hilger Uvispek with tungsten lamp and glass prism. As seen in Fig. 3 the complex formation has the greatest effect on the absorption band at 580 mμ. The band is shifted towards longer wave length, and the extinctiometric measurements were therefore performed at 586 mμ. At this wave length the slope of the absorption band has approximately the same value for all the solutions measured, and therefore the errors caused by the imperfect monochromaticity of the light are reduced (*cf.* Fronæus [6], p. 98). The effective band width was about 5 Å.

In order to reduce the error caused by the partial reflection of the light at the end plates of the absorption cells (*cf.* Fronæus [6], p. 98), solutions with the same value of ε should

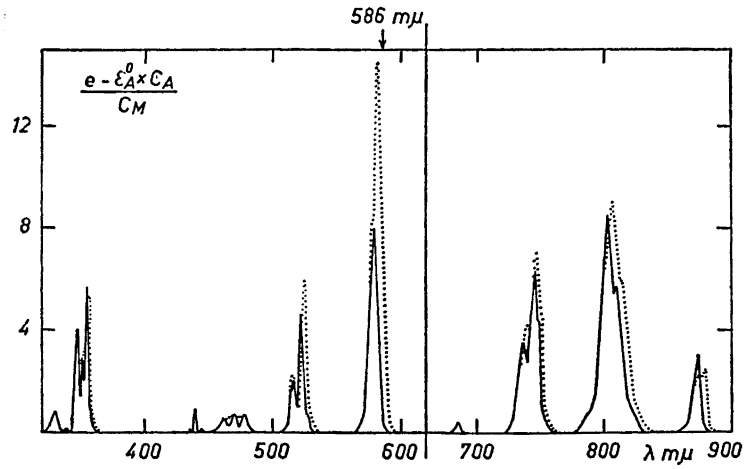


Fig. 3. The extinction curves for a neodymium perchlorate solution (the fulldrawn curve) and a complex solution with $C_{Nd^{3+}} = 50$ mM and $C_A = 500$ mM (the dotted curve). The ionic strength of both solutions was 2.0 M.

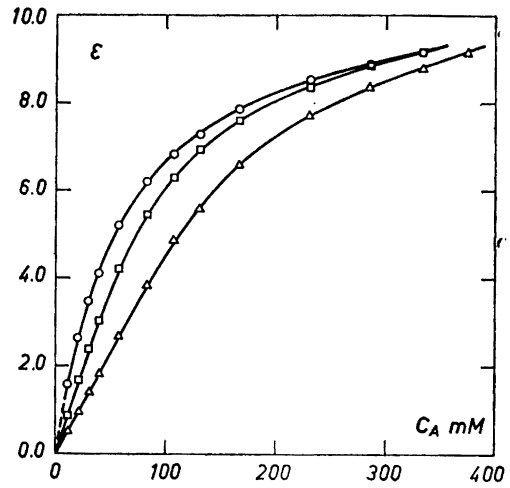


Fig. 4. ε for the neodymium glycolate system as a function of the glycolate concentration at different values of d. O : d = 4 cm, □ : d = 2 cm, △ : d = 1 cm. The relation between C_M, C'_A and d is given by eqn. (6). By correcting C'_A according to the formula $C_A = C'_A + [H^+] - C_H$ the true total concentration C_A of glycolate ions is obtained.

have the same total extinction (E). This condition has been fulfilled approximately by holding the following relation between the rare-earth concentration (C_M mM), the glycolate concentration (C'_A mM) and the length of the absorption cell (d in cm):

$$C_M = \frac{50}{d}\,(1 - C'_A \times 10^{-3}); \qquad (C_M \text{ and } C'_A \text{ in mM}) \tag{6}$$

The solutions to be measured have been prepared by mixing different volumes of two solutions:

$$S_1 : \frac{50}{d} \text{ mM } Nd(ClO_4)_3 + \left(2\,000 - 6 \cdot \frac{50}{d}\right) \text{ mM } NaClO_4$$

$$S_2 : 1\,000 \text{ mM } HAcOH + 1\,000 \text{ mM } NaAcOH + 1\,000 \text{ mM } NaClO_4$$

In the calculation of the true total concentration of glycolate ions ($C_A = C'_A + [H^+] - C_H$) the potentiometrically obtained values of the correction terms $[H^+]$ and C_H have been used. C_H depends on the presence of free perchloric acid in the rare-earth perchlorate solution ($C_H = 1.34 \times 10^{-3} \times C_M$ mM).

The relation between the lengths of the absorption cells was found to be 4.00:2.00:0.998 by means of extinction measurements performed with an alkaline solution of sodium picrate.

The validity of Beer's law was checked for neodymium perchlorate solutions with $C_M \leq 50$ mM.

The relation between

$$\varepsilon = \frac{e - \varepsilon_M^\circ \cdot C_M - \varepsilon_A^\circ \cdot C_A}{C_M}$$

and the glycolate concentration C_A has been determined experimentally for three values of d in eqn. (6). The three functions $\varepsilon = f(C_A)$ are shown in Fig. 4. By means of the three curves and eqn. (6) the pairs of values $(C_M, C_A)_\varepsilon$ — that is, the corresponding values of C_M and C_A at a given value of ε — have been calculated for the three values of d (Table 4).

According to eqn. II(13) the following equation is valid if only mononuclear complexes are formed:

$$\varepsilon = \frac{\xi}{X} = \frac{1}{X} \cdot \sum_{n=1}^{N} (\varepsilon_n - \varepsilon_M^\circ - n \cdot \varepsilon_A^\circ)\beta_n \cdot [A]^n \qquad (7)$$

Therefore solutions with the same value of ε must have the same concentration [A] of free glycolate ions. Thus if $\bar{n}_\varepsilon$ is the ligand number, the relation between $(C_A)_\varepsilon$ and $(C_M)_\varepsilon$ is given by the straight line

$$(C_A)_\varepsilon = \bar{n}_\varepsilon \cdot (C_M)_\varepsilon + [A] \qquad (8)$$

The pairs of values $(C_M, C_A)_\varepsilon$ given in Table 4 for $2.0 \leq \varepsilon \leq 6.5$ represent approximately a straight line. Therefore it is possible to calculate $\bar{n}_\varepsilon$ and [A] according to eqn. (8). The determinations of $\bar{n}_\varepsilon$ and [A] have been performed graphically. The accordance between $\bar{n}_\varepsilon$/[A] and $\bar{n}$/[A] obtained potentiometrically is relatively good. On account of the method of extrapolation, the determination of $\bar{n}_\varepsilon$/[A] begins to be very uncertain for [A] < 4 mM.

Table 4. Determination of the corresponding values of [A] and $\bar{n}_\varepsilon$ for the neodymium glycolate system and a comparison between $\bar{n}_\varepsilon$/[A] and the potentiometrically obtained values of $\bar{n}$/[A].

ε	d = 1 cm		d = 2 cm		d = 4 cm		$C_M = 0$	$\bar{n}_\varepsilon$	$\frac{\bar{n}_\varepsilon}{[A]}$ M^{-1}	$\frac{\bar{n}}{[A]}$ M^{-1}
	C_A mM	C_M mM	C_A mM	C_M mM	C_A mM	C_M mM	[A] mM			pot.
2.0	43.5	47.9	24.5	24.4	13.8	12.4	4.0	0.82	205	192
2.25	48.5	47.6	28.0	24.3	16.0	12.4	5.0	0.93	185	175
2.5	54.0	47.4	31.0	24.2	18.5	12.3	6.0	1.02	170	165
2.75	59.0	47.0	34.5	24.1	21.0	12.2	8.0	1.10	138	144
3.0	65.0	46.7	38.5	24.0	24.0	12.2	9.5	1.20	126	133
3.25	70	46.5	42.0	24.0	27.0	12.2	11.2	1.28	114	122
3.5	76	46.2	46.0	23.9	30.0	12.1	13.5	1.35	100	110
3.75	81	45.9	50.0	23.8	33.5	12.1	16.2	1.40	87	98
4.0	87	45.7	54	23.7	37	12.0	19.0	1.50	79	89
4.5	98	45.1	62.5	23.5	44.5	11.9	25	1.62	65	75
5.0	111.5	44.4	72.5	23.1	52.5	11.8	30.5	1.83	60	66
5.5	128	43.6	84	22.9	62	11.7	38	2.05	54	57
6.0	145	42.8	99	22.6	76	11.6	49	2.25	46	48
6.5	163	41.8	116	22.2	93	11.3	65	2.3	35	38

Table 5. The complexity constants of the rare-earth glycolate systems with their random errors (temperature = 20°C, ionic strength = 2 M).

Rare earth	β_1 M^{-1}	$\beta_2 \times 10^{-3}$ M^{-2}	$\beta_3 \times 10^{-4}$ M^{-3}	$\beta_4 \times 10^{-5}$ M^{-4}	$\beta_5 \times 10^{-5}$ M^{-5}
A. Potentiometric investigation					
57 La	155 ± 8	5.7 ± 0.4	6.5 ± 0.6	1.2 ± 0.3	≲ 0.7
58 Ce	225 ± 10	10.5 ± 0.7	14 ± 1.5	3.5 ± 0.7	≲ 2
59 Pr	270 ± 15	15.5 ± 1	25 ± 3	9 ± 2	≲ 5
60 Nd	320 ± 20	22 ± 2	37 ± 5	11 ± 4	≲ 5
62 Sm	360 ± 20	34 ± 2	72 ± 8	28 ± 5	≲ 10
64 Gd	300 ± 15	27 ± 2	62 ± 8	26 ± 5	≲ 10
66 Dy	330 ± 20	30 ± 3	79 ± 10	30 ± 5	≲ 20
67 Ho	350 ± 20	30 ± 3	81 ± 10	27 ± 5	≲ 30
68 Er	400 ± 20	38 ± 3	98 ± 15	30 ± 5	≲ 30
70 Yb	520 ± 30	66 ± 5	210 ± 25	60 ± 10	≲100
B. Extinctiometric investigation					
60 Nd	350 ± 50	25 ± 5	~ 20		

As the function $\bar{n}/[A] = f([A])$ is very steep for low values of [A] for the rare-earth glycolate systems, it is not possible to extrapolate $\bar{n}_\varepsilon/[A]$ to [A] = 0 from [A] = 4×10^{-3} M, the lowest value at which $\bar{n}_\varepsilon/[A]$ could be determined. In the computation of the X-function (*cf.* eqn. II(7)), [A] = 0.004 M has been chosen as the inferior limit of the integration

$$\ln \frac{X([A])}{X(0.004)} = \int_{0.004}^{[A]} \frac{\bar{n}_\varepsilon}{[A]} \, d([A]) \qquad (9)$$

In this way $X([A])/X(0.004)$ has been computed. The value of $X(0.004)$ was obtained by extrapolation of the function $X([A])/X(0.004) = f([A])$ to [A] = 0. As $X(0) = 1$ according to eqn. II(5), we have $X(0)/X(0.004) = 1/X(0.004)$. The extrapolation gave $1/X(0.004) = 0.35 \pm 0.03$. After the calculation of the X-function the complexity constants have been computed in the same way as before (Table 5).

The complexity constants have also been computed directly by means of eqn. II(4a) from the values of $\bar{n}_\varepsilon$ obtained for [A] = 4, 6, 15, and 30 mM ($\bar{n}_\varepsilon$ = 0.82, 1.02, 1.37, and 1.82, respectively). Assuming that only three complexes are formed at these low concentrations of the free ligand, we get $\beta_1 = 350$, $\beta_2 = 28 \times 10^3$, and $\beta_3 \approx 2 \times 10^5$, in good accordance with the constants in Table 5.

RESULTS

The complexity constants of the rare-earth glycolate systems are found in Table 5. The stability constants and the ratios for consecutive stability constants are shown in Table 6.

Table 6. The stability constants ($b_1 = \beta_1$, $b_n = \beta_n/\beta_{n-1}$ for $n \geq 2$) and the ratio b_n/b_{n+1} between the consecutive stability constants of the rare-earth glycolate systems.

Rare earth	b_1	b_2	b_3	b_4	b_1/b_2	b_2/b_3	b_3/b_4
La	155	37	11.5	1.8	4.2	3.2	6
Ce	225	46.5	13.3	2.5	4.8	3.5	5.5
Pr	270	57.5	16.1	3.6	4.7	3.6	4.5
Nd	320	69	16.8	3.4	4.6	4.1	5
Sm	360	94	22.5	3.9	3.8	4.2	5.5
Gd	300	90	23.0	4.2	3.3	3.9	5.5
Dy	330	91	26.5	3.8	3.6	3.5	7
Ho	350	86	27	3.3	4.1	3.2	8
Er	400	95	26	3.1	4.2	3.7	8
Yb	520	127	32	2.9	4.1	4.0	11

A comparison between the complexity constants of the glycolate and the acetate systems (Tables II:13 and II:14) shows that the glycolate systems, especially those of the heavier rare earths, are complex to a much higher degree than the corresponding acetate systems.

The complexity constants β_1, β_2 and β_3 of the higher rare-earth glycolates vary with the atomic number of the rare earth in another way than the constants of the corresponding acetates, as shown in Fig. 5. β_1, β_2 and β_3 of the glycolate systems increases from Gd to Yb, contrary to the corresponding constants of the acetate systems.

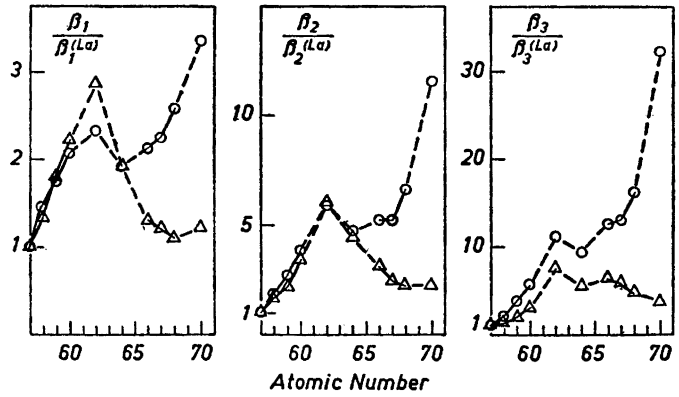


Fig. 5. The variation of β_1, β_2 and β_3 for the acetate and the glycolate systems with the atomic number of the rare earth. In order to facilitate a comparison between the variations of the complexity constants of the two systems, the quotients $\beta_n/\beta_n^{(La)}$ have been chosen as ordinates, $\beta_n^{(La)}$ denotes the complexity constant of the lanthanum system in question. ---- O: the glycolate systems; △: the acetate systems. The symbols have been linked together with a broken line if the complexity system has not been examined for a rare earth lying between them.

Statistical calculations indicate, according to Bjerrum [7], that we ought to have the following relation between the stability constants for systems with simply bound ligands

$$b_1 : b_2 : b_3 : b_4 : b_5 : b_6 = 6 : \frac{5}{2} : \frac{4}{3} : \frac{3}{4} : \frac{2}{5} : \frac{1}{6}$$

It is expected from purely statistical reasons that the quotients b_n/b_{n+1} should have greater values in systems with chelating ligands than in systems with only simply bound ligands, as the chelating ligand leaves fewer sites vacant for coordination, where the next ligand can be bound, than does the simply bound ligand. If the central ion has six octahedrically-arranged coordination sites and if the ligand occupies two adjacent coordination sites, we ought to have, according to Bjerrum [7]

$$b_1 : b_2 : b_3 = 12 : 2 : 1/3$$

The values of b_n/b_{n+1} of the glycolate systems are not so high as six, and moreover they do not differ much from the quotients b_n/b_{n+1} of the corresponding acetate systems. It may, however, be noticed that b_1/b_2 and b_2/b_3 for the glycolate systems of Dy, Ho, Er, and Yb are somewhat higher than the corresponding values for the acetate systems.

Because of the high values of β_n for the glycolate systems relative to β_n for the corresponding acetate systems, although glycolic acid is stronger than acetic acid, it is probable that the glycolate ion acts as a chelating ligand, even if the quotients b_n/b_{n+1} do not have the expected values.

I wish to express my thanks to Professor Sven Bodforss and to Professor Sture Fronæus for their kind interest in this work.

This investigation has been supported by a grant from the *Swedish Natural Science Research Council*, which is gratefully acknowledged.

REFERENCES

1. Sonesson, A. *Acta Chem. Scand.* **12** (1958) 165 (= I).
2. Sonesson, A. *Acta Chem. Scand.* **12** (1958) 1937 (= II).
3. Sundén, N. *Svensk Kem. Tidskr.* **65** (1953) 257.
4. Ahrland, S. *Acta Chem. Scand.* **7** (1953) 485.
5. Jantsch, G. and Grünkraut, A. *Z. anorg. Chem.* **79** (1913) 305.
6. Fronæus, S. *Komplexsystem hos koppar* (Diss.), Lund 1948.
7. Bjerrum, J. *Metal Ammine Formation in Aqueous Solution* (Diss.), Copenhagen 1941, p. 40.

Received March 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1011–1018

High Molecular Weight Enzyme Inhibitors

IV. Polymeric Phosphates of Synthetic Estrogens

E. DICZFALUSY, O. FERNÖ, H. FEX, B. HÖGBERG and P. KNEIP

Research Department, AB Leo, Hälsingborg and Hormone Laboratory, Department of Women's Diseases, Karolinska Sjukhuset, Stockholm, Sweden

The preparation and some of the biochemical and pharmacological properties of polydiethylstilbestrol phosphate and of some other polymeric phosphates of closely related synthetic estrogens are described. These compounds are very potent inhibitors of certain enzymes, such as acid and alkaline phosphatases, hyaluronidase and β-glucuronidase.

When injected systemically to spayed mice, the polymeric phosphates studied, especially polydiethylstilbestrol phosphate, exhibited a prolonged duration of estrogenic activity. When administered by the oral route, their estrogenic effect was negligible.

In the third paper of these series, the synthesis and properties of polyestradiol phosphate (P.E.P.) were described [1]. The greatly prolonged estrogenic activity of this compound [2] suggested a study of similar polymers prepared from synthetic estrogens. The synthesis of one of these polymers has previously been reported from our laboratories [3].

In the present paper the polymeric phosphates of diethylstilbestrol [3,4-bis(*p*-hydroxyphenyl)-3-hexene], dienestrol [3,4-bis(*p*-hydroxyphenyl)-2,4-hexadiene], hexestrol [3,4-bis(*p*-hydroxyphenyl)-hexane] and benzestrol [3-ethyl-2,4-bis(*p*-hydroxyphenyl)-hexane] are described. These polymers were prepared practically in the same way as P.E.P. Some of the properties of these compounds are shown in Table 1. In Table 2 the antienzymic activity and the duration of estrogenic action of these compounds are compared with that of P.E.P.

It appears that among the polymers of synthetic estrogens, polydiethylstilbestrol phosphate has by far the most prolonged duration of activity. Therefore the present investigation will mainly deal with this compound. The polydiethylstilbestrol phosphate preparation obtained following phosphorylation with 1.1 mole phosphoryl chloride for 70 h will hereafter be denoted as P.S.P.

Table 1. Some of the chemical properties of polymeric phosphates prepared from synthetic estrogens.

No. Compound	Molar ratio $POCl_3$: estrogen	Reaction time, h	% Non-dialyzable $P_{org.}$	% P a)	% Free Hydroxyl groups b)	% Hydrolyzable phosphorus c) $[-P(O)(OH)_2]$	% Phosphorus as d) $\rangle PO$	$>P(O)OH$	$-P(O)(OH)_2$
1. P.E.P. Standard	1.1	70	88–90	9.5	0	28–30	10	60	30
2. Polydiethylstilbestrol phosphate	0.8	70	89	7.6	10	3	23	72	5
3. Polydiethylstilbestrol phosphate	0.9	70	85	8.2	10	7	17	75	8
4. Polydiethylstilbestrol phosphate	1.0	70	87	8.9	4	14	11	76	13
5. Polydiethylstilbestrol phosphate	1.1	5	66	9.0	9	30	11	59	30
6. Polydiethylstilbestrol phosphate	1.1	25	79	9.6	—	25	9	65	26
7. Polydiethylstilbestrol phosphate (P.S.P.)	1.1	70	83	9.8	0	25	8	68	24
8. Polydiethylstilbestrol phosphate	1.2	70	79	10.4	—	35	6	61	33
9. Polydienestrol phosphate	1.1	70	75	9.4	—	23	13	63	24
10. Polyhexestrol phosphate	1.1	70	77	9.5	—	24	16	60	24
11. Polybenzestrol phosphate	1.1	70	81	8.4	—	24	—	—	—
12. Diethylstilbestrol diphosphate	—	—	—	14.7	0	100	0	0	100
13. Honvan (R)	—	—	—	—	—	—	—	—	—

a) Calculated on samples free from moisture, pyridine and chlorine.
b) According to B. Baggett *et al.*[14] Calculated as % of 'estrogen oxygens'.
c) Hydrolysis at 100° for 27 h at pH 5.0 (0.1 M acetate buffer).
d) From titration experiments. Results corrected for pyridine and chlorine.

The molecular weight of P.S.P. was found to be about 14 000 (ultracentrifuge) indicating that the polymer contains approximately 40 diethylstilbentrol moieties. The viscometric behavior of P.S.P. was found to be the same as that of P.E.P.[1], that is typical for linear polyelectrolytes.

Hydrolysis experiments with P.S.P. performed in the same way as previously described for P.E.P.[1] seem to indicate that also in this case the liberated inorganic phosphorus is formed from primary phosphoric acid esters only.

Titration experiments indicated that a certain degree of branching occurs (Table 1).

EXPERIMENTAL

A. Preparation of polymers

1. Preparation of polydiethylstilbestrol phosphates. This was carried out practically in the same way as previously described for the preparation of P.E.P. Standard[1]. In the

preparation of different batches the molar ratio $POCl_3$/diethylstilbestrol was varied between 0.8 and 1.2. Dialysis was carried out against dist. water containing 1 % NaCl, and the polydiethylstilbestrol phosphate preparations were precipitated by HCl without any addition of ethanol. Using molar ratios of $POCl_3$/diethylstilbestrol between 0.8 and 1.0 it was found that the mixture had changed into a jelly after 10 to 20 h reaction time. After hydrolysis of these jellies with ice it was necessary to heat on a steam bath for 1—2 h with stirring in order to obtain a clear solution (no inorganic phosphorus was liberated during heating). No jelly formation took place when the molar ratio of $POCl_3$/diethylstilbestrol was higher than 1.0. With a reaction time of 70 h the yields from 5 g diethylstilbestrol varied between 5.5 and 5.7 g of crude polymer. The various polydiethylstilbestrol phosphates were analyzed for moisture, *(in vacuo* 100°; found 1—2 %), pyridine (determined spectrophotometrically after alkalization and distillation; found 1—3 %), chlorine (found 0—0.5 %) and phosphorus (Table 1).

The polydiethylstilbestrol phosphate obtained with 1.1 mole $POCl_3$ and with a reaction time of 70 h (P.S.P.) has a melting range of 195—205°.

Analysis of P.S.P.: Moisture: 1.7 %; pyridine: 2.9 %; chlorine: 0.3 %; P, C and H (calculated on sample free from moisture, pyridine and chlorine): 9.8; 63.1 and 5.8 %, respectively. P.S.P. (free from moisture, pyridine and chlorine) should thus contain 78.3 % diethylstilbestrol. Molar ratio P/diethylstilbestrol: 1.08.

P.S.P. is very soluble in aqueous pyridine, soluble in aqueous alkali, slightly soluble in dry pyridine, ethanol and ethanol-water (1:1) and very slightly soluble in water, dioxane, acetone and chloroform.

2. Polydienestrol phosphate, polyhexestrol phosphate and polybenzestrol phosphate. All these polymers were prepared in the same manner as described for P.S.P. (1.1 mole $POCl_3$, 70 h at room temperature). No jelly was formed.

3. Diethylstilbestrol diphosphate. This was prepared by the method of Miescher *et al.*[4] It was purified over the sodium salt. M.p. 213—215°. [Found: P 14.7 %; equiv. wt 108 (as a dibasic acid) and 105 (as a tetrabasic acid). Calc. for $C_{18}H_{22}O_8P_2$: P 14.5 %; equiv. wt 107.]

Hydrolysis experiments. These were run at 100° between pH 3 and 10 (0.1 M buffer solution) and in 0.2 N sodium hydroxide solution. The rate of hydrolysis was followed by determining the amount of inorganic phosphorus (P_o) liberated. The formation of P_o was most rapid at pH values between 3 and 6, practically all P_o being liberated within 10 h. Practically no P_o was liberated on hydrolysis in 0.2 N NaOH solution.

At pH 5.0 (acetate buffer) the hydrolysis of diethylstilbestrol diphosphate was found to follow a first order reaction with $k = 0.87$ h^{-1}. Under the same conditions when calculated on hydrolyzable phosphorus only (Table 1), the rate of hydrolysis for various polydiethylstilbestrol phosphate preparations was found to be virtually the same. Polydienestrol phosphate, polyhexestrol phosphate and polybenzestrol phosphate were found to have k-values of 0.94, 0.71 and 0.71 h^{-1}, respectively.

Molecular weight, viscosity measurements and potentiometric titration. All these measurements have been carried out in the same manner as previously described for P.E.P. Standard [1]. For P.S.P. the following values were found. In 0.2 M phosphate buffer solution at pH 7.1, $S^\circ_{20} = 1.4 \times 10^{-13}$; $D_{20} = 7.8 \times 10^{-7}$; $V_{20} = 0.69$ *. The molecular weight was calculated to be approximately 14 000. The intrinsic viscosity of the sodium salt was found to be $[\eta] = 0.04$ in 0.25 M NaCl solution at pH 7.3. The amount of primary phosphoric acid esters found in titration experiments corresponded well to the values obtained in hydrolysis experiments (Table 1).

B. Antienzymic properties

The enzymes studied were acid phosphatase, alkaline phosphatase, hyaluronidase and β-glucuronidase. With the exception of the last mentioned enzyme, the experimental conditions and the preparations used have been described in detail in a previous communication [1].

* The estimation of the sedimentation constant has been carried out in the Carlsberg Laboratories, Copenhagen, through the courtesy of Professor K. Linderstrøm-Lang.

Table 2. Some of the biochemical and pharmacological properties of the compounds studied.

No.	Rel. amount (P.E.P. = 1.0) of inhibitor necessary for 50 % inhibition				Duration of estrogenic effect a)
	Hyaluronidase	Alkaline phosphatase	Acid phosphatase	β-Glucuronidase (bacterial)	
1. (P.E.P.)	1.0	1.0	1.0	1.0	100
2.	1.0	113	1.0	0.36	63
3.	0.7	40	0.6	0.13	100
4.	0.6	27	—	0.12	100
5.	0.7	10	1.0	—	97
6.	0.7	17	0.7	—	104
7. (P.S.P.)	0.6 b)	17 b)	0.6 b)	0.14	102
8.	0.7	15	0.5	0.12	107
9.	0.7	43	1.2	0.20	41 c)
10.	0.7	87	0.6	0.28	24 c)
11.	1.0	57	1.1	0.39	30 c)
12.	800	19	1 000	>12	—
13.	160	18	46	>12	1 c)

a) Potency is expressed as a percentage of the effect of P.E.P. Standard (Preparation No. 1).
b) Practically unchanged after hydrolysis (27 h, pH 5.0, 100°).
c) When assayed against P.S.P. these compounds show a significant deviation from parallelism. Thus these assays are statistically not valid, and the potency estimates are only approximate values calculated from the results obtained at the same dose levels (5 and 10 μg, respectively). These data are included only to demonstrate that the potency of these compounds is much less than that of P.S.P.

β-Glucuronidase. For the measurement of enzyme activity the liberation of phenolphtalein from 0.0005 M phenolphtalein glucuronide in 0.05 M acetate buffer was estimated by a modification [5] of the method of Talalay, Fishman and Huggins [6]. The volume of the incubation mixture was 1.5 ml and after 30 min at 37° the reaction was stopped and the colour developed by the addition of 5 ml of 0.2 M glycine buffer (pH 10.4).

Enzyme. Bacterial β-glucuronidase was a purified commercial product from Sigma Chemical Co., St. Louis, U.S.A. Another enzyme prepared from the common limpet *(Patella vulgata)* according to the method of Levvy, Hay and Marsh [7] was also used in some of the experiments.

Results. The relative amounts of inhibitors necessary for 50 % inhibition of the enzymes studied are shown in Table 2. In all cases the enzyme inhibition by P.S.P. could be reversed by basic proteins, *e. g.* protamine sulphate. The type of inhibition was studied by preparing classical Lineweaver plots [8]. The results are summarized in Table 3.

Table 3. Type of inhibition of different enzymes studied.

Compound	Alkaline phosphatase at pH 9.3	Acid phosphatase at pH 4.5	Bacterial β-glucuronidase at pH 7.0
Polyestradiol phosphate (P.E.P.)	competitive	non-competitive	competitive
Polydiethylstilbestrol phosphate (P.S.P.)	»	non-competitive	»
Polyhexestrol phosphate	»	—	»
Diethylstilbestrol-4,4′-diphosphate	»	competitive	»
Estradiol-3,17-diphosphate	»	»	»

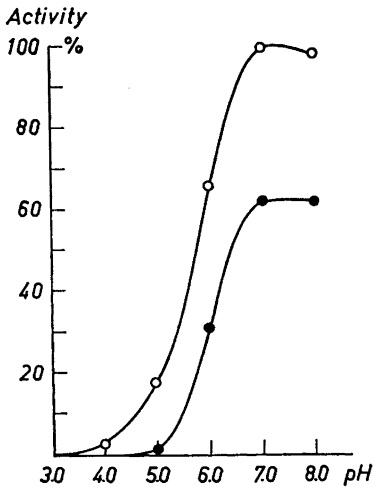


Fig. 1. Hydrolysis of 0.0005 M phenolphtalein glucuronide by bacterial β-glucuronidase (1.15 mg) at various pH values in 0.05 M acetate buffer in the absence (○) and presence (●) of polydiethylstilbestrol phosphate (100 μg P.S.P./1.5 ml).

1. Acid phosphatase. The inhibition of acid phosphatase was studied at pH 4.5 using disodium *p*-nitrophenyl phosphate as a substrate.

It appears from the data of Table 2 that the inhibitory potency of the different polymeric phosphates of synthetic estrogens is of the same order as that of P.E.P. On the other hand, diethylstilbestrol diphosphate is a very poor inhibitor of the enzyme, a fact which is in close agreement with previously reported data on estradiol diphosphate [1]. It is of interest, however, that a commercial diethylstilbestrol diphosphate preparation (Honvan®, Asta-Werke A-G.) exhibited a considerably stronger inhibition than the product synthetized by us.

The inhibition of acid phosphatase by P.S.P. is non-competitive. This is in agreement with previous data on the type of inhibition by P.E.P. On the other hand, diethylstilbestrol diphosphate, and also estradiol-3,17-diphosphate [1] inhibit the enzyme in a substrate-competitive manner.

2. Alkaline phosphatase. The enzyme used was a highly purified commercial product of Sigma Chemical Co., St. Louis, U.S.A., prepared from calf intestinal mucosa. It appears from Table 2 that the polymeric phosphates of synthetic estrogens are much weaker inhibitors of alkaline phosphatase than of acid phosphatase, when compared to P.E.P. It is of interest to note that in contrast to the results obtained with acid phosphatase the inhibition of alkaline phosphatase by diethylstilbestrol diphosphate is of the same order as that by P.S.P. It is also of interest that the use of smaller amounts of $POCl_3$ in the phosphorylation of diethylstilbestrol seems to result in compounds exhibiting a decreased antienzymic activity with regard to alkaline phosphatase, but not to acid phosphatase. The inhibition of alkaline phosphatase by P.S.P. as well as by diethylstilbestrol diphosphate and also by polyhexestrol phosphate is substrate-competitive.

3. Hyaluronidase. It is of interest to note the close correlation between the inhibitory activity of the different compounds on hyaluronidase on the one hand, and on acid phosphatase on the other hand.

4. β-Glucuronidase. The inhibition of bacterial β-glucuronidase by P.S.P. at different pH-values is illustrated in Fig. 1. The same type of curve was obtained when P.E.P. was used as an inhibitor. The schematic curves showing per cent inhibition at pH 7.0 as a function of inhibitor concentration are presented in Fig. 2. Finally Fig. 3 shows the variation in enzymic activity of the limpet β-glucuronidase at different pH-values and the inhibitory effect of P.S.P. and P.E.P. thereupon.

As far as the type of enzyme inhibition is concerned, it was found that all the compounds studied (P.S.P., P.E.P., polyhexestrol phosphate, diethylstilbestrol diphosphate) inhibited the bacterial β-glucuronidase (at pH 7.0) in a competitive manner.

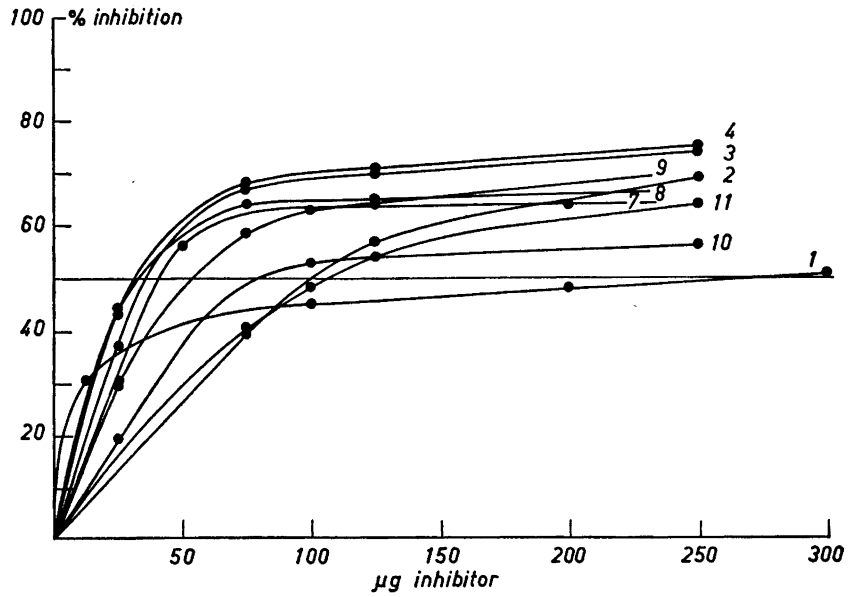


Fig. 2. Per cent inhibition of bacterial β-glucuronidase (1.5 mg) by the substances listed in Table 1. 0.05 M acetate buffer pH 7.0. Substrate 0.0005 M phenolphtalein glucuronide. Incubation time 30 min at 37°.

C. Pharmacological properties

Toxicity. The acute toxicity of P.S.P. was estimated in spayed female mice 48 h following a single intravenous injection. LD 50 was found to be 252 mg/kg, with 95 % fiducial limits of error at 232 and 273 mg/kg, respectively.

When male albino rats were injected intramuscularly with doses up to 10 mg/kg of P.S.P., and sacrificed 10 weeks after the injection, no gross pathological changes could be seen either at autopsy or on microscopical examination, except those attributable to the typical action of an efficient and prolonged estrogen such as decrease in the weight of accessory reproductive organs and gynaecomastia in the highest dosages administered. Thus, the toxicity of P.S.P. seems to be of the same order as that of P.E.P.[1] The low toxicity of P.S.P. suggests that it can probably be administered also to human beings without any major harmful effects.

Biological activity. The duration of action of the various polymeric phosphates was estimated by the use of a previously described method based on the duration of vaginal cornification in spayed mice [9]. The relative potencies of the different compounds when compared to the activity of P.E.P. are summarized in Table 2. It appears from the data of Table 2 that polymeric phosphates prepared from dienestrol, hexestrol or benzestrol exhibit a much shorter duration of estrogenic activity than similar compounds prepared from diethylstilbestrol. It also appears that the commercial preparation of diethylstilbestrol diphosphate (Honvan ®) is virtually devoid of prolonged estrogenic activity. When P.S.P. was hydrolyzed at 100° for 27 h at pH 7.6 (21.2 % P_o liberated), its biological potency remained unchanged. It is also of interest that the polymeric phosphates prepared from dienestrol, hexestrol or benzestrol are much weaker long-acting estrogens than P.S.P. These compounds also exhibit a greatly reduced *anti*-alkaline-phosphatase activity, and a somewhat reduced *anti*-β-glucuronidase activity when compared to P.S.P. However, their effect on hyaluronidase and acid phosphatase appears to be of the same order. It also appears from Table 2 that polyestradiol phosphate and polydiethylstilbestrol phosphate are equipotent long-acting estrogens.

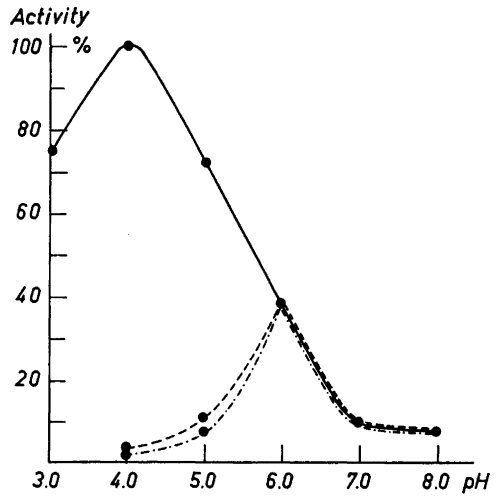


Fig. 3. Hydrolysis of 0.0005 M phenolphtalein glucuronide by limpet β-glucuronidase (112 Fishman units) at various pH values in 0.05 M acetate buffer in the absence and presence of inhibitors; ●——● no inhibitor; ● - - - ● P.E.P. Standard 100 μg/1.5 ml, ● — . — ● P.S.P. 100 μg/1.5 ml. Incubation time 30 min.

Attempts have also been made to estimate the ED 50 of P.S.P. when given orally. The ED 50 of P.S.P. by the oral route was found to be 3.9 mg/kg, that of diethylstilbestrol diphosphate 0.36 mg/kg, whereas that of diethylstilbestrol was 0.1 mg/kg. When P.S.P. was submitted to hydrolysis at 100° for 74 h at pH 6.7 (24.3 % P_o liberated), its ED 50 by the oral route was still 3.6 mg/kg, indicating that previous hydrolysis has very little, if any, effect on the absorption of the P.S.P. molecule.

In other experiments P.S.P. was administered to spayed mice by the oral route in dosages of 5 mg/kg and 10 mg/kg, respectively. Vaginal smears taken from these animals either could not reveal any estrogenic activity, or showed just a very short, transient estrogenic reaction with a mean duration of 0.8 and 1.1 days, respectively. It seems therefore safe to conclude that orally administered P.S.P. is not absorbed to any appreciable extent. These results have also been confirmed in rats using tritium-labelled P.S.P.[10]

DISCUSSION

In a previous communication[1] where the preparation of P.E.P. was described the molar ratio between $POCl_3$ and estradl-1-io7β was kept constant at 1.1. Under those conditions it was found necessary to employ a reaction time of 20 h or more, in order to obtain compounds exhibiting maximally prolonged estrogenic activity. It appears from the data of the present study that a reaction time of 5 h is already sufficient to obtain polydiethylstilbestrol phosphate preparations of maximal activity. It was also found that preparations with maximally prolonged activity could be obtained in all experiments, where the $POCl_3$/diethylstilbestrol ratio was kept between 0.9 and 1.2. Ratios below 0.9 resulted in compounds with diminished activity. On the other hand, highly active non-dialyzable preparations could be obtained even by the use of $POCl_3$/diethylstilbestrol ratios much higher than 1.2.

As to P.E.P. we have already suggested[1] the possibility that the molecular weight of the compound might be considerably lower than indicated by the

results of ultracentrifugation. A similar analysis of the present data seems to speak very much in favour of the possibility that also P.S.P. might occur in form of an association of smaller molecules.

A comparison between polydiethylstilbestrol phosphate and polyestradiol phosphate reveals marked differences as far as the antienzymatic properties are concerned. Whereas the antihyaluronidase or *anti*-acid-phosphatase activities appear to be approximately equal, polydiethylstilbestrol phosphate is a much weaker inhibitor of alkaline phosphatase than polyestradiol phosphate. As far as the inhibition of β-glucuronidase is concerned, the opposite relationship can be observed, polydiethylstilbestrol phosphate being a much stronger inhibitor than polyestradiol phosphate.

The inhibition of acid phosphatase by P.S.P. was found to be non-competitive. It is of interest that Hummel *et al.* found that the inhibition of this enzyme by polyxenyl phosphate is also of the non-competitive type [11].

As to the type of enzyme inhibition by the various compounds the inhibition of alkaline phosphatase by all compounds studied is competitive. This seems to be at variance with previously reported data from our laboratories [12], where the inhibition of kidney alkaline phosphatase by estradiol diphosphate was described as non-competitive. It should be noted. however, that neither the source of enzyme, nor the experimental conditions were identical in the present investigation with those published by Aldman *et al.*[12]

It also appears from the data of the present investigation that the majority of compounds studied are very potent inhibitors of β-glucuronidase. The inhibition is of the substrate-competitive type. This inhibition seems to be of interest in view of the fact that higher amounts of β-glucuronidase have been found in human cancer tissue than in normal tissues [13].

Acknowledgement. The excellent technical assistance of Miss Leila Nilsson, Mr. S.-Å. Johansson, Mr. K.-E. Lundvall and Mr. A. Olsson is gratefully acknowledged.

REFERENCES

1. Fernö, O., Fex, H., Högberg, B., Linderot, T., Veige, S. and Diczfalusy, E. *Acta Chem. Scand.* **12** (1958) 1675.
2. Diczfalusy, E. *Endocrinology* **54** (1954) 471.
3. Fernö, O., Fex, H., Högberg, B., Linderot, T. and Rosenberg, T. *Acta Chem. Scand.* **7** (1953) 921.
4. U.S. Patent 2 234 311 (1941).
5. Sigma Bulletin 105.
6. Talalay, P., Fishman, W. H. and Huggins, C. *J. Biol. Chem.* **166** (1946) 757.
7. Levvy, G. A., Hay, A. J. and Marsh, C. A. *Biochem. J.* **65** (1957) 203.
8. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
9. Diczfalusy, E., Magnusson, A.-M., Nilsson, L. and Westman, A. *Endocrinology* **60** (1957) 581.
10. Perklev, T. *To be published.*
11. Hummel, J., Anderson, D. and Patel, C. *J. Biol. Chem.* **233** (1958) 712.
12. Aldman, B., Diczfalusy, E., Högberg, B. and Rosenberg, T. *Biochem. J.* **49** (1951) 218.
13. Fishman, N. H., in Sumner, I. B. and Myrbäck, K. *The Enzymes*, Academic Press, New York 1950, Vol. 1 p. 635.
14. Baggett, B., Engel, L. and Fielding, L. *J. Biol. Chem.* **213** (1955) 87.

Received February 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1019–1030

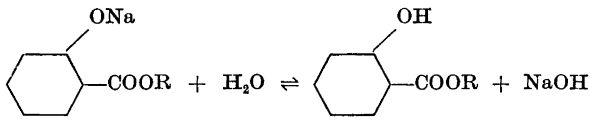
The Hydrolysis of Alkyl Salicylates in Alkaline Solutions

LAURI PEKKARINEN and EERO TOMMILA

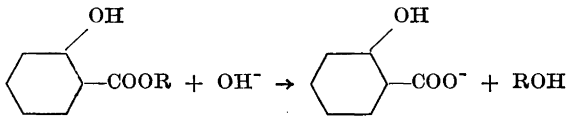
Institute of Chemistry, University of Helsinki, Helsinki, Finland

The alkaline hydrolysis of methyl, ethyl and *iso*propyl salicylates has been investigated in pure water and in various methanol-water, ethanol-water, *iso*propanol-water and acetone-water mixtures at different temperatures. All facts sustain the proposition suggested by Goldschmidt and Scholtz in 1907 that in solutions the reacting substance is the free ester, which is in equilibrium with its ionized form. Beside this reaction, a simple bimolecular process between the ester ion and hydroxyl ion has a subordinate importance. The alkaline hydrolysis of *p*- and *m*-hydroxybenzoates takes place practically solely as a reaction between the ester ion and hydroxyl ion.

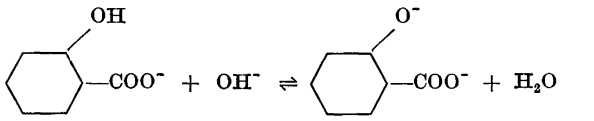
In 1907, Goldschmidt and Scholtz [1] observed that methyl salicylate in alkaline solution undergoes hydrolysis by first-order kinetics. On the basis of Goldschmidt's earlier investigations on the hydrolysis of sodio-acetoacetate [2], they concluded that the reacting substance is not the sodium compound but the free ester with which the former is in equilibrium:



I



II



III

They showed mathematically that this mechanism leads to first-order kinetics. Suppose that a is the initial total concentration of the ester and b that of the alkali hydroxide, ξ the concentration of the free ester and η that of the disodi-

um salt at any instant, and x the decrease in the concentration of the ester after a lapse of time t. Then according to the law of mass action, since the concentration of water can be taken as constant, we have

$$\frac{[\text{Free ester}] \times [OH^-]}{[\text{Ester ion}] \times [H_2O]} = \frac{\xi\ (b+\xi-\eta)}{(a-x-\xi)} = K \qquad (1)$$

The velocity equation is then given by

$$\frac{dx}{dt} = k\xi[OH^-_{\text{A}}] = kK(a-x-\xi) \qquad (2)$$

Taking into account that $\xi \ll (a-x)$, and writing $kK = k_1$, we obtain

$$\frac{dx}{dt} = k_1(a-x) \qquad (3)$$

i.e. an equation of the first order.

There is a considerable body of evidence in support of Goldschmidt's hypothesis. To-day, however, the well-known ability of salicylic acid and its esters to form chelate rings must be taken into account. The purpose of the present investigation is to study this reaction from different points of view and so gain insight into its mechanism. The reaction was investigated with different salicylic esters and under various conditions, and some kinetic measurements were also carried out with ethyl *m*- and *p*-hydroxybenzoates.

EXPERIMENTAL

Esters. The esters were prepared from the corresponding acid and alcohol by the usual method. The salicylic esters were purified by repeated distillation at reduced pressure. Their refractive indices were identical with the best values reported in the literature. The *m*- and *p*-hydroxybenzoates were crystallized from ethanol. Their melting points (72° and 115.2°) agreed with those given in the literature.

The alkali hydroxides were of a high degree of purity. The alkali solutions used were as carbonate-free as possible.

Solvents. The water used in the kinetic experiments was freshly distilled and freed from carbon dioxide by brief boiling. The methanol and ethanol were purified by the method of Walden [3]. The *iso*propanol was treated with solid sodium hydroxide and distilled. Merck's acetone "pro analysi" was used as received.

Method. The reaction vessels and method used in most of the kinetic experiments have been previously described [4]. In general, equal volumes of an a M ester solution and an $(a + b)$ M alkali hydroxide solution were used, so that after mixing the concentration of the ester salt was $a/2$ and that of the hydroxyl ions $b/2$. The reaction was stopped with an excess of 0.02 N HCl and the mixture titrated back with 0.02 N baryta solution, cresol red being used as indicator. For ethyl *m*- and *p*-hydroxybenzoates, however, methyl red was used as indicator, and the titration carried to a standard tint. The experiments at the greatest dilution were conducted in large flasks from which the samples were withdrawn with a pipette. The experiments in methanol-water and ethanol-water at 50° were carried out in sealed ampoules of Pyrex glass and filled with nitrogen. The reaction mixtures were carefully protected from atmospheric carbon dioxide.

The electrically controlled thermostats were constant to within ±0.01°. The thermometers were checked against a thermometer calibrated at the National Bureau of Standards (U.S.A.).

RESULTS AND DISCUSSION

1. The order of the reaction. Goldschmidt's observation concerning the order of the reaction was confirmed. The first-order formula gave a good constancy for k, as is exemplified by the following run (solvent: water; [sodium salt of the ester] = [NaOH] = 0.04 mole/l; temperature 25°):

Time, min.	50	82	100	120	144 ½	195	272	346
Change, %	19.0	29.5	34.5	38.5	45.5	55.5	68.5	76.5
10^7 k, s^{-1}	725	710	705	680	700	690	700	697

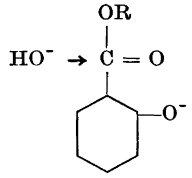
Moreover, within the limits of experimental error, in dilute solutions, the time of half completion of the reaction is independent of the initial concentrations, as the following figures show (water, 25°):

$[C_6H_4(ONa)COOEt]$ = [NaOH], mole/l	0.001	0.0025	0.005	0.01	0.02	0.04
t, min.	175	174	171	165	168	165

For the alkaline hydrolysis of ethyl *p*-hydroxybenzoate ion and ethyl *m*-hydroxybenzoate ion, the second order formula always gave a good constancy for k.

The results obtained with the ester salts are summarised in Tables 1—3.

2. The mechanism of the reaction. As already mentioned, many facts are in conformity with the reaction mechanism proposed by Goldschmidt according to which the reaction occurs between the free ester and hydroxyl ion. As is easily seen, a reaction between the ester ion (or chelate) and hydroxyl ion would exhibit second-order kinetics. The experimental results, however, as we have seen, reveal that the reaction follows a first-order rate law. In fact, the free charge on the oxygen atom of the ionized phenol group in the *ortho*-position generates a large negative charge on the carbonyl carbon in the ester ion, and this causes a very powerful repulsion between this carbon atom and the attacking hydroxyl ion:



This results in a high activation energy and small reaction velocity. A similar case occurs in the alkaline hydrolysis of ethyl *p*-hydroxybenzoate [5]. In the case of the ethyl *m*-hydroxybenzoate, the negative charge on the carbonyl carbon is less, because only the inductive effect is operative, and the effect on the activation energy is correspondingly weaker. The values in pure water [6] and in 56 % aqueous acetone [5] are seen from the following figures:

	Water			*56 % acetone*		
	10^4k_{25}	E cal	log A	10^4k_{25}	E cal	log A
(H)	293	12 680	7.76	26.9	14 560	8.18
p-O^-	2.63	13 510	6.32	0.0870	17 300	7.61
m-O^-	40.2			4.73	15 450	8.04

Table 1. Alkaline hydrolysis of ethyl salicylate. A = acetone, E = ethanol, % = per cent by weight; a = initial concn. of the ester, b = excess of alkali, c = concn. of added salt, mole/l.

Solvent	a	b	c	$10^6\ k_1\ s^{-1}$ 0.00°	15.00°	25.00°	40.00°	50.00°	E_a cal	log A
Water	0.02	0.02 NaOH	—	4.12	13.6 (10°)	69.2	323	—	18 500	9.416
»	0.02	0.06 »	—	—	—	70.4	—	—	—	—
»	0.02	0.02 »	0.1 LiCl	—	—	60.4	—	—	—	—
»	0.02	0.02 »	0.1 NaI	—	—	68.0	—	—	—	—
»	0.02	0.02 KOH	0.9 KCl	—	—	58.8	—	—	—	—
»	0.05	0.2 »	—	—	—	84.7	—	—	—	—
»	0.10	0.4 »	—	—	—	111	—	—	—	—
»	0.15	0.6 »	—	—	—	133	—	—	—	—
»	0.20	0.8 »	—	—	—	164	—	—	—	—
»	0.20	1.1 »	—	—	—	188	—	—	—	—
»	0.30	1.4 »	—	—	—	240	—	—	—	—
»	0.40	1.6 »	—	—	—	244	—	—	—	—
20 % A	0.02	0.02 NaOH	—	2.13	7.41 (10°)	43.4	210	—	19 540	9.959
»	0.04	0.04 »	—	—	—	45.3	—	—	—	—
»	0.04	0.02 »	—	—	—	44.9	—	—	—	—
50 % A	0.02	0.02 »	—	0.880	6.61	21.4	115	—	20 680	10.502
»	0.04	0.04 »	—	—	—	19.6	—	—	—	—
»	0.04	0.02 »	—	—	—	20.5	—	—	—	—
20 % E	0.02	0.02 »	—	2.84	16.6	49.9	226		18 600	9.339
50 % E	0.02	0.02 »		—	6.69	22.2	118	298	20 170	10.136
80 % E	0.02	0.02 »	—	—	2.58	8.73	46.7	131	20 740	10.145
»	0.02	0.04 »	—	—	—	—	45.4	—	—	—
»	0.02	0.02 »	0.1 NaI	—	—	—	29.0	—	—	—
»	0.02	0.02 »	0.5 »	—	—	—	15.5	—	—	—
80 % E	0.02	0.02 NaOH	0.1 KBr	—	—	—	36.9	—	—	—
»	0.02	0.02 KOH	—	—	—	—	51.1	—	—	—
»	0.02	0.02 »	0.069 KBr	—	—	—	43.1	—	—	—
95 % E	0.02	0.02 NaOH	—	—	—	1.04	5.60	15.4	20 620	9.142
»	0.02	0.04 »	—	—	—	—	—	15.7	—	—
»	0.02	0.02 »	0.1 NaI	—	—	—	—	11.2	—	—
»	0.01	0.02 »	—	—	—	—	—	16.3	—	—
»	0.01	0.03 »	—	—	—	—	—	15.0	—	—
»	0.02	0.02 »	0.05 NaI	—	—	—	—	12.2	—	—
»	0.02	0.02 »	0.3 »	—	—	—	—	9.16	—	—
»	0.02	0.02 »	0.5 »	—	—	—	—	7.99	—	—
»	0.02	0.04 »	0.5 »	—	—	—	—	10.4	—	—
»	0.02	0.04 »	0.3 »	—	—	—	—	10.6	—	—
»	0.02	0.04 »	0.05 »	—	—	—	—	13.4	—	—
»	0.01	0.02 KOH	—	—	—	—	—	30.8	—	—
»	0.02	0.02 »	—	—	—	—	—	30.2	—	—
»	0.02	0.04 »	—	—	—	—	—	27.9	—	—
»	0.02	0.02 LiOH	—	—	—	—	—	2.83	—	—
»	0.02	0.04 »	—	—	—	—	—	4.50	—	—
»	0.02	0.02 »	0.1 LiCl	—	—	—	—	2.57	—	—
»	0.02	0.04 »	0.08 »	—	—	—	—	4.71	—	—
»	0.02	0.02 »	0.02 »	—	—	—	—	2.74	—	—
»	0.01	0.02 »	0.01 »	—	—	—	—	2.65	—	—
»	0.01	0.03 »	—	—	—	—	—	3.68	—	—

Table 2. Alkaline hydrolysis of methyl salicylate. A = acetone, M = methanol.

Solvent	*a*	*b*	*c*	$10^6\ k_1\ s^{-1}$					E_a cal	log *A*
				0.00°	15.0°	25.00°	40.00°	50.00°		
Water	0.02	0.02 NaOH	—	4.23	25.7	78.1	363	—	18 910	9.759
»	0.02	0.04 »	—	—	—	76.8	—	—	—	—
20 % A	0.02	0.02 »	—	—	—	52.7	—	—	—	—
50 % A	0.02	0.02 »	—	—	—	30.8	—	—	—	—
»	0.02	0.04 »	—	—	—	32.0	—	—	—	—
20 % M	0.02	0.02 »	—	2.76	16.7	54.3	251	—	19 220	9.819
50 % M	0.02	0.02 »	—	—	7.94	26.9	140	370	20 060	10.331
95 % M	0.02	0.02 »	—	—	—	1.52	8.53	23.9	21 100	9.653
»	0.02	0.06 »	—	—	—	—	—	24.4	—	—
»	0.02	0.04 »	—	—	—	—	—	25.8	—	—
»	0.02	0.02 »	0.04 NaCl	—	—	—	—	21.0	—	—
»	0.02	0.02 LiOH	—	—	—	—	—	6.23	—	—
»	0.02	0.04 »	—	—	—	—	—	5.30	—	—

In the case of the *ortho*-compound, owing to the close proximity between the charged oxygen atom and the carbonyl carbon, the repulsion of the hydroxyl ion must be still greater than in the case of the *para*-compound. Further, the electromeric displacement $C{=}\overset{\curvearrowright}{O}$ will be somewhat hindered by the direct field effect of the charged oxygen atom. Thus the bimolecular reaction between the salicylate ion and hydroxyl ion must be very slow. This is clearly seen from the following argument. If we assume that part of the salicylic ester is hydrolysed by the Goldschmidt mechanism and part changed by a bimolecular reaction between the ester ion and hydroxyl ion, the total rate equation is, instead of (3)

$$dx/dt = k_1'(a-x) + k_2[OH^-](a-x) \quad (4)$$

or

$$dx/dt = (k_1' + k_2[OH^-])(a-x) \quad (5)$$

Table 3. Alkaline hydrolysis of *iso*propyl salicylate. A = acetone, P = *iso*propanol.

Solvent	*a*	*b*	$10^6\ k_1\ s^{-1}$					E_a cal	log *A*
			0.00°	15.00°	25.00°	40.00°	50.00°		
Water	0.02	0.02 NaOH	2.08	11.8	35.3	155	—	18 340	8.982
»	0.02	0.04 »	—	—	34.8	—	—	—	—
20 % A	0.02	0.02 »	—	—	19.3	—	—	—	—
50 % A	0.02	0.02 »	—	—	7.47	—	—	—	—
»	0.02	0.0354 »	—	—	7.85	—	—	—	—
20 % P	0.02	0.02 »	—	6.97	19.4	85.5	212	18 090	8.562
50 » P	0.02	0.02 »	—	1.90	6.70	38.4	103	21 200	10.365
»	0.02	0.04 »	—	—	—	—	103	—	—
80 » P	0.02	0.02 »	—	0.763	2.32	13.2	37.6	20 720	9.586

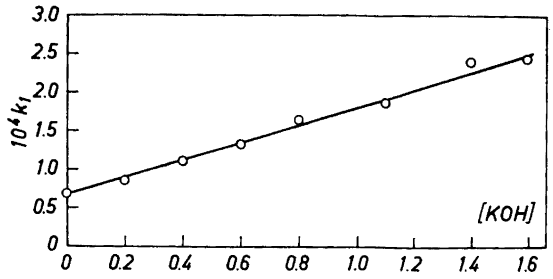


Fig. 1. Plot of k_1 *vs.* KOH in water at 25°.

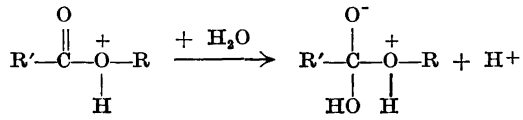
If the alkali is used in sufficient excess, the hydroxyl ion concentration remains practically constant during the reaction and the experimental rate constant, k_1, is

$$k_1 = k_1' + k_2[OH^-] \quad (6)$$

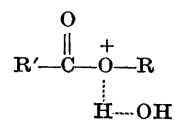
Accordingly, k_1 as a function of the hydroxyl ion concentration should yield a straight line. Fig. 1 shows that this is indeed the case. From the slope of this straight line we obtain for the bimolecular reaction in pure water at 25° $k_2 = 1.1 \times 10^{-4}$ litre mole^{-1} s^{-1}. The corresponding value for ethyl *p*-hydroxybenzoic ester ion is 2.63×10^{-4} litre mole^{-1} s^{-1}. The result shows that, beside the hydrolysis of the free ester, the bimolecular reaction between the salicylic ester ion and hydroxyl ion also takes place, although its velocity is low. The result is further interesting in that it shows that the velocity of this reaction is of the same order of magnitude as that of the *para*-compound and also that the relation of the rate constants is in the direction to be expected. The experiments were carried out with potassium hydroxide, since in this case the salt effect is at a minimum (Section 3).

Any possible role played by chelate formation is treated in Section 3.

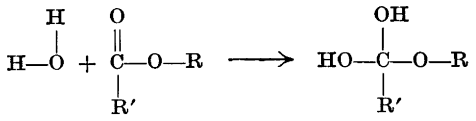
Apart from Goldschmidt's mechanism, a "water hydrolysis" would also display first-order kinetics. It is now generally accepted that the acid-catalyzed hydrolysis begins with primary addition of a proton to the ethereal oxygen, and this step is followed by addition of hydroxyl from the water to the carbonyl carbon, which is now less negative,



In the case of "water hydrolysis", the first step could correspondingly be assumed to be the transfer of a proton from the water to the ethereal oxygen,

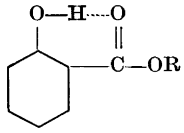


after which the reaction would proceed as in the acid-catalyzed hydrolysis. This mechanism, however, and likewise one with primary addition of water to the carbonyl group *cf.* [7],



is excluded by the fact that the acid-catalyzed hydrolysis of ethyl salicylate is extremely slow [8].

The bimolecular rate constant for the reaction $C_6H_4(OH)\cdot COOEt + OH^-$ in water at 25°, calculated from the measured first-order rate constant ($k_1 = 69.2 \times 10^{-6}$ s^{-1}) and the ionization constant of ethyl salicylate (log $K = -10.5$)[9], is $k_2 = 0.23$ l mole^{-1} s^{-1}. The corresponding value for methyl salicylate ($k_1 = 78.1 \times 10^{-6}$ s^{-1}, log $K = -10.19$) is $k_2 = 0.51$ l mole^{-1} s^{-1}. For ethyl benzoate in water at 25°, $k_2 = 0.0293$ [6]. As a comparison, it may be mentioned that the bimolecular rate constants for ethyl *o*-, *m*- and *p*-nitrobenzoates [5] in water at 25°, estimated from the measurements in 56 % acetone and supposing that the effect of the change of solvent is about the same as for ethyl benzoate [6], are $k_2 = 0.12$, 1.5 and 2.5, respectively. In the same manner, for ethyl *p*-chlorobenzoate and ethyl *p*-bromobenzoate we obtain $k_2 = 0.13$ and $k_2 = 0.15$, respectively. In 50 % ethanol the values at 25° are: ethyl salicylate ($k_1 = 22.2 \times 10^{-6}$ s^{-1}, log $K = -11.6$)[9] $k_2 = 0.0089$, ethyl benzoate $k_2 = 0.0017$ l mole^{-1} s^{-1}. We see that the unionized phenyl group acts as a rather powerful electron-attracting substituent. No doubt also the internal hydrogen bond formation [11]



which makes the carbonyl carbon more positive, plays an important role here. However, it is noteworthy that ethyl lactate, in which internal hydrogen bond formation is very weak [11], is hydrolysed much faster [12] than ethyl propionate [13] or ethyl acetate [14].

The ionization constants of the ethyl esters of *m*- and *p*-hydroxybenzoic acids are appreciably larger (log $K = -9.1$ and -8.3, respectively [9]) than that of ethyl salicylate (log $K = -10.5$). This implies that the concentration of the free ester in their alkali salt solutions is much less than that in the salicylate solutions. Further, as mentioned above, the reaction of the free salicylic ester is favoured by internal hydrogen bond formation. We have also seen that the reaction between the ions of *m*- and *p*-hydroxybenzoic esters and hydroxyl ion is less hindered than in the case of salicylate. The result is that the esters of the *m*- and *p*-isomerides undergo alkaline hydrolysis mainly by a simple bimolecular process between the ester ion and hydroxyl ion.

In one experiment with water as solvent equal equivalents of the ester and sodium hydroxide were mixed, [ester] = [NaOH] = 0.02 mole/l, 25°. The result was as follows:

Time, min.	52.5	82	118	160	260	287	320	361	420
Change, %	18.5	27.0	35.5	44.5	61.5	65.0	68.5	72.5	77.5
$10^5 k_1$, s^{-1}	6.50	6.40	6.20	6.11	6.11	6.10	6.01	5.94	5.93

As is seen, first-order kinetics are approximately obeyed in this case also. Since the ester is a very weak acid, the solution now contains a considerable concentration of the free ester and hydroxyl ions:

$$C_6H_4(O^-)COOR + H_2O \rightleftharpoons C_6H_4(OH)COOR + OH^-$$

Consequently, ξ in eqn. (2) cannot now be ignored; the neglect of ξ in the calculations has caused a small continuous decrease in the rate constant and also made its value somewhat less than the value obtained in the presence of excess of alkali ($k_1 = 6.92 \times 10^{-5}$ s^{-1}, Table 1). Thus this result of Goldschmidt and Scholtz has likewise been confirmed.

The experimental activation energies and frequency factors, as given in Tables 1—3, are strikingly high. Their values, as is shown by the data given on p. 1021, are remarkably higher than the corresponding values for the reaction between hydroxyl ion and ethyl *p*-hydroxybenzoate ion or ethyl *m*-hydroxybenzoate ion. However, we must bear in mind that the activation energies, E_a given in Tables 1—3 are only apparent, as the rate constants from which they are obtained include the change of the equilibrium constant K of eqn. (1) with change in temperature. We have

$$\frac{\mathrm{d}\ \ln\ k}{\mathrm{dT}} = \frac{E}{RT^2} \quad \text{and} \quad \frac{\mathrm{d}\ \ln\ K}{\mathrm{d}T} = \frac{\Delta H}{RT^2}$$

Thus, if E and ΔH are independent of temperature,

$$\ln k_{\mathrm{exp.}} = -E_a/RT + \ln A \text{ and } \ln K = -\Delta H/RT + \ln B$$

where k_{exp} is the experimental rate constant and E_a the (apparent) activation energy calculated from its dependence on temperature; ln A and ln B are integration constants. From (p. 1020) follows that

$$k_{\mathrm{exp.}} = k_1 = kK,\ \ln\ k = \ln\ k_1 - \ln\ K = -(E_a - \Delta H)/RT + \ln\ (A/B)$$

or

$$k = (A/B)e^{-(E_a - \Delta H)RT} \tag{7}$$

We see that the true activation energy for the bimolecular reaction between the free ester and hydroxyl ion is

$$E' = E_a - \Delta H \tag{8}$$

The values of ΔH for the reaction I are not known, but it is, perhaps, legitimate to assume that they do not differ essentially from those for phenol. For phenol (log K = —9.92), *e.g.* in ethanol-water mixtures, the values of ΔH are seen from the following table [15]:

wt. % of EtOH	0	20	50	80	95
ΔH, kcal	7.20	8.15	5.40	4.42	3.20
E', ethyl salic., kcal	11.3	10.5	14.8	16.3	17.4
E, ethyl benz., kcal	12.7	11.5	15.7	17.2	19.1

If the values of ΔH are substracted from E_a given in Table 1, the figures in the third line are obtained for ethyl salicylate. These values are of the same order of magnitude as those found for the alkaline hydrolysis of ethyl benzoate [6]. Furthermore, in accordance with the fact that the unionized phenol hydroxyl acts as an electron-attracting substituent, the activation energy for ethyl salicylate is somewhat smaller than that for ethyl benzoate in the same solvent. Corresponding results are obtained in other cases:

Methyl salicylate in methanol-water

wt. % of MeOH	0	20	50	95
ΔH for phenol, kcal	7.20	6.57	3.60	1.40
E', kcal	11.7	12.7	16.5	19.7

Iso*propyl salicylate in* iso*propanol-water*

wt. % of *i*-PrOH	0	20	50	80
ΔH for phenol, kcal	7.20	9.25	6.70	5.17
E', kcal	11.1	8.8	14.5	15.6

From the values of k_2 calculated on p. 1025 log A can also be estimated for reactions in pure water and in 50 % ethanol. The values obtained are 7.65 and 8.80, respectively. The values of log A for the alkaline hydrolysis of ethyl benzoate in the same solvents are 7.76 and 8.70, respectively. The result is very striking, for it is known that in the alkaline hydrolysis of ethyl benzoates the substituents exert their main influence on the rate of the reaction through changes in the activation energy, whereas the frequency factor often remains almost constant if the reactions are carried out in exactly the same solvent. All this is in excellent agreement with earlier experiences concerning esterhydrolysis. However, it must be reiterated that the results are based on the assumption that ΔH for salicylates is equal to that for phenol in the same solvent.

The reaction mechanism propounded obtains additional support from the fact that if the values of E' are plotted against the composition of the solvent, the graphs have the form usual for alkaline ester hydrolysis; for instance, they pass through a minimum, except in the case of methanol-water [13,14,16] (Fig. 2). On the other hand, if E_a is plotted against the composition of the solvent, the curves have a decidedly different shape.

3. Salt effects. The dependence of the rate constants of the alkaline hydrolysis of ethyl *m*- and *p*-hydroxybenzoates on the initial concentration of the reactants in water at 25° is seen from the following experimental data obtained in reaction mixtures prepared by mixing equal volumes of a $2a$ molar ester solution and a $4a$ molar sodium hydroxide solution:

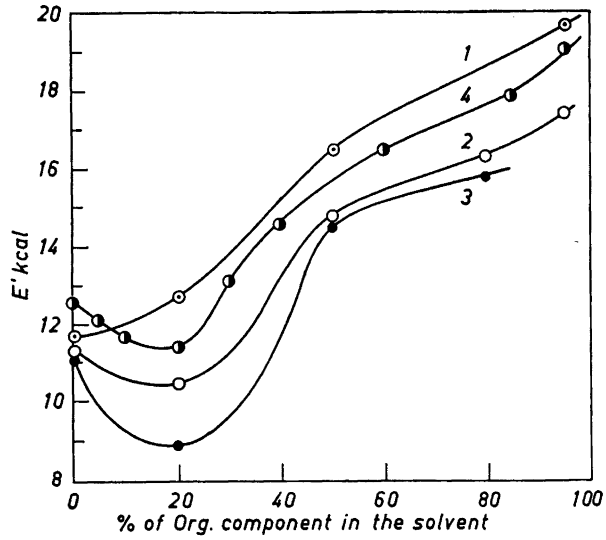


Fig. 2. Variation of the activation energy E' with the composition of the solvent.
1. Methyl salicylate in methanol-water
2. Ethyl salicylate in ethanol-water
3. *Iso*propyl salicylate in *iso*propanol-water
4. Ethyl benzoate in ethanol-water.

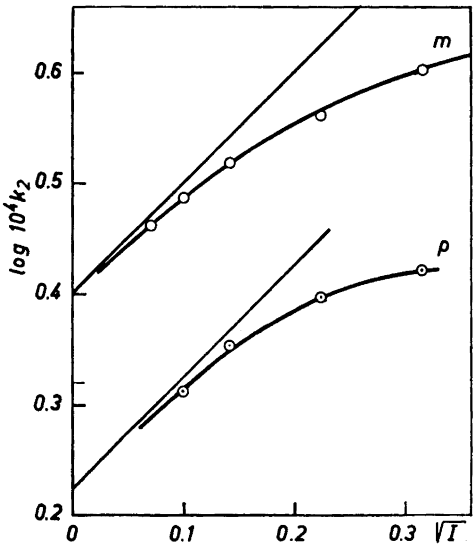


Fig. 3. Influence of the ionic strength I on the rate of the alkaline hydrolysis of ethyl *m*- and *p*-hydroxybenzoates.

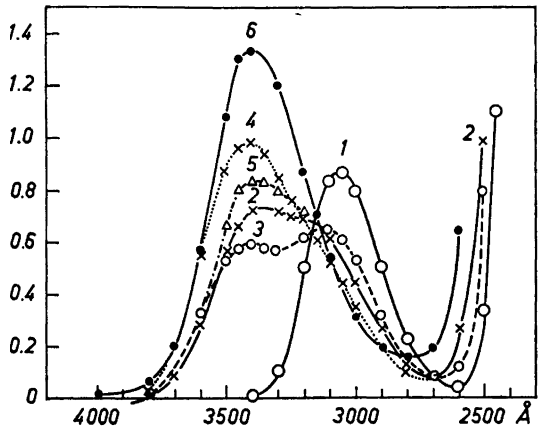


Fig. 4. Absorption curves, 95 wt. % ethanol, 1 cm cell. *1.* $c_{est.} = 0.0002$; *2.* $c_{est.} = 0.0002$, $c_{KOH} = 0.002$; *3.* $c_{est.} = 0.0002$, $c_{LiOH} = 0.0004$; *4.* $c_{est.} = 0.0002$, $c_{LiOH} = 0.0004$, $c_{LiCl} = 0.0023$; *5.* $c_{est.} = 0.0002$, $c_{NaOH} = 0.0016$, $c_{NaI} = 0.0013$; *6.* $c_{est.} = 0.0002$, $c_{NaOH} = 0.15$, $c_{NaI} = 0.09$.

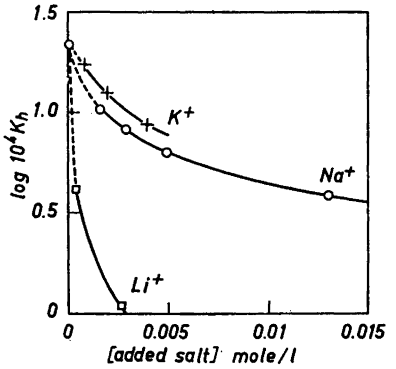


Fig. 5. The relative hydrolysis constants of the ester as a function of salt concentration.

a	0.0025	0.005	0.01	0.025	0.05
$\sqrt{I}$	0.071	0.1	0.142	0.224	0.316
m-, 10^5 k, 1 mole^{-1} s^{-1}	290	307	331	365	402
p-, 10^5 k, 1 mole^{-1} s^{-1}		20.5	22.6	25.0	26.3

Fig. 3 shows that these values approximate to the equation

$$\log k = \log k_0 + 1.02\, z_A z_B \sqrt{I}$$

in a manner which shows that we have here a reaction between two univalent ions of the same sign. Thus this result also shows that the reaction takes place between the ester ion and the hydroxyl ion.

In the hydrolysis of alkyl salicylates the rate constants are only very slightly dependent on the initial concentration of the reactants, as is seen from Tables 1—3. (The effect of excess alkali concentration has been treated in Section 2). An increase in salt concentration always decreases the reaction velocity (Table 1). In water the salt effect is very weak, except in the case of lithium salts. In 95 % ethanol, for the initial concentration 0.02 mole/l of the ester and alkali, the rate constant at 50° is 30.2×10^{-6} s^{-1} when KOH is used and 15.4×10^{-6} s^{-1} when NaOH is used. The lithium ion reduces the reaction rate still more than the sodium ion. A very interesting point is that in aqueous alcohol even at a relatively low lithium ion concentration the reaction becomes sensitive to hydroxyl ions, and as the lithium salt concentration increases the reaction finally approaches one of second-order rate.

In general, addition of alkali metal ions shifts the equilibrium (I) towards the left, which causes a decrease in the reaction velocity. This shift of the equilibrium was spectrometrically verified with a Beckman spectrophotometer. Some typical absorption curves are presented in Fig. 4. The relative hydrolysis constants, K_h, of the ester as a function of salt concentration are graphically represented in Fig. 5. As is seen, the effect of lithium ion is much greater than that of potassium or sodium ions. This corresponds to the greater effect of the lithium ion on the reaction velocity *.

One possible explanation of the powerful effect of the lithium ion is that owing to its small size this ion can be fitted between the charged oxygen and the oxygen of the carbonyl group in the salicylic ester, thus forming a chelate. Chelate formation should render the carbonyl carbon more positive and also facilitate the electrometric displacement $C=\overset{\frown}{O}$ and thus facilitate the bimolecular reaction between the ester ion and hydroxyl ion. Thus chelate formation on the one hand reduces the part of the ester changed by the Goldschmidt mechanism, and on the other hand increases the amount changed by the reaction between the ester ion and hydroxyl ion.

* The maximum for the salt form is shifted towards longer wave lengths in the order K, Na, Li, and furthermore, the extinction is greatest for the Li compound and weakest for the K compound. This was taken into account in estimating the concentration of the salt form.

REFERENCES

1. Goldschmidt, H. and Scholtz, V. *Ber.* **40** (1907) 624.
2. Goldschmidt, H. and Oslan, L. *Ber.* **32** (1899) 3390; **33** (1900) 1140.
3. Walden, P. *Z. physik. Chem.* **114** (1925) 281.
4. Tommila, E. and Lindholm, M. *Acta Chem. Scand.* **5** (1951) 647.
5. Tommila, E. *Suomen Kemistilehti A* **17** (1944) 1.
6. Tommila, E. *Suomen Kemistilehti B* **25** (1952) 37.
7. Bender, M. L. *J. Am. Chem. Soc.* **73** (1951) 1626.
8. Pekkarinen, L. *Ann. Acad. Sci. Fennicæ A II* **85** (1957).
9. Larsson, E. *Z. anorg. u. allgem. Chem.* **183** (1929) 30.
10. Ågren, A. *Acta Chem. Scand.* **9** (1955) 39, 49.
11. Pauling, L. *The Nature of the Chemical Bond.* Cornell University Press, Ithaca, N.Y. 1945, p. 328.
12. Krige, G. J. R. and Hollow, K. *Trans. Faraday Soc.* **30** (1934) 645; Salmi, E. J. and Leino, E. *Suomen Kemistilehti B* **17** (1944) 19; Vogel, K. H. and Warner, J. C. *J. Am. Chem. Soc.* **75** (1953) 6072.
13. Tommila, E. and Hietala, S. *Acta Chem. Scand.* **8** (1954) 257.
14. Tommila, E., Koivisto, A, Lyyra, J. P., Antell, K. and Heimo, S. *Ann. Acad. Sci. Fennicæ A II* **47** (1952).
15. Koivisto, A. *Ann. Acad. Sci. Fennicæ A II* **73** (1956).
16. Tommila, E. and Maltamo, S. *Suomen Kemistilehti B* **28** (1955) 73, 118.

Received February 20, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1031–1050

Short Communications

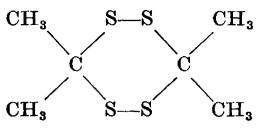
Reactions between Ketones and Ammonium Polysulphide

BENGT MAGNUSSON

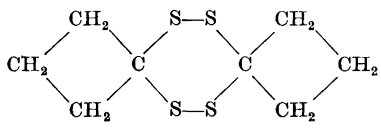
Chemical Institute, University of Uppsala, Sweden

Duplodithioacetone (I) and related compounds have been prepared by the reaction of a ketone and excess of aqueous ammonium polysulphide [1,2]. Asinger obtained 5- and 6-membered rings by allowing the carbonyl compound to react with elementary sulphur and an amine, saturated with hydrogen sulphide [3,4]. The author found that duplodithioacetone was obtained without disturbing byproducts by running the reaction in alcohol-water solution at about +4°C for one to two days. As this is a way to avoid resinous material, attempts were made with other ketones under the same conditions.

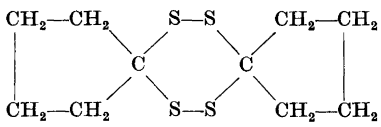
With *cyclo*hexanone white crystals were obtained, which were recrystallized from a mixture of chloroform and ethanol. However, the purified product was not the expected duplodithio*cyclo*hexanone (IV) obtained by Fredga [2] but a compound with the composition of (V), bis-4,4,7,7-pentamethylene-1,2,3,5,6-pentathiepane. Such a pentathiepane (VI) was first obtained by Fredga from *cyclo*heptanone and ammoniumpolysulphide [2].



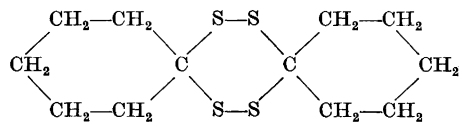
I



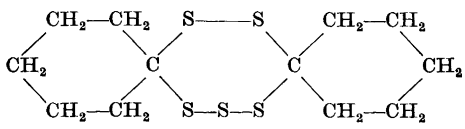
II



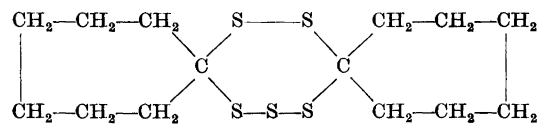
III



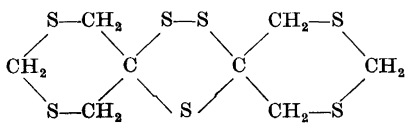
IV



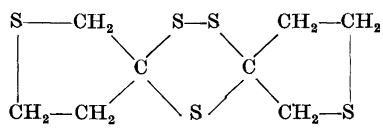
V



VI



VII



VIII

*Cyclo*pentanone gives (III) after reaction in alcoholic or aqueous solution.

*Cyclo*butanone gives the compound (II) in aqueous solution [5].

1,3-Dithian-5-on and 3-ketothiophane give five-membered rings (VII, VIII) of the type obtained by Asinger [3,4] in aqueous and alcohol-water solutions, respectively.

From the results it is apparent that the ring size is greatly dependent on the reaction conditions and the starting materials. More experiments to obtain 6-membered and larger rings are planned; results will be reported at a later date.

Experimental. Duplodithioacetone (3,3,6,6-tetramethyl-1,2,4,5-tetrathiane) (I). In a 300 ml Erlenmeyerflask 45 ml of acetone was mixed with 100 ml of ethanol and cooled to +4°C. A cooled solution of ammonium polysulphide (prepared by dissolving 20 g of ammoniumchloride in 77 ml of 2.5 M Na_2S_2) was added. The flask was closed, shaken for a minute and left in a refrigerator at about 0°C for 3 days. The crystals were then separated, washed with water to dissolve inorganic salts, dried and recrystallized 3 times from ethanol. In the first recrystallisation some Norit was added to remove colloidal sulphur. 6.57 g of duplodithioacetone with a m.p. of 94°—95° was found. On dilution of the motherliquor with water roughly 0.6 g of inpure tetrathiane was obtained.

Bis-,4,4,7,7-pentamethylene-1,2,3,5,6-pentathiepane (V). In a 200 ml Erlenmeyer flask 4 g of *cyclo*hexanone was dissolved in 100 ml of ethanol and was allowed to react with ammonium disulphide as described above. The crude material (4.74 g) was recrystallised several times from chloroform and ethanol. Colourless flakes with m.p. 85°—87°C were obtained. The product is readily soluble in chloroform, carbontetrachloride and benzene. (Found: C 44.05, 44.29, 44.80; H 5.90, 6.53, 6.24; S 49.17, 49.20; Mol. wt: 316, 316. Calc.for $C_{12}H_{20}S_5$: C 44.40; H 6.21; S 49.39; Mol. wt: 324.6).

The author is greatly indebted to Professor Arne Fredga for suggesting the present work, for stimulating discussions and for some starting materials. Thanks are further due to Dr. Göran Bergson and to Dr. Sven-Olov Lawesson for valuable advice during the preparation of this manuscript.

1. Willgerodt, C. *Ber.* **20** (1887) 2467.
2. Fredga, A. *Acta Chem. Scand.* **12** (1958) 891.
3. Asinger, F. *Angew. Chem.* **70** (1958) 372.
4. Asinger, F. *Angew. Chem.* **70** (1958) 667.
5. Fredga, A. *Private Communication.*

Received April 27, 1959.

Mass Spectrometric Demonstration of Extensive Replacement of Hydrogen by Deuterium During Catalytic Deuteration of Methyl Oleate, Methyl 9,10-Dibromostearate, and Methyl 12-Chlorostearate

NGUYEN DINH-NGUYEN

Department of Medical Biochemistry, University of Gothenburg,

and RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden

In connection with work on the determination of the position of double bonds in unsaturated fatty esters by mass spectrometric analysis [1], methyl stearate obtained by catalytic deuteration of methyl oleate over Adams' platinum catalyst at room temperature was submitted to analysis. The high-mass end of the mass spectrum reproduced in Fig. 1 shows clearly that deuterium has entered the chain not only in position 9,10. Instead of a single molecule-ion peak at $m/e = 300$ and its isotope-peak, corresponding to the entrance of two deuterium atoms, we have a whole series of molecule-ion peaks, the intensities of which decrease in a regular manner with increasing mass number. It is evident from Fig. 1 that molecules of mass 330 have been present. In these molecules all hydrogen atoms except six have been replaced by deuterium. This extensive replacement is probably closely related to the double bond shifts which are known [2] to occur during catalytic hydrogenation of olefins. Exchange reactions with the saturated hydrocarbon chain seems excluded, as no deuterium could be detected in methyl stearate which had been submitted to the same treatment as methyl oleate above.

Attempted replacement of bromine by deuterium in methyl 9,10-dibromostearate, using Adams' platinum catalyst, failed until sodium carbonate was added, and then gave a debrominated ester with mass spectrum practically identical with that of the ester obtained by catalytic deuteration of methyl oleate. A reaction similar to the debromination of vicinal dibromides by zink to give olefins [3,4] is probably involved.

*Cyclo*pentanone gives (III) after reaction in alcoholic or aqueous solution.

*Cyclo*butanone gives the compound (II) in aqueous solution [5].

1,3-Dithian-5-on and 3-ketothiophane give five-membered rings (VII, VIII) of the type obtained by Asinger [3,4] in aqueous and alcohol-water solutions, respectively.

From the results it is apparent that the ring size is greatly dependent on the reaction conditions and the starting materials. More experiments to obtain 6-membered and larger rings are planned; results will be reported at a later date.

Experimental. Duplodithioacetone (3,3,6,6-tetramethyl-1,2,4,5-tetrathiane) (I). In a 300 ml Erlenmeyerflask 45 ml of acetone was mixed with 100 ml of ethanol and cooled to +4°C. A cooled solution of ammonium polysulphide (prepared by dissolving 20 g of ammoniumchloride in 77 ml of 2.5 M Na_2S_2) was added. The flask was closed, shaken for a minute and left in a refrigerator at about 0°C for 3 days. The crystals were then separated, washed with water to dissolve inorganic salts, dried and recrystallized 3 times from ethanol. In the first recrystallisation some Norit was added to remove colloidal sulphur. 6.57 g of duplodithioacetone with a m.p. of 94°—95° was found. On dilution of the motherliquor with water roughly 0.6 g of inpure tetrathiane was obtained.

Bis-,4,4,7,7-pentamethylene-1,2,3,5,6-pentathiepane (V). In a 200 ml Erlenmeyer flask 4 g of *cyclo*hexanone was dissolved in 100 ml of ethanol and was allowed to react with ammonium disulphide as described above. The crude material (4.74 g) was recrystallised several times from chloroform and ethanol. Colourless flakes with m.p. 85°—87°C were obtained. The product is readily soluble in chloroform, carbontetrachloride and benzene. (Found: C 44.05, 44.29, 44.80; H 5.90, 6.53, 6.24; S 49.17, 49.20; Mol. wt: 316, 316. Calc.for $C_{12}H_{20}S_5$: C 44.40; H 6.21; S 49.39; Mol. wt: 324.6).

The author is greatly indebted to Professor Arne Fredga for suggesting the present work, for stimulating discussions and for some starting materials. Thanks are further due to Dr. Göran Bergson and to Dr. Sven-Olov Lawesson for valuable advice during the preparation of this manuscript.

1. Willgerodt, C. *Ber.* **20** (1887) 2467.
2. Fredga, A. *Acta Chem. Scand.* **12** (1958) 891.
3. Asinger, F. *Angew. Chem.* **70** (1958) 372.
4. Asinger, F. *Angew. Chem.* **70** (1958) 667.
5. Fredga, A. *Private Communication.*

Received April 27, 1959.

Mass Spectrometric Demonstration of Extensive Replacement of Hydrogen by Deuterium During Catalytic Deuteration of Methyl Oleate, Methyl 9,10-Dibromostearate, and Methyl 12-Chlorostearate

NGUYEN DINH-NGUYEN

Department of Medical Biochemistry, University of Gothenburg,

and RAGNAR RYHAGE

Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm, Sweden

In connection with work on the determination of the position of double bonds in unsaturated fatty esters by mass spectrometric analysis [1], methyl stearate obtained by catalytic deuteration of methyl oleate over Adams' platinum catalyst at room temperature was submitted to analysis. The high-mass end of the mass spectrum reproduced in Fig. 1 shows clearly that deuterium has entered the chain not only in position 9,10. Instead of a single molecule-ion peak at $m/e = 300$ and its isotope-peak, corresponding to the entrance of two deuterium atoms, we have a whole series of molecule-ion peaks, the intensities of which decrease in a regular manner with increasing mass number. It is evident from Fig. 1 that molecules of mass 330 have been present. In these molecules all hydrogen atoms except six have been replaced by deuterium. This extensive replacement is probably closely related to the double bond shifts which are known [2] to occur during catalytic hydrogenation of olefins. Exchange reactions with the saturated hydrocarbon chain seems excluded, as no deuterium could be detected in methyl stearate which had been submitted to the same treatment as methyl oleate above.

Attempted replacement of bromine by deuterium in methyl 9,10-dibromostearate, using Adams' platinum catalyst, failed until sodium carbonate was added, and then gave a debrominated ester with mass spectrum practically identical with that of the ester obtained by catalytic deuteration of methyl oleate. A reaction similar to the debromination of vicinal dibromides by zink to give olefins [3,4] is probably involved.

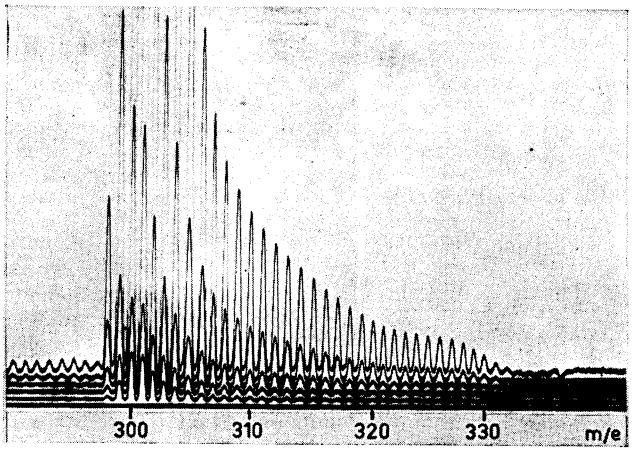


Fig. 1. High-mass end of mass spectrum of catalytically deuterated methyl oleate.

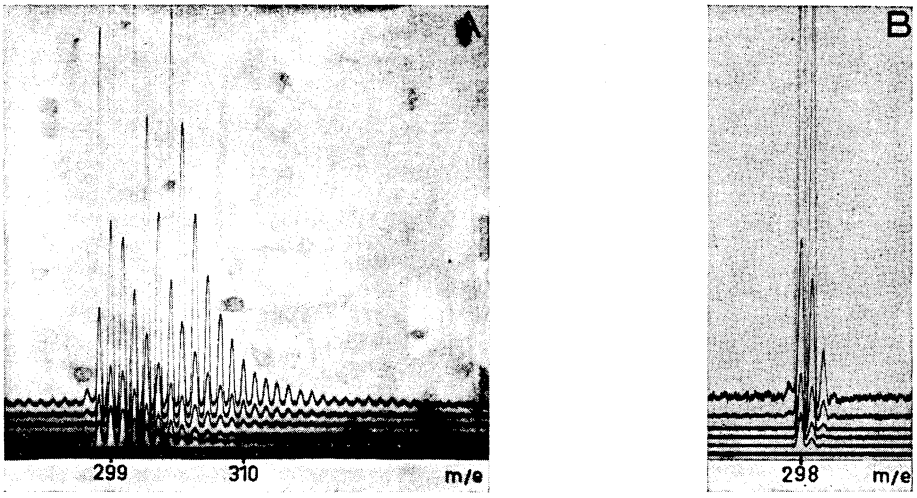


Fig. 2. A) High-mass end of mass spectrum of catalytically deuterated methyl 12-chlorostearate; B) High-mass end of mass spectrum of methyl stearate.

Deuteration of methyl 12-chlorostearate over Adams' platinum catalyst gave a dehalogenated ester without the use of sodium carbonate. The high-mass end of the mass spectrum of the ester is reproduced in Fig. 2A and shows that the molecule-ion peak at $m/e = 299$, corresponding to the replacement of chlorine by deuterium, is followed by about sixteen molecule-ion peaks of decreasing intensity. Molecules where deuterium have replaced nearly half the number of hydrogen atoms in the hydrocarbon chain have therefore been present.

Full details will be published later.

This work was made possible by grants from *Statens naturvetenskapliga forskningsråd* to S. Ställberg-Stenhagen. The hospitality of *AB Hässle* is gratefully acknowledged.

1. Dinh-Nguyen, Ng. *Acta Chem. Scand.* **12** (1958) 1350.
2. *Cf.* Taylor, T. I. in Emmett, P. H. *Catalysis*, Reinhold Publishing Corp., New York 1957, vol. 5, chap. 5; Feuge, R. O. *Ibid.* 1955, vol. 3, p. 431.
3. Nicolet, B. H. *J. Am. Chem. Soc.* **43** (1921) 2122.
4. Kaufmann, H. P. and Mestern, H. E. *Ber.* **69 B** (1936) 2684.

Received May 11, 1959.

The Deswelling Action of Hydrazine on Nitrile Rubber

SVEN SÖNNERSKOG

A-B Svenskt Konstsilke, Borås, Sweden

In a previous paper [1] an outline was given of the primary and side reactions taking place when hydrazine or hydrazine hydrate reacts with linear homopolymers of acrylonitrile. Favourable conditions were found in homogeneous systems, as indicated by gel formation and disappearance of the strong infrared band at 2 260 cm^{-1}, measuring the amount of nitrile groups in the sample. The primary polymer particle does not show this high degree of accessibility, but insolubilization occurs in both cases due to formation of interlinking units between macromolecules. The interlinking structural unit is 4-N-amino-1,2,4-triazole.

Such a reactivity is not found in copolymers of acrylonitrile and butadiene. Still more, *solubility* is limited already by the formation of microgel in the growing polymer particle at increasing degree of conversion during polymerization. Standard nitrile rubber latex with an acrylonitrile content of 37—40 % dissolves in acetone, *cyclo*hexanone or dioxane, yet with difficulty. The last mentioned solvent, however, shows a low solvent power, why phase separation instantly takes place when hydrazine is added (three phases!). Acetone and *cyclo*hexanone are obviously unsuitable due to their reactivity.

A convenient way to study the action of an aqueous solution of hydrazine hydrate on the polymer is simply to add said reagent into a nitrile rubber latex with a low particle size. Commercial grades of butadiene-acrylonitrile latices are available for this purpose, *e. g.* Hycar Latex 1561, showing a medium particle size of 500 Å. The change of solubility characteristics can be observed at arbitrary intervals by casting a rubber film, preferably at reduced pressure or in a stream of dry nitrogen, followed by thorough washing in distilled water, soaking in acetone (if possible) and drying at 80°C in an inert atmosphere before use.

The weight swelling ratios of such a rubber film in a selected series of swelling agents is shown in Table 1. The degree of swelling is — rather arbitrarily — measured after an immersion time of 24 h in a large excess of liquid.

Table 1. Weight swelling ratios of nitrile rubber films, prepared at room temperature from a latex showing a solid content of 0.25 $g.ml^{-1}$. Conc. of hydrazine hydrate: 0.85 $g.ml^{-1}$. All samples preextracted in acetone at room temperature and dried at 80°C one hour before use. Time of reaction in the rubber latex: 24 h. Grade of nitrile rubber: High acrylonitrile content (37—40 %), density in pure state 1.00, medium particle size 500 Å.

Swelling agent	Weight swelling ratio, g/g
Benzene	3.15
Toluene	2.82
Xylene (techn. grade)	2.25
*Cyclo*hexane	1.10
Dipentene	1.52
Decalin	1.17
Chlorobenzene	6.77
Pyridine	8.42
Dioxane	4.92
Acetone	3.93
*Cyclo*hexanone	8.08
2-Pyrrolidone	5.82
Dimethylformamide	7.61
Dimethylsulfoxide	4.73

A glance at the figures in Table 1 makes evident that the degree of swelling is qualitatively related to the molar volume of the solvent and its specific interaction coefficient with parent polymer. Such a

This work was made possible by grants from *Statens naturvetenskapliga forskningsråd* to S. Ställberg-Stenhagen. The hospitality of *AB Hässle* is gratefully acknowledged.

1. Dinh-Nguyen, Ng. *Acta Chem. Scand.* **12** (1958) 1350.
2. *Cf.* Taylor, T. I. in Emmett, P. H. *Catalysis*, Reinhold Publishing Corp., New York 1957, vol. 5, chap. 5; Feuge, R. O. *Ibid.* 1955, vol. 3, p. 431.
3. Nicolet, B. H. *J. Am. Chem. Soc.* **43** (1921) 2122.
4. Kaufmann, H. P. and Mestern, H. E. *Ber.* **69 B** (1936) 2684.

Received May 11, 1959.

The Deswelling Action of Hydrazine on Nitrile Rubber

SVEN SÖNNERSKOG

A-B Svenskt Konstsilke, Borås, Sweden

In a previous paper [1] an outline was given of the primary and side reactions taking place when hydrazine or hydrazine hydrate reacts with linear homopolymers of acrylonitrile. Favourable conditions were found in homogeneous systems, as indicated by gel formation and disappearance of the strong infrared band at 2 260 cm^{-1}, measuring the amount of nitrile groups in the sample. The primary polymer particle does not show this high degree of accessibility, but insolubilization occurs in both cases due to formation of interlinking units between macromolecules. The interlinking structural unit is 4-N-amino-1,2,4-triazole.

Such a reactivity is not found in copolymers of acrylonitrile and butadiene. Still more, *solubility* is limited already by the formation of microgel in the growing polymer particle at increasing degree of conversion during polymerization. Standard nitrile rubber latex with an acrylonitrile content of 37—40 % dissolves in acetone, *cyclo*hexanone or dioxane, yet with difficulty. The last mentioned solvent, however, shows a low solvent power, why phase separation instantly takes place when hydrazine is added (three phases!). Acetone and *cyclo*hexanone are obviously unsuitable due to their reactivity.

A convenient way to study the action of an aqueous solution of hydrazine hydrate on the polymer is simply to add said reagent into a nitrile rubber latex with a low particle size. Commercial grades of butadiene-acrylonitrile latices are available for this purpose, *e. g.* Hycar Latex 1561, showing a medium particle size of 500 Å. The change of solubility characteristics can be observed at arbitrary intervals by casting a rubber film, preferably at reduced pressure or in a stream of dry nitrogen, followed by thorough washing in distilled water, soaking in acetone (if possible) and drying at 80°C in an inert atmosphere before use.

The weight swelling ratios of such a rubber film in a selected series of swelling agents is shown in Table 1. The degree of swelling is — rather arbitrarily — measured after an immersion time of 24 h in a large excess of liquid.

Table 1. Weight swelling ratios of nitrile rubber films, prepared at room temperature from a latex showing a solid content of 0.25 $g.ml^{-1}$. Conc. of hydrazine hydrate: 0.85 $g.ml^{-1}$. All samples preextracted in acetone at room temperature and dried at 80°C one hour before use. Time of reaction in the rubber latex: 24 h. Grade of nitrile rubber: High acrylonitrile content (37—40 %), density in pure state 1.00, medium particle size 500 Å.

Swelling agent	Weight swelling ratio, g/g
Benzene	3.15
Toluene	2.82
Xylene (techn. grade)	2.25
*Cyclo*hexane	1.10
Dipentene	1.52
Decalin	1.17
Chlorobenzene	6.77
Pyridine	8.42
Dioxane	4.92
Acetone	3.93
*Cyclo*hexanone	8.08
2-Pyrrolidone	5.82
Dimethylformamide	7.61
Dimethylsulfoxide	4.73

A glance at the figures in Table 1 makes evident that the degree of swelling is qualitatively related to the molar volume of the solvent and its specific interaction coefficient with parent polymer. Such a

relation is strictly valid for isotropic swelling of nonionic networks, why an obvious conclusion would be to anticipate the existence of some kind of crosslinking, at least partly responsible for swelling behaviour. As a pure thermal curing reaction at the carbon-carbon double bonds must be excluded at the low temperature in question, only two cases can be considered, *viz.*

a. Reaction between hydrazine hydrate and nitrile groups at the polymer-water interface.
b. Formation of a highly active redox system between hydrazine hydrate and activator residues, capable of initiating a continued polymerization (crosslinking) to a low extent.

Said types of reaction are mutually independent but cooperate in reducing the degree of swelling of the copolymer. As the parent polymer itself shows only a moderate affinity to solvents cited above, a very slight degree of crosslinking or reduction of the number of solvogenic groups would be sufficient to produce a gel fraction. In fact the infrared spectrum of cured rubber films gives no definite information of chemical changes but a new band at 3 250 cm^{-2}, possibly due to formation of amide groupings.

An increase of reaction temperature to 80°C, as well as a prolonged time of curing, does not principally change the swelling behaviour. This remarkable insensitivity would be unintelligible if the curing effect were solely caused by a propagating chain reaction within the polymer particles, initiated by free radicals at the polymer-water interface. It is easily demonstrated that addition of a reactivating system, such as potassium persulphate-sodium pyrosulphite to a rubber latex, buffered with sodium carbonate-sodium bicarbonate, causes a gradual deswelling of the polymer due to crosslinking at the carbon-carbon double bonds. The rate is dependent of temperature and concentration of initiator. Analogous experiments on emulsions of butadiene-styrene copolymer have been reported [2].

Scope of reaction in anhydrous systems. A carefully purified and dried rubber film reacts with anhydrous hydrazine at elevated temperature. A gradual deswelling takes place at 80°C, as seen from Table 2. The rate of deswelling rapidly decreases after 24 h. The reaction is too slow at room temperature to be practically applicable.

Table 2. Weight swelling ratios of nitrile rubber films, prepared at room temperature, purified in water, dried in a current of carbon dioxide, cured in a large excess of anhydrous hydrazine at 80°C. Swelling agent: dioxane. Temperature of swelling: 20.0°C.

Time of curing, h	Weight swelling ratio, g/g
12	4.32
24	3.74
48	3.45
72	3.24
120	3.18
144	3.00
168	2.94

The cured films were not preextracted in acetone before use, because a preliminary test revealed no significant amount of soluble polymer. Remaining hydrazine in the samples was removed by soaking in methanol, followed by drying *in vacuo* for 48 h. During this drying operation a distinct pink color is gradually developed in the specimens. The intensity is related to the time of curing. This color formation is wellknown from the reaction between hydrazine and homopolymers of acrylonitrile [1].

Deswelling of a rubber film merely by heating in air at the actual temperature gives a limiting swelling ratio of 10.27 after 168 h (preextracted sample). A blank, heated in dry carbon dioxide gas in the same way, is too readily redispersed in the swelling agent to be used for quantitative measurements. Even an immersion of a film in 0.1 M potassium persulphate solution for an identical period gives a moderate decrease of swelling (weight swelling ratio = 7.92, preextracted sample). It is apparent that contributions from chemical reactions between polymer and hydrazine are of primary importance in reducing swellability. Work is continued to find more favourable working conditions, as infrared spectra also in this case give but scanty information on the extent of conceivable reactions.

1. Sönnerskog, S. *Acta Chem. Scand.* **12** (1958) 1241.
2. Bardwell, J. and Winkler, C. A. *Can. J. Research* **27B** (1949) 128.

Received May 12, 1959.

Syntheses of Some Thiolacetic Acid Derivatives [1]

II. Phenyl- and 1-Naphthylthiolacetic Acids

BERTIL SJÖBERG

Högre Tekniska Läroverket, Malmö, Sweden

Phenylacetic acid has earlier been prepared by Johnson [2] from phenylacetyl chloride and a water solution of potassium hydrosulfide. Johnson describes the acid as an oil but gives no physical data. Sakurada [3] reports the synthesis of phenylthiolacetic acid from the O-ethyl ester of the phenylthiolacetic acid through hydrolysis with an alcoholic solution of potassium hydroxide. Sakurada, however, describes the acid as yellow crystals, which definitely must be wrong (see below). 1-Naphthylthiolacetic acid does not seem to have been prepared before.

In our investigation of the plant growth activity of some thiolacetic acids [4,5] these two acids seemed to be of interest and therefore were prepared. Phenyl- and 1-naphthylacetic acids were transformed to the corresponding acetyl chlorides by phosphorus pentachloride in carbon tetrachloride solution. The acid chlorides were thoroughly purified by vacuum distillation and then reacted with sodium hydrosulfide in water solution. Both thiolacetic acids were purified by vacuum distillation and the 1-naphthylthiolacetic acid was recrystallized as well. The physical data for the acetyl chlorides and the thiolacetic acids are given in Table 1.

The infra-red spectra of the phenylthiolacetic acid in a solution of carbon disulfide (Fig. 1) and of the 1-naphthylthiolacetic acid in KBr (Fig. 2) have been recorded.

Experimental results. Preparation of phenylacetyl chloride 45 g phenylacetic acid dissolved in 180 ml carbon tetrachloride was mixed with 70 g phosphorus pentachloride (equiv.amount) at 60°C. After completed reaction the carbon tetrachloride was distilled off. By vacuum distillation we obtained at 92–98°C and 7 mm Hg 40 g phenylacetyl chloride (yield 78 %). The product was redistilled at 90–92°C and 7 mm Hg. (Found: Cl 22.5. Calc. for C_8H_7OCl: Cl 22.9.)

Preparation of phenylthiolacetic acid. 31 g phenylacetyl chloride was slowly added to a sodium hydrosulfide solution (20 g sodium hydroxide dissolved in 150 ml water and saturated with hydrogen sulfide) at 70°C. After about an hour the reaction solution was cooled to room temperature and filtered. After acidifying with hydrochloric acid the precipitated oil was extracted with ether. The ether was dried with anhydrous sodium sulfate and distilled. At 90–93°C and 2 mm Hg we obtained 22 g phenylthiolacetic acid (59 %). The acid was further purified by distillation at 92°C and 2 mm Hg. (Found: S 20.9; equiv.wt. 151.0. Calc. for C_8H_8OS: S 21.06; equiv.wt. 152.22.)

Preparation of 1-naphthylacetyl chloride. 30 g 1-naphthylacetic acid (from PAL. Ltd, London, M. P. 132.3–132.7°C) dissolved in 110 ml carbon tetrachloride was reacted with 37 g phosphorus pentachloride as described above. At 152°C and 4 mm Hg 26 g 1-naphthylacetyl chloride was obtained (yield 79 %). (Found: Cl 18.1. Calc. for $C_{12}H_9OCl$: Cl 17.3.)

Preparation of 1-naphthylthiolacetic acid. 25 g 1-naphthylacetyl chloride was reacted with sodium hydrosulfide in the same way as phenylacetyl chloride. By acidifying after completed reaction a yellow oil was obtained, which was washed with water. By drying in vacuum the oil crystallized. The yield of crude 1-naphthylthiolacetic acid was 23 g (94 %). The acid was distilled in vacuum at 170–173°C and 1 mm Hg. Recrystallization from chloroform-petroleum ether gave light yellow crystals with m. p. 59.6–60.2°C. (Found: S 15.5; equiv.wt. 204.7. Calc. for $C_{12}H_{10}OS$: S 15.85; equiv.wt. 202.28.)

Table 1. Physical data.

Compound	Boiling point °C/mm Hg	Melting point °C	n_D^{20}	n_4^{20}	MR_D
Phenylacetyl chloride	90– 92/7	colorless oil	1.5328	1.1697	141.00
Phenylthiolacetic acid	92/2	» »	1.5722	1.1409	43.91
1-Naphthylacetyl chloride	152/4	» »	1.6209	1.2281	58.60
1-Naphthylthiolacetic acid	170–173/1	59.6–60.2			

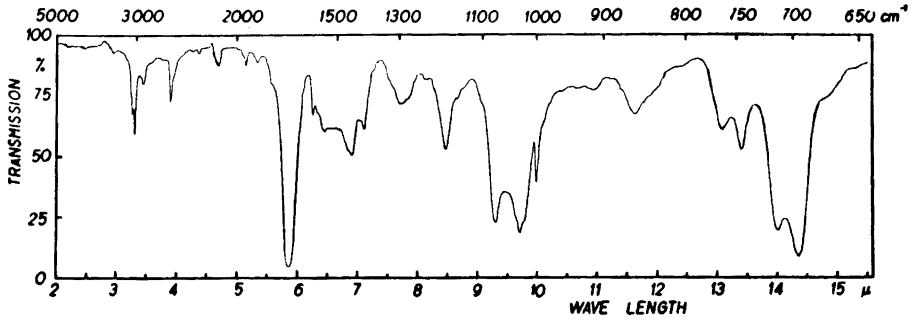


Fig. 1. The infra-red spectrum of phenylthiolacetic acid in CS_2-solution.

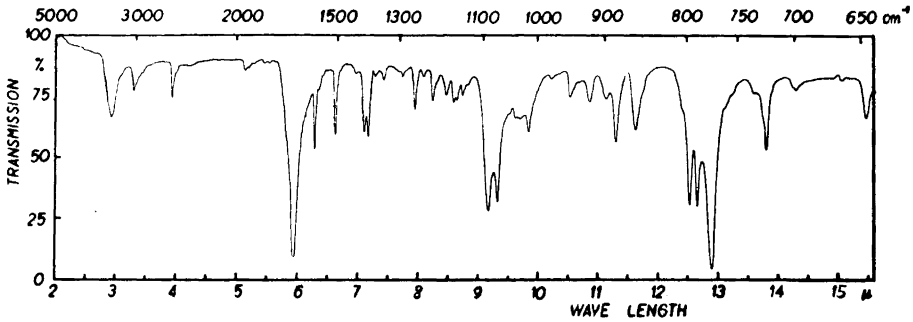


Fig. 2. The infra-red spectrum of 1-naphthylthiolacetic acid in KBr.

Infrared spectra were recorded with a Perkin-Elmer spectrometer, Model 21a, with a rock-salt prism. Slit schedule, corresponding to program 4. Speed 3 min/μ. The frequencies of the absorption bands are given below in cm^{-1}.

Phenylthiolacetic acid (Fig. 1) 133.7 g/l CS_2-solution. Cell: 0.1 mm 3 035 (w), 2 574 (w), 1 708 (s), 1 601 (w), 1 409 (m), 1 300 (w), 1 180 (m), 1 075 (s), 1 030 (s), 1 003 (m), 860 (m), 764 (m), 745 (m), 714 (s), and 697 (s).

1-Naphthylthiolacetic acid (Fig. 2) 2.0 mg/0.35 g KBr. 3 431 (w), 3 044 (w), 2 554 (w), 1 685 (s), 1 596 (w), 1 512 (w), 1 411 (w), 1 398 (w), 1 260 (w), 1 091 (m), 1 075 (m), 1 017 (w), 886 (w), 861 (w), 798 (m), 791 (m), 776 (s), 725 (w) and 648 (w).

This work was carried out 1954—1955 at the Research Laboratories, Skoghallsverken, Uddeholms AB, Skoghall, Sweden. The author gratefully acknowledges the assistance of ing. Bengt Axelsson and Torvald Olofsson (syntheses), ing. Bengt Berglund (infra-red spectrometry) and ing. Esse Zetterqvist (chemical analyses).

1. Sjöberg, B. *Acta Chem. Scand.* **11** (1957) 945.
2. Johnson, B. *J. Am. Chem. Soc.* **28** (1906) 1457.
3. Sakurada, Y. *Mem. Coll. Sci. Univ. Kyoto, Ser A* **10**, 76; *Chem. Zentr.* **1927** I, 1301.
4. Sjöberg, B., Sw. P. 158 352 (1953).
5. Burström, H., Sjöberg, B. and Hansen, B. *Acta Agr. Scand.* **VI:2** (1956) 155.

Received May 6, 1959.

Ternary Complex Formation of Fatty Acids and Fatty Acid Amides with Horse Liver Alcohol Dehydrogenase-Coenzyme Complexes

ALFRED D. WINER* and HUGO THEORELL

Nobel Medical Institute, Biochemical Department, Stockholm, Sweden

In 1955 Theorell *et al.*[1] concluded from reaction velocity constant measurements that formate reacts with the ADH—DPN ** complex but not with ADH—DPNH and suggested that it enters into the binding site for ethanol in ADH—DPN. More recent studies by Theorell and Winer [2] have shown that while formate has a small effect on D_{red}, it exerts a very strong effect on D_{ox} and suggested that the effect of formate depends on the formation of a ternary ADH—DPN-formate complex.

Since the fluorometric method appeared to offer a very rapid procedure for scanning the effects of potential inhibitors on both D_{ox} and D_{red} it was decided to investigate the effects of other saturated fatty acids. All fatty acids studied (C_1—C_{20}) form ternary complexes with ADH—DPN and compete with the ethanol binding site. It is possible to calculate the dissociation constants describing the effects of ADH-fatty acid, $K_{E,I}$, ADH—DPN-fatty acid, $K_{EO,I}$, and ADH-fatty acid-DPN, $K_{EI,O}$. The formation of fluorescent ternary complexes of ADH—DPNH-amides is also reported and it is suggested that these complexes, just as the fluorescent lactic dehydrogenase-DPNH-anion complexes recently described by Winer and Schwert [3], are similar in structure to the activated complex involved in the conversion of ADH—DPNH-aldehyde to ADH—DPN-alcohol.

Experimental. Fluorometric measurements of emission spectra and the calculation of dissociation constants have been previously described [2,4] and were made at 23.5°C in 0.1 ionic strength phosphate buffer, pH 7.03. Pure crystalline ADH was prepared as described earlier [3]. DPN and DPNH were commercial products from the Sigma Chemical Company. Fatty acids, C_1—C_8, were reagent grade chemicals further purified by distillation under suitable pressures; C_{10}—C_{20} acids were gifts of Professor E. Stenhagen and solubilized after lyophilization of the potassium salts. Amides were Eastman Kodak products.

Discussion and results. Long chain saturated fatty acids exhibit very strong ternary complex formation with ADH—DPN. The dissociation constant for the ternary complex, ADH—DPN-fatty acid, $K_{EO,I}$, decreases from 2 800 μM for formate to 0.7 μM for C_{15} fatty acid. While the dissociation constant for fatty acid and ADH, $K_{E,I}$, decreases from 40 000 μM for formate to 6 μM for C_{15} acid, the dissociation constant reflecting the effect of DPN on the ADH-fatty acid complex, $K_{EI,O}$, is rather constant at about 5 μM to C_{11} acid and then increases to 2—7 times this value from C_{11}—C_{15}. The ability of the fatty acids to form complexes of the type EI and EIO disappears abruptly from C_{16} and upwards but the dissociation constant reflecting the formation of ternary complex, $K_{EO,I}$, remains constant at about 25 μM from C_{16}—C_{20}. The formation of ADH—DPN-fatty acid results in a further quenching of the ADH—DPN emission spectra measured at 350 mμ when activated at 289 mμ. The acids show no effect on the ADH—DPNH emission spectra when measured and activated at the same wave lengths. From initial velocity measurements of the enzymatic reaction obtained kinetically, the acids compete with ethanol for the same binding site.

The corresponding fatty acid amides form fluorescent ternary complexes with ADH—DPNH but not with ADH—DPN. A representative emission spectrum with acetamide in shown in Fig. 1. The curve labelled ADH—DPNH-acetamide was obtained when acetamide at a final concentration of 0.2 M was added to ADH and DPNH present at the concentrations used for the other curves. While the wave length of maximum emission for ADH—DPNH is shifted to 450 mμ, the wave length for the ternary complex, ADH—DPNH-acetamide, is shifted to 440 mμ.

---

* U.S. Public Health Service Research Fellow of the National Institute of Neurological Diseases and Blindness, 1958—60.

** Abbreviations. ADH—DPN and ADH—DPNH, ½ molecule of horse liver alcohol dehydrogenase (E) and oxidized (O) or reduced (R) diphosphopyridine nucleotide, respectively; D_{ox}, the dissociation constant of ADH—DPN; D_{red}, the dissociation constant of ADH—DPNH; I, fatty acid or fatty acid amide.

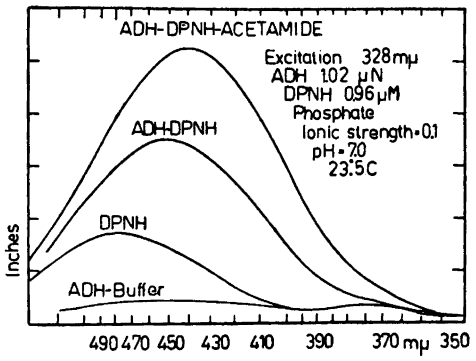


Fig. 1.

Similar wave length shifts have been observed with the lactic dehydrogenase-DPNH-carboxylic acid ternary complexes [3]. The lower amides, through *iso*butyramide, fluoresce more intensely than the ADH—DPNH complex, but higher molecular weight amides, though actually quenching, to varying extents, the fluorescence of ADH—DPNH, still exhibit the wave length shift. By analogy with kinetic experiments with formate [1], it is presumed that the very strong ternary complex formation observed with the longer chain amides has its effect on the "off" velocity of the ternary complex. The dissociation constants of the ternary complex, ADH—DPNH-amide, $K_{ER,I}$, and the ADH-amide complex, $K_{E,I}$, vary from 5 000 μM and 50 000 μM, respectively, for acetamide to 11 μM and 160 μM, respectively, for *n*-hexamide. $K_{EI,R}$ varies from 0.05 μM for acetamide to 0.02 μM for *n*-hexamide. From emission spectral data at 350 mμ the amides show a further quenching of the ADH—DPNH fluorescence when the ternary complex is formed. No effect, however, is seen with the ADH—DPN complex. As indicated by initial velocity measurements, the amides compete for the aldehyde binding site.

Ternary complex formation with the longer chain amides and acids is a logical consequence of the substrate specificity studies on liver ADH with different aldehydes and alcohols recently reported by one of us (A.D.W.) [6]. If it is considered that aldehydes and amides, and alcohols and acids, in this system, function as structural analogs, there is strong suggestion that the ADH—DPNH-amide and ADH—DPN-fatty acid complexes, in which no net reaction can occur, represent abortive complexes formed between ADH, DPNH, aldehyde and ADH, DPN, alcohol.

The complete data will be given in a forthcoming paper in this journal.

1. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
2. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys. In press.*
3. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1155.
4. Winer, A. D. and Schwert, G. W. *Science* **128** (1958) 660.
5. Theorell, H. *Advances in Enzymol.* **20** (1958) 31.
6. Winer, A. D. *Acta Chem. Scand.* **12** (1958) 1695.

Received May 14, 1959.

Isolation of Palustric Acid from the Oleoresins of North-European Pine and Spruce by Partition Chromatography

HENRIK H. BRUUN, STEIN GÅSLAND and GUNNAR LUNDQVIST

Institute of Wood Chemistry, Åbo Akademi, Åbo, Finland

Partition-chromatographic analyses of the resins of North-European (Scotch) pine, *Pinus silvestris*, and (Norway) spruce, *Picea abies*, have shown that these contain palustric acid. Palustric acid was discovered in 1954 by Loeblich, Baldwin and Lawrence in the gum and wood rosins of two American pine species, *Pinus palustris* and *Pinus caribaea* [1]. The acid has subsequently been isolated from the rosin of a further American pine, *Pinus elliotti*, and from gum rosin, wood rosin and tall oil rosin isolated from this species [2]. The acid has also been found as a component in tall oil rosin from North-European pine (Bruun and Gåsland [3,4]), but has not yet been isolated from the natural rosins of North-European tree species or other non-Ameri-

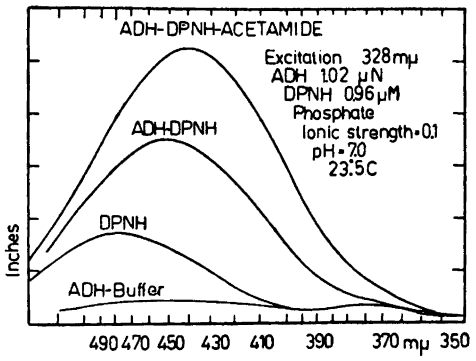


Fig. 1.

Similar wave length shifts have been observed with the lactic dehydrogenase-DPNH-carboxylic acid ternary complexes [3]. The lower amides, through *iso*butyramide, fluoresce more intensely than the ADH—DPNH complex, but higher molecular weight amides, though actually quenching, to varying extents, the fluorescence of ADH—DPNH, still exhibit the wave length shift. By analogy with kinetic experiments with formate [1], it is presumed that the very strong ternary complex formation observed with the longer chain amides has its effect on the "off" velocity of the ternary complex. The dissociation constants of the ternary complex, ADH—DPNH-amide, $K_{ER,I}$, and the ADH-amide complex, $K_{E,I}$, vary from 5 000 μM and 50 000 μM, respectively, for acetamide to 11 μM and 160 μM, respectively, for *n*-hexamide. $K_{EI,R}$ varies from 0.05 μM for acetamide to 0.02 μM for *n*-hexamide. From emission spectral data at 350 mμ the amides show a further quenching of the ADH—DPNH fluorescence when the ternary complex is formed. No effect, however, is seen with the ADH—DPN complex. As indicated by initial velocity measurements, the amides compete for the aldehyde binding site.

Ternary complex formation with the longer chain amides and acids is a logical consequence of the substrate specificity studies on liver ADH with different aldehydes and alcohols recently reported by one of us (A.D.W.) [6]. If it is considered that aldehydes and amides, and alcohols and acids, in this system, function as structural analogs, there is strong suggestion that the ADH—DPNH-amide and ADH—DPN-fatty acid complexes, in which no net reaction can occur, represent abortive complexes formed between ADH, DPNH, aldehyde and ADH, DPN, alcohol.

The complete data will be given in a forthcoming paper in this journal.

1. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
2. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys. In press.*
3. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1155.
4. Winer, A. D. and Schwert, G. W. *Science* **128** (1958) 660.
5. Theorell, H. *Advances in Enzymol.* **20** (1958) 31.
6. Winer, A. D. *Acta Chem. Scand.* **12** (1958) 1695.

Received May 14, 1959.

Isolation of Palustric Acid from the Oleoresins of North-European Pine and Spruce by Partition Chromatography

HENRIK H. BRUUN, STEIN GÅSLAND and GUNNAR LUNDQVIST

Institute of Wood Chemistry, Åbo Akademi, Åbo, Finland

Partition-chromatographic analyses of the resins of North-European (Scotch) pine, *Pinus silvestris*, and (Norway) spruce, *Picea abies*, have shown that these contain palustric acid. Palustric acid was discovered in 1954 by Loeblich, Baldwin and Lawrence in the gum and wood rosins of two American pine species, *Pinus palustris* and *Pinus caribaea* [1]. The acid has subsequently been isolated from the rosin of a further American pine, *Pinus elliotti*, and from gum rosin, wood rosin and tall oil rosin isolated from this species [2]. The acid has also been found as a component in tall oil rosin from North-European pine (Bruun and Gåsland [3,4]), but has not yet been isolated from the natural rosins of North-European tree species or other non-Ameri-

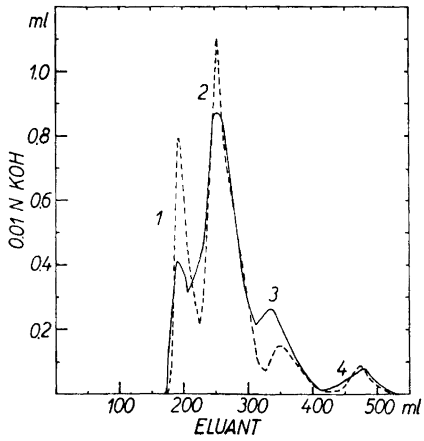


Fig. 1. Chromatogram of the rosin acids in the oleoresins of North-European pine (———) and spruce (— — —). Ordinate gives consumption of 0.010 N alcoholic potassium hydroxide in the titration of 5-ml eluent volumes employing phenolphthalein as indicator. Peak 1 contains palustric acid. For the compositions of the other peaks, see text.

can species. Similarly as other abietic acids, palustric acid may be transformed by isomerization into other rosin acids and may also be formed by similar molecular rearrangements from other rosin acids, for instance, from levopimaric acid by heat treatment [1,5].

Experimental. The analyzed samples consisted of rosin that had recently oozed from the trunks of 60-year-old living trees of pine and spruce from which the bark had been removed to increase the oleoresinous exudation. The acid value of the pine rosin was 125 mg and that of the spruce rosin 110 mg of potassium hydroxide per g, which figures indicate that the samples contained 67 and 59 % rosin acids (based on an assumed mean equivalent weight of 302). The respective saponification values were 137 and 124 mg of potassium hydroxide per g.

In order to obtain correct analytical data on the rosin acid contents of the natural, unchanged rosins, the samples were chromatographed without prior separation of the rosin acids from other components, primarily terpenes, present in the samples. These latter components were not found to cause any difficulties in the chromatographic analyses.

The chromatographic analyses were performed according to Ref.[1] with aminopyridine and furfuryl alcohol as the immobile phase on silicic acid and *iso*octane as the eluent. For details, see Ref.[3]

Results. The chromatograms of the pine and spruce resins are reproduced in Fig. 1. Each chromatogram exhibits four peaks. The peak volumes and peak areas are given in Table 1.

Table 1. Chromatographic data for the rosin-acids in oleoresins from North-European pine (*Pinus silvestris*) and spruce (*Picea abies*).

Peak No. in Fig. 1	Peak volume ml	Peak area % of total area
	Pinus silvestris	
1	190	18
2	250	59
3	ca. 340	18
4	ca. 470	5
	Picea abies	
1	190	25
2	250	60
3	ca. 340	10
4	ca. 470	5

According to our earlier studies [3] peak 1 consists of palustric acid, peak 2 may include pimaric acids, *l*-abietic acid and levopimaric acid, peak 3 contains *neo*abietic acid and peak 4 dehydrogenated rosin acids.

In order to confirm the identity of the acid in peak 1, the respective eluant fractions were combined, the *iso*octane removed by reduced pressure, and the resulting acid dissolved in acetone and reprecipitated with *cyclo*hexylamine [6]. A sample of palustric acid was also precipitated as its *cyclo*hexylamine salt under the same conditions. The *cyclo*hexylamine salts, which were isolated in a yield of 85—90 %, were dissolved in optically pure alsolute ethanol and the ultraviolet absorption spectra of the solutions recorded. The recorded ultraviolet spectra (Fig. 2) showed that the *cyclo*hexylamine salts of the acids in peak 1 of pine and spruce were identical with the spectrum for the *cyclo*hexylamine salt of the sample of palustric acid. The specific extinction coefficient (a) for the latter salt at 265—266 mμ is 25.0, while the value

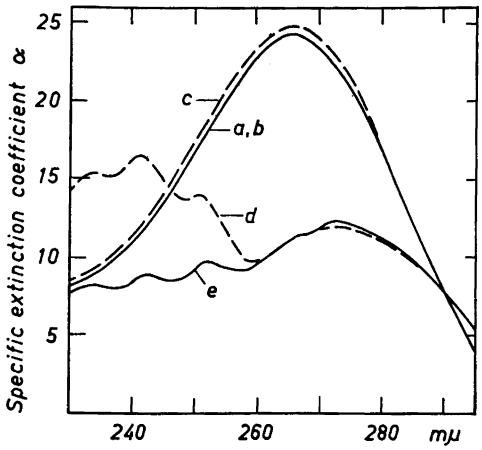


Fig. 2. Ultraviolet absorption spectra of palustric acid (in form of its *cyclo*hexylamine salt) isolated by partition chromatography from oleoresins of North-European pine (*a*) and spruce (*b*) and the spectrum of the *cyclo*hexylamine salt of a palustric acid sample (*c*). Curves *d* and *e* gives the absorption spectra of the *cyclo*hexylamine salts of the acids in peak 2 (in Fig. 1) from pine and spruce respectively. The specific extinction coefficients α were calculated to refer to the acids rather than to the salts.

of very pure palustric acid reported by Loeblich, Baldwin and Lawrence [1] is 30.1. This implies that the palustric acid sample we have employed as reference compound contained 83 % palustric acid. (The ultraviolet spectrum of the latter acid in absolute ethanol gave the same value for the specific extinction coefficient.) The palustric acid content of the pine resin was thus 15 % and that of the spruce rosin 21 % of the total rosin acids in the sample. Palustric acid hence represents a major component in the rosin of North-European pine and spruce. The palustric acid content is higher in the spruce rosin than in any pine species, where its content has been found to vary between 7 and 12 % (see Ref.[2]).

We have also isolated by *cyclo*hexylamine precipitation the acids in peak 2 and determined the ultraviolet absorption of the precipitate in absolute ethanol. The recorded spectrum for the acids in peak 2 for pine rosin (Fig. 2d) exhibits a maximum absorption, $\alpha = 16.6$, at 241 mμ and another, $\alpha = 12.0$, at 272 mμ. These maxima are characteristic for *l*-abietic and levopimaric acids, respectively; the absorption values indicate that the *l*-abietic acid content in peak 2 for the pine is about 18 % or 11 % of the total rosin acid fraction and the levopimaric acid content in the same peak is 63 % or 37 % of the total rosin acid fraction (in these calculations the contribution of levopimaric acid to the α-value of *l*-abietic acid has been taken into account). The corresponding values of α for spruce (Fig. 2 e) are 9.0 and 12.4, which imply that the abietic acid content is 7.5 %, or 4.5 % of the total rosin acids, and the levopimaric acid content 65 %, or 39 % of the total rosin acids. In American pine species [2] the abietic acid content is near 10 % and the levopimaric acid content between 24 and 31 %. The distribution of rosin acids in the oleoresin of the North-European spruce thus differs essentially, particularly with respect to palustric acid, but also with respect to abietic acid, from that in the pine species.

The authors are indebted to Dr. E. L. Patton, Naval Stores Research Station, Olustee, U.S., for the sample of palustric acid. One of us (H.B.) has received a grant from the *State Commission for Natural Sciences of Finland* and one (S.G.) a grant from the *Royal Norwegian Council for Scientific and Industrial Research.* This aid is gratefully acknowledged.

1. Loeblich, V. M., Baldwin, D.E. and Lawrence, R.V. *J. Am. Chem. Soc.* **77** (1955) 2823.
2. Baldwin, D. E., Loeblich, V. M. and Lawrence, R. V. *Ind. Eng. Chem Data Series* **3** (1958) 342.
3. Bruun, H. H. and Gåsland, S. *Acta Chem. Scand.* **13** (1959). 598.
4. Bruun, H. H. *Paper and Timber (Finland)* **41** (1959) 73.
5. Loeblich, V. M., Baldwin, D. E., O'Connor, R. T. and Lawrence, R. V. *J. Am. Chem. Soc.* **77** (1955) 6311.
6. Harris, G. C. and Sanderson, T. F. *J. Am. Chem. Soc.* **70** (1948) 334.

Received June 1, 1959.

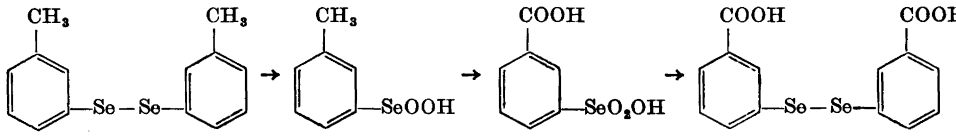
Diphenyldiselenide-3,3'-dicarboxylic Acid

ARNE FREDGA and
CHRISTINA EVERTSDOTTER

Chemical Institute, University of Uppsala, Uppsala, Sweden

The diphenyldiselenide-2,2'-dicarboxylic acid is easily obtained from diazotised anthranilic acid and alkali diselenide [1]. The 4,4'-isomer has been prepared in the same way [2] and also *via* the 4-selenocyanobenzoic acid [3], but all attempts to obtain the 3,3'-compound (IV) from diazotised 3-aminobenzoic acid have failed [2,4]. It is known that the corresponding sulphur compounds behave in the same manner, but here the 3,3'-isomer is easily obtained from 3-chlorosulphonyl-benzoic acid [5].

As the isomeric diphenyldiselenide-dicarboxylic acids and their methyl esters were needed for spectrochemical studies, we have tried to prepare the 3,3'-isomer by the following route:



(I) (II) (III) (IV)

The difficulty is to oxidise the methyl group without loss of selenium and destruction of the molecule. It was found that careful oxidation with permanganate on a small scale and subsequent reduction with tin and hydrochloric acid gave the desired acid (IV) in very good yield. It was not necessary to isolate the intermediate III.

The methyl ester was easily obtained from the acid and diazomethane.

Experimental: The *3,3'-ditolyldiselenide (I)* was prepared from 3-tolylmagnesium bromide and elementary selenium. The procedure of Foster [6] was followed with some minor modifications. The compound was obtained as an orange-coloured oil, which did not crystallise even after standing at −15°C for 8 months.

3-Tolylseleninic acid (II) was obtained by oxidation of the diselenide with perhydrol in ethereal solution according to Mc Cullough and Gould [7].

Diphenyldiselenide-3,3'-dicarboxylic acid (IV). In a 250 ml three-necked flask, fitted with a stirrer, a reflux condenser and a dropping funnel, was placed a solution of 2.03 g 3-tolylseleninic acid and 0.85 g sodium hydrogen carbonate in 30 ml of water. A solution of 2.99 g potassium permanganate in 45 ml of water was added dropwise with stirring, after which the solution was heated gently for 15 min. 5.08 g of solid potassium permanganate were then added, and the stirring and gentle heating continued for 1 h. The excess permanganate was then destroyed by adding 20 ml of ethanol. The manganese oxides were filtered off and washed with water. The combined filtrates were then concentrated to 100 ml and heated with 10 g tin and 100 ml concentrated hydrochloric acid. A current of air was passed through the solution to oxidise any selenol groups which might have been formed. The acid separated as a yellow microcrystalline powder which was filtered off, dissolved in sodium hydrogen carbonate solution, and reprecipitated with hydrochloric acid. The yield was nearly quantitative. M. p. 238° (decomp.). (Found: Se 39.4. Calc. for $C_{14}H_{10}O_4Se_2$: Se 39.47.)

Dimethyl diphenyldiselenide-3,3'-dicarboxylate. 1.0 g acid was dissolved in 50 ml of ether, and an ethereal solution of diazomethane was added drop by drop until nitrogen was no longer evolved. The ether was evaporated and the residue recrystallised from methanol. The ester was obtained as small yellow needles. M. p. 74—75°. (Found: Se 36.8. Calc. for $C_{16}H_{14}O_4Se_2$: 36.88.)

A grant from *AB Astra* is gratefully acknowledged.

1. Schoeller, A. *Ber.* **52** (1919) 1517.
2. Lesser, R. and Weiss, R. *Ber.* **46** (1913) 2651.
3. Gaythwaite, W. R., Kenyon, J. and Phillips, H. *J. Chem. Soc.* **1928** 2286.
4. Bergson, G. *Private communication.*
5. Smiles, S. and Stewart, J. *J. Chem. Soc.* **119** (1921) 1792.
6. Foster, G. *Org. Syntheses* **24** (1944) 89.
7. Mc Cullough, J. D. and Gould, E. S. *J. Am. Chem. Soc.* **71** (1949) 674.

Received June 2, 1959.

On the Transfer of L-5-Vinyl-2-thiooxazolidone from the Rumen to the Milk

ARTTURI I. VIRTANEN, MATTI KREULA and MARTTI KIESVAARA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

When the method for microdetermination of goitrogenous L-5-vinyl-2-thiooxazolidone (VTO) was further developed in this laboratory, it was found that this compound disappears from the milk even at low temperatures*. This disappearance takes place in different milk samples with varying speed. If the milk is heated immediately after milking, no disappearance occurs even within several days at low temperature. Also at room temperature retention is then fairly good for one day. Kreula and Kiesvaara will present the analytical method for determination of VTO both in cruciferous plants and in milk in this journal.

Taking the above observations on the retention of VTO in milk into consideration, we have made numerous feeding experiments with cows fed with green turnip rape, green rape, and marrow kale. The results showed that VTO is transferred to the milk in very small amounts [1]. When a cow was fed with 15 kg of marrow kale twice a day, 5 μg of VTO per litre was found in the milk (daily milk production 6 l). Altogether 0.05 % of the total amount of VTO formed in marrow kale (2.2 mg of VTO per kg fresh plants) was transferred to the milk. In another experiment in which 10 kg of green rape (28 mg of VTO per kg fresh plants) was once fed per cow to a group of four cows (milk production 12 h after the feeding was 5 l per cow), 27 μg of VTO per litre was found in the milk. Also in this case 0.05 % of the total VTO in fodder was transferred to the milk.

When 500 mg of crystalline VTO was fed to a cow, and the cow was milked 2, 6.5, 10.5, 24, and 48 h after feeding, the following amounts of VTO were found in the milk (Table 1).

The experiment showed that VTO is very rapidly transferred from the rumen to the milk. In milk samples taken 24 h after feeding, VTO could not be found with certainty in the milk. Of the 500 mg crystalline VTO fed, about 0.05 % was found in the milk. It is remarkable that the same percentage of VTO is transferred to the milk, on the one hand when the cow is fed with green plants which contain the glycoside from which VTO is formed in the rumen, and on the other hand when feeding crystalline VTO.

In another feeding experiment 1.6 g of the precursor (glycoside of 2-hydroxy-3-butenyl*iso*thiocyanate compound isolated from rape seeds) of VTO was fed to a cow. Only traces of VTO could be found in the milk. The results show that the glycoside is not appreciably hydrolyzed in the rumen without the enzyme present in cruciferous plants. The quantitative, or almost quantitative, enzymatic hydrolysis of the glycoside in the rumen by the influence of the enzyme present in

* Later it has been noticed that the VTO reappears in the milk on treatment with H_2S.

Table 1. Transfer of VTO from rumen to milk when the cow is fed with 500 mg of crystalline VTO. The feeding took place immediately after morning milking, and the cow was then milked after the hours given in the table.

	Before VTO feeding	After 2 h	6.5 h	10.5 h	24 h	48 h
VTO content of milk μg/l	0	190	35	17	possibly traces	0
VTO transferred to milk, % of total fed	—	0.035	0.013	0.006		
			0.054			

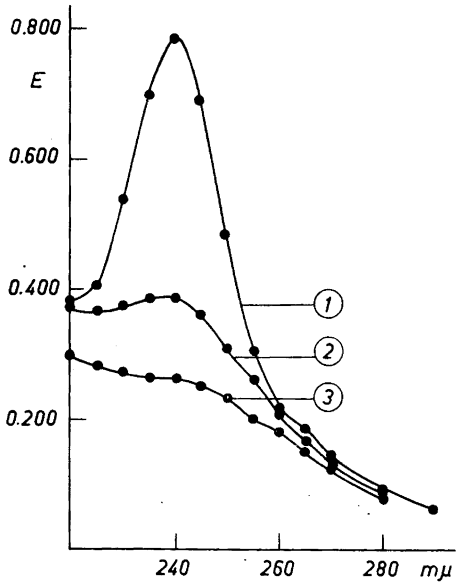


Fig. 1. UV-spectra of VTO in cow's milk after feeding of 500 mg of crystal VTO. The curves show the relative values after 2 h (1), 6.5 h (2), and 48 h (3).

the plants in question is supported by these results.

The earlier performed feeding experiment with 0.5 kg of crushed moistened rape seeds, whereby so large an amount of VTO as 4.5 g could be formed in the seeds, gave a negative result as to the transfer of VTO to the milk [2]. The result probably partly depended on that the milk came to stand unheated for several hours before the analysis. Another cause may, however, be the physiologically abnormally large amount of VTO which could be formed in the rape seeds fed. This unfavourable effect was shown by the fact that the cow lost her appetite for some days after the feeding. A new experiment with 100 g of crushed moistened rape seeds in which 900 mg of VTO could be formed, showed that 12 h after feeding the milk sample contained 100 μg of VTO per litre. 0.07 % of the total amount of VTO formed in the crushed seeds was thus transferred to the milk.

On the basis of the experiments performed, it can be said that about 0.05 % of the VTO present in the fodder, or formed in it during chewing and in the rumen, regularly seems to be transferred to the milk. When feeding cows on farms with green cruciferous plants, the amount of VTO can hardly exceed 100 μg of VTO per litre of milk. It is therefore not very likely that the cruciferous plants in the feeding of cows could increase the VTO content of the milk to such a degree that the milk could cause disturbances in the functioning of the thyroid gland of man. The problem requires a thorough elucidation, however. Investigations on the presence of substances which possibly disturb the functioning of the thyroid gland, in plants used by man and animals, as well as on their mode of action, are in progress in this laboratory.

1. Communication at the meeting of Societas Biochemica, Biophysica et Microbiologica Fenniae on October 27, 1958. *Suomen Kemistilehti A* **32** (1959) 45.
2. Virtanen, A. I., Kreula, M. and Kiesvaara, M. *Acta Chem. Scand.* **12** (1958) 580.

Received April 30, 1959.

Correction to "Hydrolysis of Carboxylic Acid Esters of Thiocholine and its Analogues" *

EDITH HEILBRONN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

In Table 7, p. 1503, first line from above *for* B_{OH^-} 2 760 ± 350 *read* B_{OH^-} 2 890 ± 350 and for *E* 12.6 kcal *read E* 13.2 kcal.

Received May 22, 1959.

* *Acta Chem. Scand.* **12** (1958) 1492.

New Syntheses of 3-Bromothiophene and 3,4-Dibromothiophene

SALO GRONOWITZ

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

3-Bromothiophene is a key intermediate in the preparation of 3-substituted thiophenes [1]. It has been obtained from 2,3,5-tribromothiophene by removal of the α-bromines through the Grignard, entrainment method [2] with ethyl bromide as the auxiliary halide or through halogen metal interconversion with *n*-butyllithium [3]. In both cases the metal-organic compounds were hydrolyzed with water. It has also been obtained from 4,5-dibromo-2-thiophenecarboxylic acid through simultaneous debromination and decarboxylation with copper powder in quinoline [4].

Zinc-dust in acid media is a classical reagent for the removal of halogen and has been used for the preparation of alkanes from alkyl halides [5]. It appears however, that this reagent has been considered rather unspecific and therefore has not been used for selective dehalogenation of polyhalogen compounds. The effectiveness of this reagent is evident from the results of Bak *et al.*[6] They reduced 3-iodo- and 3,4-diiodothiophene to the corresponding deuterated thiophenes in 75 min at 100°C using Zn-dust in CH_3COOD and D_2O.

For other investigations 2,3- and 2,4-dideuterothiophene were needed [7]. It was, however, found that when the corresponding bromine compounds were subjected to dehalogenation with Zn-dust in acetic acid under conditions similar to Bak's only small yields of the desired dideuterated compounds were obtained [7]. The main products (70—80 %) were the corresponding deuterated 3-bromothiophenes. With ordinary acetic acid 3-bromothiophene free from the 2-isomer was obtained.

This prompted the present author to investigate the possibility of obtaining isomer-free 3-bromothiophene from 2,3,5-tribromothiophene and 3,4-dibromothiophene from tetrabromothiophene. It was also found that refluxing 2,3,5-tribromothiophene with Zn-dust in acetic acid gave an 80 % yield of 3-bromothiophene free from the 2-isomer (< 0.5 %). This fact was established by the absence in the product's IR-spectrum of the very strong 2-bromothiophene absorption band [2] at 10.25 μ.

It is interesting to note that in the dehalogenation of tetrabromothiophene under similar conditions a 65 % yield of 3-bromothiophene and only 10 % of 3,4-dibromothiophene was obtained. The IR-analysis proved the absence of other isomeric bromothiophenes. These results indicate that one β-bromine facilitates the removal of another. By changing the experimental conditions it was, however, possible to obtain 3,4-dibromothiophene in 68 % yield.

This preparation of 3-bromothiophene is more convenient and gives better yield than earlier methods.

Experimental. 3-Bromothiophene. To a mixture of 55 g of 2,3,5-tribromothiophene, 32 ml of acetic acid and 100 ml water, 65 g of Zn-dust was added. The mixture which grew hot was frequently shaken during the first half hour and then refluxed for 16 h. Thiophene 3-bromothiophene and water were directly distilled off until the temperature of the vapour had risen to 103°. The organic layer was separated, dried with anhydrous sodium carbonate and fractionated. After a small forerun consisting of mainly thiophene (1.2 g), 22.4 g of 3-bromothiophene, b.p. 159—161° $n_D^{20} = 1.5919$, was obtained, which had the same IR-spectrum as authentic 3-bromothiophene [2].

3,4-Dibromothiophene. In a two-necked flask fitted with a refluxcondenser and a dropping-funnel was placed 65 g of tetrabromothiophene 65 ml of acetic acid 200 ml of water and 65 g of Zn-dust. The mixture was heated until a vigorous reaction started. The heating mantle was removed and when the reaction had somewhat subsided the flask was arranged for distillation. The reaction mixture was rapidly distilled. After each 50 ml of distillate the heavier organic layer was separated and the aqueous layer returned to the reaction mixture through the dropping-funnel. After 3 h 35 g of organic phase had been obtained and no more distilled with the water. The organic phase was dried with sodium carbonate and fractionated *in vacuo*. After a forerun of 3.2 g, b.p. 45—94°/12 mm Hg, consisting of 3-bromo- and 3,4-dibromothiophene, 26,5 g (68 %) of pure 3,4-dibromothiophene, b.p. 94—95°/12 mm Hg, $n_D^{20} = 1.6380$ was obtained. The IR-spectrum was identical to that of the authentic 3,4-dibromothiophene [8] and the strong characteristic lines of 2,3-, 2,4- and 2,5-dibromothiophene in the 10.0—10.3 μ region were absent in its spectrum.

1. Gronowitz, S. *Arkiv Kemi* **13** (1959) 295.
2. Gronowitz, S. *Arkiv Kemi* **7** (1954) 267.
3. Lawesson, S. O. *Arkiv Kemi* **11** (1957) 373.
4. Motoyama, R., Nishimura, S., Imoto, E., Murakami, Y., Hari, K. and Ogawa, J. *J. Chem. Soc. Japan* **78** (1957) 950.
5. Wislicenus, J., *Ann.* **219** (1883) 312.
6. Bak, B., Christensen, P., Rastrup-Andersen, J. and Tannenbaum, E. *J. Chem. Phys.* **25** (1956) 892.
7. Hoffman, R. A. and Gronowitz, S. *Arkiv Kemi. In print.*
8. Steinkopf, W., Jacob, H. and Penz, M. *Ann.* **512** (1934) 136.

Received June 9, 1959.

The Identification of Drugs

VI. Nitration of Acetarsol *

K. ILVER, J. BERGER and Aa. JACKEROTT

Laboratory of the Pharmacopoeia Commission, and The Royal Danish School of Pharmacy, Chemical Laboratory B, Copenhagen, Denmark

In order to obtain a suitable derivative of acetarsol (3-acetamido-4-hydroxyphenylarsonic acid) as an identity test in the Scandinavian Pharmacopoeia nitration was tried. Burschkies and Rothermundt [1] state that acetarsol treated with a mixture of concentrated nitric acid and concentrated sulphuric acid at a temperature below 0°C yields 5-nitroacetarsol (m.p. 218–220°C).

In the present work 2 M nitric acid was added to acetarsol and the solution was boiled for 15 sec and then cooled. A yellow derivative immediately crystallised and it did not contain arsenic.

We have now proved that the derivative is identical with 2-acetamido-4,6-dinitrophenol which means that besides the expected introduction of a nitro group *ortho* to the hydroxyl group another nitro group has replaced the arsonic group to give a dinitro derivative. The identity was confirmed by quantitative titration with titanous chloride and mixed melting point determination with an authentic sample prepared by nitration of 2-acetamidophenol. Furthermore, the free phenol group of the derivative was acetylated and the resulting product was found to be identical with O,N-diacetylpicramic acid. This compound was prepared by acetylation of picramic acid.

The proposed derivative is specific for acetarsol as none of the other arsenicals used as drugs will give this derivative.

Experimental. 2-Acetamido-4,6-dinitrophenol from acetarsol. 0.1 g acetarsol is added 5 ml of 2 M nitric acid and the solution is boiled about 15 sec. and then immediately cooled with tap-water. The yellow precipitate is recrystallised from 10 ml of water. M.p. (corr.) 206–207°C (lit.[2] 205–206°C). Titanometric equiv. weight, found: 20.2, 20.1. Calc. for $C_8H_7O_6N_3$: 20.1. If the mixture is boiled for 5 min no precipitate is formed, probably due to an oxidation.

Acetylation of picramic acid and 2-acetamido-4,6-dinitrophenol. The substance is refluxed for 30 min with acetic anhydride and a few drops of pyridine. After cooling water is added and the precipitate is recrystallised from glacial acetic acid. Nearly colourless crystals, m.p. (corr.) 191°C (lit.[2] 190–191°C).

1. Burschkies, K. and Rothermundt, M. *Arch. Pharm.* **276** (1938) 226.
2. King, H. *J. Chem. Soc.* **1927** 1049.

Received June 11, 1959.

* Part V. *Dansk Tidsskr. Farm.* **32** (1958) 81.

Correction to "Least Square Method for Computor Calculations of Stability Constants" *

JAN RYDBERG and JAMES C. SULLIVAN

Chemistry Division, Argonne National Laboratory, Lemont, Illinois, USA

P. 187, right column, first line from above,

for $L = 35$ *read* $L = 46$.

* Acta Chem. Scand. **13** (1959) 186.

Received March 11, 1959.

1. Gronowitz, S. *Arkiv Kemi* **13** (1959) 295.
2. Gronowitz, S. *Arkiv Kemi* **7** (1954) 267.
3. Lawesson, S. O. *Arkiv Kemi* **11** (1957) 373.
4. Motoyama, R., Nishimura, S., Imoto, E., Murakami, Y., Hari, K. and Ogawa, J. *J. Chem. Soc. Japan* **78** (1957) 950.
5. Wislicenus, J., *Ann.* **219** (1883) 312.
6. Bak, B., Christensen, P., Rastrup-Andersen, J. and Tannenbaum, E. *J. Chem. Phys.* **25** (1956) 892.
7. Hoffman, R. A. and Gronowitz, S. *Arkiv Kemi. In print.*
8. Steinkopf, W., Jacob, H. and Penz, M. *Ann.* **512** (1934) 136.

Received June 9, 1959.

The Identification of Drugs

VI. Nitration of Acetarsol *

K. ILVER, J. BERGER and Aa. JACKEROTT

Laboratory of the Pharmacopoeia Commission, and The Royal Danish School of Pharmacy, Chemical Laboratory B, Copenhagen, Denmark

In order to obtain a suitable derivative of acetarsol (3-acetamido-4-hydroxyphenylarsonic acid) as an identity test in the Scandinavian Pharmacopoeia nitration was tried. Burschkies and Rothermundt [1] state that acetarsol treated with a mixture of concentrated nitric acid and concentrated sulphuric acid at a temperature below 0°C yields 5-nitroacetarsol (m.p. 218–220°C).

In the present work 2 M nitric acid was added to acetarsol and the solution was boiled for 15 sec and then cooled. A yellow derivative immediately crystallised and it did not contain arsenic.

We have now proved that the derivative is identical with 2-acetamido-4,6-dinitrophenol which means that besides the expected introduction of a nitro group *ortho* to the hydroxyl group another nitro group has replaced the arsonic group to give a dinitro derivative. The identity was confirmed by quantitative titration with titanous chloride and mixed melting point determination with an authentic sample prepared by nitration of 2-acetamidophenol. Furthermore, the free phenol group of the derivative was acetylated and the resulting product was found to be identical with O,N-diacetylpicramic acid. This compound was prepared by acetylation of picramic acid.

The proposed derivative is specific for acetarsol as none of the other arsenicals used as drugs will give this derivative.

Experimental. 2-Acetamido-4,6-dinitrophenol from acetarsol. 0.1 g acetarsol is added 5 ml of 2 M nitric acid and the solution is boiled about 15 sec. and then immediately cooled with tap-water. The yellow precipitate is recrystallised from 10 ml of water. M.p. (corr.) 206–207°C (lit.[2] 205–206°C). Titanometric equiv. weight, found: 20.2, 20.1. Calc. for $C_8H_7O_6N_3$: 20.1. If the mixture is boiled for 5 min no precipitate is formed, probably due to an oxidation.

Acetylation of picramic acid and 2-acetamido-4,6-dinitrophenol. The substance is refluxed for 30 min with acetic anhydride and a few drops of pyridine. After cooling water is added and the precipitate is recrystallised from glacial acetic acid. Nearly colourless crystals, m.p. (corr.) 191°C (lit.[2] 190–191°C).

1. Burschkies, K. and Rothermundt, M. *Arch. Pharm.* **276** (1938) 226.
2. King, H. *J. Chem. Soc.* **1927** 1049.

Received June 11, 1959.

* Part V. *Dansk Tidsskr. Farm.* **32** (1958) 81.

Correction to "Least Square Method for Computor Calculations of Stability Constants" *

JAN RYDBERG and JAMES C. SULLIVAN

Chemistry Division, Argonne National Laboratory, Lemont, Illinois, USA

P. 187, right column, first line from above,

for $L = 35$ *read* $L = 46$.

* Acta Chem. Scand. **13** (1959) 186.

Received March 11, 1959.

Isolation of ^{32}P-labeled Phosphorylserine from a Purified Preparation of Alkaline Bone Phosphatase Incubated with Radioactive Inorganic Phosphate

GUNNAR ÅGREN, ÖRJAN ZETTERQVIST and MAIE OJAMAE

Department of Medical Chemistry, University of Uppsala, Sweden

In a previous paper from this laboratory it was reported that ^{32}P-labeled phosphorylserine could be isolated from an acid hydrolysate of a commercial preparation of intestinal alkaline phosphatase [1]. This seemed to indicate that an intermediate enzyme-phosphate was formed in the enzyme reaction, as we had earlier suggested for the hexokinase reaction [2]. The results with intestinal alkaline phosphatase have recently been confirmed with a highly purified preparation of the enzyme [3]. Meanwhile similar results have also been obtained with preparations of alkaline bone phosphatase. Bone tissue is a poor source of phosphatase when compared with intestinal tissue [4].

The enzyme was first prepared from calf foreleg bones as described by Volkin [5] and for subsequent purification the method of Portmann [6] was followed. The fraction corresponding to step 14 was further purified by chromatography on a diethylaminoethyl cellulose column [3]. The buffer used was Tris-hydrochloric acid buffer pH 7.8. The column was loaded with 1 mg of protein per ml of column volume. The elution was performed with a stepwise increase of magnesium acetate concentration. The enzyme appeared in a protein peak at a magnesium acetate concentration of 0.04 M as determined by its ultraviolet absorbancy at 2 800 Å. The specific activity of the most active enzyme preparation, calculated according to Portmann, was 70 000 PU (Portmann units). In this way the enzyme was purified about 300 times as compared with the five-fold increase in purity obtained by Martland and Robison[7].

6 300 PU with the specific activity of 70 000 PU/mg N in Tris-buffer pH 7.0 was incubated with 0.75 mC of ^{32}P in the presence of 0.03 M magnesium acetate. After 5 min the enzyme was precipitated by adding 50 % trichloroacetic acid to a final concentration of 10 % and acetone was added to complete precipitation. The enzyme was hydrolyzed with 2 N HCl and radioactive phosphorylserine isolated as previously described [2] and identified by scanning of paper strips of electropherograms.

In a series of experiments 600 mg samples of enzyme with a specific activity of 420 PU/mg N and 5 mC ^{32}P were incubated at different pH for 5 min at room temperature. The total radioactivity of the isolated phosphorylserine fractions measured in L.K.B. Robot Scaler was the following:

pH 5 839 000 cpm	pH 8 629 000 cpm
(counts per min)	
pH 6 885 000 cpm	pH 9 126 000 cpm

In a second series of experiments similar samples of enzyme were incubated for 3, 6 12, 24, and 48 sec. with 5 mC ^{32}P. The total activities of the isolated phosphorylserine fractions were the same in all cases. The results indicate that inorganic phosphate is rapidly incorporated into the bone phosphatase molecule by covalent linkage. Calculations showed that the affinity of radioactive inorganic phosphate to bone alkaline phosphatase was of the same size as that of intestinal alkaline phosphatase. The incorporation of phosphate into the bone enzyme also increased within the same pH range as previously found with the intestinal enzyme [1]. The rapid incorporation and the high affinity of phosphate to the bone enzyme may be of interest with regard to its possible rôle as a transphosphorylating agent in bone tissue.

1. Engström, L. and Ågren, G. *Acta Chem. Scand.* **12** (1958) 357.
2. Ågren, G. and Engström, L. *Acta Chem. Scand.* **10** (1956) 489.
3. Engström, L. *Acta Soc. Med. Upsaliensis* (1959). *In Press.*
4. Kay, H. D. *Biochem. J.* **22** (1928) 855.
5. Volkin, E. *Methods in Enzymology* **2** (1955) 539.
6. Portmann, P. *Z. physiol. Chem. Hoppe-Seyler* **309** (1957) 87.
7. Martland, M. and Robison, R. *Biochem. J.* **23** (1929) 237.

Received June 11, 1959.

The Phosphotransferase Activity of Alkaline Phosphatases. Studies with Dephosphorylated Phosphopeptone as Phosphate Acceptor

GUNNAR ÅGREN

Department of Medical Chemistry, University of Uppsala, Sweden

In a recent paper it was reported that alkaline bone phosphatase catalysed the synthesis of phosphorylated amino acids and amines when labeled glycerophosphate was used as phosphate donor [1]. The present report describes similar experiments with dephosphorylated phosphopeptone as phosphate acceptor.

Radioactive glycerophosphate was obtained by use of AT^{32}P and glycerokinase prepared according to Bublitz and Kennedy [2]. Dephosphorylated phosphopeptone was obtained from a caseinphosphopeptone preparation made according to Mellander [3] and further separated in a four cell electrodialysing apparatus [4]. Material from cell 2 with a phosphorus content of 5.5 % was dephosphorylated with phosphoprotein-phosphatase purified in this institute [5] and at pH 5.8 in the presence of thioglycolic acid [6]. Dephosphorylated phosphopeptone was separated from phosphopeptone on a Dowex 1 formate column. Dephosphorylated phosphopeptone eluted from the column with water appeared in two fractions, one containing considerable amounts of sodium phosphate and a second rather saltfree fraction. This second fraction, II, was used in the following. It was not homogeneous. Electropherograms carried out at pH 3 showed the presence of at least 6 fractions.

A comparison was made between the transphosphorylating activity of alkaline phosphatases from milk [7] and bone [8]. 16 mg of milk phosphatase with a total activity of 1 120 Portmann units (PU)[9], and 23 mg of bone phosphatase with a total activity of 1 960 PU were each incubated with 25 mg of dephosphorylated phosphopeptone in bicarbonate buffer of pH 9.5 for 10 min at room temperature and in a total volume of 2 ml. To each solution 25 mg of glycerophosphate labeled with 1 mC of radioactivity had also been added. The heat-inactivated solutions were separated by gradient elution with 0 → 1 M formic acid on small Dowex 1 formate columns. Only in the experiment with milk phosphatase was it possible to isolate phosphopeptides where radioactivity moved parallel with ninhydrin colour on electropherograms. One of the 4 fractions gave sufficient amount of material for amino acid analysis and the one-dimensional chromatogram gave spots corresponding to aspartic acid, serine, glycine, glutamic acid, alanine and valine.

While the detailed sequence has not been established, it is to be noted that the composition of the peptide is in agreement with the composition of phosphopeptides obtained from the phosphate-catching "active sites" in chymotrypsin and phosphoglucomutase, respectively [10]. In a further attempt to elucidate Koshland's hypothesis [10] 90 mg of dephosphopeptone II were incubated in the presence of 0.01 M magnesium acetate with 5 mC of ^{32}P at pH 6 for 10 min [8]. The hydrolysed portion was separated on a Dowex 50 column. After inorganic phosphate three small radioactive peaks were obtained. The last one ran parallel with unlabeled phosphorylserine on electropherograms. The first fraction after a second hydrolysis with 2 N HCl for 20 h at 120°C showed an identical pattern of amino acids when run parallel on the chromatogram with the hydrolysate of the phosphopeptide isolated from the enzymatic transphosphorylation. The result supports Koshland's hypothesis [10] of a more common sequence of amino acids around serine making the otherwise inert CH_3OH side chain reactive to the phosphoryl group and a second amino acid sequence responsible for the enzyme specificity.

1. Ågren, G. *Acta Chem. Scand. To be published.*
2. Bublitz, C. and Kennedy, E. P. *J. Biol. Chem.* **211** (1954) 951.
3. Mellander, O. *Upsala Läkareförens. Förh.* **152** (1947) 107.
4. Ågren, G. and Glomset, J. *Acta Chem. Scand.* **7** (1953) 1071.
5. Glomset, J. *Biochim. et Biophys. Acta* **32** (1959) 349.
6. Hofman, T. *Biochem. J.* **69** (1958) 139.
7. Morton, K. *Biochem. J.* **55** (1953) 795.
8. Ågren, G., Zetterqvist, Ö. and Ojamäe, M. *Acta Chem. Scand.* **13** (1959) 1047.
9. Portmann, P. *Z. physiol. Chem. Hoppe-Seyler* **309** (1957) 87.
10. Koshland, D. E. and Erwin, M.Y. *J. Am. Chem. Soc.* **79** (1957) 2659.

Received June 11, 1959.

On the Crystal Structure of β-ZnOHCl

H.-E. FORSBERG and W. NOWACKI*

Department of Inorganic Chemistry, The Royal Institute of Technology, Stockholm 70, Sweden; Institut für anorganische, analytische und physikalische Chemie, Institut für Mineralogie und Petographie, Universität, Berne, Switzerland

Recently single crystals of β-ZnOHCl have been prepared from ZnO and $ZnCl_2$ by Feitknecht and Oswald [1], who kindly supplied us with specimens suitable for X-ray investigations. From rotation and Weissenberg photographs taken around the *a*- and *b*-axes with Cu*K* radiation, it was concluded that the crystals are orthorhombic with the following cell dimensions.

$a \approx 5.86$ Å $b \approx 6.58$ Å $c \approx 11.33$ Å $V \approx 436.9$ Å^3

The unit cell contains eight formula units.

The extinctions *h0l* for *h* odd, *hk*0 for *k* odd and 0*kl* for *l* odd are in agreement with the centrosymmetric space group *Pcab* (No. 61 in the *International Tables for X-ray Crystallography* [2]) which also has been confirmed by the structure determination.

The following preliminary parameters of the atoms were determined mainly from Patterson and Fourier projections of *h0l* and 0*kl*:

8 Zn in 8(*c*) with $x = 0.250$ $y = 0.131$ $z = 0$

8 O in 8(*c*) with $x = 0.594$ $y = 0.126$ $z = 0.071$

8 Cl in 8(*c*) with $x = 0.583$ $y = 0.107$ $z = 0.357$

8(*c*) $\pm$ (x,y,z), $\pm$ $(\frac{1}{2}-x, \frac{1}{2}+y, \bar{z})$,
$\pm$ $(\frac{1}{2}+x, \bar{y}, \frac{1}{2}-z)$, $\pm$ $(\bar{x}, \frac{1}{2}-y, \frac{1}{2}+z)$.

Figs. 1 and 2 show the structure of β-ZnOHCl. The zinc atoms are surrounded by three oxygen and three chlorine atoms, so that a coordination Zn-6X in the shape of an octahedron is obtained. The structure can also be described as pseudohexagonal zinc layers separated by an ordered layer of oxygen and chlorine atoms.

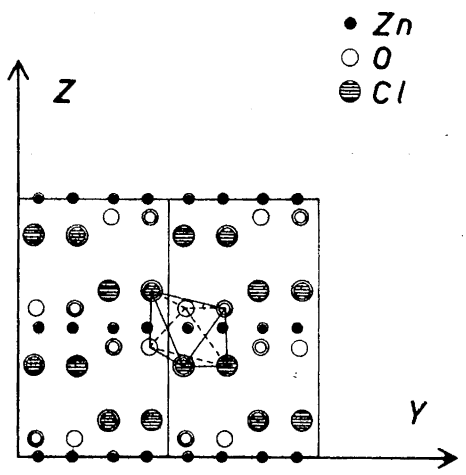


Fig. 1. The structure of β-ZnOHCl, viewed along [100]. (The range for the *x* parameter is $-0.406 \leq x \leq 0.594$.)

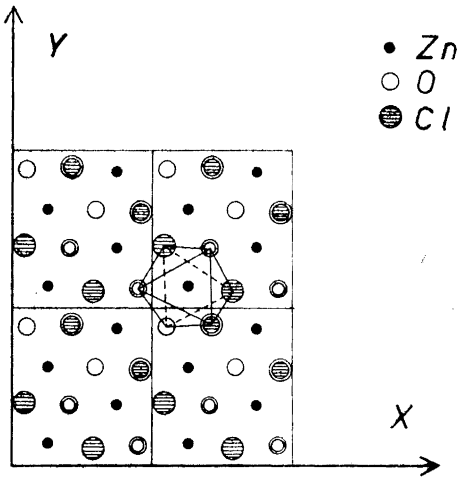


Fig. 2. The figure shows the pseudohexagonal zinc layer at $z = \frac{1}{2}$.

The following ranges of interatomic distances were found.

Zn – Zn	3.29 – 3.40 Å
Zn – O	2.01 – 2.17 Å
Zn – Cl	2.46 – 2.56 Å
O – O	2.56 – 3.36 Å
O – Cl	3.08 – 3.51 Å
Cl – Cl	3.48 – 4.08 Å

* Communication No. 110 of Abteilung für Kristallographie und Strukturlehre, Mineralogisches Institut Universität Bern.

The complete structure determination will be published later.

1. Feitknecht, W. and Oswald, H. R. *Private communication.*
2. *International Tables for X-ray Crystallography*, Kynoch Press, Birmingham 1952, Vol. I, p. 150.

Received June 12, 1959.

Unit Cell and Space Group of VS_4

BJØRN PEDERSEN

Kjemisk institutt, Universitetet i Oslo, Blindern, Norway

In an X-ray and magnetic study of the system V/S by Klemm and Hoschek [1] a polysulphide of vanadium was discovered. By means of vapour pressure measurements the composition of this compound was decisively determined as VS_4 by Biltz and Køcher [2]. A polysulphide of vanadium is also found in nature as patronite first described by Hewett [3]. On the basis of analytical data given by Hillebrand [4], Biltz and Køcher [2] concluded that patronite is VS_4. A sample of this mineral was kindly placed at our disposal by the Curator of Harvard Museum, Dr. C. Frondel. Powder photographs showed that the major phase present was the same as synthetic VS_4. — No crystallographic data on VS_4 seems to have been reported.

In a recent investigation of the vanadium sulphides [5], single crystals of VS_4 were obtained by heating a mixture of $VS_{1.5}$ and sulphur, corresponding to the formula VS_4, in an evacuated and sealed silica tube for four months at 400°C and slowly cooled to 100°C over another four months. The reaction was not complete, however, as some sulphur was still present in the tube. The crystals had grown as clustres of long, black needles with metallic lustre. Most of them were unsuitable for X-ray work, but after some search a crystal with very good properties was found. Its dimensions were: $0.33 \times 0.06 \times 0.07$ mm^3.

Oscillation and Weissenberg photographs taken with CuK-radiation ($\lambda_{\alpha_1} = 1.5405$ Å) around the needle axis (the c-axis) showed the crystal to be monoclinic. This was confirmed by precession photographs taken with MoK-radiation of the $h0l$-plane. From Guinier powder photographs the cell dimensions were found to be:

$a = 12.67$ Å $b = 10.41$ Å $c = 12.11$ Å
$\beta = 148.37°$

Missing reflexions were: hkl when $h+k = 2n+1$, $h0l$ when $l = 2n+1$ and $(h = 2n+1)$ and $0k0$ when $(k = 2n+1)$. This is characteristic of the space groups No. 9, Cc and No. 15, $C2/c$. The density was determined by flotation to be 2.80 g/cm^3, which indicates the presence of eight VS_4 groups in the unit cell ($d_{calc} = 2.835$ g/cm^3).

As the basis for a structure determination, the intensities of the $hk0$- and $h0l$-reflexions were estimated visually. The intensities were corrected for Lorentz and polarisation effects, but not for absorption and extinction.

This intensity material was used to decide between the two characteristic space groups by means of the Wilson ratio for detection of centers of symmetry, $\varrho = \langle|F_0|\rangle^2/\langle|F_0|^2\rangle^6$. For the 48 $hk0$- and 38 $h0l$- reflexions ϱ was calculated as 0.77 and 0.71, respectively. The predicted value for ϱ is 0.635 for centrosymmetric crystals and 0.785 for noncentrosymmetric crystals. Thus the Wilson ratio indicates that the crystal is noncentrosymmetric and the space group accordingly No. 9, Cc.

Some crystals were kindly tested for piezoelectricity by Dr. W. G. Perdok of the University of Groningen, but with negative results. This might perhaps be due to the smallness of the crystals (< 0.06 mm) in the direction of the polar axis.

1. Klemm, W. and Hoschek, E. *Z. anorg. u. allgem. Chem.* **226** (1936) 359.
2. Biltz, W. and Køcher, A. *Z. anorg. u. allgem. Chem.* **242** (1939) 324.
3. Hewett, F. *Eng. Mining J.* **1906** 385.
4. Hillebrand, W. F. *J. Am. Chem. Soc.* **29** (1912) 1019.
5. Pedersen, B. *Thesis*, University of Oslo, 1958.
6. Wilson, A. J. C. *Acta Cryst.* **2** (1949) 318.

Received June 5, 1959.

The complete structure determination will be published later.

1. Feitknecht, W. and Oswald, H. R. *Private communication.*
2. *International Tables for X-ray Crystallography*, Kynoch Press, Birmingham 1952, Vol. I, p. 150.

Received June 12, 1959.

Unit Cell and Space Group of VS_4

BJØRN PEDERSEN

Kjemisk institutt, Universitetet i Oslo, Blindern, Norway

In an X-ray and magnetic study of the system V/S by Klemm and Hoschek [1] a polysulphide of vanadium was discovered. By means of vapour pressure measurements the composition of this compound was decisively determined as VS_4 by Biltz and Køcher [2]. A polysulphide of vanadium is also found in nature as patronite first described by Hewett [3]. On the basis of analytical data given by Hillebrand [4], Biltz and Køcher [2] concluded that patronite is VS_4. A sample of this mineral was kindly placed at our disposal by the Curator of Harvard Museum, Dr. C. Frondel. Powder photographs showed that the major phase present was the same as synthetic VS_4. — No crystallographic data on VS_4 seems to have been reported.

In a recent investigation of the vanadium sulphides [5], single crystals of VS_4 were obtained by heating a mixture of $VS_{1.5}$ and sulphur, corresponding to the formula VS_4, in an evacuated and sealed silica tube for four months at 400°C and slowly cooled to 100°C over another four months. The reaction was not complete, however, as some sulphur was still present in the tube. The crystals had grown as clustres of long, black needles with metallic lustre. Most of them were unsuitable for X-ray work, but after some search a crystal with very good properties was found. Its dimensions were: $0.33 \times 0.06 \times 0.07$ mm^3.

Oscillation and Weissenberg photographs taken with CuK-radiation ($\lambda_{\alpha_1} = 1.5405$ Å) around the needle axis (the c-axis) showed the crystal to be monoclinic. This was confirmed by precession photographs taken with MoK-radiation of the $h0l$-plane. From Guinier powder photographs the cell dimensions were found to be:

$a = 12.67$ Å $b = 10.41$ Å $c = 12.11$ Å
$\beta = 148.37°$

Missing reflexions were: hkl when $h+k = 2n+1$, $h0l$ when $l = 2n+1$ and $(h = 2n+1)$ and $0k0$ when $(k = 2n+1)$. This is characteristic of the space groups No. 9, Cc and No. 15, $C2/c$. The density was determined by flotation to be 2.80 g/cm^3, which indicates the presence of eight VS_4 groups in the unit cell ($d_{calc} = 2.835$ g/cm^3).

As the basis for a structure determination, the intensities of the $hk0$- and $h0l$-reflexions were estimated visually. The intensities were corrected for Lorentz and polarisation effects, but not for absorption and extinction.

This intensity material was used to decide between the two characteristic space groups by means of the Wilson ratio for detection of centers of symmetry, $\varrho = \langle|F_0|\rangle^2/\langle|F_0|^2\rangle^6$. For the 48 $hk0$- and 38 $h0l$- reflexions ϱ was calculated as 0.77 and 0.71, respectively. The predicted value for ϱ is 0.635 for centrosymmetric crystals and 0.785 for noncentrosymmetric crystals. Thus the Wilson ratio indicates that the crystal is noncentrosymmetric and the space group accordingly No. 9, Cc.

Some crystals were kindly tested for piezoelectricity by Dr. W. G. Perdok of the University of Groningen, but with negative results. This might perhaps be due to the smallness of the crystals (< 0.06 mm) in the direction of the polar axis.

1. Klemm, W. and Hoschek, E. *Z. anorg. u. allgem. Chem.* **226** (1936) 359.
2. Biltz, W. and Køcher, A. *Z. anorg. u. allgem. Chem.* **242** (1939) 324.
3. Hewett, F. *Eng. Mining J.* **1906** 385.
4. Hillebrand, W. F. *J. Am. Chem. Soc.* **29** (1912) 1019.
5. Pedersen, B. *Thesis*, University of Oslo, 1958.
6. Wilson, A. J. C. *Acta Cryst.* **2** (1949) 318.

Received June 5, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1051–1063

Transition Point Depression and its Structural Interpretation in the Binary System Sodium Sulfate-Potassium Sulfate

T. FÖRLAND

N.T.N.F.'s Institutt for Silikatforskning, N.T.H., Trondheim, Norway,

and J. KROGH-MOE

Svenska Silikatforskningsinstitutet, Göteborg, Sweden

The heat content curves of K_2SO_4 and of $NaKSO_4$ have been determined in the temperature range from 25°C to 850°C. The depression of the transition point of sodium sulfate by the addition of potassium sulfate has been determined experimentally. The equilibrium lines of transitions in the phase-diagram of the binary system, sodium sulfate-potassium sulfate have been calculated from the heats of transition of these two compounds together with the heat of transition found for $NaKSO_4$.

A consistent treatment of the system is shown to require that in the high temperature solid solution of sodium sulfate with potassium sulfate there is a cation disorder. The second order transition in $NaKSO_4$ is established as an order-disorder transition.

For regions of solid solution high in potassium sulfate the data available indicate that the sodium ions occur in groups, probably consisting of two ions occupying neighbouring cation positions.

The system sodium sulfate-potassium sulfate exhibits a continuous series of solid solutions in a limited temperature range below the liquidus curve. This is shown in the phase diagram in Fig. 1 taken from the work of Nacken [1]. For compositions from 50 to 75 mole-% potassium sulfate, the hexagonal (high temperature) structure is preserved below the transition temperature [2]. Several authors observed a thermal effect in this composition range [1-5]. Bredig states that the thermal effect is due to a second order transition, since he found only a change in the axial ratio of the hexagonal crystals on passing the transition point [2]. He further suggests that this transition is due to the excitation of at least one rotational degree of freedom of the sulfate ion in the high temperature range. The possibility of an order-disorder reaction is

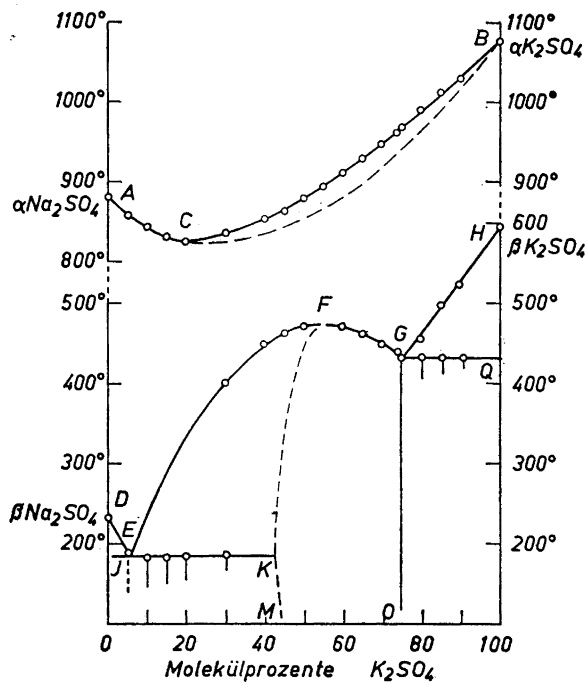


Fig. 1. Phase diagram of the system sodium sulfate-potassium sulfate according to Nacken.

also considered, but is believed to be less likely because of the speed of the transition. An order-disorder reaction is not ruled out by this argument, however. The average diffusion distance necessary to establish the ordered state and the activation energy of diffusion are probably small in this case, so that the reaction should be expected to proceed rapidly. In the following it will be shown that the phase diagram of the system sodium sulfate-potassium sulfate, observed by Nacken [1,3], can be interpreted on the basis of an order-disorder transition.

In the high temperature solid solution it is reasonable to assume that the sodium and potassium ions can replace each other on any one of the cation positions, since the solid solution extends across the whole diagram. Just below the transition point the solid solution extends only from about 50 to 75 mole-% potassium sulfate. This indicates that only in one fourth of the cation positions can sodium and potassium ions replace each other. Thus in the low temperature solid solution one-half of the cation positions have become permanent potassium ion positions and one-fourth of the positions are permanently filled with sodium ions. Hence an ordering has taken place.

The crystal structures of $NaK_3(SO_4)_2$ and $Na_2K_2(SO_4)_2$ determined at room temperature give additional evidence for this particular ordering. In these structures the cations are distributed over three different kinds of positions.

One kind of cation position takes potassium ions, another takes sodium ions and, finally, the last cation position takes either a sodium or a potassium ion [6-8]. There are twice as many of the former cation positions as of either of the two others.

Hilmy [8] investigated the composition of crystals formed from a water solution of sodium and potassium sulfate, and he found that below 35°C the solid solubility range is very narrow and close to the composition $NaK_3(SO_4)_2$.

The lines in the phase diagram corresponding to the transition are analogous to the liquidus lines in a binary system with limited solid solution, and the depression of the transition temperature can be treated in the same way as the depression of a freezing point.

On the basis of the structural model outlined above, the change in configurational entropy with the sodium potassium ratio may be calculated in the high temperature and low temperature modifications. With an additional knowledge about the change in heat content by the transition from high to low temperature modification, the equilibrium lines of the phase diagram may be calculated. In the following, equilibrium lines of the phase diagram calculated on the basis of the structural model and calorimetric measurements of heat of transitions, will be compared with experimental points on the equilibrium lines.

DEPRESSION OF THE TRANSITION POINT OF SODIUM SULFATE

The depression of the transition point of sodium sulfate by addition of potassium sulfate can, according to classical thermodynamics, be derived briefly as follows:

At the transition point of a solid solution of potassium sulfate in sodium sulfate the partial free energy of sodium sulfate in the high temperature state ($\overline{F}_{Na_2SO_4}$) is equal to the free energy of pure sodium sulfate in the low temperature state ($F_{Na_2SO_4(low)}$):

$$\overline{F}_{Na_2SO_4} = F_{Na_2SO_4(low)}$$

Here an eventual small solubility of potassium sulfate in the low temperature phase is disregarded.

We will now choose pure sodium sulfate in the high temperature state as standard state. Referring to this state the above equation can be written:

$$\overline{F}_{Na_2SO_4} - F_{Na_2SO_4(high)} = \Delta\overline{F}_{Na_2SO_4}$$
$$= F_{Na_2SO_4(low)} - F_{Na_2SO_4(high)} = -\Delta F_{tr}$$

where $F_{Na_2SO_4(high)}$ is the free energy of sodium sulfate in the standard state and ΔF_{tr} is the free energy of transition. Neglecting a minor correction term due to the difference in specific heat of sodium sulfate in the two states, we can introduce

$$\Delta F_{tr} = \Delta H_{tr} - T\Delta S_{tr} = \Delta H_{tr}\left(1 - \frac{T}{T_{tr}}\right) = -\Delta\overline{F}_{Na_2SO_4}$$

where ΔH_{tr} is the heat of transition, T is the temperature of the equilibrium and T_{tr} is the transition temperature of pure sodium sulfate. If further $\Delta\bar{F}_{Na_2SO_4}$ is expressed by the partial entropy $\Delta\bar{S}_{Na_2SO_4}$ and the partial heat content $\Delta\bar{H}_{Na_2SO_4}$, we finally obtain

$$\Delta\bar{S}_{Na_2SO_4} = \Delta H_{tr}\left(1 - \frac{T}{T_{tr}}\right) + \frac{\Delta\bar{H}_{Na_2SO_4}}{T} \quad (1)$$

Introducing the assumption of complete sodium-potassium disorder in the solid solution, the entropy of mixing due to this disorder can be expressed as

$$\Delta S = -R(n_{Na}\ln N_{Na} + n_K \ln N_K) \quad (2)$$

where n_{Na} and n_K are the molar amounts of sodium and potassium ions in the mixture and N_{Na} is defined by

$$N_{Na} = \frac{n_{Na}}{n_{Na} + n_K}$$

and N_K is defined correspondingly.

Differentiating with respect to n_{Na}, one obtains

$$\frac{\partial \Delta S}{\partial n_{Na}} = \Delta\bar{S}_{Na} = -R\ln N_{Na}$$

and as

$$\frac{\partial \Delta S}{\partial n_{Na_2SO_4}} = 2\,\frac{\partial \Delta S}{\partial n_{Na}}$$

one obtains the partial entropy of sodium sulfate

$$\Delta\bar{S}_{Na_2SO_4} = -2\ R\ln N_{Na} \quad (3)$$

Introducing this into eqn. (1) one obtains

$$-2\ R\ln N_{Na} = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{Na_2SO_4}}{T} \quad (4)$$

For mixtures of this kind the partial heat content of a component will generally be proportional to the square of the fraction of the second component. If N_{Na} is close to unity, N_K^2 will be small, and $\Delta\bar{H}_{Na_2SO_4}/T$ will be small compared to $-2\ R\ln N_{Na}$. The change in partial heat content of sodium sulfate may, therefore, be expected to be insignificant for systems with a high sodium sulfate content.

Calorimetric measurements of the heat content of sodium sulfate by Coughlin [9] give 1.68 kcal for the heat of transition. The transition temperature is 234°C according to Nacken. The same value was found in the present investigation. Using these data, the transition temperature T of the solid solution

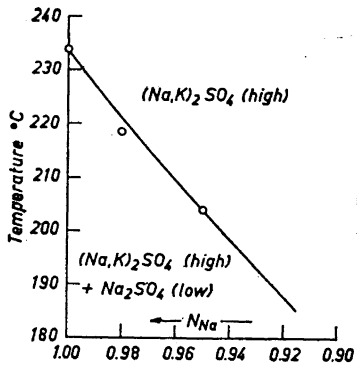


Fig. 2. Transition point depression of sodium sulfate by potassium sulfate. Circles represent observed points. Solid line is calculated.

may be calculated as a function of the composition N_{Na} and a comparison with the experimental data can be done.

No good previous determination of the depression of the transition point of sodium sulfate by addition of potassium sulfate was found in the literature. (One point on the depression curve indicated by Nacken [7] is more likely to be the eutectic point). Therefore, measurements of the transition point depression were carried out by the present authors.

Experimental

The transition point was determined by ordinary thermal analysis. A sample of approximately 200 g with a Pt-PtRh thermo-couple in the center was cooled at a speed of about 0.1° C/min, and the break in the temperature-time curve was observed. The accuracy of the temperature measurement was of the order ± 1°C. The results are given in Table 1.

Table 1.

Mole % Na_2SO_4 in the solid solution in the high temperature phase	Temperature at which transition starts
100	234°C
98	218°C
95	204°C

On Fig. 2 is shown a plot of the transition temperature, T, as a function of N_{Na} as given by eqn. (4) omitting the term $\Delta\bar{H}_{Na_2SO_4}/T$. The circles represent the observed transition temperature for different compositions. As is seen from the figure, the agreement between calculated and observed values is good. One may conclude that the K^+ ions introduced into the high temperature modification of sodium sulfate are randomly distributed over all positions which are available for the K^+ ions. Further, one may conclude that the partial heat of mixing of sodium sulfate is small in this composition range.

It should be emphasized that the above results do not give information about whether the K^+ ions are randomly distributed over all cation positions

or a fraction of them. Assuming, *e.g.*, that in this concentration range only half of the cation positions can be occupied by K^+ ions, one will have a system where $1/2\ (n_K + n_{Na}) - n_K$ moles of Na^+ ions and n_K moles of K^+ ions are distributed over $1/2\ (n_K + n_{Na})$ moles of cation positions. If this distribution is random, the entropy of mixing due to this disorder can be expressed by the equation

$$\Delta S = -R\left[1/2\ (n_{Na} - n_K)\ln \frac{1/2\ (n_{Na} - n_K)}{1/2\ (n_{Na} + n_K)} + n_K \ln \frac{n_K}{1/2\ (n_{Na} + n_K)}\right] \quad (5)$$

$$\Delta\bar{S}_{Na_2SO_4} = -R\ln(1 - 2N_K) \quad (6)$$

Comparing eqns. (3) and (6) one can see that in both cases will the calculated partial entropy of sodium sulfate be approximately equal to $2\ RN_K$ when N_K is small. Thus, in the concentration range in question it is not possible to determine whether the entropy of the system corresponds to eqn. (3) or to eqn. (6) when only $\Delta\bar{S}_{Na_2SO_4}$ is known.

THE DEPRESSION OF THE TRANSITION POINT OF POTASSIUM SULFATE

Analogous to the above treatment, the depression of the transition point of potassium sulfate by additions of sodium sulfate can be expressed by the equation

$$\Delta\bar{S}_{K_2SO_4} = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{K_2SO_4}}{T} \quad (7)$$

where $\Delta\bar{S}_{K_2SO_4}$ and $\Delta\bar{H}_{K_2SO_4}$ are partial entropy and heat content of potassium sulfate in the high temperature modification, and T_{tr} and ΔH_{tr} are the transition temperature and heat of transition of pure potassium sulfate. As for the region high in sodium sulfate, one may assume that also in this concentration region the cations are randomly distributed over all cation positions giving an entropy of mixing

$$\Delta S = -R(n_{Na}\ln N_{Na} + n_K \ln N_K)$$

and

$$\Delta\bar{S}_{K_2SO_4} = -2\ R\ln N_K \quad (8)$$

Introducing this into eqn. (7), one obtains

$$-2\ R\ln N_K = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{K_2SO_4}}{T} \quad (9)$$

The transition temperature is according to Nacken [1] 595°C, and calorimetric measurements by Shomate and Naylor [10] give 1.94 kcal for the heat of transition of pure potassium sulfate. Assuming that the term $\Delta\bar{H}_{K_2SO_4}/T$ is of minor importance, at least for mixtures high in potassium sulfate, one can thus calculate the transition temperature as a function of composition.

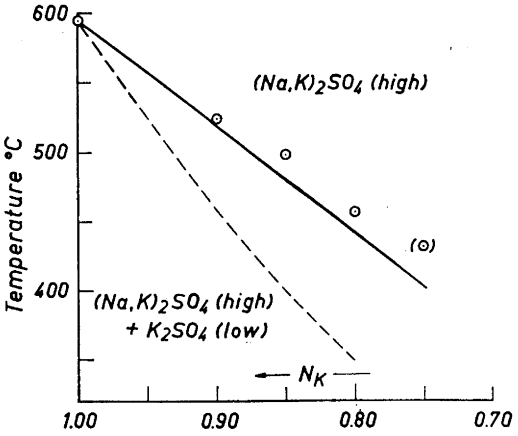


Fig. 3. Transition point depression of potassium sulfate by sodium sulfate. Circles represent points observed by Nacken. Dotted line is calculated assuming independent sodium ions and solid line is calculated assuming sodium pairs.

This is shown on Fig. 3 where the transition temperature is plotted as a function of N_K, giving the dotted line. This calculated line may be compared with the transition temperatures observed by Nacken and given as small circles in the figure. As is seen from the figure, there is a large discrepancy between observed and calculated values.

One will notice that the deviations are very closely proportional to N_{Na}. This indicates that the discrepancy is not due to a change in the partial heat content of potassium sulfate, as this term generally would give a deviation proportional to N_{Na}^2. The partial entropy of potassium sulfate, on the other hand, would vary approximately proportionally to N_{Na} as $-\ln N_K \approx N_{Na}$ in the concentration range in question. Actually the observed change in transition temperature by addition of sodium sulfate is only one-half of the calculated. This corresponds to a partial entropy of potassium sulfate, only one-half of that expressed by eqn. (8).

The only reasonable interpretation of this is that the Na^+ ions occur in groups probably consisting of two ions. The entropy of mixing for a mixture of K^+ and Na^+ ions where the Na^+ ions occur in groups of two ions occupying neighboring positions can be expressed by the equation (compare Flory [11]):

$$\Delta S = -R(n_K \ln \frac{n_K}{n_{Na} + n_K} + \frac{n_{Na}}{2} \ln \frac{n_{Na}}{n_{Na} + n_K}) \quad (10)$$

From this the partial entropy of potassium sulfate is:

$$\Delta \bar{S}_{K_2SO_4} = -2\ R \ln N_K - R(1 - N_K) \quad (11)$$

Introducing this into eqn. (7), the equation for the transition temperature, T, as a function of composition N_K, will be:

$$-2\,R\ln N_K - R(1-N_K) = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{K_2SO_4}}{T} \qquad (12)$$

A plot of this function (omitting the term $\Delta\bar{H}_{K_2SO_4}/T$) is shown on Fig. 3 as the solid curve. As can be seen, the agreement between this calculated curve and the observed transition temperatures is good. One may conclude from this that for solid solutions with a high content of potassium sulfate the Na^+ ions occur in groups of two, and these groups are randomly distributed over the positions available for them.

One could also assume that the cation positions themselves occur in groups of two which either can be occupied by two Na^+ ions or two K^+ ions. The entropy of mixing for such a mixture is given by

$$\Delta S = -R(1/2\,n_K \ln \frac{1/2\,n_K}{1/2\,n_K+1/2\,n_{Na}} + 1/2\,n_{Na}\ln \frac{1/2\,n_{Na}}{1/2\,n_K+1/2\,n_{Na}}) \qquad (13)$$

Differentiation gives for the partial entropy of potassium sulfate

$$\Delta\bar{S}_{K_2SO_4} = -R\ln N_K \qquad (14)$$

It can be easily shown that this expression for mixtures high in potassium sulfate is almost identical with the previous entropy expression (eqn 11), so that a distinction between the two types of disorder is not possible on the basis of the above data.

The inversion point depression could also be explained by the assumption of groups consisting of 3 Na^+ ions. This model would, however, give a larger difference between observed and calculated inversion point at the lowest content of sodium sulfate. As one should expect the agreement to be better in this region, groups of 3 Na^+ may be considered less probable.

The formation of pairs of Na^+ ions on neighbouring sites in the sodium-potassium sulfate mixture was certainly not expected. The conclusion depends on the applied value of the heat of transition of potassium sulfate (besides on the phase diagram measurements), and thus it could possibly be due to an error in this quantity. For this reason the heat of transition was redetermined.

Experimental

The heat content of potassium sulfate as a function of temperature was obtained with a calorimeter fairly similar to that first used by Oelsen, Rieskamp and Oelsen [12] and later modified by Tashiro [13]. This apparatus is designed to allow a simultaneous recording of the sample temperature and the water temperature of the calorimeter. For details of the experimental technique employed, Tashiro's paper should be consulted. Our calorimeter differed from his mainly in having two more radiation shields around the sample container to decrease the rate of cooling of the sample.

A sample of 65.8 g K_2SO_4 was fused in the sample container, and then kept at a temperature of about 1 000°C before being placed in the calorimeter. During the cooling in

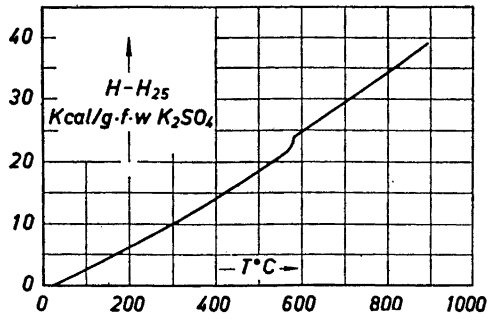


Fig. 4. Experimental heat content curve for K_2SO_4.

the calorimeter the temperature of the sample was recorded at intervals of about 15°C. The observed heat content as a function of temperature is shown in Fig. 4. The heat of transition was found to be

$$H_{tr} = 1\,920 \text{ cal/g.f.w. } K_2SO_4$$

Average specific heat for the following temperature regions are:

25 to 595°C: $\bar{c}_p = 39.2$ cal/deg.g.f.w.
595 to 850°C: $\bar{c}_p = 48.4$ cal/deg.g.f.w.

As is seen from the figure, the specific heat of the low temperature modification increases considerably with increasing temperature.

The value for the heat of transition (1.92 kcal) agrees very well with the previous value (1.94 kcal) obtained by Shomate and Naylor, and thus it confirms our conclusion that groups of Na^+ ions are formed in the high temperature modification of potassium sulfate.

DEPRESSION OF THE TRANSITION POINT OF $NaKSO_4$ BY ADDITIONS OF SODIUM SULFATE

Analogous to the two preceding calculations the depression of the transition point of $NaKSO_4$ by additions of sodium sulfate can be expressed by the equation

$$\Delta\bar{S}_{NaKSO_4} = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{NaKSO_4}}{T} \qquad (15)$$

where $\Delta\bar{S}_{NaKSO_4}$ and $\Delta\bar{H}_{NaKSO_4}$ are the partial entropy and the partial heat of mixing of $NaKSO_4$ in a mixture in the high temperature modification, the standard state being pure $NaKSO_4$ in the same modification. T_{tr} and ΔH_{tr} are the transition temperature and the heat of transition of $NaKSO_4$, respectively. The equation is based on the assumption that there is no sodium-

potassium disorder in the low temperature modification of $NaKSO_4$. (The phase diagram by Nacken indicates that $NaKSO_4$ in the low temperature modification can only have a very small amount of excess sodium. It should be noted, as indicated by the dotted curves for this region of the phase diagram shown in Fig. 6, that $NaKSO_4$ does not have a sharp transition point, but rather a transition region. However, the major change appears (*e.g.*, from the heat content curve) to take place over a fairly short temperature interval so that the expression $\Delta S_{tr} = \Delta H_{tr}/T_{tr}$ may be considered to be a good approximation.)

Assuming a complete sodium potassium disorder in the high temperature modification, the entropy due to this disorder is equal to

$$S = -R(n_{Na}\ln N_{Na} + n_K \ln N_K)$$

Differentiation with respect to n_{Na} and n_K gives:

$$\frac{\partial S}{\partial n_{NaKSO_4}} = \frac{\partial S}{\partial n_{Na}} + \frac{\partial S}{\partial n_K} = -R(\ln N_{Na} + \ln N_K)$$

As we have chosen pure $NaKSO_4$ in the high temperature modification as the standard state, the partial entropy of $NaKSO_4$ in the mixture referring to this standard state will be:

$$\Delta\bar{S}_{NaKSO_4} = -R\ln(N_{Na}N_K) + R\ln\ 1/4) \qquad (16)$$

Introducing (16) into (15) one obtains

$$-R\ln N_{Na}N_K + R\ln\ 1/4 = \Delta H_{tr}\left(\frac{1}{T} - \frac{1}{T_{tr}}\right) + \frac{\Delta\bar{H}_{NaKSO_4}}{T} \qquad (17)$$

Neglecting a change in the heat of mixing in the concentration range in question, one may thus calculate the transition temperature as a function of composition if the transition temperature and heat of transition of pure $NaKSO_4$ is known.

From Nacken's measurements the transition temperature is equal to 470°C. No data were available for the heat of transition, however, and a calorimetric determination was, therefore, carried out.

Experimental

The heat content of $NaKSO_4$ as a function of temperature was determined by the method described in the previous part of this paper. A sample of 63 g $NaKSO_4$ was made by fusing together equal molar amounts of *pro analysi* potassium and sodium sulfate. The observed heat content curve is given in Fig. 5. The heat of transition was found to be:

$$\Delta H_{tr} = 1\,750 \text{ cal/g.f.w. } NaKSO_4$$

at 470°C. The heat of fusion was found to be

$$\Delta H_f = 4\,650 \text{ cal/g.f.w.}$$

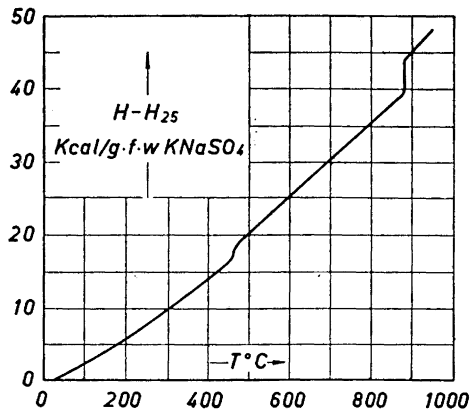


Fig. 5. Experimental heat content curve for $NaKSO_4$.

with a fusion point of 884°C. Average specific heats for the following two regions are:

25 to 450°C $\bar{c}_p$ = 37.4 cal/deg.g.f.w.
500 to 850°C $\bar{c}_p$ = 50.3 cal/deg.g.f.w.

The specific heat in the low temperature region increases considerably with the temperature, so that the specific heat just below the transition is not very different from the specific heat above the transition. This speaks against the view that the transition releases new degrees of freedom.

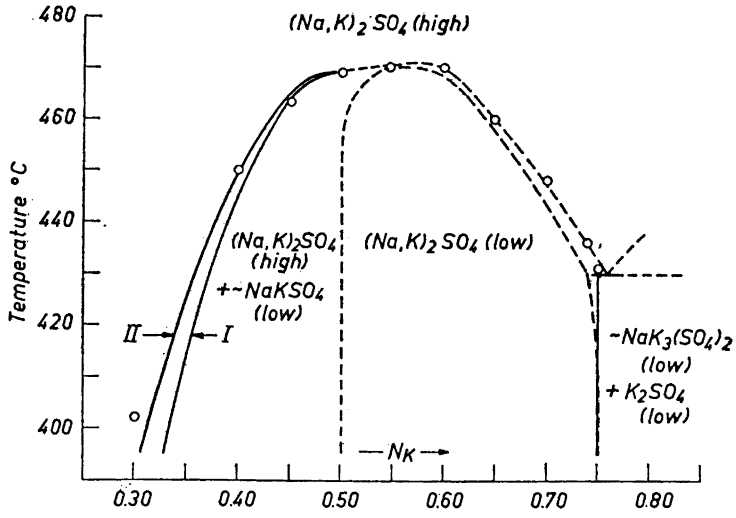


Fig. 6. Part of the phase diagram of the sodium potassium sulfate system in the composition range around the compound $NaKSO_4$. Circles represent points observed by Nacken. The solid lines are calculated on the basis of certain models for the structure of the solid solution.

Knowing the heat of transition of $NaKSO_4$ it is now possible to calculate the transition temperature of mixtures having an excess of Na_2SO_4, according to eqn (17).

A plot of such a calculation is shown on Fig. 6, curve I. The circles represent the transition temperature observed by Nacken. As can be seen from the figure, the agreement between calculated and observed transition temperature is good. Before concluding that this structural model is therefore the most likely, one should however calculate the partial entropy of $NaKSO_4$ based on other postulates about the cation disorder in the solid solution.

Previously it was shown that in solid solutions with a high sodium sulfate content the K^+ ions were randomly distributed over certain cation positions. But on the basis of measurements one could not say whether this applied to all cation positions or only a fraction (*e.g.*, 1/2) of them. Assuming that only half of the positions can be occupied by K^+ ions, the entropy of the solid solution is given by eqn (5). Differentiation gives the following expression for the partial entropy of $NaKSO_4$:

$$\Delta\bar{S}_{NaKSO_4} = -R\ln N_K - R\ln 2$$

This calculated entropy is in distinct disagreement with the experimental results.

The formation of pairs of Na^+ ions may possibly persist for $N_{Na} > 1/2$. It is, therefore, of interest to calculate the depression of the transition point of $NaKSO_4$ on this basis. The entropy of mixing for such a system is given by eqn (10). Differentiation gives the following expression for the partial entropy of $NaKSO_4$:

$$\Delta\bar{S}_{NaKSO_4} = -R\ln\left(N_K\sqrt{N_{Na}}\right) - \frac{R}{2}(N_{Na} - N_K) + R\ln\tfrac{1}{2}\sqrt{\tfrac{1}{2}}$$

Substituting this entropy expression into eqn (15) the change in transition temperature for $NaKSO_4$ as a function of composition can be calculated (omitting the term $\Delta\bar{H}_{NaKSO_4}/T$). A plot of this function is shown on Fig. 6, curve II. As is seen from the figure, the agreement between the calculated and observed transition temperature is even better than for the previous calculation based on complete $Na^+ - K^+$ disorder. However, without more accurate information on the thermodynamic properties of the solid solution, a distinction between the two kinds of disorder cannot be made. However, the possibility that the cation positions as well as the cations occur as pairs may be excluded in this concentration range. According to eqn (13) we have:

$$\Delta S = -1/2\ R(n_K\ln N_K + n_{Na}\ln N_{Na})$$

Differentiation with respect to the $NaKSO_4$ content gives

$$\Delta\bar{S}_{NaKSO_4} = -1/2\ R\ln N_K N_{Na} + 1/2\ R\ln\ 1/4 \qquad (18)$$

which gives a transition point depression only half of that experimentally found.

In region $1/2 < N_K < 3/4$ a solid solution exists both in the high and the low temperature state of the mixture. A calculation of the change in transition temperature with composition could be based on an assumption about the disorder in the high temperature state as discussed in the preceding paragraphs combined with a limited disorder over one-quarter of the positions in the low temperature state. Such a calculation would give curves of the type as indicated by the dotted lines on Fig. 6. However, without more experimental data than available now, such calculations would not be of much value. One would have to know the compositions of both the high and low temperature phase in equilibrium. Further, more thermal data should be available, particularly the heat of transition of $K_3Na(SO_4)_2$. Without these data the calculations would contain at least one freely chosen parameter and, thus, an agreement between experiment and calculation could always be obtained.

CONCLUSION

The preceding discussion has shown that consistent values for the structural entropies can be obtained by assuming a random distribution of K^+ ions and a random distribution of pairs of Na^+ ions over all cation positions in the high temperature solid solution. This proves that the second order transition of $NaKSO_4$ is an order-disorder transition.

Acknowledgement. Thanks are expressed to *Norges Teknisk-Naturvitenskapelige Forskningsråd* for financial support.

REFERENCES

1. Nacken, R. *Neues Jahrb. Mineral. Geol.* **24A** (1907) 1.
2. Bredig, M. A. *J. Phys. Chem.* **47** (1943) 587.
3. Nacken, R. *Zentr. Mineral.* **1910** 262.
4. Jänecke, E. *Z. physik Chem.* **64** (1908) 343.
5. Perrier, C. and Bellanca, A. *Periodico Mineral. (Rome)* **11** (1940) 163.
6. Gossner, B. *Neues Jahrb. Mineral. Geol.* **57A** (1928) 89.
7. Bellanca, A. *Periodico Mineral. (Rome)* **14** (1943) 67.
8. Hilmy, M. E. *Am. Mineralogist* **38** (1953) 118.
9. Coughlin, J. P. *J. Am. Chem. Soc.* **77** (1955) 868.
10. Shomate, C. H. and Naylor, B. F. *J. Am. Chem. Soc.* **67** (1945) 72.
11. Flory, J. P. *J. Chem. Phys.* **10** (1942) 51.
12. Oelsen, W., Rieskamp, K. H. and Oelsen, O. *Arch. Eisenhüttenw.* **26** (1955) 253.
13. Tashiro, M. *Glass Ind.* **37** (1956) 549.

Received February 19, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1064–1068

Evidence of the Different Nature of Human Plasminogen and Proactivator

MOGENS LASSEN

From the Biological Institute of the Carlsberg Foundation, and the University Institute of Physical Chemistry, Copenhagen, Denmark

Human plasminogen and proactivator were found to have different thermal stabilities at 50° and pH 10.6. This observation supports the assumption that these components are chemically different.

The fibrinolytic activity induced in human blood by streptokinase, an extracellular streptococcal product, is due the conversion of plasminogen into the proteolytic enzyme, plasmin. Studies by Christensen [1] and Christensen and MacLeod [2] indicated a direct catalytic effect of streptokinase on plasminogen. Müllertz and Lassen [3] suggested that streptokinase does not react directly with human plasminogen, but that an activator of plasminogen is formed through a reaction between streptokinase and a substance, termed proactivator, which is present in large amounts in human plasma. Troll and Sherry [4] and Müllertz [5] independently suggested the reaction between streptokinase and proactivator to be a stoicheiometric and reversible reaction, while the effect of the activator on plasminogen was catalytic:

$$\text{Streptokinase} + \text{proactivator} \rightleftharpoons \text{activator}$$

$$\text{Plasminogen} \xrightarrow{\text{activator}} \text{plasmin}$$

This concept was rejected again by Sherry. He now assumes identity between plasminogen and proactivator and supposes the activator to be a complex of streptokinase with plasminogen or plasmin (Sherry and Alkjaersig [6,7]), and his concept has been accepted by several investigators. The present work gives evidence for the original suggestion that proactivator and plasminogen are two different factors.

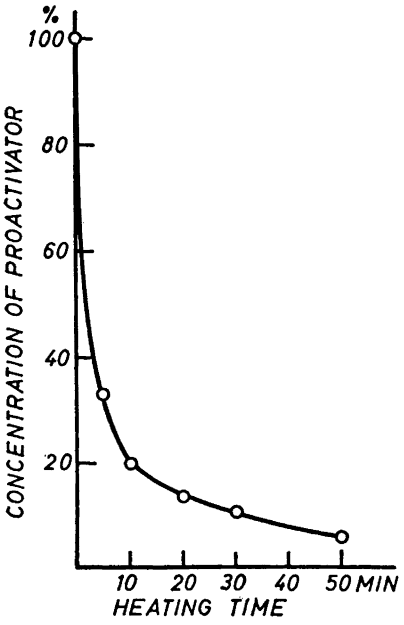


Fig. 1. Inactivation of proactivator at 50° and pH 10.6. Abscissa: heating time. Ordinate: residual concentration of proactivator in per cent of the concentration in the unheated solution.

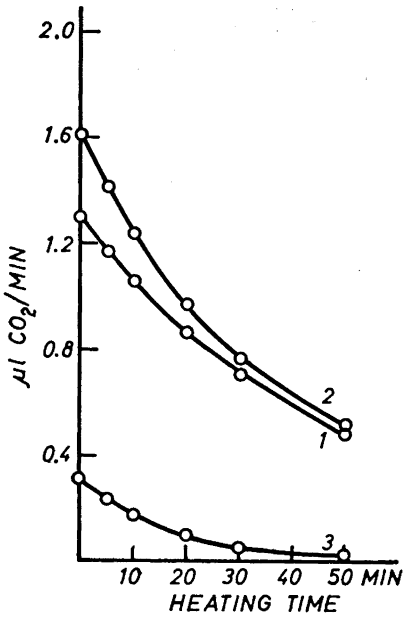


Fig. 2. Inactivation of plasminogen at 50° and pH 10.6. Abscissa: heating time. Ordinate: residual concentration of spontaneously occurring plasmin (curve 1), of plasminogen + plasmin (curve 2), and of plasminogen (curve 3). The last curve was obtained by subtracting curve 1 from curve 2. The activation of plasminogen with urine activator is described in the text. The estimations were performed by the manometric method using 0.25 M $NaHCO_3$ in the liquid phase and 50 % carbon dioxide in the gas phase, giving the pH 7.34. In the side arms: 25 mg BAEe. In the main compartment of flasks: Curve 1; 40 µl of the heated solution + 1 960 µl 0.25 M $NaHCO_3$. Curve 2; 40 µl of the heated solution + 20 µl of the urine activator solution + 1 940 µl 0.25 M $NaHCO_3$.

MATERIALS AND METHODS

Streptokinase. "Varidase" was kindly supplied by the Lederle Laboratories Division, American Cyanamid Company. The units are those stated on the packages as determined according to Christensen [8].

Urine activator. A preparation of human urokinase (Ploug and Kjeldgaard [9]) was kindly supplied by Løvens kemiske Fabrik, Copenhagen. By means of the lysis time method (Lassen [10]) the activating power of this preparation was compared with that of streptokinase activated human plasma. 1 mg urine activator preparation was found to

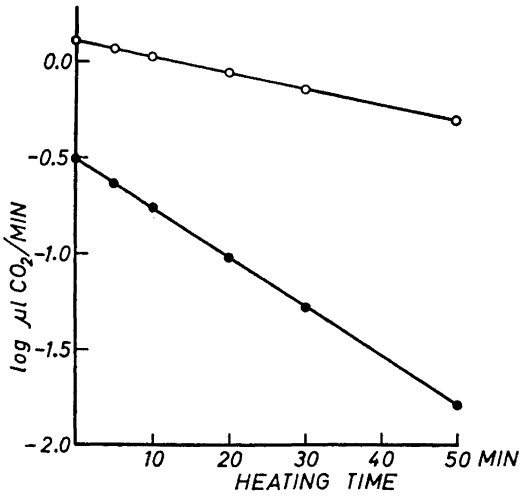


Fig. 3. Denaturation of plasminogen (○) and plasmin (●) at 50° and pH 10.6. First order rate constants: plasminogen, 0.0198 min^{-1}; plasmin 0.059 min^{-1}. $t_{\frac{1}{2}}$: plasminogen, 36 min; plasmin 12.5 min.

correspond in activity to approximately 1 500 units of streptokinase mixed with 1 ml of human plasma (excess).

Plasminogen and proactivator. 100 ml freshly prepared human serum was diluted with 1 900 ml icecold distilled water and adjusted to pH 5.5 with acetic acid. After standing for 30 min at 0° the precipitate was collected by centrifuging and dissolved in 0.9 % NaCl to a final volume of 16 ml. This solution was adjusted to pH 2.0 with 1 M HCl and heated at 70° for 30 min. The heated solution was then mixed with 4 ml veronal-gelatin buffer (Lassen [10]), adjusted to pH 7.7, and clarified by centrifuging. The procedure caused some loss in plasminogen and proactivator, but it removed the bulk of inhibitory substances.

Manometric determination of plasmin. Plasmin was measured by the method previously reported (Lassen [11]), using benzoyl-L-arginine ethylester (BAEe) as substrate.

Determination of proactivator. Proactivator was measured by the lysis time method as described by Lassen [10].

RESULTS

Advantage was taken of the difference between the stabilities of the components at alkaline reaction. The heat inactivation was performed as follows:

10 ml of the plasminogen and proactivator solution was mixed with 0.5 ml 0.1 M NaOH. The resulting pH was 10.6. 1 ml aliquots were then heated at 50° for different periods of time, whereafter they were cooled, neutralized by adding 50 µl 0.1 M HCl, and cleared by centrifuging. The final determinations of proactivator and plasminogen were performed as follows:

Proactivator was estimated as described using the unheated solution for the standard curve (Fig. 1). Plasminogen was converted into plasmin (see below) and estimated by the manometric method. Because of the low content of proactivator in the heated solutions, streptokinase was considered unsuitable for the activation of plasminogen. Instead 200 µl of the samples

were activated for 30 min at room temperature with 100 μl of a solution containing 4 mg urine activator per ml. The unheated solutions of proactivator and plasminogen contained small amounts of plasmin. The activity of this spontaneously occurring plasmin was determined separately, and subtracted from the activities obtained after activation with urine activator (Fig. 2).

The figures show that by heating at pH 10.6 proactivator disappears more rapidly than plasminogen, indicating that we are concerned with two different compounds. The denaturations of plasminogen and of the spontaneously occurring plasmin are first order reactions (Fig. 3), while the denaturation of proactivator seems to be more complicated. The relative decreases of plasmin and proactivator during heating for 30 min are in accordance with the results obtained by Müllertz [5,12].

DISCUSSION

The experimental results indicate the presence of at least two fibrinolytic precursors in human plasma. One of these is plasminogen. About the other can only be said that it is essential for the formation of the activator by streptokinase. On the basis of our present knowledge it seems most natural to identify this factor with the proactivator presented in the introductory scheme.

Experimental evidence of different heat stabilities of human plasminogen and proactivator has been presented by Troll, Sherry and Wachman [13] and by Troll and Sherry [4]. The first authors found proactivator to be labile in plasma at pH 7.0 and a temperature of 50°, while plasminogen was found to be stable. The present author has tried to reproduce this experiment, but found both precursors to be stable at the above conditions. A less marked, although obviously still significant difference between the heat stabilities of the two precursors were found at 100° and pH 2 and at 50° and pH 9 by Troll and Sherry [4]. The results of the last experiment resemble the results presented in this paper (50° and pH 10.6). However, Sherry later abandoned the two component-theory, which means that he questions the results of these experiments (Sherry and Alkjaersig [6,7]). Ablondi and Hagan [14] who used a more purified preparation of human plasminogen found identical heat stabilities of plasminogen and proactivator at 50° and pH 6.4. However, they filtered the heated aliquots before the assays. In a similar experiment at 50° and pH 7.0 where filtration was omitted, a greater stability of plasminogen than of proactivator was apparent from their results. The authors ascribe this deviation to a different solubility of the heated proteins in different assay methods, and suggest that this phenomenon might be interfering in similar experiments made by other investigators. In the present investigation the neutralized aliquots were cleared by centrifuging, and no precipitation occurred during the assays.

Acknowledgements. The author is indebted to Dr. Tage Astrup for his interest and helpful advice. The investigation was made possible by grants from *Statens almindelige Videnskabsfond*, Denmark. It forms part of the investigations on fibrinolysis for which Dr. Astrup receives support from the *Josiah Macy, Jr. Foundation*, New York, and the *National Danish Association against Rheumatic Diseases.*

REFERENCES

1. Christensen, L. R. *J. Gen. Physiol.* **28** (1945) 363.
2. Christensen, L. R. and MacLeod, C. M. *J. Gen. Physiol.* **28** (1945) 559.
3. Müllertz, S. and Lassen, M. *Proc. Soc. Exptl. Biol. Med.* **82** (1953) 264.
4. Troll, W. and Sherry, S. *J. Biol. Chem.* **213** (1955) 881.
5. Müllertz, S. *Biochem. J.* **61** (1955) 424.
6. Sherry, S. and Alkjaersig, N. *J. Clin. Invest.* **35** (1956) 735.
7. Sherry, S. and Alkjaersig, N. *Thromb. Diath. Haem.* **1** (1957) 264.
8. Christensen, L. R. *J. Clin. Invest.* **28** (1949) 163.
9. Ploug, J. and Kjeldgaard, N. O. *Biochem. et Biophys. Acta* **24** (1957) 278.
10. Lassen, M. *Scand. J. Clin. Lab. Invest.* **10** (1958) 384.
11. Lassen, M. *Biochem. J.* **69** (1958) 360.
12. Müllertz, S. *Thrombosis and Embolism, I. Int. Conf. Basel* (1954) 75.
13. Troll, W., Sherry, S. and Wachman, J. *J. Biol. Chem.* **208** (1954) 85.
14. Ablondi, F. N. and Hagan, J. J. *Proc. Soc. Exptl. Biol. Med.* **95** (1957) 195.

Received April 3, 1959.

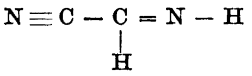
ACTA CHEMICA SCANDINAVICA 13 (1959) 1069–1074

Studies on a Polymerization Product of Hydrogen Cyanide

TOMMY WADSTEN and STEN ANDERSSON

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

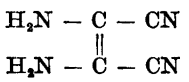
The crystalline compound formed by the polymerization of hydrogen cyanide has been studied by X-ray diffraction methods and infrared absorption measurements. The compound has been found to be a dimer of HCN and not a tetramer as suggested by previous authors. The following structure of the molecule is proposed:



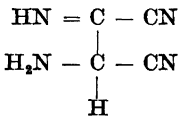
Strong intermolecular hydrogen bonds should be present in the structure.

It has long been well-known that pure hydrogen cyanide or hydrogen cyanide to which a small amount of an alkaline substance has been added polymerizes more or less rapidly depending on the concentration of the alkaline compound and on the temperature. The polymerization of pure hydrogen cyanide is very slow. The product is a dark, almost black, coal-like solid which cannot be reconverted to monomeric hydrogen cyanide. In the dark solid obtained, reddish brown, rodshaped crystals can be found. These crystals can be extracted by means of their solubility in ether. The dark residue, which is X-ray amorphous, is thought to consist of a mixture of several phases.

The structure of the crystalline polymerization compound has been discussed by several authors. From ebullioscopic measurements in methanol, Bedel [1] concluded the compound to be a tetramer of hydrogen cyanide. He suggested the structural formula $CN{\cdot}CH(CN){\cdot}NH_2{\cdot}HCN$. Gryszkiewicz-Trochimowski [2] considered it to have the following structure



while Hinkel and co-workers [3] thought this structure to be wrong and proposed instead the tautomeric structure:



Webb and co-workers [4] studied the compound by means of its ultraviolet absorption spectra, dipole moment measurements and infrared absorption spectra. They considered their data to support the structure proposed by Gryszkiewicz-Trochimowski.

By means of single crystal X-ray data, Sass and Donohue [5] found the unit cell dimensions of the polymer to be

$$a = 6.47 \text{ Å}, \quad b = 18.20 \text{ Å}, \quad c = 5.23 \text{ Å}, \quad \beta = 123°.$$

They did not, however, determine the structure.

EXPERIMENTAL

The polymerization products were obtained from a HCN gas bomb that had been kept for several years. The polymerization in the bomb was complete. From the dark solid, a lot of rod-shaped, reddish brown crystals could be picked out by hand. The crystals were almost colourless when recrystallized from ether. The X-ray patterns obtained from the crystals before and after the purification were identical.

The polymerization product was also prepared according to Linstead and co-workers [6]. The specimen obtained in this way was identical with the above mentioned one.

The purified crystals gave the following analysis:

	C	N (by residue)	H
weight % obs.	44.6	51.6	3.8
weight % calc.	44.4	51.9	3.7

The "melting point" (decomposition temperature) was found to be 180—181°C in good agreement with earlier observations.

The X-ray powder pattern was obtained using a Guinier camera with strictly monocromatized CuKα_1 radiation. The single crystal data were registered by the Weissenberg techniques with CuK radiation.

The infrared absorption spectrum was obtained on a Perkin-Elmer Spectrometer, Model 21. The sample was prepared for the measurements by the potassium bromide technique.

Table 1. The Guinier powder pattern of HCN dimer. Dimensions of the monoclinic unit cell:

$a = 6.511$ Å, $b = 18.31$ Å, $c = 5.208$ Å, $\beta = 121.9°$

I	$\sin^2\Theta_{obs}$	hkl	$\sin^2\Theta_{calc}$
vw	0.00700	020	0.00708
w	0.01941	100	0.01941
st	0.02121	110	0.02118
w	0.02653	120	0.02649
m	0.02836	040	0.02832
m	0.03122	12$\bar{1}$	0.03122
vw	0.03181	011	0.03210
w	0.03540	130	0.03534
w	0.04012	13$\bar{1}$	0.04007
m	0.04612	031	0.04626
m	0.05260	14$\bar{1}$	0.05246
vst	0.05869	041	0.05865

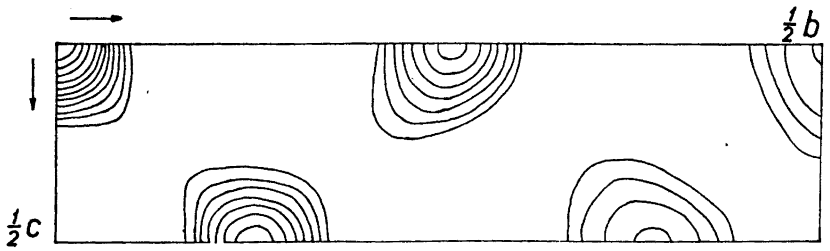


Fig. 1. HCN dimer: Patterson function. Projection along [100].

X-RAY STUDIES

A crystal was picked out from the dark solid and rotated around the rod-axis in the Weissenberg-camera. The identity period in this direction was found to be about 6.5 Å. Complete single crystal data were collected with this crystal setting. The crystal was monoclinic. Using the single crystal data, the powder pattern could be indexed as shown in Table 1. The following unit cell dimensions were thus derived:

$$a = 6.511 \text{ Å}, \quad b = 18.31 \text{ Å}, \quad c = 5.208 \text{ Å} \quad \beta = 121.9°.$$

The volume, 527.3 Å^3, gives a calculated density of 1.36 for 16 formula units HCN in the cell in fair agreement with the observed value of 1.31. The density was determined with the flotation method. The deviation from the value 1.41 determined by Sass and Donohue [5] is remarkable.

The reflexions which were systematically absent were $0k0$ with k odd and $h0l$ with l odd. This is in agreement with the space group $P2_1/c$, but the reflexion material may be insufficient (because of the high termal motion of the atoms) to draw definite conclusions.

The Patterson projection $P(vw)$ was calculated (*cf.* Fig. 1). It shows only eight strong, sharp and well resolved maxima. The spacing between the maxima in the projection is never less than 3.1 Å, which greatly exceeds the intramolecular distances of a compound of this kind. The structure must thus contain chain molecules running parallel to the a axis and distributed in the bc plane just like the maxima of Fig. 1. The length of the molecules must therefore correspond to the dimer (HCN_2). Adjacent molecules are probably connected by hydrogen bonds — this may also be inferred from the somewhat distorted appearance of the maxima in $P(vw)$.

Further studies on the crystal structure, which also include low-temperature investigations, are in progress.

DISCUSSION OF THE MOLECULAR STRUCTURE

In addition to the available X-ray data, some physical data concerning this substance are of particular interest. According to Webb and co-workers [4], the compound is a strong dipole and hence the molecule must be asymmetric.

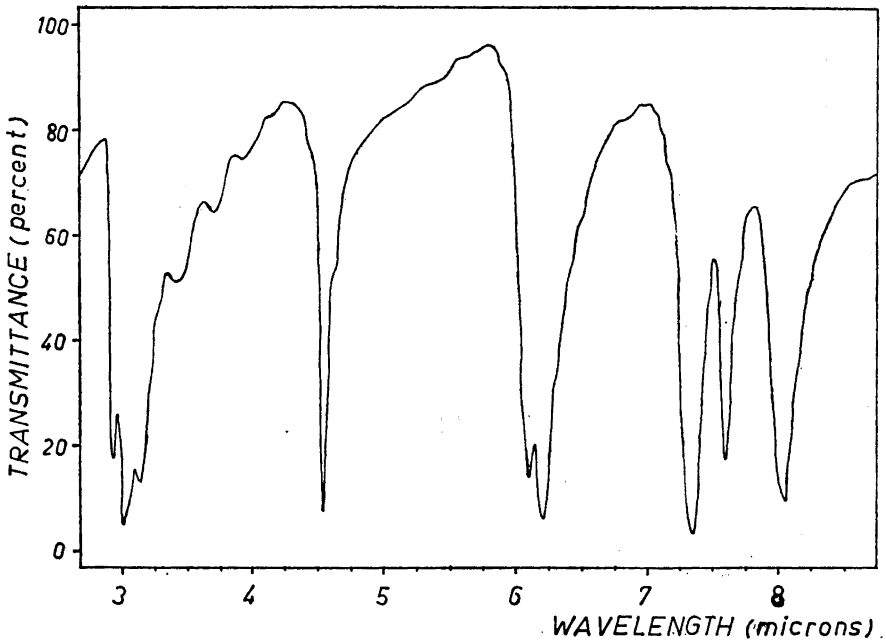


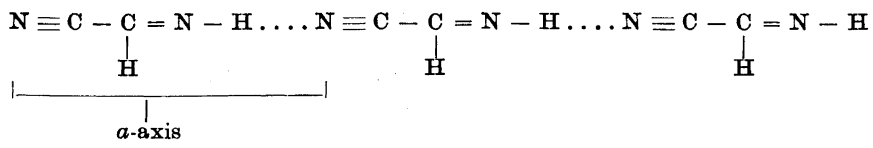
Fig. 2. Infrared spectra of HCN dimer.

Like Webb and co-workers, we have also studied the infrared absorption spectrum of the compound. However, our interpretation of the spectrum is not in agreement with theirs. As is seen in Fig. 2, the diffuse, weak absorption maxima at 3.42 μ (2 924 cm^{-1}), 3.70 μ (2 703 cm^{-1}) and 3.92 μ (2 551 cm^{-1}) clearly demonstrate the presence of strong hydrogen bonds. Furthermore, there is a characteristic maximum at 4.54 μ (2 203 cm^{-1}) corresponding to a carbon-nitrogen triple bond. The maxima at 6.08 μ (1 645 cm^{-1}) may correspond to a carbon-nitrogen double bond. The maxima at 2.93 μ (3 413 cm^{-1}), 3.01 μ (3 322 cm^{-1}) and 6.20 μ (1 613 cm^{-1}) correspond to nitrogen-hydrogen bonds. The maxima at 3.15 μ (3 175 cm^{-1}) and 7.35 μ (1 361 cm^{-1}) and possibly also at 7.60 μ (1 316 cm^{-1}) can probably be assigned to carbon-hydrogen bonds. The cause of the maxima at 8.05 μ (1 242 cm^{-1}) is uncertain.

A molecule satisfying the above mentioned physical data can thus be written:

$$N \equiv C - \underset{\displaystyle H}{\overset{\displaystyle |}{C}} = N - H$$

A suitable name for the compund seems to be iminoacetonitrile. The sum of the three bond lengths N $\equiv$ C, C—C and C = N is about 3.8 Å, if conventional bond distances are used. The chains are arranged along the *a*-axis as follows:



The bond distance N—H....N will thus be quite short, indicating strong hydrogen bonds in agreement with the infrared data. (The result obtained by Bedel in his molecular weight determination may be due to these hydrogen bonds.)

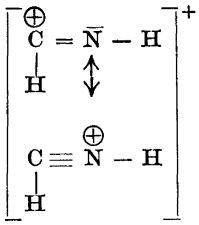
The high "melting" point, 181°C, for such a small molecule supports this suggestion concerning the presence of strong hydrogen bonds.

PROPOSED MECHANISM FOR THE POLYMERIZATION

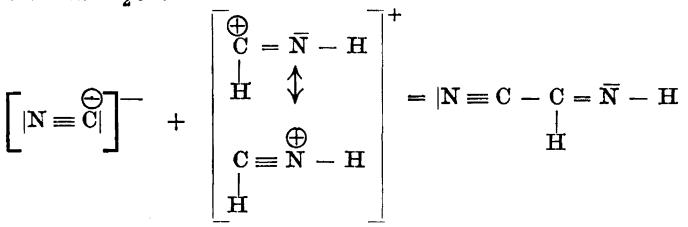
Pure hydrogen cyanide exhibits autoprotolyse according to the scheme

$$HCN + HCN = H_2CN^+ + CN^-$$

The ion H_2CN^+ can be written in two resonance forms:



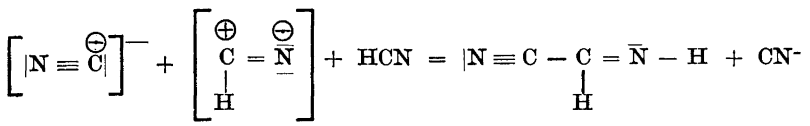
The dimer may thus be formed by the combination of the CN^--ion and the upper resonance form of H_2CN^+



In the presence of hydroxyl ions, the protolysis of hydrogen cyanide follows the scheme

$$HCN + OH^- = CN^- + H_2O$$

The cyanide ion may react with another molecule of hydrogen cyanide according to



The process evidently regenerates the cyanide ion and the polymerisation may thus be caused by a very small quantity of an alkaline substance.

It is interesting to note that no real melting occurs when the dimer is heated at temperatures above its "melting point". Some decomposition occurs and a black, X-ray amorphous residue is left which is stable up to several hundred degrees. The black residue resembles the dark solid formed originally during the polymerization of hydrogen cyanide. Further studies on this subject are in progress.

Acknowledgements. The authors wish to thank Dr. Arne Magnéli for his never failing support and many valuable discussions. Thanks are also due to Dr. Lennart Larsson for his help with the interpretation of the infrared absorption spectrum and to Miss Kerstin Stenberger for valuable discussions in the field of organic chemistry.

REFERENCES

1. Bedel, C. *Compt. rend.* **176** (1923) 168.
2. Gryszkiewicz-Trochimowski, E. *Roczniki Chem.* **8** (1928) 165.
3. Hinkel, L. E., Richards, G. O. and Thomas, O. *J. Chem. Soc.* **1937** 1432.
4. Webb, R. L., Frank, S. and Schneider, W. C. *J. Am. Chem. Soc.* **77** (1955) 3491.
5. Sass, R. L. and Donohue, J. *Acta Cryst.* **10** (1957) 375.
6. Linstead, R. P., Noble, E. G. and Wright, J. M. *J. Chem. Soc.* **1937** 920.

Received April 28, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1075–1080

Determination of Starch Solubilized by Means of Irradiation

JANUSZ TRZEBIŃSKI* and LARS EHRENBERG

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

Solubilization of starch by irradiation can be utilized for analytical purposes. Water, chlorophyll, sugars, and other alcohol-soluble compounds must be removed before irradiation by means of extraction of the material with hot ethanol. After irradiation, starch is extracted with water and may be determined colorimetrically as the iodine complex or as glucose after hydrolysis. Since the method involves only simple and well-defined procedures, it seems well suited for rapid analysis of great numbers of samples. For application of the method a suitable radiation source is required, *e. g.* a (50—) 100—1 000 curie ^{60}Co gamma-source or a van de Graaff accelerator.

In routine investigations of plants and plant materials, the analysis of starch has always been an intriguing problem. The quantitative determination of this inexactly defined compound is laborious and time-consuming, especially owing to difficulties in extracting and solubilizing the compound in a reproducible manner. It has previously been found that irradiation makes starch more easily soluble in water, chiefly due to the induction of carboxyl groups [1]. This investigation is primarily concerned with the question, whether irradiation can facilitate a rapid and reproducible quantitative determination of starch.

The methods in use for the determination of starch involve the extraction and dissolution by means of HCl, $HClO_4$, $CaCl_2$, or alkalis. These chemicals are either so reactive that the structure of starch is changed, or, as in the case of $CaCl_2$, their dissolving capacity is rather small. Since the chemicals often also dissolve contaminating compounds, great difficulties arise which hinder subsequent procedure.

For the determination of starch several methods have been used. Determination of glucose after acid hydrolysis gives correct values only if the starch has first been isolated. The determination of glucose after hydrolysis by means of enzymes is also circumstantial, depending on, *i.a.*, variations in the amylose/

* Present address: Instytut Hodowli Roslin, Bydgoszcz, Poland.

amylopectin ratio. Between starches of different origin, differences in the proportions of these starch components also prevent the general use of a colorimetric determination of the starch-iodine complex. Since amylose and amylopectin exhibit different spectra with iodine, and perhaps also for other less well-defined reasons, the iodine complexes of starches of different origin exhibit different spectra and intensities of extinction [2,3]. Similarly, as in the case of enzyme hydrolysis, such factors may be corrected for, if the method is standardized by means of starch from the plant material under investigation [4]. Apart from the genetically controlled variation of the composition of starch, there is also a variation within the plant during its vegetative development [5]. The latter variation is rather small ($\pm$ 2 %) and has but an insignificant influence on the colorimetric determination of the iodine complex.

Polarometric methods [6] give reliable values only when fairly large amounts of starch are determined.

In summarizing this short review of methods for the determination of starch, it can be stated that the quantitative extraction and purification followed by acid hydrolysis permits the most exact determination [7]. This method is laborious, however, and not suited to routine determinations.

PROCEDURES

From the studies by Ehrenberg, Jaarma, and Zimmer [1] on the effects of roentgen- and gamma-radiations on starches it can be concluded that different physico-chemical changes are induced in the material leading to a greater solubility in water of the latter. This effect seems partly to be due to a disintegration of the molecules but especially to the introduction of acidic -COOH groups, which can be titrated and which, in neutral milieu, occur as the negatively charged base groups, $-COO^-$. (Such changes are already obtained when monosaccharides are irradiated [8]; the true nature of the groups in the case of polysaccharides has not yet been determined.)

Two criteria must be fulfilled to permit the use of these radiation-induced changes for analytical purposes:

(1) Irradiation should not interfere with opportunities for determining starch colorimetrically (or, in tests aiming at a calibration, as glucose after hydrolysis);

(2) Irradiation of the plant material should permit the extraction and dissolution of starch.

Chemical changes which might be of importance to the first criterion were studied by irradiating starches of different origin (potato, rice, arrow root, maize) with doses 10^5—10^7 rad of γ-radiation from a 60 curie ^{60}Co-source. Before irradiation, the starches were desiccated with aceton to a water content of 5 %.

Throughout the investigation, 0.1 % solutions of starch were prepared by dissolving 250 mg in 150 ml boiling water, and, after another 15 min of boiling and cooling, diluting to the mark of a 250 ml volumetric flask. For the colorimetric investigation of the iodine complex, 2 ml of the 0.1 % solution (*i. e.*, 2 mg starch) were introduced in a 100 ml volumetric flask, followed by 18 ml water, 2 ml 2 N acetic acid, 1 ml 10 % KI solution, and,

Table 1. Influence of gamma radiation on the properties of potato starch; *cf.* text.

Radiation dose Mrad	Extinction at λ = 590 mμ	Reducing sugar, % of starch	Specific viscosity	pH of 0.1 % solution
0	0.295	< 1	0.520	6.92
0.12	0.295	< 1	0.220	6.90
0.19	0.295	< 1	0.220	6.90
0.50	0.295	< 1	0.166	6.72
0.86	0.295	< 1	0.154	6.70
1.6	0.295	< 1	0.135	6.60
10	0.281	10	0.070	5.60

finally, 10 ml 0.01 N KIO_3. Water was then added to the mark. The extinction of the greenish solution was determined in a Beckman spectrophotometer at wave-lengths 500—650 mμ.

The radiation effects on the starch were also characterized by determinations of reducing sugars (5 ml samples of the 0.1 % solution were determined according to the method of Somogyi [9]), specific viscosities, and pH of the solutions.

The data demonstrate that up to doses of 1—2 × 10^6 rad, no changes occur which could be suspected to appreciably affect the analytical properties of the starches. In Table 1 this is demonstrated for potato starch; the other starches behaved identically in all respects. Higher doses, around 10 × 10^6 rad, are required to change the extinction. At the same time great amounts of reducing groups are introduced, and the viscosity indicates a considerable breakdown of the molecules [1]. The decreased extinction has to be ascribed, to a great extent, to a displacement towards red of the colour of the iodine complex (Fig. 1 and earlier publication [1]).

For the elucidation of factors pertinent for the second criterion it was important to include materials widely different in composition in general and in their starch content. Tissues storaging starch (potato tubers and barley seeds), as well as vegetative parts of plants with a low starch content (leaves of potato and maple, *Acer platanoides*) were investigated. In the following, the pilot experiments with potato tubers and maple leaves will be described.

Roughly disintegrated parts of the material were dehydrated in boiling ethanol. This treatment extracts chlorophyll and other alcohol-soluble substances (simple sugars, glycosides, alkaloids, fats, and waxes). The enzymes become inactivated, which is of extreme importance in the determination of starch in green, vegetative parts of plants,

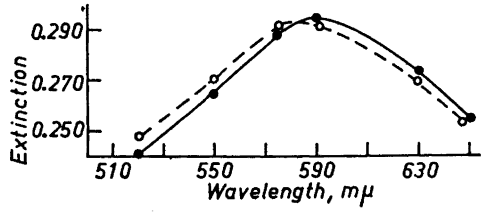


Fig. 1. Extinction curves of the iodine complex of potato starch (2 mg/100 ml). ● unirradiated and irradiated with doses up to 1.6 × 10^6 rad; ○ irradiated with 10 × 10^6 rad.

Table 2. Starch content in the materials studied. Classic methods.

Material	1 Residue after alcohol extraction %	2 Reducing sugars in residue (1) %	3 Pentosanes in residue (1) %	4 (2−3) × 0.9 %	5 Starch according to Pucher %
Maple leaves I	9.1	16.4	8.7	6.9	6.7
» » II	10.3	22.3	10.9	10.7	10.3
Potato tubers	24.8	66.4	2.1	57.9	55.3

where the (low) starch content undergoes rapid changes. Two extractions were performed at a final alcohol concentration of 75 %, and a third one at 80 % alcohol.

The dry material was ground in a Wiley mill (sieve 60). Parts of the samples were then very finely ground in a ball mill and sifted through silk-fabric.

The ball mills were prepared from thick-walled 100 ml cream bottles, each with 80—100 ball bearings 0.4—0.6 cm in diameter. In each bottle 0.5—0.8 g plant material was ground by placing the rubber-stoppered bottles in a shaking-machine. Gentle shaking (about 50 times per min) for 4—5 h was sufficient to disintegrate the material beyond a limit where no cells could be identified in the microscope.

Table 3. Colorimetrically determined starch in water extract of residue (column 1 in Table 2) after alcohol extraction. a = grinding in Wiley mill; b = grinding in ball mill (see text). Values related to residue after alcohol extraction and to values determined according to the method of Pucher *et. al.*

Material	Radiation dose Mrad	Starch found			
		a: Wiley mill		b: Ball mill	
		% of extraction residue	% of value acc. to Pucher	% of extraction residue	% of value acc. to Pucher
Maple leaves I	0	1.70	25	2.00	30
	0.12	2.10	31.5	5.80	86
	0.5	2.35	35	6.60	98
	1.6	2.70	40	6.65	99
Maple leaves II	0	2.68	26	3.50	34
	0.13	2.99	29	3.92	38
	0.21	3.81	37	6.50	63
	0.52	3.90	38	10.1	98
	0.93	4.45	43	9.95	96
	1.7	5.35	52	10.4	101
Potato tubers	0	40.4	73	43.6	79
	0.12	46.1	83	47.5	86
	0.5	51.5	93	55.7	101
	1.6	52.5	95	54.8	99

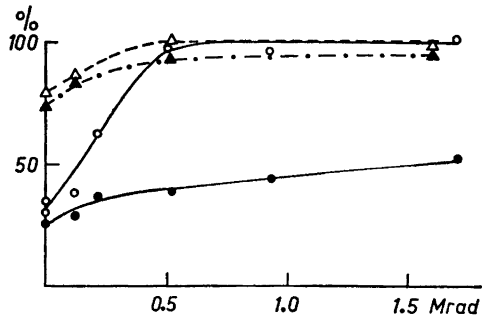


Fig. 2. Colorimetrically determined starch as a function of radiation dose. 100 % = starch content determined according to Pucher *et al.* ● Maple leaves: Wiley mill; ○ Maple leaves: ball mill; ▲ Potato tubers: Wiley mill; △ Potato tubers: ball mill.

200—500 mg material, depending on the starch content, is weighed, suspended in 25 ml water and then poured quantitatively into 150 ml of boiling water. The boiling is continued under reflux for 30 min. The addition of a few drops of *n*-octanol prevents foaming. After cooling, the solution is diluted to 250 ml, and after clearing by centrifugation, the iodine compound is determined colorimetrically at 590 mμ and in accordance with the method given above. The starch concentration is calculated from a calibration curve, which is prepared utilizing isolated starch of the type to be determined [10]. (Since in some cases, *e. g.*, maple leaves, absolutely pure starch could not be easily prepared, the calibration curve was based on the amount of glucose determined after hydrolysis.)

In order to compare the data from irradiated materials with exact values for their starch contents, the latter were determined according to Pucher *et al.*[7]. In addition, a generally applied laboratory method was used, involving determination of total reducing sugars after 2.5 h hydrolysis in 0.7 N HCl, followed by subtraction of pentosanes, determined according to Tollens and Kröber [11]. The difference, multiplied by 0.9, was regarded as starch.

When the starch content had to be related to total dry or fresh weight, the loss of original material by the alcohol extraction was determined in a special sample. (1 g of material was weighed into a Soxhlet extraction thimble, extracted with alcohol as described above, dried and weighed again.)

In Tables 2—3 data are presented for three materials which were subjected to thorough study, *viz.* maple leaves I (harvested about June 1st), maple leaves II (harvested a month later), and potato tubers. In principle the other materials behaved identically. Table 2 gives the weight of the material after extraction with alcohol, as per cent of original fresh weight (column 1), as well as the starch values, as percentages of the extraction residue, determined as the difference between total reducing sugars and pentosanes, or according to Pucher. These two methods give acceptably agreeing values for the starch contents.

For the same materials, the results of the colorimetric analyses after irradiation are presented in Table 3. The influences of the radiation dose and of the grinding method are clearly demonstrated, as illustrated by the summarizing graph, Fig. 2. The following conclusions can be drawn from the data:

(1) that with both methods of grinding, irradiation increases the possibility of extracting starch with water;
(2) that effective extraction is reached, however, only in the finely ground samples (ball mill), *i.e.*, where all cells were destroyed;
(3) that the dose required to make the starch fully soluble and extractable, amounts to about 500 000 rad. At this and higher doses good agreement was obtained with the data given in Table 1.
(4) It was further demonstrated that irradiation of thermally dried samples, which had not been extracted by means of alcohol, did not lead to corresponding solubilization of the starch, not even at higher doses ($> 10^6$ rad).

The stay of J. Trzebiński at the University of Stockholm was financed by a fellowship granted by the *Rockefeller Foundation*. The investigation was further supported by the *Swedish Agricultural Research Council*.

REFERENCES

1. Ehrenberg, L., Jaarma, M. and Zimmer, E. C. *Acta Chem. Scand.* **11** (1957) 950.
2. Mc Cready, R. M., Guggolz, J., Silviera, U. and Owens, H. S. *Anal. Chem.* **22** (1950) 1156.
3. Lansky, S., Kooi, M. and Schoch, J. *J. Am. Chem. Soc.* **71** (1954) 4066.
4. Nielsen, J. P. and Gleason, P. C. *Ind. Eng. Chem. Anal. Ed.* **17** (1945) 131.
5. Halsall, T. G., Hirst, E. L., Jones, J. K. N. and Sansome, F. W. *Biochem. J.* **43** (1948) 70.
6. Steiner, E. T. and Guthrie, J. D. *Ind. Eng. Chem. Anal. Ed.* **16** (1944) 736.
7. Pucher, G. W., Leavenworth, C. S. and Vickery, H. B. *Anal. Chem.* **20** (1948) 850.
8. Phillips, G. O. and Moody, G. J. *Proc. U.N. 2nd Intern. Conf. Peaceful Uses Atomic Energy Geneva 1958*, P/47.
9. Somogyi, M. *J. Biol. Chem.* **160** (1945) 61.
10. Porter, H. K. and Martin, R. V. *J. Exptl. Botany* **3** (1952) 326.
11. *Cf.* Winston, A.L. and Winton, K.B. *The Analysis of Foods*, New York, 1945.

Received March 5, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1081–1096

Electrophoresis Studies on Blood Plasma Esterases

II. Avian, Reptilian, Amphibian and Piscine Plasmata

KLAS-BERTIL AUGUSTINSSON

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

The esterase activity of the plasmata from birds, reptiles, amphibians and fish was investigated using as substrates a series of aliphatic, aromatic, heterocyclic and choline esters. The distribution of esterase activity and total protein after electrophoresis on cellulose columns are reported for the plasmata of cock, duck, turtle, two frogs *(Rana, Xenopus)*, and five teleostian fishes (pike, cod, eel, wrasse, frogfish). The electrophoretic patterns for the plasma proteins of one Elasmobranch *(Squalus)* and one Cyclostome *(Myxine)* are also demonstrated.

Of the three types of esterases present in most mammalian plasmata, only the aliesterases (B-esterases) and cholinesterases (C-esterases) were present in the plasmata of lower vertebrates, with the single exception of the plasma from female frogs which also contained an arylesterase (A-esterase) in addition to a propionylcholinesterase. Cock and chicken plasmata were characterized by a propionylcholinesterase which was the only esterase present and differed from other cholinesterases in many respects. Duck plasma contained a propionyl-B-esterase in addition to a propionylcholinesterase. One esterase only was present in turtle plasma; it had the characteristics of both a B- and C-esterase and was highly sensitive to physostigmine although non-choline esters were hydrolysed more rapidly than choline esters. More than one B-type esterase were probably present in the plasma of *Xenopus laevis*, which also contained a butyrylcholinesterase in low concentration.

In all the teleostian plasmata, an acetylcholinesterase was present in low concentration. The main esterases present in these plasmata were acetyl- or propionyl-B-esterases. Eel plasma contained such an esterase in very high concentration. The plasma of spiny dogfish had low B- and C-esterase activities, and the esterase activity of hagfish plasma with most substrates was negligible.

The esterase patterns of mammalian plasmata were discussed in the first paper of this series [1]. This paper considers the plasmata from lower vertebrates from a comparative point of view.

MATERIAL AND METHODS

Plasmata were prepared from heparinized blood, obtained by heart puncture or by cutting the ventral aorta (fish).

Substrates and inhibitors were the same as in the first report of this series. The following abbreviations are employed: acetyl, Ac; propionyl, Pr; butyryl, Bu; benzoyl, Bz; succinyl, Su; *iso*amyl, Am; triglycerides, *e. g.*, TBu; phenyl, Ph; choline, Ch; acetyl-β-methylcholine, MeCh; polyoxyethylene sorbitan monolaurate, Tween 20; tetra-*iso*propylphosphoramide, *iso*-OMPA; bis-mono*iso*propylamine fluorophosphine oxide, mipafox; 10-(1-diethylaminopropionyl) phenothiazine hydrochloride, Astra 1397; 1,5-*bis* (4-trimethylammoniumphenyl) pentane-3-one diiodide, 62C47; *bis*- (piperidinomethyl coumaranyl-5)ketone, 3318CT.

Electrophoresis was carried out on cellulose columns (3 cm × 40 cm) in veronal buffer solution (pH 8.4, $I = 0.1$) at 5—15°C, using 5 ml of buffered plasma [1]. The protein concentration of each eluted fraction (2.8 ml) was estimated by a modified Folin procedure [2].

Esterase activity was determined by the Warburg technique and expressed in b_{30} values [3]. The efficiency of esterase inhibition by various compounds was expressed in pI_{50} values, extrapolated from curves of inhibition (%) — inhibitor concentration (pI) where sufficient inhibitor was used at the higher pI levels to establish the point of complete inhibition.

RESULTS

The hydrolysis rates of various substrates by original plasma samples are summarized in Table 1. Activity values (b_{30} per 0.1 ml plasma) obtained with additional substrates are given in the text. The electrophoretic patterns presented in Figs. 1—11 show the rate of elution of the esterases in relation to various protein constituents. In the legends to these figures, relative substrate specificity and sensitivity to certain inhibitors of each esterase component are summarized in tabular form. The peaks of esterase activity are designated B and C, corresponding to aliesterase and cholinesterase activities, respectively. Arylesterases (A) were not present in the plasmata studied, except for a sample from frog (*Rana*).

Cock and chicken (Fig. 1). The esterase pattern of cock plasma was characterized by the comparatively high hydrolysis rate of propionylcholine which was the highest value observed among the esters used as substrates. This plasma was unique among those examined in being devoid of both A- and B-esterases. The B-type esterase described by Aldridge [4] was actually identical with the propionylcholinesterase present, hydrolysing acetyl-β-methylcholine at a comparatively high rate. This enzyme was a typical physostigmine-sensitive propionylesterase hydrolysing both tripropionin and phenyl propionate at a higher rate than their other acyl homologues. No other esterases were detected on electrophoresis in the separated plasma. The hydrolysis rates of four different substrates were all found to be influenced at the same degree by the eight inhibitors tested (Table 2), providing additional evidence that the same single esterase hydrolysed these substrates. This esterase was found to be extremely sensitive to mipafox.

The propionylcholinesterase of the cock differed from both the acetylcholinesterases present in most animal erythrocytes and from the butyrylcholinesterases present in several mammalian plasmata. This difference included the unusual substrate specificity, *i.e.*, high rate of hydrolysis of acetyl-β-methylcholine (also observed previously [5,6]) and various types of non-choline esters,

Table 1. Enzymic hydrolysis of various esters by avian, reptilian, amphibian and piscine plasmata. Samples from at least three animals of each species were analysed. The activity values (b_{30}) refer to initial hydrolysis rates with a representative sample and are expressed in μl CO_2 per 0.10 ml of plasma per 30 min, corrections made for nonenzymic hydrolysis. Where values are indicated in brackets, initial hydrolysis rate was determined by extrapolation. A question mark indicates that such extrapolation could not be done because of a too rapid decrease of the hydrolysis rate. An asterisk denotes that the activity varies greatly from individual to individual. Code names are explained in the text.

Species	AcCh	PrCh	BuCh	MeCh	BzCh	SuCh	AmAc	AmPr	AmBu	TAc	TPr	TBu	PhAc	PhPr	PhBu
Cock	37	71	36	19	2	<1	4	6	7	10	36	22	36	62	38
Duck	43	74	67	7	8	2	?	?	?	30	220	112	350	505	360
Turtle, *Testudo*	14	103	27	7	1	0	(450)	(500)	(300)	700	1 500	800	650	1 030	320
Frog, *Rana*	40	90	87	2	10	7	10	21	59	12	53	33	180*	265*	220*
Clawed frog, *Xenopus*	2	5	9	—	—	—	34	51	70	108	402	309	900	—	1 020
Pike, *Esox*	12	2	1	1	1	<1	(40)	(65)	(120)	120	400	280	370	—	255
Cod, *Gadus*	6	4	3	2	1	1	?	(4)	(4)	390	107	20	570	—	27
Eel, *Anquilla*	10	4	3	2	<1	21	?	?	?	800	4 250	4 750	8 000	—	20 300
Wrasse, *Labrus*	4	2	2	1	1	<1	5	(20)	16	28	160	130	160	—	345
Frogfisch, *Lophius*	13	6	4	2	5	3	10	7	6	523	175	31	770	—	33
Dogfish, *Squalus*	4	3	1	<1	<1	<1	2	2	2	3	10	4	14	—	11
Hagfish, *Myxine*	0	0	0	0	<1	0	0	0	0	0	0	0	<1	—	<1

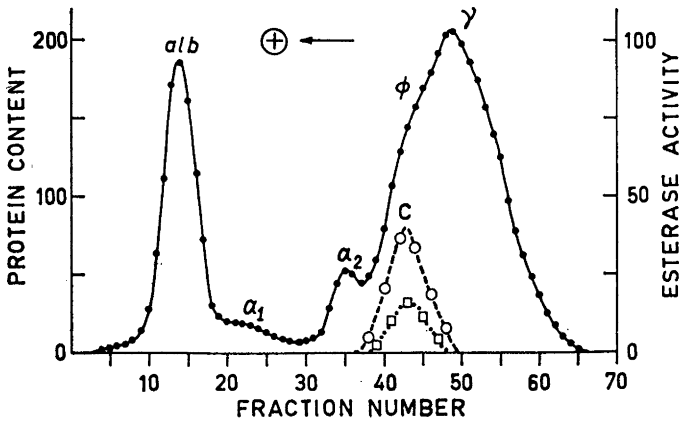


Fig. 1. Cock plasma. Distribution of esterase activity and total protein after column electrophoresis (veronal buffer, pH 8.4; I = 0.1; 260 V; 60 mA; 30 h). The protein content is expressed as relative amount based on extinction values. Esterase activity is expressed in b_{30} values with the ml aliquots from each fraction as indicated below: ●——●, protein concentration; □·····□, PhAc (0.40) and TPr (0.40); O— — —O, PrCh (0.40).

Esterase activities (b_{30}/0.10 ml) against various substrates and esterase inhibition (pI_{50}) by physostigmine (Phys) and an organophosphorus compound of the peak fraction from each esterase component.

Esterase component	PhAc	PhPr	PhBu	TPr	AcCh	PrCh	pI_{50}	
							Phys	Mipafox
C	4	8	5	4	5	10	7.8	9.0

and a unique pattern of susceptibility to various esterase inhibitors since those being selective inhibitors of acetylcholinesterase (62C47 and 3318CT) had very little effect in this case (Table 2). The low inhibiting effect of such compounds on the chicken serum cholinesterase was observed previously [7,8]. Rat plasma contains a propionylcholinesterase based on substrate specificity, but the propionylcholinesterase of cock plasma differs from that of the rat in showing no inhibition by excess substrate (AcCh or PrCh), as was demonstrated also in previous papers [5,9]. The cock (chicken) plasma cholinesterase, therefore, has to be regarded as a special type of physostigmine-sensitive esterase, differing from the cholinesterases of other vertebrate plasmata in many respects. It is another example of the view [1] that no clear-cut distinction between various groups of cholinesterases can be made.

Chicken plasma gave the same results both regarding the properties of the propionylcholinesterase present and the lack of other types of esterases. The sex difference in plasma cholinesterase activity observed by Caridroit *et al.*[10] was not further investigated.

Table 2. Susceptibility of cock-plasma esterase to various inhibitors. Values refer to negative logarithm of molar inhibitor concentration producing 50 % esterase inhibition. The enzyme preparation (whole plasma and separated esterase fractions, respectively) was incubated for 50 min with the inhibitor prior to addition of substrate.

Inhibitor	Propionyl-choline	Tri-propionin	Phenyl-acetate	Phenyl-butyrate
Physostigmine	7.8	7.8	7.85	7.6
iso-OMPA	6.2	6.0	6.25	6.25
Mipafox	9.0	8.9	8.9	8.9
Astra 1397	5.3	5.4	5.25	4.55
3318CT	<3.5	<3.5	<3.5	<3.5
62C47	<3.5	<3.5	<3.5	<3.5
Atoxyl	<3.5	<3	<3	<3
Quinine sulphate	<3.5	<3.5	<3	<3

Duck (Fig. 2). The plasma from duck hydrolysed the phenyl esters and the triglycerides at a much higher rate than did cock plasma. With these substrates and with choline esters, the propionates were hydrolysed more rapidly than the corresponding acetates and butyrates. The hydrolysis rate with

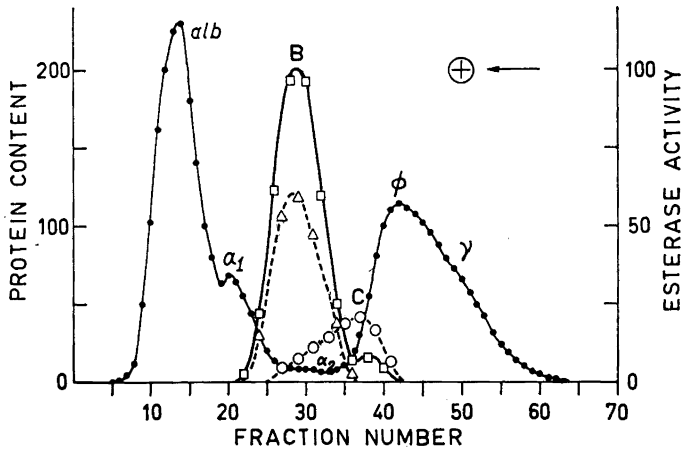


Fig. 2. Duck plasma. Conditions as for Fig. 1. □———□, PhBu (0.20) and PhPr (0.10); △– – –△, TPr (0.20); O– – –O, PrCh (0.40).

Esterase component	PhAc	PhPr	PhBu	TPr	PrCh	BuCh	pI_{50}			
							Phys	*iso*-OMPA	Astra 1397	Atoxyl
B	45	98	49	30	(2)	(1)	5.6	5.5	<3	<3
C	1	5	4	<1	5	4	8.5	8.8	5.5	5.7

acetyl-β-methylcholine was relatively high, but lower than that for cock plasma. On the other hand, the carbon analogue of acetylcholine (3,3-dimethylbutyl acetate) was hydrolysed at a comparatively high rate (b_{30} 20). The hydrolysis rates with the *iso*amyl esters dropped off very rapidly with time although the initial rates were as high as with the triglycerides.

In contrast to cock plasma, the duck plasma gave two esterase peaks on electrophoresis. The main peak was due to a B-estrase which was sensitive to *iso*-OMPA (Fig. 2) and mipafox (pI_{50} 8.5). This esterase differed from the B-type esterases of the mammalian plasmata in being sensitive to 10^{-5} M physostigmine as is also characteristic of certain B-esterases of lower vertebrates (turtle, frog, pike). This propionyl-B-esterase was similar to a cholinesterase in regard to the susceptibility to inhibitors. However, a propionylcholinesterase (C) was found in a second slower-moving peak. Some of the esterase activity was also found in the B-esterase peak as determined by comparing the hydrolysis of propionylcholine and phenyl propionate by these fractions. Except for differences in sensitivity to physostigmine, the B- and C-esterases were best differentiated by using Astra 1397 (or atoxyl) which selectively inhibited the C-esterase in a concentration of 10^{-5} M.

Indoxyl acetate was hydrolysed by the B-esterase only.

Turtle, Testudo graeca (Fig. 3). The propionyl esters of all series of substrates were hydrolysed at the highest rate. Particularly high rates were observed with tripropionin and phenyl propionate. This plasma hydrolysed heroin

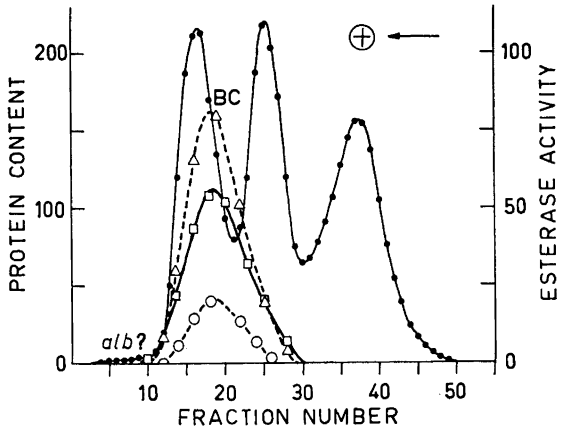


Fig. 3. Turtle plasma. Conditions as for Fig. 1. △— — —△, TPr (0.025); □————□, heroin (0.10); ○— — —○, PrCh (0.20).

Esterase component	PhPr	PhBu	TPr	PrCh	Heroin	pI_{50}	
						Phys	*iso*-OMPA
BC	210	60	320	10	55	6.3	6.6

Table 3. Susceptibility of turtle-plasma esterase to various inhibitors. *Cf.* Table 2 for details.

Inhibitor	Tri-propionin	Phenyl-propionate	Heroin	Propionyl-choline
Physostigmine	6.4	6.3	6.1	6.3
iso-OMPA	5.2	6.6	5.2	6.6
3318CT	<3.5	≈ 4.0	<3.5	≈ 4.0

(b_{30} 220) and the carbon analogue of acetylcholine (b_{30} 160) more rapidly than the choline esters.

Turtle plasma was almost free of albumin-like proteins, confirming a recent observation made by Cohen and Stickler [11]. Only one esterase peak was obtained on electrophoresis and this had about the same electrophoretic mobility as was found for most B-esterases of vertebrate plasmata. Most results obtained were consistent with the view that only one esterase was present in this plasma. Summation experiments with tripropionin, heroin and propionylcholine showed that all three substrates were probably split by the same esterase. This was also indicated by the inhibitory experiments, although the 50 % inhibition values (Table 3) obtained with the original plasma showed some discrepancies for the compounds *iso*OMPA and 3318CT.

The properties of this esterase were unique in many respects. It hydrolysed the propionates in the following order of decreasing rates: TPr > PhPr > AmPr > Heroin > PrCh. This esterase was sensitive to both physostigmine and the organophosphorus compounds used (Table 3). Therefore, it had the characteristics of both a B-and C-esterase (*cf.* Ref.[1]), and is the first example of a physostigmine-sensitive esterase hydrolysing non-choline esters more rapidly than choline esters. This esterase, therefore, may represent an intermediate stage in the phylogenetic evolution of plasma esterases (see further the third paper of this series [12]).

Frog, Rana temporaria (Fig. 4). The hydrolysis rate with phenyl esters varied considerably from animal to animal. The plasma from female frogs generally had much greater activity than that from male frogs against these esters. Phenyl acetate, for instance, was hydrolysed at about a 40 times higher rate by female plasma compared with male plasma. This sex difference was due to the presence of a propionyl- (or acetyl-) arylesterase (A-esterase; see Fig. 4, inserted graph) which was absent in the male frog plasmata investigated in this study. Heroin was hydrolysed at a higher rate by frog plasma (both sexes, b_{30} 25) than by most mammalian plasmata.

On electrophoresis the B- and C-esterases migrated close together, the latter having a somewhat lower mobility than the former, in contrast to most other plasma esterases of these types. A fast-migrating esterase was probably identical with the B-esterase of the main esterase peak as was demonstrated by substrate specificity and inhibitor experiments. This esterase was a propionylesterase which was more sensitive to physostigmine (pI_{50} 4.5) than the B-esterases of mammalian plasmata.

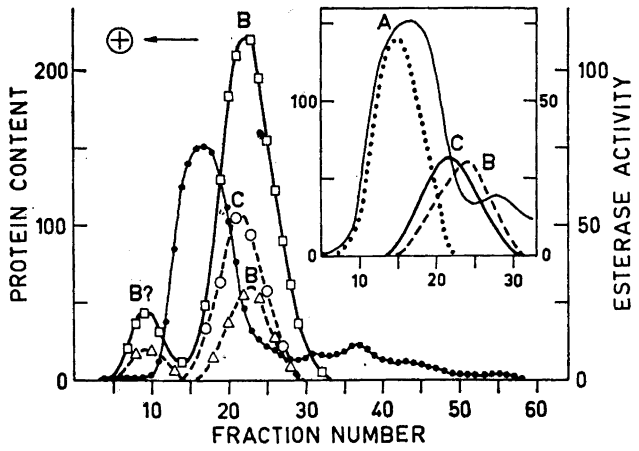


Fig. 4. Frog (male) plasma. Conditions as for Fig. 1. □———□, PhBu (0.40); △– – –△, TPr (0.40); O– – –O, PrCh (0.40).

Esterase component	PhAc	PhBu	TPr	PrCh	BuCh	pI_{50}	
						Phys	*iso*-OMPA
B	210	30	7	0	0	4.5	6.2
B?	3	5	2	0	0	4.5	6.2
C	?	?	?	14	13	7.2	7.2

The insert (of the same scale as the main graph) is the electrophoretic pattern of the plasma from a female frog with high activity against phenyl esters (PhAc, $b_{30} > 5\,000$). ———, protein; ....., PhAc (0.02); – – – –, TPr (0.20); ———, BuCh (0.20).

The C-esterase of frog plasma was a propionylcholinesterase which hydrolysed butyrylcholine at a relatively high rate and was less sensitive to mipafox (pI_{50} 5.2) but more sensitive to atoxyl (pI_{50} 5.1) and quinine (pI_{50} 4.2) than are most other plasma cholinesterases. Atoxyl was a useful selective inhibitor of cholinesterase of this plasma, because both B- and A-esterases (when present) were resistant to this agent. Plasma cholinesterase of male frogs was responsible for about 30 % of the total hydrolysis of the phenyl esters.

The propionylcholinesterase was inhibited by high concentrations of propionylcholine and butyrylcholine with a substrate optimum of 7×10^{-3} M. In this respect this esterase resembles the group of acetylcolinesterases present in the erythrocytes of various animals.

South African clawed frog, Xenopus laevis (Fig. 5). The esterases present in the plasma sample analysed, collected from five females, differed greatly from those of the plasma of *Rana*. An arylesterase was absent because the

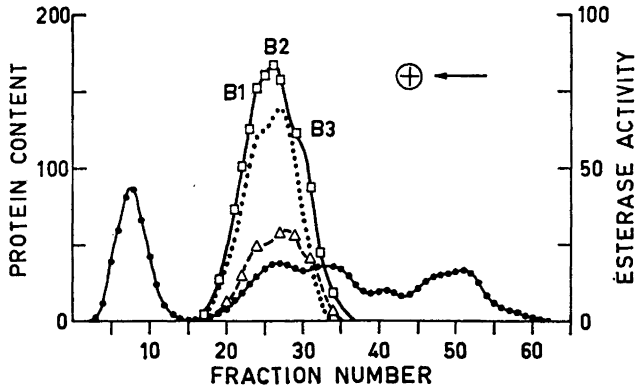


Fig. 5. Clawed-frog plasma. Conditions as for Fig. 1. □———□, PhBu (0.05); ....., PhAc (0.05); △———△, TPr (0.05).

Esterase component	PhAc	PhBu	AmBu	TPr	Tween	BuCh	pI_{50}	
							Phys	Mipafox
B1	120	150	3	50	2	0	3.5	6
B2	140	170	6	60	<7	0	3.5	6
B3	?	120	5	40	13	0	4.5	6
C	?	?	?	?	?	<1	7	7

hydrolysis of phenyl esters was completely inhibited by low concentrations of both mipafox and *iso*-OMPA. There were probably more than one B-type esterase with different substrate specificities. The complexity of the esterase peak obtained on electrophoresis was in conformity with this hypothesis. The peaks B1 and B2 could possibly be due to the same enzyme, but the esterase of peak B3 was characterized by relatively higher rate of Tween-20 hydrolysis and greater sensitivity to physostigmine than the B1- and B2-esterase fractions. The B-esterases were all of the butyryl- or propionyl-esterase type and hydrolysed heroin at high rates (b_{30} per 0.1 ml plasma, 110).

The C-esterase, present in too low concentration to be detected after electrophoresis, was a butyrylcholinesterase which was highly sensitive to Astra 1397 ($pI_{50} \approx 5$) as are most of the plasma butyrylcholinesterases.

This plasma contained in high concentration a protein which migrated faster on electrophoresis than most albumin-like proteins of other plasmata.

Pike, Esox lucius (Fig. 6). Plasma from this fish hydrolysed triglycerides more rapidly than phenyl esters, tripropionin being split at the highest rate. The main esterase present was a B-esterase being highly sensitive to mipafox ($pI_{50} \approx 9$) and less so to physostigmine (pI_{50} 4.8) when tested with tripropionin,

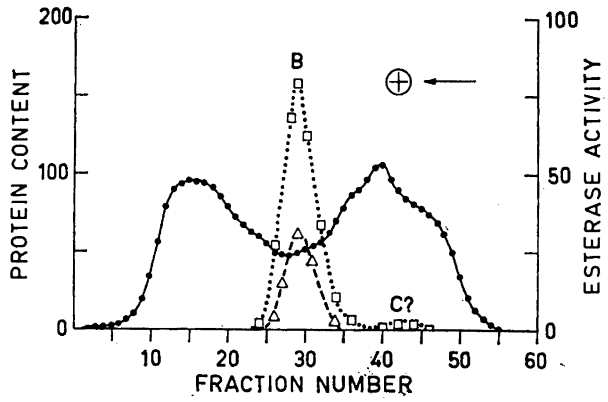


Fig. 6. Pike plasma. Conditions as for Fig. 1. □·····□, PhAc (0.10); △———△, AmBu (0.10).

Esterase component	PhAc	PhBu	AmBu	TPr	AcCh	pI_{50}	
						Phys	Mipafox
B	80	75	30	100	0	4.8	≈9
C	?	0	0	0	<1	6.5	≈4

phenyl acetate and phenyl butyrate respectively as substrates. This enzyme hydrolysed heroin at a low rate (b_{30} 7) in contrast to the B-esterases of cod and eel plasmata, and the carbon analogue of acetylcholine at a relatively high rate (b_{30} 34).

The C-esterase present in low concentration was an acetylcholinesterase which was much less sensitive to mipafox than the B-esterase. In this respect the pike cholinesterase was similar to an acetylcholinesterase of higher animals.

Cod, Gadus callarias (Fig. 7). An acetyl-B-esterase was characteristic of this plasma. Heroin was hydrolysed at a high rate (b_{30} 220), and mipafox was an inhibitor but not *iso*-OMPA. The esterase appeared as a uniform peak on electrophoresis.

An acetylcholinesterase was present but in too low concentration to be detected in the fractions after electrophoresis.

Eel, Anguilla anguilla (Fig. 8). This plasma was characterized by its unusually high activity in hydrolysing aromatic esters and triglycerides, especially those of butyric acid. This material seems to be the richest animal source of "lipase" activity ever described. It is 100 times more active than human plasma in hydrolysing these esters. Heroin was hydrolysed at a high rate (b_{30} 210) and so also was Tween 20 (b_{30} 60). The high "lipase" activity tested with tributyrin has been reported previously [13].

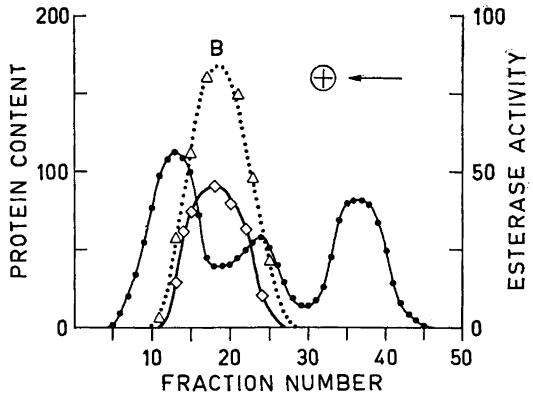


Fig. 7. Cod plasma. Conditions as for Fig. 1. △·····△, TAc (0.20); □———□, heroin (0.20).

Esterase component	PhAc	*p*-Nitro-PhAc	TAc	Heroin	AcCh	pI_{50}	
						Phys	Mipafox
B	60	40	42	23	0	≈3.5	5.3
C	?	?	?	?	<1	≈6	—

The main esterase present was a butyryl- or propionylesterase (B1), which hydrolysed the aromatic esters at a higher rate than the triglycerides. This enzyme was highly sensitive to mipafox and *iso*-OMPA (see legends to Fig. 8), and as many esterases of this type present in the plasmata of lower vertebrates it was relatively sensitive to physostigmine. A minor esterase peak with a lower electrophoretic mobility was also observed. It was due to an esterase which differed from the main esterase in hydrolysing the acetates more rapidly than the butyrates, in being much less sensitive to mipafox and *iso*-OMPA and highly resistant to physostigmine. This enzyme has to be regarded as a B-esterase, although the aliphatic esters were hydrolysed at a lower rate than the aromatic ones.

As with most fish plasmata, eel plasma contained a C-esterase, the specificity and sensitivity to inhibitors of which were characteristic of an acetylcholinesterase. High concentration of acetylcholine inhibited the activity.

Wrasse, Labrus berggylta (Fig. 9). The plasma from females contained a B-esterase which hydrolysed butyrates and propionates at a higher rate than acetates. It was similar to the B2-esterase of eel plasma in being sensitive to mipafox but not to *iso*-OMPA. Tween 20 was hydrolysed at a relatively high rate by this enzyme (b_{30} 100). Choline esters were hydrolysed at very low rates, due probably to an enzyme similar to an acetylcholinesterase.

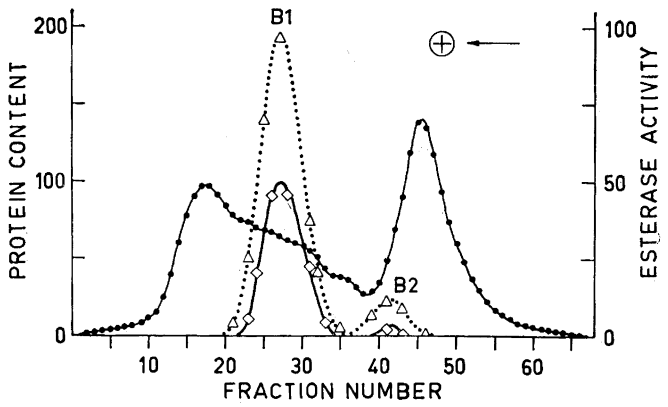


Fig. 8. Eel plasma. Conditions as for Fig. 1. △·····△, TAc (0.05); ◇———◇, heroin (0.1).

Esterase component	PhAc	PhBu	AmBu	TAc	TPr	TBu	Heroin	AcCh
B1	2 000	5 000	65	190	1 580	1 500	50	0
B2	30	1	0	21	7	2	3	0
C	?	?	?	<1	?	?	?	<1

pI_{50}				
Phys	Mipafox	*iso*-OMPA	Astra 1397	3318CT
3.8	7.5	7.1	4.5	3.5
<3	3.2	<3	<3	<3
7.7	—	4.1	—	5.2

The plasmata of Labrides are characterized by the presence of a blue-coloured dye [14,15]. On electrophoresis, this dye of protein nature was easily separated from the other three main protein constituents by its comparatively low mobility. The coloured protein was probably a chromoprotein which gave absorption maxima at 390, 600 and 645 mμ. Part of the dye with the same absorption maxima was found also in two other protein fractions (see Fig. 9).

The four protein peaks obtained on electrophoresis were recently observed also by Fine and Drilhon [16] on electrophoresis on paper and gelatine.

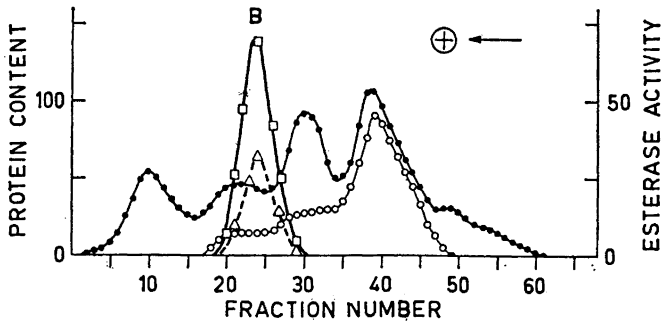


Fig. 9. Wrasse plasma. Conditions as for Fig. 1. □———□, PhBu (0.05); △ – – – △, TPr (0.1); ○———○, blue colour measured spectrophotometrically at 645 mμ.

Esterase component	PhAc	TPr	Tween	AcCh	pI_{50}			
					Phys	Mipafox	*iso*-OMPA	3318CT
B	140	32	30	0	<3	4.1	<3	3.4
C	0	0	0	<1	≈ 6	≈ 6	—	—

Frogfish, Lophius piscatorius (Fig. 10). A B-esterase was present in high concentration and had the properties of an acetylesterase. This esterase hydrolysed heroin at a higher rate (b_{30} 535) than any other plasmata studied in this series. In contrast to most other esterases of this type, *para*-substitution in phenyl acetate with a nitro group increased the enzymic hydrolysis. Mipafox was an inhibitor but not *iso*-OMPA. Like the B-esterase of *Labrus* plasma, the frog-fish esterase was sensitive to physostigmine and 3318CT in 10^{-4} M or higher concentrations. An acetylcholinesterase was probably present in low concentration.

This plasma was characterized by the very low content of albumin-like proteins.

Other teleostians. Blood plasma samples from sea-scorpion (*Cottus scorpius*) and mackerel (*Scomber scomber*) were also analysed. Both plasmata contained a B-esterase in low concentration which was of a butyryl- or propionylesterase type. Choline esters were hydrolysed at very low rates, due to the presence of an acetylcholinesterase. The low content of such an esterase in teleostian plasmata was reported previously [5,17].

The electrophoretic patterns showed three or four protein peaks, the mackerel plasma having higher concentration of fast-running proteins than most other fish plasmata studied. The green dye characteristic of the Cottied plasmata was probably a chromoprotein which migrated electrophoretically more rapidly than the other main plasma proteins. The electrophoretic patterns of these two plasmata will be published in a following paper [12].

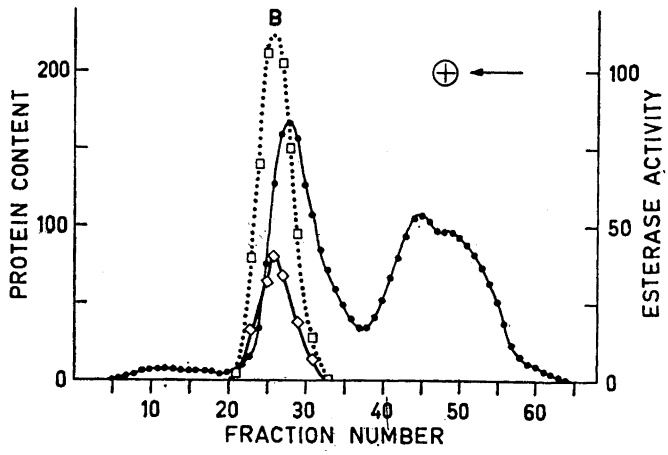


Fig. 10. Frogfish plasma. Conditions as for Fig. 1. □·····□, PhAc (0.05); ◇————◇, heroin (0.025).

Esterase component	PhAc	TAc	Heroin	pI_{50}			
				Phys	Mipafox	*iso*-OMPA	3318CT
B	225	155	160	4.0	5.5	<3	3.4
C	?	?	?	≈ 7	—	—	—

Spiny dogfish shark, Squalus acanthias. The plasma sample obtained from this Elasmobranch had very low esterase activity, too low to be detected after electrophoresis. The activity was due to a propionyl-B-esterase and an acetylcholinesterase. The low content of the latter enzyme was previously reported [5,17]. The protein curve obtained on electrophoresis is shown in Fig. 11.

Hagfish, Myxine glutinosa. The sample collected from several animals had negligible esterase activity when tested with most substrates. Aromatic esters were hydrolysed at very low rates and this reaction was probably due to an acetyl- or propionyl-B-esterase. A cholinesterase was probably not present. Fig. 11 shows the electrophoretic pattern of the protein components which were present in low concentrations (3.5 mg of total protein per 100 μl plasma).

The low lipase activity of the plasma from another Cyclostome (*Petromyzon marinus*) was noticed previously [13].

A discussion of the results reported above in relation to those of the first report of this series will be presented in a concluding paper [12].

This investigation was supported by a grant from the *Swedish Natural Science Research Council*. A grant from *Stiftelsen Lars Hiertas Minne* made it possible to work during the summer 1957 at the *Kristineberg Zoological Station*, Sweden, on fresh material from

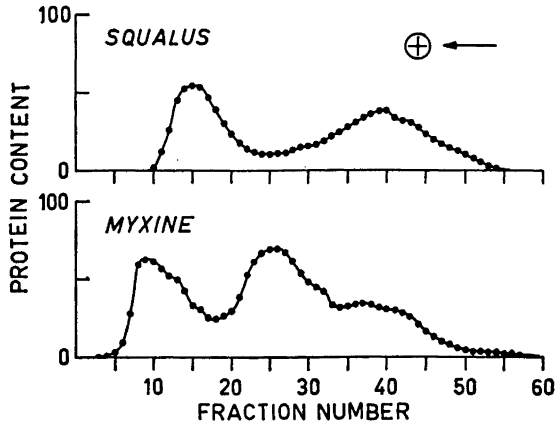


Fig. 11. Plasmata from spiny dog-fish shark and hagfish. Conditions as for Fig. 1. Esterase activities were too low to be detected after electrophoresis.

Species	Esterase component	pI_{50} Phys	
Squalus	B	4	(PhAc)
	C	< 7.0	(AcCh)
Myxine	B?	< 4	(*p*-nitro-PhAc)

marine fish, and I wish to express my sincere gratitude to Dr. Gunnar Gustafson for placing the facilities of the station at my disposal. I also thank Dr. Claes-Henry Hansson, Dr. Bertil Olsson, Dr. Torsten Fredriksson, Assistent Johan Lundin and Dr. Tore Hultin for blood samples of various animals. Grateful acknowledgements for the kind supply of chemicals are due to Dr. Lars-Erik Tammelin (for *iso*-OMPA, mipafox, and 3,3-dimethylbutyl acetate), Dr. Richard Dahlbom (for the compound Astra 1397), Dr. F. C. Copp (for the compound 62C47), and Dr. J. Jacob (for the compound 3318CT).

The skilled technical assistance of Mrs. Toril Rode and Mrs. Gunnel Jonsson is gratefully acknowledged.

REFERENCES

1. Augustinsson, K.-B. *Acta Chem. Scand.* **13** (1959) 571.
2. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. *J. Biol. Chem.* **193** (1951) 265.
3. Augustinsson, K.-B. *Methods of Biochem. Anal.* **5** (1957) 1.
4. Aldridge, W. N. *Biochem. J.* **53** (1953) 110.
5. Augustinsson, K.-B. *Acta Physiol. Scand.* **15** (1948) Suppl. 52.
6. Earl, C. J. and Thompson, R. H. S. *Brit. J. Pharmacol.* **7** (1952) 261.
7. Austin, L. and Berry, W. K. *Biochem. J.* **54** (1953) 695.
8. Ferrari, W. *Nature* **180** (1957) 144.

9. Myers, D. K. *Biochem. J.* **55** (1953) 67.
10. Caridroit, F., Kaswin, A. and Serfaty, A. *Ann. endocrinol. (Paris)* **9** (1948) 213.
11. Cohen, E. and Stickler, G. B. *Science* **127** (1958) 1392.
12. Augustinsson, K.-B. *Acta Chem. Scand.* **13** (1959) 1097.
13. Fontaine, M. and Callamand, O. *Bull. inst. océanog.* **46** (1949) No. 943–944.
14. Nolf, P. *Arch. intern. physiol.* **4** (1906) 98.
15. Fontaine, M. *Compt. rend. soc. biol.* **117** (1934) 420.
16. Fine, J. and Drilhon, A. *Compt. rend.* **246** (1958) 3183.
17. Serfaty, A. *Semaine hôp.* **1950**, 1647; *Bull. soc. hist. nat. Toulouse* **92** (1957) 205.

Received March 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1097–1105

Electrophoresis Studies on Blood Plasma Esterases

III. Conclusions

KLAS-BERTIL AUGUSTINSSON

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

A discussion is given of the results obtained in a comparative study of the esterases present in the plasmata from 27 various vertebrates. Each species has its own esterase pattern, and within each of the three groups of esterases, arylesterases (A-esterases), aliesterases (B-esterases) and cholinesterases (C-esterases), there are variations in the properties of individual enzymes. Arylesterase is the predominant esterase in mammalian plasmata, and is absent in the plasmata of birds, reptiles, amphibians and fish. Aliesterase (lipase) is the main esterase of lower vertebrate plasmata, and is not present in human, monkey, dog, ruminant, cock, or turtle plasmata. A propionyl-aliesterase is considered to be the most primitive type of esterase present in vertebrate plasmata, and other esterases including the various types of aryl- and cholinesterases are suggested to be phylogenetically evolved from such an esterase. The esterase present in turtle plasma is of an intermediate stage. The physiological function and origin of the plasma esterases are briefly discussed.

The plasma protein patterns of little piked whale, sea-scorpion and mackerel are illustrated.

A number of investigations have demonstrated that there are appreciable and characteristic variations in plasma (serum) protein patterns with animal species. Significant differences in the electrophoretic patterns for various species have been observed in respect of mobilities, number and relative proportion of protein components, and presence or absence of components [1–5]. Such electrophoresis studies were performed either with the Tiselius apparatus or with the paper electrophoresis technique. Only rarely were the biochemical properties of individual proteins investigated in these comparative studies. In the present investigation, the results of which were reported in the first two papers of this series [6] and in a preliminary paper [7], preparative electrophoresis on cellulose columns was used to separate esterase-active components for studies of the properties of individual enzymes with respect to specificity and sensitivity to various selective esterase inhibitors. Such investigations were regarded to be of importance because previous differentiation of these enzymes

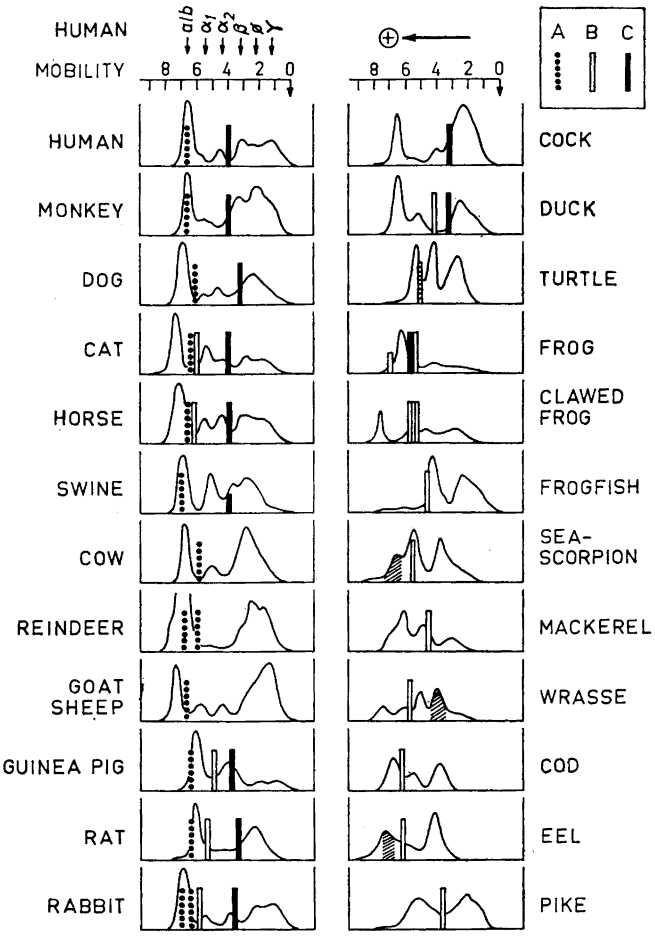


Fig. 1. Comparison of the electrophoretic mobilities of proteins and esterases of vertebrate plasmata relative to those of human plasma proteins (marked at the top of the graph). Experimental conditions: 5 ml plasma fractionated on cellulose columns (3 cm × 40 cm) for 30 h in veronal buffer (pH 8.4, $I = 0.1$) at an applied voltage of 260 V and 60 mA. The mobilities of human plasma proteins expressed in $cm^2V^{-1}s^{-1} \times 10^{-5}$ are those obtained with the Tiselius apparatus in the same buffer solution[8]. ————, protein; A, arylesterase; B, aliesterase and/or lipase; C, cholinesterase. The unique properties of the esterase of turtle plasma is specifically marked. Shaded areas refer to coloured proteins: blue for wrasse, green for sea-scorpion and eel.

was based mainly on work with original plasma (serum) or crude enzyme preparations. These previous studies have led to much difference of opinion as to the classification of plasma esterases.

Protein patterns. The absolute electrophoretic mobilities of various protein components were difficult to evaluate with the technique used. However, all

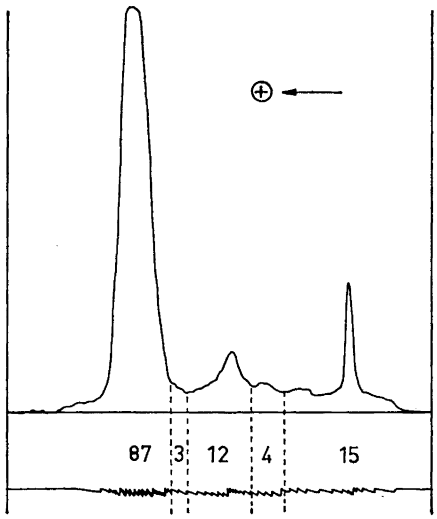


Fig. 2. Electrophoretic pattern of a plasma sample from *Balaenoptera acutorostrata* (little piked whale). Integrated curve from a paper electropherogram; veronal buffer (pH 8.6, $I = 0.1$), 150 V, 5 mA, 8 h. Numbers refer to relative protein concentrations.

electrophoresis runs and elutions were carried out under identical conditions, and therefore the relative mobilities of the components of various plasmata could be compared relative to those of human plasma proteins. Such a comparison of the results obtained with most of the plasmata studied is shown in Fig. 1. The results presented for individual species are based on a relatively low number of animals, but the patterns obtained are reproducible enough to demonstrate the species variation of protein patterns of the plasmata investigated. It will be remembered in this connection that individual variations in these patterns have been observed [1] in certain species. In the experience of the present author, such variations are particulary frequent for the esterase patterns of rabbit and swine plasmata. In addition, sex differences for certain vertebrate plasmata, *e. g.*, fowl plasma [2], have been reported, and alterations in anatomy and ecology during metamorphosis and growth has been shown to be accompanied by radical biochemical changes, also in the blood plasma proteins [9,10]. These individual variations, however, were not further investigated in the present investigation, since the electrophoretic plasma patterns of each species were generally reproducible with a few exceptions.

The electrophoretic patterns of all mammalian plasmata studied were characterized by a sharp albumin peak, the mobility of which varied relatively little from animal to animal. Albumin was also the predominant protein in a plasma sample from a whale, *Balaenoptera acutorostrata*, the electrophoretic pattern of which is shown in Fig. 2; the esterase activity of this sample was too low to be detected after electrophoresis. Proteins with similar mobilities to those of mammalian albumins were present in avian and amphibian plasmata, but not in the plasma of turtle. The absence of albumin-like serum proteins

in turtles was reported previously [11,12]. The influence of genetic and physiologic factors on plasma protein composition are obvious for amphibian and reptilian plasmata [13], and is particularly striking for teleostian plasmata [14,15]. Considerable differences were thus observed in the present investigation for two *Rana* species, the clawed frog *(Xenopus laevis)* which is a member of the peculiar family Aglossa, and seven teleostian fishes. Frogfish plasma was characterized by a sharp protein peak with a mobility similar to that of human plasma α_2-globulin. Other teleostian plasmata contained albumin-like proteins in varying amounts. It is of possible significance that the phylogenetically lowest teleostian studied, pike, had a very low amount of albumin-like protein, which was absent in the plasmata of both the spiny dogfish shark (an Elasmobranch), confirming a previous observation [16], and the hagfish (a Cyclostome). The electrophoretic pattern of pike plasma was also in other main characteristics similar to that of the elasmobranch plasma.

In certain species (cat, reindeer, rat, cock, duck, frog, eel) a small amount of protein, migrating somewhat faster than albumin, was present. These components had no relation to the esterases studied, and their relation to other plasma proteins is unknown. A fast-moving protein was present in a relatively high amount in the plasma of clawed frog.

Among all plasmata studied much variation was observed in the globulins, both those migrating between albumins and fibrinogen and the γ-globulins with lower mobility relative to that of fibrinogen. No distinct separation of β- and γ-globulins and fibrinogen was obtained in most cases. The α-globulins of several plasmata were more complex than those of human plasma. Turbidity, probably associated with lipoid, appeared to be caused in most cases by these components, especially the fast-migrating α-globulins. The amount of γ-globulins in mammalian plasmata varied considerably with species, and a component comparable to this protein was absent, or present in low amount, in most teleostian plasmata. Great variation in fibrinogen concentration was also observed. The amount of this component was particularly low in animals belonging to lower phylogenetic orders. In the whale plasma sample analysed, fibrinogen was apparently absent (Fig. 2).

Esterase patterns. Electrophoretic separation of plasma esterases has been reported only in a few cases. Human plasma *cholinesterase*, which precipitates together with albumin on ammonium sulphate fractionation [17,18], migrates on electrophoresis between the α_2- and β-globulin components [19–21]. Horse plasma cholinesterase behaved similarly on paper electrophoresis [22,23]. It was demonstrated in the present series of investigations that there was very little variation from animal to animal in the electrophoretic mobility of this type of esterase, when present. Frog plasma cholinesterase was an exception in this respect, with an unusually high electrophoretic mobility. Although the biochemical properties of these esterases varied considerably with species, the cholinesterases present in mammalian and avian plasmata behaved similarly on electrophoresis, with mobilities close to those of α_2 and/or β-globulins of human plasma. The low cholinesterase activity of ruminant and fish plasmata could not be detected after electrophoresis, but by indirect methods it was found that the cholinesterase present had an electrophoretic mobility close to those of other plasma cholinesterases. The unique single esterase of turtle

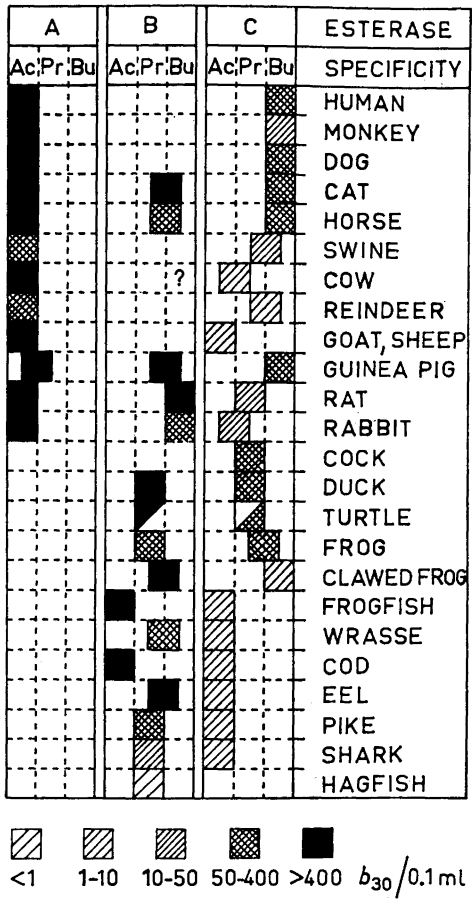


Fig. 3. Plasma-esterase patterns of vertebrates. Groups of esterases: A, arylesterase; B, aliesterase, lipase; C, cholinesterase. Relative esterase specificity based on the acyl radical of the substrate, selectively hydrolysed by the enzyme in each group: Ac, acetyl; Pr, propionyl; Bu, butyryl. Activity values in b_{30} per 0.1 ml plasma (μl CO_2 evolved from a bicarbonate — CO_2 buffer in 30 min at 25°C corrections made for the spontaneous substrate hydrolysis) refer to the hydrolysis rates observed with the substrate which is hydrolysed at the highest rate by each esterase. Monkey plasma is a sample from a macaque monkey. The properties of the esterase of turtle plasma are those of a propionyl-B- *and* propionyl-C-esterase. This histogram includes revisions in addition to that previously reported [7].

plasma moved at a higher rate than other plasma cholinesterases, but more slowly than the aliesterases of mammalian plasmata. The electrophoretic behaviour, as well as other properties of turtle-plasma esterase, are intermediate between the C- and B-esterases.

Great variation in the properties of cholinesterases present in various vertebrate plasmata was observed (Fig. 3). The cholinesterases of human, monkey,

dog, cat, horse and guinea-pig plasmata had the specificity of butyrylcholinesterases. A similar esterase was also present in low amounts in clawed-frog plasma. The ruminant plasmata had low cholinesterase activity, and the specificity of these esterases varied considerably from animal to animal, but in no case was a butyrylcholinesterase detected. Swine plasma cholinesterase had unique properties, and was present in high concentration in sow's colostum and milk [24,25]. An acetylcholinesterase was present in low concentration in the plasmata of goat and sheep and in those of all teleostians studied. Rat and rabbit plasmata were characterized by propionylcholinesterases, the properties of which were different with respect to sensitivity to quaternary ammonium compounds and organophosphorus compounds, respectively [6]. The plasmata of cock and duck also contained propionylcholinesterase, differing from both the rat and rabbit cholinesterases, *e. g.*, in the activity-substrate concentration relationship for choline esters. The esterase in turtle plasma had the properties of a cholinesterase in being highly sensitive to physostigmine, but differed from all other esterases of this type by hydrolysing choline esters at a lower rate than non-choline esters. It is suggested that this esterase is of a phylogenetically intermediate stage between the B-esterases present in high concentration in the plasmata of lower vertebrates, and the C-esterases of vertebrates belonging to higher orders. It is also of probable significance that the plasma esterase of turtle is a propionylesterase, since such an esterase type may be the most primitive one from a phylogenetic point of wiew. During the biochemical evolution, the propionyl- and butyrylcholinesterases might have originated from the B-esterases, the former being more specialized forms of the latter. The acetylcholinesterases of goat, sheep and fish plasmata and the propionylcholinesterase of rabbit plasma, the properties of which differed from those of most propionylcholinesterases of other vertebrates, might have a different source of origin. The similarity of these esterases to the acetylcholinesterases present in nervous and muscle tissues and in erythrocytes is suggestive evidence for this hypothesis.

Plasma cholinesterases therefore are regarded as a group of esterases with much divergent properties, with regard to specificity against various choline and non-choline esters, sensitivity to inhibitors, and activity-substrate concentration relationships. No clearly cut classification can be made, and it is advisable to state the enzyme source in any work with these esterases. It is not possible to apply results obtained with the plasma of one species to any other species. Intermediate types between "specific" cholinesterases, as butyryl-, propionyl- and acetylcholinesterases, also exist, and in most vertebrate plasmata the cholinesterase present is characteristic of the species.

Aliesterase seems to be the main esterase of lower vertebrate plasmata. It was absent in human and monkey plasmata, and its presence in ruminant plasmata is questionable. This esterase was not detected in cock (and chicken) plasma, but was present in high concentration in fish plasmata, and was the only detectable esterase in cyclostome plasma. The electrophoretic mobility of aliesterase varied considerably with species, but the enzyme migrated in most cases together with α-globulins, at a higher rate than cholinesterases but more slowly than arylesterases. The electrophoretic separation of the A- and B-esterases of rabbit and guinea-pig plasmata was reported previously [26].

The aliesterases of vertebrate plasmata are represented by various types as far as the specificity is concerned. Acetyl-, propionyl-and butyryl-aliesterases as well as intermediate types were found in the present investigation. Although "aliesterase" is not always an adequate term for these esterases, since phenyl esters are also hydrolysed, in some cases at a higher rate than aliphatic esters, this nomenclature is still recommended. Aliesterases are in most cases the predominant esterases for the hydrolysis of aliphatic esters, including triglycerides. When these esterases are absent, the cholinesterases are the only esterases able to hydrolyse these substrates. In no case were the arylesterases found to split aliphatic esters. Most aliesterases are highly sensitive to organophosphorus compounds and, when aromatic esters are used as substrates for these esterases, certain organophosphorus compounds are the best reagents for differentiating them from plasma arylesterases, all of which are resistant to these agents. However, animal tissues contain other esterases differing from those discussed in the present series of papers [27,28]. Aliesterases differ from the cholinesterases in being unable to hydrolyse choline esters, and being in most cases resistant to 10^{-5} M physostigmine. The latter agent, however, is not an absolute selective inhibitor of cholinesterase, because the aliesterases of duck, frog, clawed frog, frogfish and pike are sensitive to physostigmine in comparatively low concentrations (pI_{50} 5.5—4.5), although they do not hydrolyse choline esters. This is indicative of a close relation between the B- and C-esterases mentioned above. The turtle-plasma esterase displays the characteristics of both a B- and C-esterase including high sensitivity to physostigmine (pI_{50} 6.3). The plasma aliesterases of the lowest vertebrates studied in this series were propionylesterases and this type of esterase is therefore suggested to represent a more primitive stage in the phylogenetic evolution of plasma esterases, of which both cholinesterases and arylesterases may be regarded as specialized forms.

Arylesterases are typical for mammalian plasmata and are absent in all other vertebrate plasmata studied. They have the highest electrophoretic mobility, in most cases migrating close to the albumins. In certain cases (human, swine, rabbit) it was not possible to separate the albumin and arylesterase peaks by the technique used. For swine plasma, the biosyntheses of albumin and arylesterase during the suckling period for piglets were found to parallel each other [25]. This does not mean, however, that the two proteins have the same origin (*e. g.*, the liver; see further below). In addition, albumin itself may have esterase activity, which was actually shown for the reaction between certain α-naphthyl esters (*e. g.*, acetate, N-methyl carbamate) and the albumin of various plasmata [29].

Arylesterases are, with few exceptions, acetylesterases. In all cases aromatic butyrates (phenyl and naphthyl) were found to be hydrolysed at a much lower rate than the corresponding acetates. These esterases are probably also responsible for the hydrolysis of aromatic phosphates, e.g., *p*-nitrophenyl phosphate and its diethyl derivative. In addition, the hydrolysis by blood plasma of other organophosphorus compounds, e. g., di*iso*propoxy-phosphoryl fluoride (DFP), dimethylamido-ethoxy-phosphoryl cyanide (tabun) and tetraethyl pyrophosphate [26,30,31] is in some cases at least partly due to arylesterase. The hydrolysis of dimethylcarbamoyl fluoride was also found to parallel the

hydrolysis of DFP [32] and tabun in separated rabbit plasma (unpublished results from this laboratory), and arylesterase may therefore be one of the esterases responsible for this reaction.

Arylesterases differed from the aliesterases and cholinesterases in their resistance to all enzyme inhibitors tested in this investigation. Introductory experiments demonstrated that *p*-hydroxymercuribenzoate (and *p*-chloromercuribenzoate) and certain metallic ions (*e. g.*, Ag^+, Hg^{2+}, Cu^{2+}) are powerful inhibitors of arylesterases. These agents are useful selective inhibitors of these esterases, because both B- and C-esterases were resistant to those concentrations of the SH-reagents, which gave at least 50 % inhibition of the arylesterase activity. It was found that the reaction between the active SH-group(s) of those enzymes and the SH-reagents (including the metallic ions) was time-dependent, and that the inhibited esterase could be reversed by cystein. A full account of these studies will be reported elsewhere.

Physiological function and origin of plasma esterases. As long as the natural substrates of the plasma esterases are unknown, the physiological function of these enzymes are not easily understood. They may be of importance in the metabolism of various types of compounds containing ester linkages. Such compounds may be natural metabolites or administered medical products. Aryl- and aliesterases are present in high amounts in liver, kidney and pancreas. Preliminary *in-vivo* experiments with swine (performed in collaboration with B. Olsson) have shown that acetylsalicylic acid is hydrolysed to salicylic and acetic acids more readily in an animal with high plasma arylesterase activity compared with one with low activity. Aliesterase (lipase) may be the predominant enzyme for the hydrolysis of triglycerides, including fats, and the high aliesterase of eel plasma may be related to the toxic properties of this plasma [33]. Plasma cholinesterase may be of importance in hydrolysing acetylcholine or other naturally occurring choline esters (*e.g.*, propionylcholine) which come out into the blood stream. Finally, it is the opinion of the author that the great variation in the properties of plasma esterases with species reflects random species-differentiation of specific properties, rather than being the result of enzyme adaptation in relation to specific natural substrates.

The distributation of plasma esterases in animal tissues and the origin of these enzymes have not been investigated in the present study, a knowledge of which should be helpful in approaching the problem with regard to the physiological function of these enzymes. It is known that various tissues, *e. g.*, liver, kidney, pancreas and intestinal mucosa, contain esterases which sometimes are identical with those present in blood plasma, sometimes have fundamentally different properties compared with the plasma esterases. The latter types of esterases may occur in the plasma under pathological conditions. Human plasma cholinesterase is produced in the liver, according to a number of previous investigations. However, the origin of plasma cholinesterases of other vertebrates may not be the same. For instance, it has been demonstrated recently in this laboratory that swine plasma cholinesterase is not produced in the liver, but in the pancreas. The arylesterase of the same species, being closely related to albumin, is probably produced in the kidney. Any of these organs (liver, kidney, pancreas) may be the site of biosynthesis of plasma

esterases. The acetylcholinesterase present in the plasmata of lower vertebrates originate probably from still other sources.

Grateful acknowledgements are due to Prof. W. N. Aldridge, of the Medical Research Council, Toxicology Research Unit, Carlshalton, England, and to Prof. Alf G. Johnels, of the Swedish Museum of Natural History, Stockholm, Sweden, for valuable comments on the presentation of this paper.

REFERENCES

1. Svensson, H. *Arkiv Kemi, Mineral. Geol.* **22 A** (1946) No. 10.
2. Deutsch, H. F. and Goodloe, M. B. *J. Biol. Chem.* **161** (1945) 1.
3. Moore, D. H. *J. Biol. Chem.* **161** (1945) 21.
4. Deutsch, H. F. and McShan, W. H. *J. Biol. Chem.* **180** (1949) 219.
5. Boguth, W. *Zentr. Veterinärmed.* **1** (1953) 168, 311.
6. Augustinsson, K.-B. *Acta Chem. Scand.* **13** (1959) 571, 1081.
7. Augustinsson, K.-B. *Nature* **181** (1958) 1786.
8. Spector, W. S. (editor) *Handbook of Biological Data.* W. B. Saunders Co., Philadelphia, p. 25.
9. Frieden, E., Hermer, A. E., Fish, L. and Lewis, E. J. C. *Science* **126** (1957) 559.
10. Augustinsson, K.-B. and Olsson, B. *Biochem. J.* **71** (1959) 484.
11. Zweig, G. and Crenshaw, J. W. *Science* **126** (1957) 1065.
12. Cohen, E. and Stickler, G. B. *Science* **127** (1958) 1392.
13. Dessauer, H. C. and Fox, W. *Science* **124** (1956) 225.
14. Drilhon, A. *Compt. rend.* **237** (1953) 1779.
15. Saito, K. *Nippon Suisan Gakkaishi* **22** (1957) 752, 760; cited from *Chem. Abstr.* **52** (1958) 14014.
16. Irisawa, H. and Irisawa, A. F. *Science* **120** (1954) 849.
17. Faber, M. *Acta Med. Scand.* **114** (1943) 72, 475.
18. Augustinsson, K.-B. *Acta Physiol. Scand.* **15** (1948) Suppl. 52.
19. Glick, D., Glaubach, S. and Moore, D. H. *J. Biol. Chem.* **144** (1942) 525.
20. Cohen, J. A. and Warringa, M. G. P. J. *Biochim. et Biophys. Acta* **25** (1957) 600.
21. Vincent, D. *Clin. Chim. Acta* **3** (1958) 104.
22. Togni, G. P. and Meier, O. *Experientia* **9** (1953) 106.
23. Gregoire, J. and Derrien, Y. *Compt. rend. soc. biol.* **146** (1952) 1491.
24. Augustinsson, K.-B. *Acta Chem. Scand.* **12** (1958) 1150.
25. Augustinsson, K.-B. and Olsson, B. *Biochem. J.* **71** (1959) 477.
26. Goutier, R. *Biochim. et Biophys. Acta* **19** (1956) 524.
27. Aldridge, W. N. *Biochem. J.* **57** (1954) 692.
28. Bergmann, F., Segal, R. and Rimon, S. *Biochem. J.* **67** (1957) 481; **68** (1958) 493.
29. Casida, J. E. and Augustinsson, K.-B. *Biochim. et Biophys. Acta. In press.*
30. Mounter, L. A. *J. Biol. Chem.* **209** (1954) 813.
31. Augustinsson, K.-B. and Heimbürger, G. *Acta Chem. Scand.* **8** (1954) 1533.
32. Augustinsson, K.-B and Casida, J. E. *Biochem. Pharmacol. In press.*
33. Wunder, W. and Toth, L. von *Z. Fischerei* **32** (1934) 381.

Received March 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1106–1108

On the Photo-Transformation of Ergot Alkaloids

III. Two New Lumi-Ergot Alkaloids

HANS HELLBERG

Chemical Department, State Pharmaceutical Laboratory, Stockholm, Sweden

The preparation and properties of lumiergometrine II and lumiergocristine II have been described.

In work performed by Stoll and Schlientz [1] and by the present author [2] there has been reported the preparation of all the four lumialkaloids of the ergotamine series and three of the four possible lumialkaloids in each of the ergometrine and ergocristine series. However, the two missing alkaloids had been seen on chromatograms by this author [2] and now they have been prepared in a crystalline form.

The lumialkaloids in question are obtained in very minute yields on irradiation of the corresponding genuine alkaloids, and the methods of preparation therefore had to be refined. The new procedures are given under "Experimental". The main improvement in the case of ergometrine is the partition chromatographic procedure for the separation, taken from our work on lumilysergic acids [3]. In the case of ergocristine the new method for removing coloured material from the alkaloids has turned out to be of general value in the peptide alkaloid group. Another improvement is here the decrease in ethanol content of the first elutrient in the chromatography. In that way the peak of lumiergocristine II is more completely separated from an impurity, making the substance more readily crystallizable.

EXPERIMENTAL

Lumiergometrine II (and I) from ergometrine

In a quartz flask under N_2 atmosphere a solution of 1 g of ergometrine maleate in 40 ml of M/5 HCl was irradiated by a mercury lamp (Hanovia S4) until the transformation ceased (c. 3 h) as judged from the quotient between the extinctions at 283 and 315 mμ. The blue solution was treated with 0.2 g of sodium phosphate and 10 g of sodium chloride

and the pH adjusted to 8—9 with sodium hydroxide. The alkaloids *etc.* were extracted with 5 × 50 ml of a mixture of ethanol and ether (1 + 2), the extract dried for several hours over desiccated sodium sulphate and finally evaporated to dryness in a vacuum (N_2 atmosphere). The residue was submitted to partition chromatography on celite. Column: 50 × 2.5 cm. Solvent: butanol, benzene and acetic acid 20 % (1 + 1 + 2). The water phase was used as a stationary phase and the organic phase as elutrient. The course of the elution was followed by the colour reaction with *p*-dimethylaminobenzaldehyde, performed as in paper chromatography [2,3] on spots of the eluate, which were applied to a sheet of filter paper. After a small fraction, containing, *i. a.*, untransformed ergometrine, two lumialkaloid fractions were obtained, the first one strongly dominating and containing lumiergometrine I, the second one containing lumiergometrine II. The two fractions were separately evaporated to dryness (vacuum, N_2 atmosphere), dissolved in a mixture of ethanol and chloroform (1 + 3) and filtered through a column of alumina, subsequently washed with the same solvent until no alkaloid appeared in the eluate. The solutions were evaporated to dryness again. The residue of lumiergometrine I was dissolved in 2 ml of methanol and treated with 12 ml of ether, when the substance crystallized. The lumiergometrine II was dissolved in 0.5 ml of methanol by heating. On cooling the material crystallized, and the crystallization was made more complete by adding 1 or 2 ml of ether.

The yield of lumiergometrine I was c. 65 % and of lumiergometrine II c. 1 %.

Lumiergometrine II, α-lumiergometrine of Ref[2], crystallized from methanol in thin needles. In accordance with the loss on drying (100°C, high vacuum) and acidimetric equivalent weight the crystals contained one mole of methanol of crystallization. They melted on the Kofler block very diffusely between 128—138°C under decomposition. The pK_a-value in 20 % ethanol obtained as in Ref[2] was 7.20. The following constants are given on a dry basis. *Light absorption:* E (1 %, 1 cm) = 189 at 285 mμ (max) in M/10 HCl. *Optical activity:* $[\alpha]_D^{20°} = -19°$ (±5°) in pyridine, $c = 0.4$*. Sufficient material for elementary analysis was not available.

Lumiergocristine II (and I) from ergocristine

A solution of 1 g of ergocristine in 10 ml of concentrated acetic acid and 90 ml of water was illuminated as described for ergometrine in the previous paragraph. To the green solution was added 30 ml of 5 M sodium hydroxide and then 5 M ammonia to pH 9. The mixture was extracted with 3 × 15 ml of chloroform and the filtered solution evaporated to dryness (vacuum, N_2 atmosphere). The strongly discoloured residue was purified from most of the coloured material by the following procedure. It was weakly heated with 3 × 15 ml of 5 % tartaric acid solution. The liquid was centrifuged or filtered until clear and put into a separator together with 250 ml of ether. 10 ml of 5 M ammonia were added in one portion and the separator immediately rotated until the precipitate had mainly dissolved and only a small residue lay in the interphase. The remaining precipitate and the water solution in the separator were washed with a further 50 ml of ether, and then not only the ether but also as much as possible of the water phase was poured out. The rest was redissolved in 5 % tartaric acid solution and the treatment with ammonia and ether repeated on a smaller scale. The collected ether solutions were dried over desiccated sodium sulphate for several hours, and evaporated to dryness (vacuum, N_2 atmosphere). The residue, now only faintly coloured, was dissolved in 20 ml of methanol by gentle heating, and from this solution pure lumiergocristine I crystallized. The mother liquor was evaporated to dryness, dissolved in absolute chloroform and chromatographed on alumina (60 × 1 cm Al_2O_3). With 0.8 % ethanol in absolute chloroform as elutrient a small fraction of unchanged ergocristine came out first and then two small fractions of transformation products were obtained, the second one containing lumiergocristine II. On changing the elutrient to 5 % ethanol in chloroform another quantity of lumiergocristine I was obtained. The lumiergocristine II was crystallized from methanol.

---

* Based on one single determination.

The yield of lumiergocristine I was c. 70 % of lumiergocristine II c. 1 %.

Lumiergocristine II, α-lumiergocristine of Ref [2], crystallized from methanol in short pillars. In accordance with the small loss on drying (1—2 % at 130°C, high vacuum) the crystals did not contain any solvent of crystallization. On the Kofler block they underwent some sort of transformation at about 70°C and melted at 171—174°C under decomposition. The following constants are calculated on a dry basis. *Light absorption:* E (1 %, 1 cm) = 104 at 285 mμ (max) in M/10 HCl. *Optical rotation:* $[\alpha]_D^{20°} = 0°$ (±3°) in pyridine, $c = 0.6$. *Elementary analysis:* Found: C 66.16; H 7.06; O 15.29; N 11.05. Calc. for $C_{35}H_{41}O_6N_5$ (627,7): C 66.96; H 6.58; O 15.29; N 11.16.

Acknowledgements. The work has been financially supported by *Statens Medicinska Forskningsråd*. The elementary analysis was performed by Dr. Alfred Bernhardt, Max-Planck-Institut für Kohlenforschung, Mühlheim (Ruhr). The English was revised by Mrs. M. Spilsbury. To all these I am very grateful.

REFERENCES

1. Stoll, A. and Schlientz, W. *Helv. Chim. Acta* **38** (1955) 585.
2. Hellberg, H. *Acta Chem. Scand.* **11** (1957) 219.
3. Hellberg, H. *Acta Chem. Scand.* **12** (1958) 678.

Received February 20, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1109–1112

The Action of Bacterial Trypsin on Tyrosine-Ethyl-Ester

S. O. ANDERSEN and JOHN GRAAE

Universitetets fysisk-kemiske institut, Copenhagen, Denmark

The splitting of tyrosine-ethyl-ester by a bacterial enzyme was studied. The reaction follows strictly the first order form to more than 99 % hydrolysis. pH optimum is found to 7.4 at 20°C and to 6.8 at 40°C. This variation can be explained by assuming that the enzyme attacks only the positively charged substrate molecules. The variation of activity with temperature is determined.

Bacterial trypsin is an enzyme isolated from *Bacillus subtilis*, closely related to, or identical with subtilisin. Subtilisin is mostly known for its proteolytic activity [1] but it has been shown that it is able to split methyl-esters of the lower fatty acids too [2,3]. In this work the hydrochloride of tyrosine-ethyl-ester is used as substrate, because it is a welldefined, reasonably soluble compound.

Crystalline bacterial trypsin was kindly placed at our disposal by *Novo Terapeutisk Laboratorium*, Copenhagen, Denmark.

The substrate was a commercial product (from Nutritional Biochemical Corporation) which was once recrystallized from ethyl-acetoacetate. Titration of the recrystallized product gave a molecular weight of 247.9 (Theoretical: 245.7).

The experiments were carried out in 50 ml N/10 KCl in which the desired amount of substrate was dissolved. Substrate concentrations varied from 8 mmoles/l to 20 mmoles/l. The enzyme was added and the hydrolysis followed by continuous automatic titration in a selfregistering pH-stat. If nothing else is stated temperature is 20°C and pH 7.0.

It was seen that the reaction strictly follows the first order form under all conditions examined. In Fig. 1 is plotted $\log_{10}(1-\alpha)$ *versus* t for an experiment followed to 99.7 % hydrolysis. For the routine determinations of the velocity constants a plot of log $(c_{t+\tau}-c_t)$ *versus* t was used, as suggested by Guggenheim [4]. $c_{t+\tau}$ is the amount hydrolysed at time $t+\tau$ and c_t is the amount hydrolysed at time t. The straight line obtained by this plot indicates that the reaction can be described by a first order form, with a velocity constant that is the slope of the line. It is experimentally proved that this constant, k', can be written as $k \cdot E$, where E is the enzyme concentration (see below).

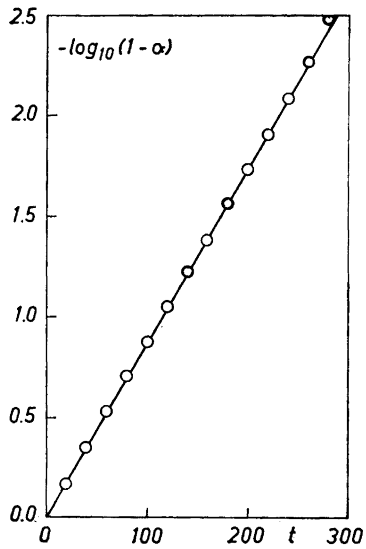


Fig. 1. As abscissa time in min and as ordinate $-\log_{10}(1-\alpha)$ for a typical experiment. Note that the degree of reaction at 260 and 280 min are 0.995 and 0.997, respectively. Temp. 20°C. pH = 7.0. Initial concentration of substrate = 17 mM. E = 8.75×10^{-4} mM. From the graph k is determined to 9 850 $min^{-1} M^{-1}$. At this experiment precipitation of tyrosine started at about 50 min. (α = 0.62.)

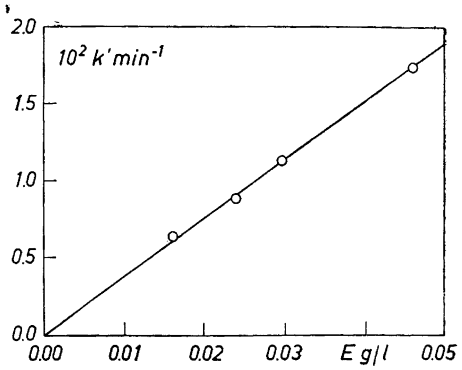


Fig. 2. As abscissa concentration of enzyme in g/l and as ordinate velocity constant k'. Temp. 20°C. pH = 7.0.

Where the constant is given per mole of enzyme it is based on a molecular weight of the enzyme of 27 500 [5]. The constant determined in this way is used for all further comparisons of experiments performed under different contions.

During the experiments it was noticed that after a period of supersaturation, tyrosine suddenly precipitated. As this precipitation in no way changed the fact that the reaction follows the first order form very strictly (*cf.* Fig. 1) it seems reasonable to conclude that the concentration of tyrosine has no influence at the reaction.

Table 1. The table gives the velocity constants determined with enzyme kept in solution in a refrigerator for the period indicated in the first column. pH = 7.0. Temp. = 20°C. Calculations based on $\log_{10}$.

Time, h	k min^{-1} l g^{-1}
0	0.396
4	0.374
23.6	0.392
26.2	0.398
47.4	0.382

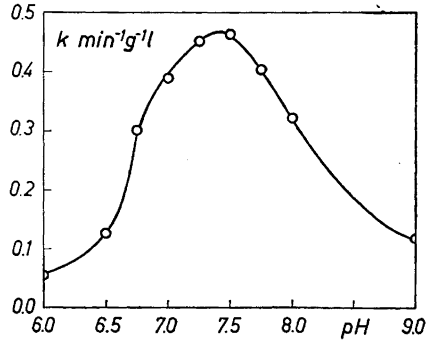


Fig. 3. Dependence of velocity constant on pH. Temp. 20°C.

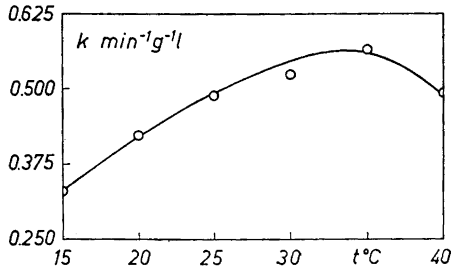


Fig. 4. Dependence of velocity constant on temperature. pH = 7.4.

As subtilisin is known to undergo selfdigestion when left alone, experiments were carried out in order to find out whether it was necessary to take special precautions to prevent selfdigestion. An enzyme sample was dissolved in water (1 g/l) and placed in a refrigerator. At suitable intervals an experiment was performed and the velocity constant determined. The results are given in Table 1. It is seen that there is no decrease in activity within 48 h.

The variation of the velocity constant with the enzyme concentration was studied. Experiments were performed at different enzyme concentrations and the constant determined in each case. From Fig. 2 it is seen that the constant is proportional to the enzyme concentration, as could be expected.

The variation of activity with pH was investigated. A pH optimum is found at 7.4 (Fig. 3). This is somewhat lower than that found when determined with other substrates [1-3].

The dependence of activity on temperature is shown in Fig. 4, where the experiments are performed at pH 7.4 at all temperatures. The pH was determined with a Sørensen phosphate buffer (80 ml M/15 Na_2HPO_4 and 20 ml M/15 KH_2PO_4) which is supposed to have pH 7.4 at all temperatures in the investigated interval. The somewhat strange decrease in activity at higher temperatures cannot be due to an irreversible denaturation of the enzyme, as the reaction still follows the first order scheme, and that indeed would not be expected if the enzyme were destroyed during the reaction. An investigation of the activity-pH curve has shown a shift in the pH-optimum with increasing temperature so that pH optimum at 40°C is lowered to pH = 6.9 (Table 2). It is most likely that this fact is the explanation of the peculiar form of the temperature-activity curve.

It is known that the pK values at 20°C of the amino group in amino acid esters is about 7—8 [6], and titration shows that the amino group in our sub-

Table 2. Variation of velocity constant with pH at 40°C.

pH	7.40	7.20	7.00	6.80	6.50
k min^{-1} l g^{-1}	0.495	0.571	0.665	0.659	0.576

strate has a pK value of 7.4 at 20°C and 6.95 at 40°C. This gives ΔH about 9 500 cal/mole in good agreement with findings for analogous compounds [6]. It therefore seems reansonable to conclude that the enzyme attacks only the positively charged substrate molecules, as this theory, combined with the found values for pK_{20} and pK_{40}, explains the finding of a pH optimum lower than that found ordinarily as well as the changing in pH optimum with temperature.

The pH optimum is usually found at about 8 [1-3] and it seems reasonable to assume that about this pH we have the highest concentration of the active form of the enzyme. But if the enzyme attacks only the positively charged tyrosine-ethyl-ester molecules this will give a lower pH optimum as the concentration of the positively charged molecules is very low at pH over and about 8. The effect of the temperature is understood in the same way as the pK for the substrate is lowered from 7.40 to 6.95 when the temperature is increased from 20°C to 40°C and therefore the concentration of the active — positively charged — form of the substrate is lowered at pH 7.4 (pH optimum at 20°C) and hence pH optimum is lowered.

REFERENCES

1. Ottesen, M. *Compt. rend. Lab. Carlsberg Ser. chim.* **30** (1958) 211 (Thesis).
2. Güntelberg, A. V. and Ottesen, M. *Compt. rend. Lab. Carlsberg Ser. chim.* **29** (1954) 36.
3. Graae, J. *Acta Chem. Scand.* **8** (1954) 356.
4. Guggenheim, E. A. *Phil. Mag.* **2** (1926) 538.
5. Marcker, K. *Personal communication.*
6. Cohn, E. J. and Edsall, J. T. *Proteins, Amino Acids and Peptides.* New York 1943.

Received April 9, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1113–1123

Eine quantitative Untersuchung über die Protolysenverhältnisse zweier sulfonierten basischen Triphenylmethanfarbstoffe

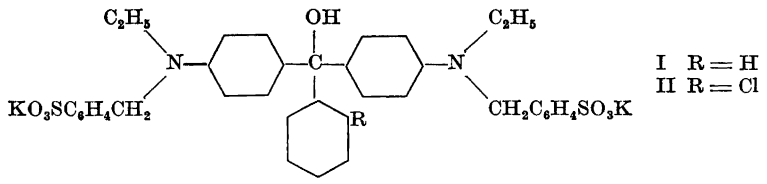
RUNE CIGÉN

Abteilung für anorganische und physikalische Chemie des Chemischen Instituts der Universität Lund, Schweden

Die Protolysenreaktionen zweier sulfonierten basischen Triphenylmethanfabstoffe wurden bei 20°C in Wasserlösungen mit konstanter Ionenstärke = 0,5 M quantitativ untersucht. Sie bilden eine Reihe gefärbter und eine Reihe farbloser Formen, die in Protonen- und Hydratationsgleichgewichten miteinander stehen. Die Reaktionen verlaufen nach demselben Protolysenschema, das früher für Malachitgrün und einige Derivate von Malachitgrün aufgestellt wurde. Die Dissoziations-, Hydratations- und Geschwindigkeitskonstanten in dem Protolysenschema wurden bestimmt. Die Messungen wurden spektrophotometrisch durchgeführt.

In einer Schrift 1954 von Bodforss, Ahrland und Cigén [1] wurden die Protolysenreaktionen von Malachitgrün untersucht. Später publizierten Bodforss und Cigén [2] eine Untersuchung über u.a. einen sulfonierten basischen Triphenylmethanfarbstoff, Lichtgrün SF. Diese beiden Untersuchungen waren indessen unvollständig, und Cigén [3] publizierte 1956 eine Dissertation, in der zehn Farbstoffe, Malachitgrün und Derivate von Malachitgrün, genau untersucht waren. In der letztgenannten Schrift wurde ein Protolysenschema aufgestellt, das nun ein einer eingehenderen Untersuchung über zwei sulfonierte malachitgrünähnliche Farbstoffe, Lichtgrün SF und Brillantsäuregrün 6 B geprüft wird. Es hat sich gezeigt, dass das Protolysenschema im Prinzip auch für solche Farbstoffe wie Kristallviolett anwendber ist (Cigén [4]).

Den Analysen von Bodforss [5] gemäss hat das Lichtgrün SF, (LG), zwei Sulfonsäuregruppen und die Konstitution I. Das Brillantsäuregrün 6 B (BG), hat die Konstitution II

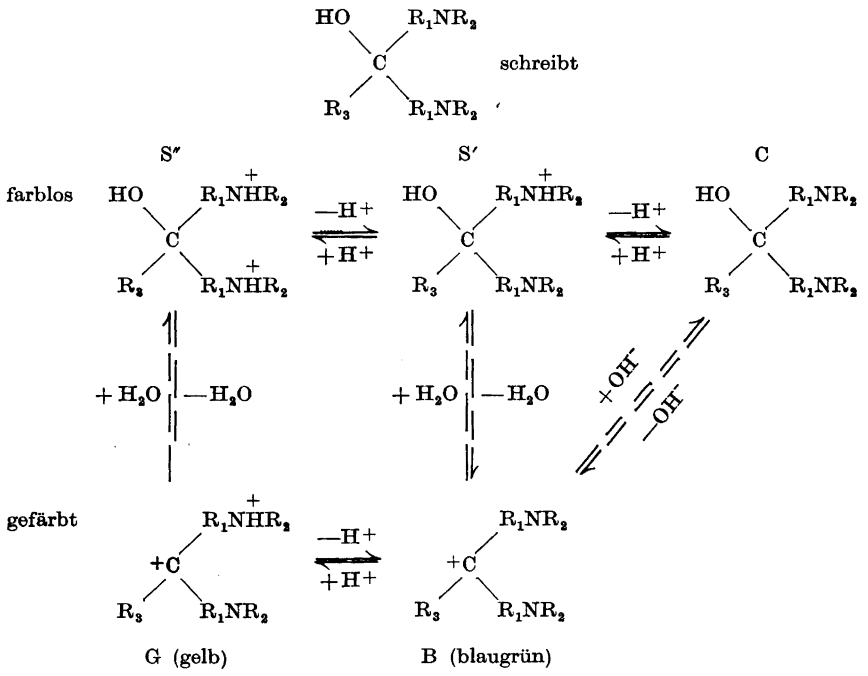


I R = H
II R = Cl

An diesen Farbstoffen durchgeführte Untersuchungen zeigten, dass ihre Protolysenreaktionen qualitativ mit denen der früher untersuchten nicht sulfonierten basischen Triphenylmethanfarbstoffe übereinstimmen. Man kann darum ein Protolysenschema derselben Art für LG und BG aufstellen wie das früher für Malachitgrün und Kristallviolett geschah.

Eine neutrale Wasserlösung von BG oder LG ist blaugrün. Bei Zusatz von Alkali wird die Lösung mit einer Geschwindigkeit entfärbt, die dem [OH^-] proportional ist. Fügt man eine starke Säure hinzu wird die Lösung augenblicklich gelb und verblasst dann wegen der Hydratation der gelben Form. Die Reaktionen sind reversibel. Die Reaktionen, die zu den Farbänderungen Veranlassung geben, finden bei den beiden Aminogruppen und bei dem zentralen Kohlenstoffatom statt. Da die Reaktionen, wenn man die Lösung sauer macht, so nahe mit den entsprechenden Reaktionen von Malachitgrün übereinstimmen, scheinen die Dissoziationen bei den Sulfonsäuregruppen die Protolysenverläufe betreffs Farbänderungen nicht zu beeinflussen.

Die Reaktionen, die stattfinden, wenn man das pH der Lösung verändert, können folgenderweise geschrieben werden, falls man BG und LG



Ausgezogene Pfeile bedeuten, dass die Reaktionen sehr rasch, "momentan", verlaufen, während gestrichelte Pfeile bedeuten, dass die Reaktionen mit messbarer Geschwindigkeit verlaufen.

Folgende Konstanten werden definiert:

$$K_1 = \frac{[G]}{[B][H^+]} \; ; \; K_3 = \frac{[S'][H^+]}{[S'']} \; ; \; K_4 = \frac{[C][H^+]}{[S']} \; ; \; K_2 = \frac{[S'']_\infty}{[G]_\infty} \; ;$$

$$K_6 = \frac{[B]_\infty}{[S']_\infty}$$

k_1 = die Geschw.keitskonst. der Reaktion $G + H_2O \rightarrow S''$
k_2 = » » » » $S'' \rightarrow G + H_2O$
k_3 = » » » » $B + H_2O \rightarrow S'$
k_4 = » » » » $S' \rightarrow B + H_2O$
k_5 = » » » » $B + OH^- \rightarrow C$
k_6 = » » » » $C \rightarrow B + OH^-$

Die Untersuchungen wurden mit einem Spektrophotometer durchgeführt, weshalb spezifische Extinktionen benutzt wurden, um die Konzentrationen der verschiedenen Farbstoffkomponenten auszudrücken. Hierbei bedeuten
ε_B, ε_G die molekularen Extinktionskoeffizienten für B und G
e_o, e_t, e_∞ die spezifische Extinktion einer Lösung bei der Zeit 0 und t und bei Gleichgewicht (die Schichtdicke $d = 1$ cm)
E_o, E_t, E_∞ die abgelesenen Extinktionen, wenn $d \neq 1$
e_B, e_G die spezifische Extinktion einer Lösung die nur B oder G enthält.
Man kan folgende Beziehungen ableiten (vgl. Ref.[3,4])

$$K_1 = \frac{e_B - e_o}{(e_o - e_G)[H^+]} \tag{1}$$

(1) kann folgenderweise umgeschrieben werden

$$\frac{[H^+]}{e_B - e_o} = [H^+] \frac{1}{e_B - e_G} + \frac{1}{K_1(e_B - e_G)} \tag{2}$$

In einem Diagramm trägt man $[H^+]/(e_B - e_o)$ als Funktion von $[H^+]$ auf und bekommt so eine Gerade, deren Abschnitt auf der Orinatenachse, y, und deren Winkelkoeffizient, l, die Gleichungen $y = 1/K_1(e_B - e_G)$ und $l = 1/(e_B - e_G)$ ergeben.

Bei Gleichgewicht wird die Extinktion

$$e_\infty = \frac{e_B + K_1[H^+] \cdot e_G}{1 + 1/K_6 + K_4/K_6[H^+] + K_1[H^+](1 + K_2)} \tag{3}$$

In sauren Lösungen, pH $\sim$ 1, gibt (3)

$$K_2 = \frac{e_G}{e_\infty} + \frac{e_B - e_\infty(1 + K_1[H^+] + 1/K_6)}{e_\infty \cdot K_1[H^+]} \tag{4}$$

und in schwach alkalischen Lösungen kann (3) folgendermassen approximiert und umgeschrieben werden

$$\frac{[H^+]}{e_\infty} = [H^+] \cdot \frac{1 + K_6}{K_6 \cdot e_B} + \frac{K_4}{K_6 \cdot e_B} \tag{5}$$

Aus (5) kann man, wenn man $[H^+]/e_\infty$ als Funktion von $[H^+]$ aufträgt, K_4/K_6 und unter gewissen Umständen K_6 erhalten.

Man kann folgende zusammenfassende Gleichung für die Kinetikreaktionen ableiten, wenn man annimmt, dass die individuellen Geschwindigkeitskonstanten $k_1 ... k_4$ bei den Hydratationen und Dehydratationen im Reaktionschema unabhängig von dem pH sind, was für früher untersuchte Farbstoffe gilt. Da die Totalkonzentration des Farbstoffes, C_M, bei den Messungen im Vergleich mit den Konzentrationen der benutzten Pufferlösungen sehr gering ist, verlaufen die Zeitreaktionen pseudomonomolekular.

$$k = \frac{1}{t} \ln \frac{e_o - e_\infty}{e_t - e_\infty} = \frac{k_2[H^+]^2 + k_4 K_3[H^+] + k_6 K_3 K_4}{[H^+]^2 + K_3[H^+] + K_3 K_4}$$

$$+ \frac{k_1 K_1[H^+] + k_3 + k_5[OH^-]}{K_1[H^+] + 1} \tag{6}$$

(6) kann in saurer Lösung, pH $\leq$ 1, folgendermassen approximiert werden

$$k = \frac{1}{t} \ln \frac{e_o - e_\infty}{e_t - e_\infty} = k_1 \cdot \frac{K_1[H^+]}{1 + K_1[H^+]} + k_2 \tag{7}$$

und in stark alkalischer Lösung, pH > 10,

$$k = k_6 + k_3 + k_5[OH^-] \tag{8}$$

Zwischen diesen beiden Extremen kann man für pH 4—8 eine kleinere Approximation von (6) vornehmen

$$k = \frac{k_4 K_3[H^+]}{[H^+]^2 + K_3[H^+] + K_3 K_4} + k_3 \tag{9}$$

Wenn man für eine grosse Anzahl von Lösungen, die man durch Mischung einer Farbstoffstammlösung mit geeigneten Puffern erhält, die Anfangs- und die Gleichgewischtsextinktionen bestimmt und ausserdem die Geschwindigkeit der Farbänderungen misst, kann man aus den oben gegebenen Gleichungen die Konstanten im Protolysenschema bestimmen.

Je nach den Grössenverhältnissen zwischen den verschiedenen Konstanten sind die vorgenommenen Approximationen mehr oder weniger gut, und schrittweise Approximationen können erforderlich werden.

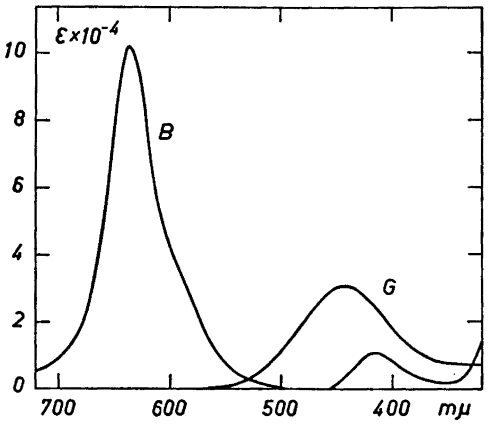


Fig. 1. Absorptionskurven von Brillantsäuregrün 6 B in Wasserlösungen. Kurve B entspricht dem blauen Farbion, B, (in 0,5 M KCl) und Kurve G dem gelben, G, (in 2 M HCl).

EXPERIMENTELLES

Chemikalien und Lösungen. Brillantsäuregrün 6 B (Farbenfabrik Bayer) und *Lichtgrün SF* (Ciba). Die Präparate wurden in Methanol fraktionsweise mit Kaliumacetat gefällt. Die besten Fraktionen wurden aus Methanol umkristallisiert und über P_2O_5 getrocknet. Man erhält die Farbstoffe als grüne Pulver, die etwas hygroskopisch sind. Von den Farbstoffen wurden Stammlösungen durch Abwägung und Auflösung in Wasser bereitet.

Zu Pufferlösungen wurden *Salzsäure, Natriumacetat, Dinatriumhydrogenphosphat, Kaliumdihydrogenphosphat, Borsäure* und *Natriumhydroxyd*, die sämtliche von pro Analysi Qualität waren, verwendet.

Man kann thermodynamische Konstanten in einem so komplizierten Reaktionsschema wie das von diesen Farbstoffe kaum bestimmen. Darum wurde die Methode gewählt, Konzentrationskonstanten zu bestimmen, die bei der Ionenstärke 0,5 M gelten, mit *Kaliumchlorid* p.a. als Neutralsalz.

Die Apparatur war dieselbe, die für die Untersuchungen über Kristallviolett benutzt wurde (Ref.[4]).

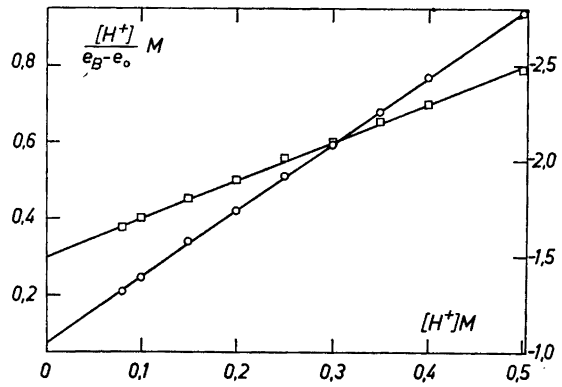


Fig. 2. $[H^+]/(e_B - e_o)$ als Funktion von $[H^+]$ für die Bestimmung von K_1 und ε_G. □ λ = 636 mμ. ○ λ = 450 mμ.

MESSUNGEN UND ERGEBNISSE

Brillantsäuregrün 6 B. Zuerst wurden Absorptionskurven für BG in 0,5 M KCl und in 2 M HCl aufgenommen; Fig. 1 Kurve B und G. Kurve B zeigt, dass die blaue Form ein Absorptionsmaximum für $\lambda = 636$ mμ, $\varepsilon_{max} = 1{,}02 \times 10^5$ und eines für $\lambda = 416$ mμ, $\varepsilon_{max} = 1{,}05 \times 10^4$, hat. Die gelbe Form G hat ein Absorptionsmaximum für $\lambda = 440$ mμ, und ε_{max} wurde später zu $3{,}0 \times 10^4$ bestimmt. In 2 M HCl liegt der Farbstoff teilweise als ein farbloses Salz, S″, vor, weshalb man aus der Absorptionskurve die molekulare Extinktion von G nicht direkt erhalten kann.

Die Bestimmung von K_1. Zwischen pH 1 und pH 0 schlägt die Farbe von BG von blau in gelbgrün um, wegen der Reaktion $B + H^+ \rightleftharpoons G$. Eine Reihe von Messlösungen wurde dadruch bereitet, dass die Farbstammlösung schnell mit verschiedenen Pufferlösungen von HCl + KCl in Y-geformten Röhren gemischt wurde. Das Gleichgewicht $B + H^+ \rightleftharpoons G$ stellt sich augenblicklich ein, aber die Lösungen verblassen danach. Deshalb wurden die Extinktionen zur Zeit $t = 0$ extrapoliert. Für die Messlösungen war $C_M = 1 \times 10^{-5}$ M, $[H^+] = 0{,}080$—$0{,}500$ M, $d = 1$ cm, $\lambda = 636$ und 450 mμ, $T = 20{,}0$°C. In Fig. 2 ist $[H^+]/(e_B - e_0)$ als Funktion von $[H^+]$ der Gleichung (2) gemäss aufgetragen. Man erhält für $\lambda = 636$ mμ: $y = 0{,}300$ M und $l = 0{,}99$ und für $\lambda = 450$ mμ: $y = -1{,}05$ und $l = -3{,}45$. Dies ergibt bei 636 mμ: $K_1 = \mathbf{3{,}30 \pm 0{,}05\ M^{-1}}$, $e_G = 0{,}010$, $\varepsilon_G = 1{,}0 \times 10^3$ und bei 450 mμ: $K_1 = \mathbf{3{,}29 \pm 0{,}05\ M^{-1}}$, $e_G = 0{,}301$, $\varepsilon_G = 3{,}0 \times 10^4$.

Die Bestimmung von K_2. Die Gleichgewichtsextinktionen wurden für eine Reihe von Lösungen abgelesen. $C_M = 2 \times 10^{-5}$ M, $[H^+] = 0{,}150$—$0{,}500$ M, $d = 5$ cm, $\lambda = 636$ mμ, $T = 20$°C. In Tab. 1 sind die aus (4) berechneten K_2-Werte. Der Mittelwert wird $\mathbf{10{,}0 \pm 0{,}2}$.

Die Bestimmung von k_1 und k_2. Die Bleichung der gelbgrünen Lösung, die man erhält, wenn man eine blaue Farbstoffstammlösung mit verschiedenen Pufferlösungen aus HCl + KCl mischt, wurde kinetisch verfolgt. In Tab. 1 finden sich die Geschwindigkeitskonstanten k und k_1, die man bekommt,

Tabelle 1. Berechnung von K_2 und k_1. Brillantsäuregrün 6 B. $\lambda = 636$ mμ, $T = 20{,}0$°C, $C_M = 2 \times 10^{-5}$ M, $d = 5$ cm.

[H+] M	e_∞	K_2	k min^{-1}	k_1 min^{-1}
0,500	0,055	9,8	0,134	0,185
0,400	0,067	9,9	0,127	0,190
0,350	0,075	10,0	0,118	0,192
0,300	0,087	10,0	0,112	0,187
0,250	0,101	10,1	0,1035	0,187
0,200	0,123	10,1	0,0945	0,189
0,150	0,160	9,9	0,0814	0,188
0,100			0,0656	0,188
0,080			0,0576	0,186
Mittelwert		10,0		0,188

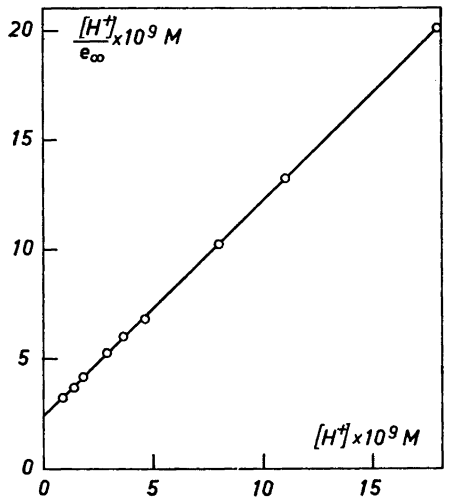


Fig. 3. $[H+]/e_\infty$ als Funktion von $[H+]$ für die Bestimmung von K_4/K_6. λ = 636 mμ.

wenn man die Gleichung (7) verwendet. Da $k_1[G]_\infty = k_2[S'']_\infty$ und $[S'']_\infty/[G]_\infty = K_2$, kann man k_1 und k_2 aus (7) berechnen, wenn man K_1 und K_2 kennt. Ganz wie für die früher untersuchten basischen Triphenylmethanfarbstoffe sind auch hier k_1 und k_2 vom pH unabhängig. Man bekommt die Mittelwerte k_1 = **0,188 ± 0,003 min^{-1}** und k_2 = **0,0188 ± 0,0006 min^{-1}.**

Die Bestimmung von K_4/K_6. In schwach alkalischen Lösungen verblasst BG langsam unter Bildung von dem farblosen Salz, S', und dem farblosen Carbinol, C. In einer Reihe von Lösungen wurde die Farbstoffstammlösung mit verschiedenen Boratpuffern gemischt. Die Gleichgewichtsextinktionen, e_∞, wurden nach drei Tagen abgelesen. $C_M = 1 \times 10^{-5}$ M, d = 1 cm, λ = 636

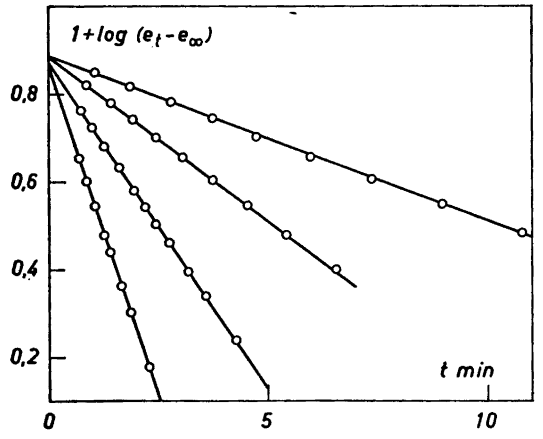


Fig. 4. Kinetikmessungen in NaOH + KCl, λ = 636 mμ, T = 20.0°C. $[OH^-]$ ist von oben nach unten 0.0316, 0.0632, 0.1264 und 0.2528 M.

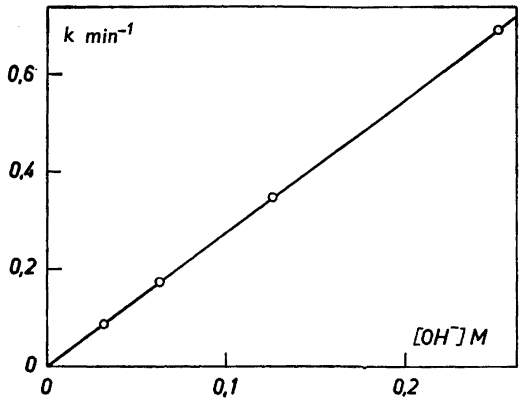


Fig. 5. Die Geschwindigkeitskonstante k als Funktion von $[OH^-]$ für die Bestimmung von k_5. $[OH^-] = 0.0316-0.2528$ M.

mμ. In Fig. 3 ist $[H^+]/e_\infty$ gemäss Gleichung (5) als Funktion von $[H^+]$ aufgetragen. Man erhält $y = 2{,}43 \times 10^{-9}$ M, $l = 0{,}98$ und $K_4/K_6 = \mathbf{(2{,}48 \pm 0{,}05) \times 10^{-9}\ M}$.

Die Bestimmung von k_5 und k_6. Die Verbleichung in stark alkalischen Lösungen wurde auch untersucht. In Fig. 4 sind Kinetikmessungen bei vier verschiedenen $[OH^-]$ graphisch dargestellt, und in Fig. 5 sind die aus Fig. 4 erhaltenen Geschwindigkeitskonstanten, k, als Funktion von $[OH^-]$ aufgetragen. Man ersieht aus Fig. 5, dass k dem $[OH^-]$ direkt proportional ist. Von der Gleichung (8) erwartet man ja auch, dass k eine lineare Funktion von $[OH^-]$ sein soll. Man kann aber $k_6 + k_3$ nicht aus Fig. 5 bestimmen, da die Gerade durch die Origo läuft. Man bekommt $k_5 = \mathbf{2{,}73 \pm 0{,}03\ min^{-1}\ M^{-1}}$ und k_6 kann dann folgenderweise bestimmt werden. Bei Gleichgewicht gilt

$$k_5[OH^-][B]_\infty = k_6[C]_\infty \tag{10}$$

Aber $[H^+][C]_\infty = K_4[S']_\infty$ und $K_6[S']_\infty = [B]_\infty$, weshalb $k_6 = k_5 K_w K_6/K_4$. Früher wurde K_4/K_6 zu $2{,}48 \times 10^{-9}$ bestimmt, und da wird $k_6 = \mathbf{(1{,}36 \pm 0{,}05) \times 10^{-5}\ min^{-1}}$, da einer anderen Untersuchung gemäss $K_w = 1{,}24 \times 10^{-14}$.

Die Bestimmung von K_3, K_4, K_6, k_3 und k_4. k_3 wurde durch Kinetikmessungen in einem Boratpuffer pH = 8,74 bestimmt; Fig. 6. In diesem pH-Gebiet kann man die Gleichung (6) folgendermassen approximieren

$$k = k_3([H^+]K_6/K_4 + 1) + k_6 + k_5[OH^-] \tag{11}$$

Da k_5, k_6 und K_6/K_4 bekannt sind, kann man k_3 bestimmen. Man erhält $k_3 = \mathbf{(4{,}79 \pm 0{,}05) \times 10^{-4}\ min^{-1}}$.

Die restlichen Konstanten wurden folgenderweise bestimmt. Eine partiell entfärbte Farbstammlösung von BG in 0,02 M HCl wurde mit verschiedenen Acetatpuffern gemischt, deren Zusammensetzung so war, dass man pH 3,92 — 6,14 bekam. Hierbei bildete sich B wieder, mit einer Geschwindigkeit, die k in der Gleichung (6) ergibt. In diesem pH-Gebiet kann man (6) zu

$$k = \frac{k_4 K_3[H^+]}{[H^+]^2 + K_3[H^+] + K_3K_4} + k_3 \tag{12}$$

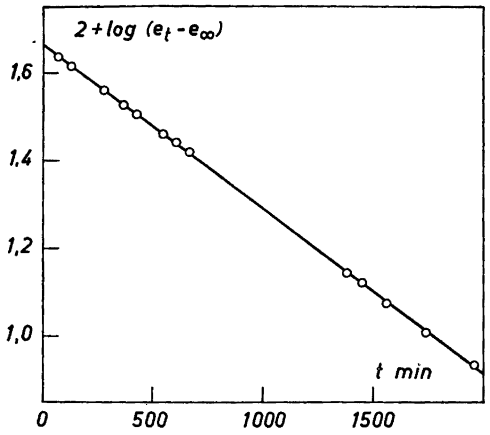


Fig. 6. Eine Kinetikreihe in Boratpuffer pH = 8.74 für die Bestimmung von k_3. λ = 636 mμ.

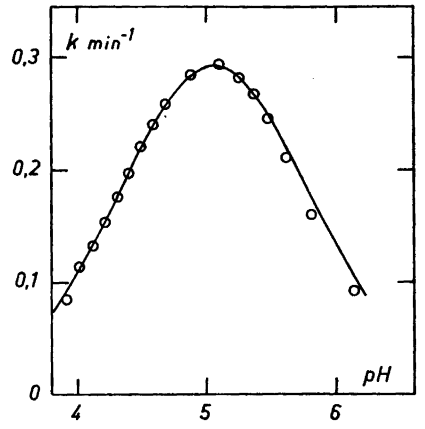


Fig. 7. Kinetikmessungen in Acetatpuffern. $C_M = 8 \times 10^{-6}$ M, λ = 636 mμ, T = 20,0°C. Die Geschwindigkeitskonstante als Funktion von pH. Die ausgezogene Kurve wurde aus der Gleichung (12) berechnet.

approximieren. Deriviert man (12) hinsichtlich $[H^+]$ erhält man

$$[H^+]_{k\,max} = \sqrt{K_3K_4} \tag{13}$$

und

$$k_{max} = \frac{k_4K_3}{K_3 + 2\sqrt{K_3K_4}} + k_3 \tag{14}$$

(14) kann wie folgt geschrieben werden

$$k_{max} = \left(\frac{K_6}{K_4} \cdot \frac{K_3K_4}{K_3 + 2\sqrt{K_3K_4}} + 1\right) \cdot k_3 \tag{15}$$

In (15) sind K_6/K_4 und k_3 früher bestimmt, und aus (13) erhält man K_3K_4. Man kann daher K_3 aus (15) bestimmen und danach K_4, K_6 und k_4 berechnen. In Fig 7 ist k gegen pH für eine Reihe von Kinetikmessungen in Acetatpuffern aufgetragen. Man bekommt $k_{max} = 0{,}297$ min^{-1} für pH = 5,06, und $[H^+] = 8{,}71 \times 10^{-6}$ ergibt $K_3K_4 = 7{,}59 \times 10^{-11}$. Dies ergibt $K_3 = \mathbf{(3{,}27 \pm 0{,}06) \times 10^{-5}}$ **M,** $K_4 = \mathbf{(2{,}32 \pm 0{,}05) \times 10^{-6}}$ **M,** $K_6 = \mathbf{(9{,}4 \pm 0{,}3) \times 10^2}$ und $k_4 = \mathbf{(0{,}45 \times 0{,}02)\ min^{-1}}$.

Hiermit sind sämtliche Konstanten im Protolysenschema bestimmt. Die ausgezogene Kurve in Fig. 7 ist aus (12) berechnet, wobei die oben gegebenen Konstantwerte benutzt wurden. Die Übereinstimmung zwischen den experimentell gefundenen k-Werten (Kreise) und den aus (12) berechneten ist gut.

Dem Protolysenschema gemäss soll $K_1K_2K_3K_6 = 1$ sein. Das experimentell gefundenen Konstantprodukt wird 1.01.

Tabelle 2. Vergleich einander entsprechender Konstantwerten bei einigen Farbstoffen.

	Malachitgrün	*p*-Chlor-Malachitgrün	*o*-Chlor-Malachitgrün	Lichtgrün SF	Brillantsäuregrün 6 B
$K_1 \times 10^{-1}$ M	28	19,3	22,1	2,5	3,30
$K_3 \times 10^{5}$ M	2,7	6,3	4,3	1,95	3,3
$K_4 \times 10^{6}$ M	5,8	5,2	3,2	3,7	2,3
K_5	22	25	1,76	113	10,0
K_6	68	33	600	193	940
k_1 min^{-1}	0,168	0,145	0,00209	1,30	0,19
k_2 »	0,0078	0,0058	0,00120	0,0115	0,019
k_3 »	0,0065	0,0060	0,000127	0,023	0,00048
k_4 »	0,44	0,20	0,076	4,5	0,45

Lichtgrün SF. In Ref.[2] wurden einige Konstanten in dem Protolysenschema des Lichtgrün SF bestimmt, nämlich $K_1 = \mathbf{2{,}5 \pm 0{,}1}$ **M**$^{-1}$, $K_2 = \mathbf{113 \pm 2}$, $k_1 = \mathbf{(1{,}30 \pm 0{,}04)}$ **min**$^{-1}$, $k_2 = \mathbf{(0{,}0115 \pm 0{,}0005)}$ **min**$^{-1}$ und $K_4/K_6 = 1/K_5 = \mathbf{(1{,}92 \pm 0{,}06) \times 10^{-8}}$ **M.** Die restlichen Konstanten wurden dort wegen experimenteller Schwierigkeiten nicht bestimmt. Die Untersuchungen sind jedoch wiederaufgenommen und glücklich zu Ende geführt worden. Die grössten Schwierigkeiten lagen darin, dass der Farbstoff sich sehr schwer reinigen liess, und dass die grossen Reaktionsgeschwindigkeiten im pH-Gebiete 4—5 die Kinetikmessungen unsicher machten. Die Reaktionen verlaufen bei pH 5 bis 50 % in nur 16 sek.

Die Messungen und Bestimmungen der Konstanten wurden in derselben Weise wie für Brillantsäuregrün 6 B durchgeführt. Die Genauigkeit der Konstantwerte sind jedoch nicht ebenso gut.

Kinetikmessungen in NaOH + KCl ergaben $k_5 = \mathbf{(59 \pm 1)}$ **min**$^{-1}$ **M**$^{-1}$, $k_6 = \mathbf{(3{,}8 \pm 0{,}2) \times 10^{-5}}$ **min**$^{-1}$ und in Boratpuffer, pH = 8,72, $k_3 = \mathbf{(0{,}0233 \pm 0{,}0005)}$ **min**$^{-1}$ Die Kinetikmessungen in Acetatpuffern ergaben pH = 5,07 für k_{max}, $k_{max} = 1{,}05$ min^{-1}, und man erhält daraus $K_3 = \mathbf{(1{,}95 \pm 0{,}10) \times 10^{-5}}$, $K_4 = \mathbf{(3{,}7 \pm 0{,}2) \times 10^{-6}}$, $K_6 = \mathbf{193 \pm 10}$, $k_4 = \mathbf{(4{,}5 \pm 0{,}3)}$ **min**$^{-1}$. Das experimentell gefundene Konstantprodukt $K_1K_2K_3K_6$ wird 1.06.

Die erhaltenen Ergebnisse zeigen, dass die beiden nun untersuchten Farbstoffe sich ähnlich verhalten wie die früher untersuchten basischen Triphenylmethanfarbstoffe. Auffallend ist die Einwirkung eines Substituenten in *orto*-Stellung zum zentralen Kohlenstoffatom. Vergleicht man die Konstanten für Malachitgrün und dessen *p*-Chlor und *o*-Chlor Derivate und ferner Lichtgrün SF und dessen *o*-Chlor Derivat, Brillantsäuregrün 6 B, ersieht man aus Tabelle 2, dass ein Chlor in *para*-Stellung verhältnismässig wenig auf die Konstantwerte einwirkt. Dasselbe gilt von den Dissoziationskonstanten K_1, K_3 und K_4 für ein Chlor in *orto*-Stellung. Dagegen wirkt ein *o*-Chlor stark auf die Gleichgewichte zwischen den gefärbten und den farblosen Formen sowie auf die Geschwindigkeitskonstanten ein. Die Reaktionen gehen wesentlich langsamer, und die Gleichgewichte werden gegen die gefärbten Formen stärker verschoben, wahrscheinlich, weil das *o*-Chloratom durch sterische Hinderung

die Anlagerung von Wassermolekeln am zentralen Kohlenstoffatom erschwert. Entsprechend sind die Verhältnisse bei den früher untersuchten Farbstoffen *p*- und *o*-Nitro-Malachitgrün. Der sterische Einfluss einer *o*-Nitrogruppe ist noch stärker als der eines *o*-Chloratoms. Die Untersuchung von ein paar anderen *orto*-Derivaten von Malachitgrün wird geplant.

Statens Naturvetenskapliga Forskningsråd (Schwedischer naturwissenschaftlicher Forschungsrat) hat einen Teil dieser Arbeit finanziell unterstützt. Herrn Mag.phil. Ingmar Grenthe danke ich für seine wertvolle Mitwirkung bei zeitraubenden Kinetikmessungen.

REFERENZEN

1. Bodforss, S., Ahrland, S. und Cigén, R. *Z. physik. Chem.* **203** (1954) 73.
2. Bodforss, S. und Cigén, R. *Kgl. Fysiograf. Sällskap. Lund Förh.* **26** (1956) No 6.
3. Cigén, R. *Protolysengleichgewichte und Reaktionsgeschwindigkeiten einiger basischer Triphenylmethanfarbstoffe* (Diss.), Universität Lund 1956.
4. Cigén, R. *Acta Chem. Scand.* **12** (1958) 1456.
5. Bodforss, S. *Kgl. Fysiograf. Sällskap. Lund Förh.* **24** (1954) No 4.

Eingegangen am 23. Februar 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1124–1128

The Identity of Thujopsene and Widdrene and of Hinokiic and Widdrenic Acids

H. ERDTMAN and T. NORIN

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Widdrene is identical with the main constituent of thujopsene. Widdrenic acid is identical with hinokiic acid. The structure of this acid requires modification. Tentative structures are suggested for thujopsene and hinokiic acid.

The wood oil of the Hiba tree, *Thujopsis dolabrata* (L.f.) Sieb. et Zucc. has been studied by Japanese chemists over a long period. Both Yano [1] and Uchida [2] concluded from their early investigations that the main neutral constituent of the oil was a tricyclic sesquiterpene $C_{15}H_{24}$. This was given the name thujopsene by Kawamura [3] who investigated it in some detail and suggested two alternative structures. Obata and Fukushi [4] reported that they had been unable to isolate thujopsene from Hiba oil but Fujita [5] definitely confirmed its presence. These investigations and especially the important contribution of Kawamura, published in Japanese, have remained largely unknown in the West.

The chemistry of thujopsene has recently attracted considerable interest in Japan and several groups are now studying its structure. A careful description of this compound was published (in English) a short time ago by Akiyoshi and Nagahama [6] in a paper which is a repetition and extension of Kawamura's work and from which most of the above details have been taken. Their paper records the UV and IR absorption of thujopsene and some results of permanganate oxidation including the isolation, together with other degradation products, of a C_{15}-ketocarboxylic acid. This on oxidation with hypobromite furnished a C_{14}-dicarboxylic acid from which an anhydride and a dimethyl ester were prepared.

During studies in this laboratory on the constituents of heartwood extracts from trees of the South African genus *Widdringtonia* [7] a tricyclic sesquiterpene $C_{15}H_{24}$ was obtained, which was called widdrene and according to its IR absorption and gas chromatographic behaviour was undoubtedly a pure compound. Further studies have demonstrated the presence of the same compound in several other conifers of the family *Cupressaceae* (*Chamaecyparis*

thyoides [8], *Biota orientalis* [9] and some species of *Juniperus* [10]) and it appears that it is a common constituent of woods that have the characteristic sweet fragrance of "pencil wood".

On ozonisation widdrene gave a C_{15}-ketocarboxylic acid that was oxidised by hypobromite to a C_{14}-dicarboxylic acid.

Table 1 shows the constants for thujopsene and widdrene and some of their degradation products.

Table 1.

Thujopsene $C_{15}H_{24}$	*Widdrene* $C_{15}H_{24}$
B. p. 121–122°/12 mm $n_D^{25} = 1.5015$, $[\alpha]_D^{20} = -79.7°$	B. p. 118–120°/10 mm $n_D^{25} = 1.5033$, $[\alpha]_D^{20} = -109°$
Ketocarboxylic acid $C_{15}H_{24}O_3$	*Ketocarboxylic acid* $C_{15}H_{24}O_3$
M. p. 164–165° Semicarbazone, m. p. 219° (decomp.) 2,4-Dinitrophenylhydrazone, m. p. 187–189°	M. p. 166–168°, $[\alpha]_D^{20} = -129°$ Semicarbazone, m. p. 220° (decomp.) 2,4-Dinitrophenylhydrazone, m. p. 153–154°
Dicarboxylic acid $C_{14}H_{22}O_4$	*Dicarboxylic acid* $C_{14}H_{22}O_4$
M. p. 209.5–210.5° Anhydride, m. p. 109–110° Dimethyl ester, m. p. 71–73°	M. p. 215°, $[\alpha]_D^{20} = -57°$ Anhydride, m. p. 113°, $[\alpha]_D^{20} = -39°$ Dimethyl ester, m.p. 71–73°, $[\alpha]_D^{20} = -59°$

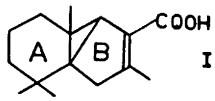
From the close similarity of these constants (apart from those of the ketocarboxylic acid 2,4-dinitrophenylhydrazones) and since widdrene is of widespread occurrence it seemed very probable that thujopsene and widdrene were identical but that the former was not a pure compound. This was confirmed by comparison of the IR spectrum of thujopsene with that of pure widdrene.

Through the courtesy of Dr. S. Katsura, Takasago Perfumery Co, Tokyo, it has been possible to investigate a large sample of Japanese hiba oil. The neutral fraction gave pure widdrene, only the small fractions at the beginning and end showing boiling point and rotation changes. The IR absorption curves of both *Thujopsis* and *Widdringtonia* widdrenes were superimposable and the crystalline oxidation products were identical. Pure widdrene shows a single sharp carbon-carbon double bond stretching vibration band at 1 682 cm^{-1}. Impure samples of widdrene from *Widdringtonia* and thujopsene from *Thujopsis* gave an additional band at 1 647 cm^{-1} together with other minor differences.

Hirose and Nakatsuka [19] arrived at the same conclusion as regards the identity of thujopsene and widdrene. Oxidation of their "thujopsene" ($[\alpha]_D$ −92°) with selenium dioxide gave an aldehyde identical with that obtained by similar oxidation of "widdrene" from *Biota orientalis*.

Since the name thujopsene has considerable priority we suggest that it should be retained but reserved for the fraction of thujopsene that corresponds to widdrene.

In the investigation of thujopsene from *Widdringtonia* it was oxidised with selenium dioxide to give a characteristic aldehyde "widdrenal" (thujopsenal) which was oxidised further with silver oxide to a monocarboxylic acid, "widdrenic acid", $C_{15}H_{22}O_2$. This acid was also isolated directly from the *Widdringtonia* heartwood extracts and has recently been found in several other woods containing thujopsene including *Chamaecyparis thyoides* [8] and several *Juniperus* species [10]. Uchida [11] long ago isolated an acid, which he called hinokiic acid, from the steam volatile oil obtained from the leaves of the Japanese hinoki tree, *Chamaecyparis obtusa* Endl. Hinokiic acid has also been shown to occur in small amounts in the leaves of *Chamaecyparis taiwanensis* Masamune et Suzuki [12] and has recently been obtained by Masamura [13] from a garden variety of hinoki, "chyabohiba". Uchida thought that the composition was $C_{15}H_{24}O_2$ but Moroe and Hara [14] have shown that the correct composition of hinokiic acid is $C_{15}H_{22}O_2$. Okuda [15a-f] has made an extensive investigation of hinokiic acid and has proposed the structure (I).



I

It was noticed that there were considerable similarities between the physical constants of hinokiic acid and widdrenic acid but there were also certain discrepancies. According to Okuda, permanganate oxidation of hinokiic acid gave a dicarboxylic acid containing only thirteen carbon atoms. However the melting point of this acid agreed with that of our C_{14}-widdrenedicarboxylic acid which has also been obtained by permanganate oxidation of widdrenic acid. The optical rotation of Okuda's dicarboxylic acid [15f] was reported to be almost exactly 100° lower than that of the widdrene dicarboxylic acid (misprint?). The analytical data recorded for the "C_{13}-dicarboxylic acid" were not very satisfactory and it seemed probable that it was not a pure compound. Okuda also obtained the dicarboxylic acid by ozonisation of hinokiic acid followed by further oxidative degradation. From the neutral fractions of the ozonisation products he isolated an oily ketone, $C_{13}H_{20}O$.

Okuda's papers are all in Japanese and we are grateful to Dr. Y. L. Chow for translating them.

Ozonisation of widdrenic acid followed by oxidation with hypobromite also gave acid and neutral products. From the former there was isolated widdrene dicarboxylic acid and from the latter a dibromoketone, m.p. 99—100°. (Found: C 44.6; H 5.2; Br 46.0. $C_{13}H_{18}OBr_2$, requires C 44.6; H 5.2; Br 45.7) which on treatment with zinc and acetic acid gave a crystalline ketone, m.p. 49.5—50.5°. (Found: C 81.3; H 10.4. $C_{13}H_{20}O$ requires C 81.3; H 10.4.)

Some constants for hinokiic acid and widdrenic acid and some of their degradation and transformation products are shown in Table 2.

From the close similarity of these constants (apart from those of the dicarboxylic acid) it seemed very probable that hinokiic acid and widdrenic acid were identical. Dr. Okuda very kindly supplied a generous sample of crude hinokiic acid from which the pure acid was obtained and direct comparison

Table 2.

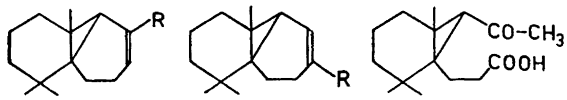
Hinokiic acid $C_{15}H_{22}O_3$	*Widdrenic acid* $C_{15}H_{22}O_3$
M. p. 166°, $[\alpha]_D^{20} = -90.4°$	M. p. 169—171°, $[\alpha]_D^{20} = -86°$
Methyl ester, m. p. 107°	Methyl ester, m. p. 109—110°, $[\alpha]_D^{20} = -99°$
Dihydroderivative ("*iso*-dihydrohinokiic acid") (Pd in EtOH), m. p. 159°, $[\alpha]_D^{20} = 77°$	Dihydroderivative (PtO_2 in EtOH), m. p. 160—161°, $[\alpha]_D^{20} = 75°$
Tetrahydroderivative (PtO_2 in HAc), m. p. 143—144°, $[\alpha]_D^{20} = 61.3°$	Tetrahydroderivative (PtO_2 in HAc), m. p. 145—146°, $[\alpha]_D^{20} = 60°$
Dicarboxylic acid $C_{13}H_{20}O_4$	*Dicarboxylic acid* $C_{14}H_{22}O_4$
M. p. 210—211°, $[\alpha]_D^{20} = -156.2°$	M. p. 215°, $[\alpha]_D^{20} = -57°$
Dimethyl ester, m. p. 69°	Dimethyl ester m. p. 71—73°, $[\alpha]_D^{20} = -59°$
Oily ketone $C_{13}H_{20}O$ Semicarbazone, m. p. 224—225°	*Ketone* $C_{13}H_{20}O$, m. p. 49.5—50.5° Semicarbazone, m. p. 225—226°

of this acid and widdrenic acid (mixed m.p., IR) showed that they were identical. The name widdrenic acid is thus redundant and should be abandoned *.

The structure (I) proposed for hinokiic acid cannot, however, be entirely correct. In ring B there can be no C=C—CH_3 grouping since the dicarboxylic acid, m.p. 215° (thujopsene dicarboxylic acid) and hinokiic acid on oxidation according to Kuhn-Roth gave almost the same yield of acetic acid (0.62 and 0.57 moles; thujopsene 1.49 mole). The oxidation of thujopsene to hinokiic acid and further to the C_{14}-dicarboxylic acid shows that there is only one substituent, the carboxyl group on the double bond.

Further investigation here has now shown that the ketone, m.p. 49.5—50.5°, from the ozonolysis products obtained from hinokiic acid is identical with a ketone formed by a Dieckmann condensation from the dimethyl ester of thujopsene dicarboxylic acid. According to the IR and UV absorption this ketone contains a bi*cyclo*[3.1.0]hexane system with a carbonyl group in conjugation with the *cyclo*propane ring ($\nu_{max}^{CCl_4}$ 1 723 cm^{-1}; λ_{max}^{EtOH} 210.5 mμ, ε 4 900). Dihydroumbellulone [16] which has a similar structure shows the same absorption ($\nu_{max}^{CCl_4}$ 1 722 cm^{-1}; λ_{max}^{EtOH} 210 mμ. ε 2 470). Dihydrolumiprednisone acetate [17] and dihydrolumisantonin [18] are also similar. The presence of both the *cyclo*propane ring and the double bond is also indicated by the results of the hydrogenation experiments of thujopsene and hinokiic acid (*cf.* Table 2). There is at present no reason to doubt Okuda's conclusions on the nature of ring A. The structure of thujopsene and hinokiic acid are therefore presumably either IIa or IIb (with R = CH_3 and COOH, respectively):

* It may in the future be found advantageous to rationalise the nomenclature of these compounds by abandoning the name hinokiic acid altogether in favour of names based on thujopsane.



IIa IIb III

The UV spectra of the ketocarboxylic acid obtained by ozonolysis of thujopsene (λ_{max}^{EtOH} 210.5 mμ, ε 4 700), of the C_{14}-dicarboxylic acid (no maximum above 205 mμ in EtOH, $\varepsilon_{205\ m\mu}$ *ca.* 800), and of a lactone, m.p. 126—128°, obtained by potassium borohydride reduction of the ketoacid (no maximum above 205 mμ in EtOH, $\varepsilon_{205\ m\mu}$ *ca.* 150) indicate that the ketogroup of the ketocarboxylic acid is conjugated with the *cyclo*propane ring (structure III). Structure IIa (R = CH_3) is therefore preferred for thujopsene and IIa (R = COOH) for hinokiic acid.

REFERENCES

1. Yano, M. *J. Soc. Chem. Ind. Japan* **16** (1913) 443.
2. Uchida, S. *J. Soc. Chem. Ind. Japan* **31** (1928) 501.
3. Kawamura, J. *Bull. Imp. For. Exp. Stn. Japan* **30** (1930) 59.
4. Obata, Y. and Fukushi, S. *J. Agr. Chem. Soc. Japan* **26** (1952) 113.
5. Fujita, N. *Bull. Imp. For. Exp. Stn. Japan* **76** (1954) 175.
6. Akiyoshi, S. and Nagahama, S. *Bull. Chem. Soc. Japan* **30** (1957) 886; Ref. *Chem. Abstr.* **52** (1958) 10964.
7. Erdtman, H. and Thomas, B. R. *Acta Chem. Scand.* **12** (1958) 267.
8. Enzell, C. *To be published shortly.*
9. Erdtman, H. and Pelchowicz, Z. *Chem. Ber.* **89** (1956) 341.
10. Runeberg, J. *To be published shortly.*
11. Uchida, S. *J. Ind. Chem. Japan* **31** (1928) 650; Ref. *Chem. Zentralbl.* **1928** *II* 1577.
12. Nozoe, T. *XIIIth Intern. Congr. Pure and Appl. Chem.*, Stockholm 1953. *Abstract of Papers*, p. 213.
13. Masamura, M. Ref. *Chem. Abstr.* **51** (1957) 17900.
14. Hara, S. and Moroe, T. *J. Pharm. Soc. Japan* **71** (1951) 503; Ref. *Chem. Abstr.* **45** (1951) 9223.
15. Okuda, O. a) *J. Pharm. Soc. Japan* **72** (1952) 703, b) *ibid.* p. 705, c) *ibid.* **73** (1953) 1, d) *ibid.* p. 4, e) *ibid.* p. 6, f) *ibid.* p. 9.
16. Eastman, R. H. *J. Am. Chem. Soc.* **76** (1954) 4115.
17. Barton, D. H. R. and Taylor, W. C. *J. Chem. Soc.* **1958** 2500.
18. Barton, D. H. R., De Mayo, P. and Shafiq, M. *J. Chem. Soc.* **1958** 140.
19. Hirose, Y. and Nakatsuka, T. *J.Jap.Wood Research Soc.* **4** (1958) 26.

Received March 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1129–1136

The Structure of the Oxazolidine-2-thione Derivatives Synthesized from Some Reducing Sugars

ALF WICKSTRÖM and JENS KRISTIAN WOLD

Universitetets farmasøytiske institutt, Blindern-Oslo, Norway

The structure of the previously described cyclic thione-carbamates of some reducing sugars has been examined by means of their oxidation by periodate and their absorption spectra in infrared and ultraviolet. Results were obtained which contribute to the determination of the structure of the compounds prepared from D-xylose, L-arabinose, and D-fructose.

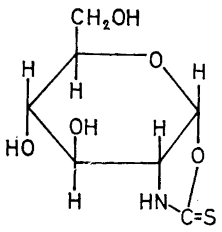
It appeared that they all contain an oxazolidine-2-thione ring fused with the pentoses (through C_1 and C_2) in their furanose form or with D-fructose (through C_2 and C_3) in its pyranose form.

These oxazolidine-2-thione derivatives crystallize in the thione form. In aqueous solution they exhibit uniform acid strength.

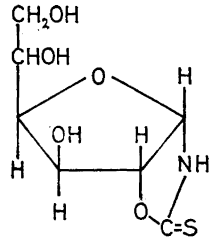
Some reducing sugars, D-glucose [1], D-fructose [2], D-galactose, D-xylose, and L-arabinose [3], have been reported to give with thiocyanic acid crystalline condensation products which presumably all contain an oxazolidine-2-thione ring. Since this cyclic thione-carbamate group in simpler compounds is known to exhibit antithyroid activity [4] and since the structure of these sugar derivatives still presents some unresolved problems, it seemed of interest to examine their structure as far as new methods of approach would allow.

In the present work infrared and ultraviolet absorption spectra as well as a periodate oxidation technique eliminating the disturbing effect of the thione group, have been used to elucidate the structure of the cyclic thione-carbamates of the following sugars: D-xylose, L-arabinose, and D-fructose. Oxazolidine-2-thione, tetrahydrogen-1,3-oxazine-2-thione, and D-gluco-oxazolidine-2-thione have served as reference compounds. All the above-mentioned substances were synthesized according to previously described methods. The D-fructose derivative, not obtainable as a completely homogeneous product by recrystallizations [2], required a chromatographic purification.

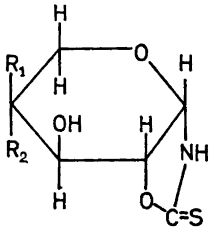
The condensation product between thiocyanic acid and D-glucose, D-gluco-oxazolidine-2-thione, was used as a reference substance because its structure is that most completely examined in this series of sugar derivatives. The formula (II) now accepted differs from the originally suggested structure [1]



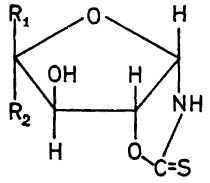
I



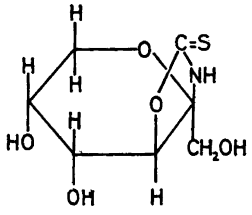
II



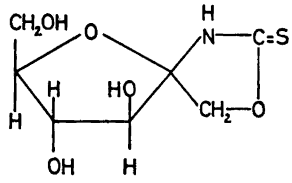
III: $R_1 = H$, $R_2 = OH$
IV: $R_1 = OH$, $R_2 = H$



V : $R_1 = CH_2OH$, $R_2 = H$
VI: $R_1 = H$, $R_2 = CH_2OH$



VII



VIII

(I) by having a furanose ring instead of a pyranose ring and by the mode of attachment of the thione-carbamate group to the sugar chain.

Schwarz [5], studying the oxidation of the D-glucose and the D-galactose derivative by periodate, found that they both yielded one molecule of formaldehyde, a result which is consistent with the furanose form only. Further, the D-glucose compound readily formed an *ortho*formate [5], proving that the thione-carbamate group is incorporated in a five-membered and not in a six-membered ring. Only the hydroxyl group on C_3 and not that on C_2 of the sugar ring is so located as to render the formation of an *ortho*formate sterically possible.

After hydrolytic cleavage of the oxazolidine ring of the D-glucose compound the presence of 2-amino-2-deoxy-D-glucose could not be demonstrated [3]. Edward and Martlew [6] found by paper chromatography of the acid hydrolysate no spots of the very acid-resistant 2-amino-2-deoxy-D-glucose, but 1-aminoglucose and diglucosylamine could be detected. They concluded that the nitrogen of the thione-carbamate group is attached to C_1 as in formula (II) and not to C_2 as previously [1] suggested (I).

All formulas discussed in the present paper are written in agreement with this conclusion, the nitrogen being attached to the anomeric carbon. Primarily, it is difficult to conceive of a reaction between a reducing sugar and thiocyanic acid resulting in the replacement of a hydroxyl group by nitrogen on any carbon other than the anomeric, whereas a similar replacement on the anomeric carbon might result from a hypothetic sequence of possible reactions already mentioned by Bromund and Herbst [3]: intermediate formation of glycosyl-*iso*thiocyanate undergoing intramolecular urethane formation to a cyclic thione-carbamate with one of the alcohol groups of the sugar moiety, *e. g.* with the adjacent hydroxyl group to an oxazolidine-2-thione ring. Further, the sugar derivatives are found to be of uniform acid strength ($pK_A^{20°} = 9.33$—9.48) and considerably stronger acids than the unsubstituted ring system, oxazolidine-2-thione ($pK_A^{20°} = 11.05$), which according to the present investigation is incorporated in all the sugar derivatives studied. This shows that the interaction of the sugar moiety on the presumably acid NH-group is of similar magnitude throughout the series, indicating that the nitrogen is probably located in an analogous position in all the derivatives, *e. g.* on the anomeric carbon.

According to Schwarz [5] the reaction of D-gluco-oxazolidine-2-thione and of D-galacto-oxazolidine-2-thione with unbuffered sodium periodate is complica-

Table 1. Oxidations by means of periodate of the condensation products between thiocyanic acid and reducing sugars.

Parent sugar	Reaction time at 20° (h)	0.5	1	2	6	22	44
D-Glucose	I [a]	5.02	5.01			5.18	
	II [b]	2.02	2.03			2.05	
D-Fructose	I [a]		3.90	4.32	4.65	5.00	5.03
	II [b]		0.38	0.45	0.83	1.50	1.50
D-Xylose	I [a]	4.05	4.05	4.17	4.32	4.32	
	II [b]	0.19	0.19	0.13	0.18	0.19	
L-Arabinose	I [a]	4.04	4.07	4.15	4.10	4.30	
	II [b]	0.18	0.27	0.27	0.26	0.25	

[a] molecules of periodate reduced.

[b] molecules of potassium cyanide reacting with the oxidation product. The figures are mean values of at least two determinations and are referred to one molecule of the sugar derivative.

ted by the oxidation of the thione group. Iodine was liberated, indicating that periodate was reduced beyond the iodate step, and in most cases the reaction mixture also became cloudy (possibly sulphur), indicating that the sulphur of the thione group was not completely oxidized to sulphate. The authors have overcome this difficulty by carrying out the oxidation with a large excess of periodate in a solution acidified by acetic acid. The organic sulphur is then completely oxidized to sulphate by periodate and no reduction of the resulting iodate occurs. This modification affords the possibility of studying the periodate oxidation of these sulphur compounds by classical analytical methods such as the arsenite titration of excess periodate followed by the cyanide titration of carbonyl groups in the oxidation product (Table 1).

Thus, D-gluco-oxazolidine-2-thione (II) consumes five molecules of periodic acid — four molecules to oxidize the sulphur to sulphate (Found: 0.97 mole) and one molecule to transform a glycol group to two aldehyde groups, one of which is formaldehyde [5].

The two pentose compounds reduce four molecules of periodate, corresponding to the oxidation of sulphur to sulphate (Found: 0.94 mole). The insignificant consumption of potassium cyanide, being apparently independent of the reaction time and the reduction of the oxidant, does not indicate a glycol-splitting reaction.

The fructose compound reduces the four molecules of periodate required to oxidize the thione group to sulphate (Found: 0.99 mole) and one additional molecule of periodate which is consumed in the cleavage of a glycol group liberating two (Found: 1.5) titratable aldehyde functions. Only two possible bicyclic formulas, (VII) and (VIII), are consistent with this result, and formula (VII) has been chosen because it agrees with other experimental evidence.

The cyclic thione-carbamates of D-xylose and L-arabinose were believed to possess a pyranose structure [3] (formulas (III) and (IV), respectively) in complete analogy with the previously suggested structure of D-gluco-oxazolidine-2-thione (formula (I)). The two pentose compounds, however, have no glycol group able to reduce periodate. Formula (IV) of the L-arabinose compound includes a *cis* glycol group, and the actual resistance of the ring system towards periodate, therefore, contradicts this structure. The two vicinal hydroxyl groups in formula (III) of the D-xylose compound are held in a *trans* position which presumably renders this glycol group relatively more resistant to glycol-splitting reagents. Complete resistance of *trans* glycol groups towards periodate has so far been observed only in extremely rigid systems, such as 1-6-anhydro-D-glucofuranose [7]. In case, however, the argument against formula (III) based upon the stability of the sugar ring when exposed to periodate might be considered less decisive, it should be mentioned that a supporting argument favouring the modified formula (V) emerged from the infrared spectrum of the D-xylose compound.

The infrared spectra of all substances enumerated in Table 2 have been studied by the potassium bromide plate technique. The main purpose of this examination was to obtain information about the size of the N-heterocyclic ring in the various sugar derivatives.

The infrared absorption of different series of oxazolidine-2-thione derivatives has been investigated by Ettlinger [8] (samples in chloroform) and Skulski *et al.*[9] (samples in Nujol).

The strong absorption band of the five-membered cyclic thione-carbamate in oxazolidine-2-thione (1 525 cm^{-1}) and in D-gluco-oxazolidine-2-thione (1 510 cm^{-1}), tentatively attributed to a NH bending vibration [9], is found in the same region of the spectra of all the sugar derivatives studied, whereas this band of the six-membered cyclic thione-carbamate, tetrahydrogen-1,3-oxazine-2-thione, is displaced to a higher frequency (1 575 cm^{-1}). This is a strong indication of the presence of a five-membered ring (oxazolidine-2-thione) not only in the D-glucose compound but in all the sugar derivatives examined.

It also emerged from the infrared spectra of the sugar derivatives that, in analogy with the unsubstituted oxazolidine-2-thione and some of its substitution derivatives [9], they crystallize in the thione form (and not in the tautomeric thiol form). Absorption due to SH stretching or to C=N bonds did not occur (Table 2).

Further, on comparing the infrared spectra of the oxazolidine-2-thiones of the two homomorphous [10] sugars D-glucose and D-xylose, it was noticed that the absorption curves exhibited a striking parallelism between 4 000 cm^{-1} and 900 cm^{-1}. This may be taken as an indication of the presence of the same fused ring system in both sugar derivatives, formula (II) and (V).

The ultraviolet absorption of the unsubstituted N-heterocyclic rings and the sugar derivatives was also recorded. Results tabulated in Table 3 support the above conclusion that the cyclic thione-carbamates of various sugars examined all contain this group in a five-membered ring.

In order to choose between the two D-fructo-oxazolidine-2-thione formulas (VII) and (VIII) the reducing product of the periodate oxidation was examined by methods which have proved to be reliable in the study of oligo-saccharides, *e. g.* by Courtois and Wickstrøm [11]: paper chromatographic analysis of carboxylic acids formed by bromine oxidation of the aldehyde groups and subsequent acid hydrolysis. D-Fructo-oxazolidine-2-thione yielded glycolic acid in about 50 % of the theoretical amount. The glycolic acid is considered to contain C_6 and C_5 of the fructose moiety of formula (VII), whereas a substance of the formula (VIII) by the same sequence of reactions should yield D-glyceric acid (from C_6, C_5, and C_4). Only one acid spot was observed, probably as a result of destruction of the N-heterocyclic moiety during hydrolysis.

All experimental results agree with the formulas (V) and (VI) for the D-xylose and the L-arabinose derivative, respectively, and with formula (VII) for the D-fructose derivative. According to these formulas the two pentose compounds have a structure analogous to that of the D-gluco-oxazolidine-2-thione (II) and not to the erroneous formula of this compound (I). The size of the rings in formula (VII) of the D-fructose compound is in agreement with the structure attributed to this substance by Zemplén *et al.*[2]

Table 2. Infrared absorption from 1 500 to 4 000 cm^{-1}.

Assignment	NH or OH stretching vibration (bonded) cm^{-1}	NH bending [a] cm^{-1}
Substance		
Oxazolidine-2-thione	3 220	1 525
Tetrahydrogen-1,3-oxazine-2-thione	3 210	1 575
D-Gluco-oxazolidine-2-thione	3 410 3 200	1 510
D-Fructo-oxazolidine-2-thione	3 370 3 180	1 505
D-Xylo-oxazolidine-2-thione	3 420 3 170	1 525
L-Arabo-oxazolidine-2-thione	3 380 3 270	1 485

Table 3. Ultraviolet absorption.

Substance	Aqueous solution		Solution in 0.1 N NaOH [b]	
	λ_{max} (mμ)	$\varepsilon \times 10^{-3}$	λ_{max} (mμ)	$\varepsilon \times 10^{-3}$
Oxazolidine-2-thione [a]	239	16.0	228	12.3
Tetrahydrogen-1,3-oxazine-2-thione	244	14.9	242	11.8
D-Gluco-oxazolidine-2-thione	241	19.7	233	14.5
D-Fructo-oxazolidine-2-thione	241	21.5	233	16.8
D-Xylo-oxazolidine-2-thione	241	20.3	233	14.6
L-Arabo-oxazolidine-2-thione	241	19.6	233	14.3

[a] the ultraviolet absorption found is in agreement with previously published curves for this compound [4].

[b] the absorption in alkaline solution is due to the ionized heterocyclics because of the occurrence of an isosbestic point between the curve in 0.1 N NaOH and curves recorded at several lower pH values.

EXPERIMENTAL

All melting points are micro melting points (Kofler's hot-stage microscope).

1. Preparations

(a) *Cyclic thione-carbamates* were prepared from the various reducing sugars (D-glucose, D-xylose, L-arabinose, D-fructose) according to methods previously described [1-3]. The products obtained from the aldoses were found to be identical with the compounds described in the literature by determination of their melting points, their optical rotation in water, and their reduction of hypoiodite [12] (8 equiv.): D-glucose compound, m. p. 170°, $[\alpha]_D^{20°} = +33.9°$ ($c = 2$), 99.7 %; D-xylose compound, m. p. 131°, $[\alpha]_D^{28°} = +13.2°$ ($c = 2$), 99.5 %; L-arabinose compound, m. p. 136°, $[\alpha]_D^{20°} = +11.8°$ ($c = 2$), 98.7 %.

The heterocyclic product obtained from D-fructose and thiocyanic acid (Zemplén's "Fructose Rhodanverbindung 2") is contaminated with variable amounts of Zemplén's

"Fructose Rhodanverbindung 1", presumably an open chain addition product, m. p. 217°, which is extremely difficult to remove completely by recrystallizations [2]. The two products may be separated on paper chromatograms with the solvent system acetone, *n*-propanol, water (45:45:10 v/v) and the spots ($R_F^{20°}$ = 0.53 (1) and 0.66 (2)) revealed by silver nitrate and ethanolic sodium hydroxide [13]. From columns packed with Whatman standard grade cellulose powder (4.6 × 37 cm) and developed with the above-mentioned solvent system, a poor yield of chromatographically pure "Fructose Rhodanverbindung 2" was obtained, *e. g.* from 0.5 g of the crude mixture (m. p. 172—76°, containing about 10 % of the "Fructose Rhodanverbindung 1") 0.05 g of chromatographically pure "Fructose Rhodanverbindung 2" was obtained. It was further purified by recrystallization from methanol, m. p. 185° (decomp.), $[\alpha]_D^{20} = -50.7°$ (c = 1, water), iodometric titration [2], 97.8 %.

(b) *Oxazolidine-2-thione*, m. p. 97.5°, was prepared from ethanolamine according to Rosen [14], and tetrahydrogen-1,3-oxazine-2-thione was synthesized by a similar procedure starting with 3-amino-propanol; the method described by Rosen [14] for the preparation of 4,4,6-trimethyl-tetrahydrogen-1,3-oxazine-2-thione from 4-amino-4-methyl-pentanol-2 was followed without any essential modification. Repeated crystallization from *n*-butanol gave long, colourless needles (only 3 % yield), m. p. 127°, titration with hypoiodite [12] 99.7 %. (Found: C 41.01; H 5.95; N 11.98; S 28.17. Calc. for C_4H_7ONS: C 41.02; H 6.03; N 11.95; S. 27.36.)

2. Oxidations with periodate

(a) *Titration of periodate uptake and liberated aldehydes.* A 0.05 M periodate reagent (A) of pH about 4 was prepared by dissolving sodium *para*-periodate (Hopkin and Williams Ltd) (7.5 g) in N acetic acid (200 ml) and water (about 200 ml), filtering the solution after some days through sintered glass and adding water to 500 ml. The samples (10 ml of 0.005 M solutions) were oxidized with this reagent (20 ml) at room temperature in the dark. Iodic acid, not iodine or hydroiodic acid, was formed by reduction of the periodic acid. After neutralization with sodium bicarbonate the periodate uptake was determined by the classical arsenite method [15] and titratable carbonyl groups were estimated according to the previously described technique [11]. For oxidation of the fructose compound 0.01 M periodate was used. Results are shown in Table 1.

(b) *Determination of sulphuric acid liberated by periodate oxidation.* The samples (5×10^{-5} molecule in 10 ml of water) were oxidized by the periodate reagent (A) (20 ml). The oxidation was stopped by adding 2 N hydrochloric acid (1.5—2 ml) and then hydrazine hydrate (50 % w/w) dropwise, stirring constantly until the colour of the reaction mixture changed from violet to yellow (0.5—1 ml). The solution was heated on a water bath till the colour (iodine) disappeared completely. Barium sulphate was precipitated from the boiling solution by adding barium chloride in slight excess. The following molecular yields of sulphuric acid per molecule of oxidized sugar derivative were found as mean values of repeated gravimetric determinations (reaction time with periodate in brackets): D-gluco-oxazolidine-2-thione, 0.97 (30 min), D-xylo-oxazolidine-2-thione, 0.94 (60 min), L-arabo-oxazolidine-2-thione, 0.94 (60 min), D-fructo-oxazolidine-2-thione, 0.99 (20 h).

(c) *Examination of the reducing product formed by periodate oxidation of the* D-*fructo-oxazolidine-2-thione.* The sample (25 mg) was oxidized with free periodic acid (500 mg) in aqueous solution at room temperature for 20 h. The solution was neutralized with strontium hydroxide to pH 5—6 and filtered. The filtrate was treated with a weak excess of bromine at room temperature until an aliquot failed to reduce the Nessler reagent (6—8 days). Bromine was then removed by aeration and the solution evaporated to dryness (reduced pressure). The residue was taken up in 2 N sulphuric acid (1.50 ml) and heated in a boiling water bath for one hour. Sulphuric acid was removed by treatment with strontium carbonate and the hydrolysate examined by paper chromatography in the following solvent systems: I, ethyl acetate-formic acid-water (10:2:5 v/v), and II, *n*-butanol-formic acid-water (10:1:5 v/v), which clearly separated authentic samples of glycolic acid ($R_F^{20°}$ = 0.52 (I) and 0.67 (II)) and D-glyceric acid ($R_F^{20°}$ = 0.35 (I) and 0.55

(II)). In repeated experiments only spots corresponding to glycolic acid were observed (bromophenol blue spray). By comparison with a series of standard solutions of glycolic acid (0.1 to 0.5 %) the amount of glycolic acid recovered from the fructose compound was evaluated to about 50 % of the theoretical, indicating its formation by a main reaction. The heterocyclic moiety of the oxidized fructose compound was probably undergoing destruction during hydrolysis (gas evolution).

3. Determination of acid strength

The apparent pK_A values at 20° were calculated from pH measurements on a Metrohm pH-meter E 196 S of 0.01 M aqueous (carbon dioxide free) solutions half neutralized with barium hydroxide. The following mean values were obtained: oxazolidine-2-thione, 11.05 (5,5-dimethyl-oxazolidine-2-thione [4], 11.05); D-glucooxazolidine-2-thione, 9.33; D-xylo-oxazolidine-2-thione, 9.48; L-arabo-oxazolidine-2-thione, 9.48; D-fructo-ozaxolidine-2-thione, 9.40.

4. Absorption spectra

The ultraviolet absorption spectra were recorded on a Beckman DU spectrophotometer using about 2×10^{-5} M solutions of the samples (Table 3). The infrared spectra of the samples (Table 2) in potassium bromide plates were recorded with a Perkin-Elmer Model 21 double beam spectrophotometer.

The authors are indebted to Mr. E. Manser, E. T. H. Zürich, for performing the elementary micro analysis and to siv.ing. J. Lothe, *Sentralinstituttet for Industriell Forskning*, Oslo, for discussion of the infrared spectra recorded in his laboratory. Gratitude is also expressed to *Norges Almenvitenskapelige Forskningsråd* for the award of a Research Fellowship to one of us (J.K.W.).

REFERENCES

1. Zemplén, G., Gerecs, A. and Rados, M. *Ber.* **69** (1936) 748.
2. Zemplén, G., Gerecs, A. and Illés, E. *Ber.* **71** (1938) 590.
3. Bromund, W. H. and Herbst, R. M. *J. Org. Chem.* **10** (1945) 267.
4. Astwood, E. B., Greer, M. A. and Ettlinger, M. G. *J. Biol. Chem.* **181** (1949) 121.
5. Schwarz, J. P. C. *J. Chem. Soc.* **1954** 2644.
6. Edward, J. T. and Martlew, E. F. *Chem. & Ind. London* **1952** 1034.
7. Dimler, R. J. *Advances in Carbohydrate Chem.* **7** (1952) 37.
8. Ettlinger, M. G. *J. Am. Chem. Soc.* **72** (1950) 4699.
9. Skulski, M., Garmaise, D. L. and McKay, A. F. *Can. J. Chem.* **34** (1956) 815.
10. Pigman, W. *The Carbohydrates*, New York, 1957.
11. Courtois, J. and Wickstrøm, A. *Bull. soc. chim. biol.* **32** (1950) 759.
12. Wojahn, H. *Arch. Pharm.* **284** (1951) 243.
13. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. *Nature* **166** (1950) 444.
14. Rosen, A. A. *J. Am. Chem. Soc.* **74** (1952) 2994.
15. Fleury, P. and Lange, J. *J. pharm. & chim.* **17** (1933) 107.

Received February 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1137–1140

On the Metabolism of Bile Acids in the Guinea Pig

Bile Acids and Steroids 86

HENRY DANIELSSON and TARO KAZUNO*

Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden

The catabolism of 4-^{14}C-cholesterol to bile acids has been studied in intact and bile fistulated guinea pigs. The only bile acid formed from cholesterol in the liver is chenodeoxycholic acid. During the enterohepatic circulation chenodeoxycholic acid is oxidized to 7-keto-lithocholic acid by the action of intestinal microorganisms. The 7-keto-lithocholic acid thus formed is reduced in the liver to chenodeoxycholic acid, as was shown by injection of 24-^{14}C-7-keto-lithocholic acid to intact and bile fistulated guinea pigs.

The bile of guinea pig is reported to contain chenodeoxycholic acid (3α, 7α-dihydroxycholanic acid) and 7-keto-lithocholic acid (3α-hydroxy, 7-keto-cholanic acid), present as conjugates with taurine [1]. The important part played by intestinal microorganisms in the metabolism of bile acids during their enterohepatic circulation has recently become apparent by work in this laboratory. Lindstedt and Sjövall [2] found, that deoxycholic acid, which makes up 90 % of the bile acids in gall-bladder bile of rabbit, is formed from cholic acid by the action of bacteria in the intestine. The same reaction has been shown also to occur in man [3], and in rat [4], that was also observed by Portman [5]. In the rat, however, the deoxycholic acid is rapidly rehydroxylated to cholic acid in the liver [6]. In the pig, hyodeoxycholic acid (3α,6α-dihydroxy-cholanic acid) is a microbial product of hyocholic acid (3α,6α,7α-trihydroxy-cholanic acid) [7]. In all above mentioned cases the intestinal microorganisms remove a 7α-hydroxyl from a trihydroxylated bile acid, whether the position of the hydroxyls is 3α,6α,7α or 3α,7α,12α. This reaction has been further studied *in vivo* by means of doubly labeled cholic acid (7β-tritio, 24-^{14}C-cholic acid) [8,9], and it has been found, that the removal of the 7α-hydroxyl occurs with retention of tritium that, however, apparently is shifted during this process from the 7β to the 7α position.

Norman and Sjövall [4] have recently made a detailed study of the metabolism of bile acids in the rat during their passage through the intestinal tract, and they have shown, that among microbially formed products of cholic acid

* Permanent address: Department of Biochemistry, Hiroshima University School of Medicine, Hiroshima, Japan.

small amounts of ketonic acids, mainly 3α,12α-dihydroxy -7-ketocholanic acid, are also absorbed from the large intestine and excreted in the bile.

Thus in the rat intestinal microorganisms are responsible for the removal of the 7α-hydroxyl from cholic acid and also for the oxidation of this group to a ketone. The latter reaction does not seem to be a quantitatively important intermediate step in the formation of deoxycholic acid, as the 7β-tritium in cholic acid is retained to a high extent in the deoxycholic acid.

In view of above mentioned data and considering that the guinea pig is reported to have no trihydroxylated bile acid, but only chenodeoxycholic and 7-keto-lithocholic acid in its bile, it was of interest to study the formation of bile acids in this species and the changes, if any, of the bile acids during their enterohepatic circulation.

EXPERIMENTAL

Male guinea pigs of the Danish State Serum Institute strain, weighing approx. 300 g, were used. Bile fistulas were prepared as described for rats [10].

Procedures for analysis of bile. Conjugated bile acids were extracted from acidified bile with *n*-butanol. Conjugates were split by hydrolysis with N sodium hydroxide at 120° for 8—12 h in a sealed steel tube, and the free bile acids were extracted with ether from the acidified reaction mixture. The bile acids were separated with reversed phase partition chromatography using phase system C for conjugated acids [11] and F for free acids [12].

Phase systems

	Moving phase	Stationary phase
C	150 ml methanol 150 ml water	15 ml chloroform 15 ml *iso*octanol
F	165 ml methanol 135 ml water	45 ml chloroform 5 ml heptane

Hostalene (Farbwerke Hoechst, G.m.b.H., West-Germany), prepared as earlier described [7], was used as supporting material for the stationary phase. Columns were prepared as described by Norman [11], using 3 ml of stationary phase per 4.5 g of Hostalene. Chromatograms were run at constant temperature of 23°. The effluent was titrated with 0.02 N methanolic sodium hydroxide, and suitable aliquots were assayed for radioactivity, which was determined with an automatic end-window counter, Frieseke und Hoepfner, West-Germany.

Labeled compounds. 4-^{14}C-cholesterol was obtained from the Radiochemical Centre, Amersham, England. Specific activity: $\sim$ 50 μC/mg. 24-^{14}C-7-ketolithocholic acid was prepared from 24-^{14}C-chenodeoxycholic acid according to the procedure described by Samuelsson [13]. Specific activity: $\sim$1 μC/mg.

RESULTS AND DISCUSSION

The formation of bile acids from cholesterol and the metabolism of these in guinea pig was studied by analyses of labeled products in gall-bladder and fistula bile after intraperitoneal injection of 4-^{14}C-cholesterol and of 24-^{14}C-7-keto-lithocholic acid. The 4-^{14}C-cholesterol in doses of 10—50 μC (0.2—1 mg) was injected as an emulsion with 1 % bovine serum albumin in saline. 1—5 days after administration of the isotope, a bile fistula was prepared and bile collected for 12—24 h or the animal was sacrificed and the gall-bladder removed.

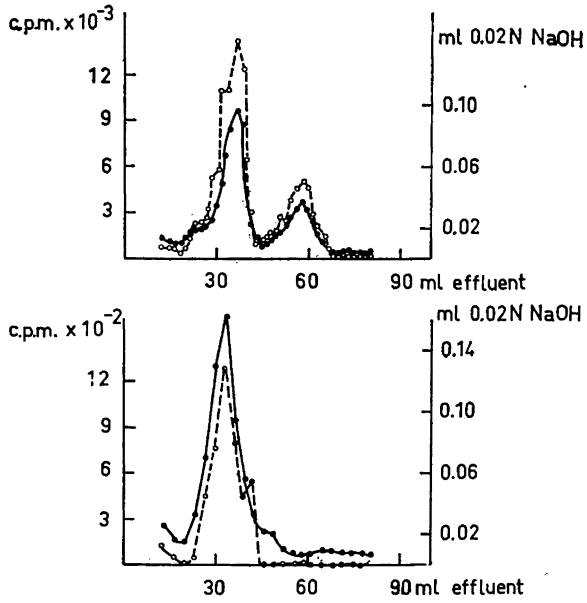


Fig. 1. Chromatogram of hydrolyzed bile collected from bile fistula between 0 and 4 h (upper curve) and between 8 and 20 h (lower curve) after fistulation. The animal had been injected with 50 μC 4-^{14}C-cholesterol 5 days before operation. Columns: 4.5 g Hostalene. Phase system F. Solid line: titration. Broken line: radioactivity.

The unhydrolyzed bile acids were chromatographed with phase system C, and it was found, that bladder bile and fistula bile collected 0—8 h after fistulation contained mainly glycine conjugates, about 80 % of total, the rest being taurine conjugates. Fistula bile collected later than 8 h after fistulation had a considerable part and sometimes all of the bile acids conjugated with taurine.

When the bile acids were chromatographed after hydrolysis on phase system F, it was found, that there appeared one or two radioactive titration peaks, depending on the time the bile samples were collected. Bile collected from bladder, and fistula bile collected during the first 4—6 h after fistulation contained two labeled acids, appearing in the chromatogram at the place for chenodeoxycholic and 7-keto-lithocholic acid, respectively, as is shown in the upper curve of Fig. 1. Bile collected later than 6 h after fistulation contained only one labeled acid with chromatographic mobility as chenodeoxycholic acid, *cf.* lower curve, Fig. 1. The identity of the labeled compounds with chenodeoxycholic, 7-keto-lithocholic and chenodeoxycholic acid, respectively, was established by co-crystallization with unlabeled material, the specific activity remaining constant through four crystallizations with a final weight of crystals about one quarter the original weight (chenodeoxycholic acid was crystallized from methanol/ethyl acetate twice and ethyl acetate twice; 7-keto-lithocholic acid from ethyl acetate, methanol/water, acetone/water and ethyl acetate).

Bladder bile and fistula bile collected 0—3 h after fistulation, when the preformed bile acids are being excreted, contained approximately the same

proportion of chenodeoxycholic to 7-keto-lithocholic acid, 3:1, whether the cholesterol had been injected 1 or 5 days before collection of bile, indicating that the microbial formation of 7-keto-lithocholic acid from chenodeoxycholic acid occurs rapidly and then reaches an equilibrium, which conceivably could be maintained by a continuous reduction of the 7-ketone to a 7α-hydroxyl in the liver. To test this, 24-^{14}C-7-keto-lithocholic acid in doses of 0.3 μC (0.3 mg) was injected intraperitoneally into intact and bile fistulated guinea pigs. The labeled products in bile were analyzed as described above.

Already after 1 day of enterohepatic circulation 73 % of the isotope in the bile was present as chenodeoxycholic acid; the remainder consisted of unchanged 7-keto-lithocholic acid. About the same distribution of isotope (83 % in chenodeoxycholic acid) was found in bile from an animal, in which the labeled 7-keto-lithocholic acid had taken part in the enterohepatic circulation for 3 days. When the 7-keto-lithocholic acid was injected into bile fistulated guinea pigs, the isotope appeared in bile rapidly, and after 4 h 70—80 % of the injected amount had been excreted. About 50 % of the isotope was present as chenodeoxycholic acid and the remainder as unchanged 7-keto-lithocholic acid.

Thus, the guinea pig liver efficiently reduces 7-keto-lithocholic acid to chenodeoxycholic acid. In this connection it is interesting, that the rat liver reduces 7-keto-lithocholic acid mainly to the 7β-epimer, ursodeoxycholic acid[13]. These experiments do not exclude the possibility, that the reduction of 7-keto-lithocholic acid to chenodeoxycholic acid is effected also by intestinal microorganisms, but this does not seem entirely likely in view of results obtained in the rat, where the microbial action on the bile acid nucleus mainly is oxidative or consists of dehydroxylations [4].

The results obtained in this investigation seem to establish, that the guinea pig, as the only species so far known, does not catabolize cholesterol in the liver to a trihydroxylated bile acid, but only to chenodeoxycholic acid. During the enterohepatic circulation of bile this acid is transformed by microbial action into 7-keto-lithocholic acid, which in turn is reduced in the liver to chenodeoxycholic acid.

Acknowledgments. This work is part of investigations supported by *Statens Medicinska Forskningsråd, Knut och Alice Wallenbergs Stiftelse*, Stockholm, and the *National Heart Institute, National Institutes of Health*, Bethesda, Maryland, USA (H 2842).

REFERENCES

1. Haslewood, G. A. D. and Wootton, V. *Biochem. J.* **47** (1950) 584.
2. Lindstedt, S. and Sjövall, J. *Acta Chem. Scand.* **11** (1957) 421.
3. Lindstedt, S. *Arkiv Kemi* **11** (1957) 145.
4. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
5. Portman, O. *Arch. Biochem. Biophys.* **78** (1958) 125.
6. Bergström, S., Rottenberg, M. and Sjövall, J. *Z. physiol. Chem. Hoppe-Seyler* **295** (1953) 278.
7. Bergström, S., Danielsson, H. and Göransson, Å. *Acta Chem. Scand.* **13** (1959) 776.
8. Bergström, S., Lindstedt, S. and Samuelsson, B. *J. Biol. Chem.* **234** (1959) 2022.
9. Lindstedt, S. and Samuelsson, B. *J. Biol. Chem.* **234** (1959) 2026.
10. Bergström, S., Sjövall, J. and Voltz, J. *Acta Physiol. Scand.* **30** (1953) 22.
11. Norman, A. *Acta Chem. Scand.* **7** (1953) 1413.
12. Sjövall, J. *Acta Physiol. Scand.* **29** (1953) 232.
13. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 236.

Received April 1, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1141–1144

On the Metabolism of Bile Acids in the Mouse

Bile Acids and Steroids 87

HENRY DANIELSSON and TARO KAZUNO*

Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden

The catabolism of 4-^{14}C-cholesterol to bile acids in the mouse has been studied. Cholic acid and small amounts of chenodeoxycholic acid are formed. The presence of chenodeoxycholic acid in mouse bile is therefore postulated. The latter acid was partially transformed into two more polar compounds having the same chromatographic mobility as the 6β-hydroxylated acids (3α,6β,7α- and 3α,6β,7β-trihydroxycholanic acid) formed from chenodeoxycholic acid in the rat.

24-^{14}C-Deoxycholic acid was hydroxylated to cholic acid in the mouse.

Recent work on the effect of intestinal microorganisms on bile acids has demonstrated, that the primary bile acids, *i. e.* those formed in the liver, often are modified during their enterohepatic circulation by the action of bacteria. Thus, deoxycholic acid in man [1] and rabbit [2] is a microbial metabolite of cholic acid. The same reaction occurs in rat [3,4], but the deoxycholic acid formed is in this species rehydroxylated to cholic acid in the liver [5]. The removal of a 7α-hydroxyl from a primary trihydroxylated bile acid by the action of intestinal microorganisms also occurs in the pig [6], where hyocholic acid is transformed into hyodeoxycholic acid.

These findings, that were discussed in more detail in the preceding paper [7], seem to suggest a general scheme of bile acid metabolism, irrespective of species. To obtain additional experimental support for such a hypothesis, and as a part of a more general survey of bile acid metabolism in different species, it was of interest to investigate the formation and metabolism of bile acids in the mouse, that is reported to have cholic acid in its bile [8].

* Permanent address: Department of Biochemistry, Hiroshima University School of Medicine, Hiroshima, Japan.

EXPERIMENTAL

White male mice of the Danish State Serum Institute strain, weighing approx. 25 g, were used. The animals were sacrificed by cervical dislocation. Bile was recovered from the gall-bladder and from 50 % ethanol washes of the upper part of the small intestine.

The analytical procedures for conjugated and free bile acids were essentially the same as those described in the preceding paper [7]. 4-^{14}C-cholesterol was obtained from Radiochemical Centre, Amersham, England, and was administered as an emulsion with 1 % bovine serum albumin in saline. 24-^{14}C-Chenodeoxycholic and 24-^{14}C-deoxycholic acid were prepared according to Bergström *et al.*[9] and were generous gifts from Dr. Arne Norman.

RESULTS AND DISCUSSION

10 μC (0.2 mg) of 4-^{14}C-cholesterol were injected intraperitoneally into each of 8 mice, and 24, 48, 72 and 120 h after injection 2 animals were sacrificed and the bile was collected. Chromatography of the unhydrolyzed bile samples revealed that the bile acids were present in bile exclusively as taurine conjugates. After hydrolysis the bile acids were chromatographed with phase system F and in Fig. 1 is shown a chromatogram of the free bile acids from 2 mice injected 48 h before sacrifice. The radioactive material in the first peak was identified as cholic acid by isotope dilution. The identity of the radioactive material in the second peak with chenodeoxycholic acid was established in the same manner. Chromatograms of hydrolyzed bile from the mice injected with 4-^{14}C-cholesterol 24, 72 and 120 h before sacrifice did not differ from the above mentioned, except that the amount of chenodeoxycholic acid varied from a few percent to 10 %.

The catabolism of cholesterol to bile acids in the mouse thus follows the same path as in rat. In the latter animal chenodeoxycholic acid is further metabolized to two 6β-hydroxylated acids (3α,6β,7α- and 3α,6β,7β-trihydroxycholanic acid), recently identified by Doisy and coworkers [10,11], and cholic acid is transformed by the action of intestinal microorganisms into deoxycholic acid [3], which in turn is rapidly rehydroxylated to cholic acid in the liver [5].

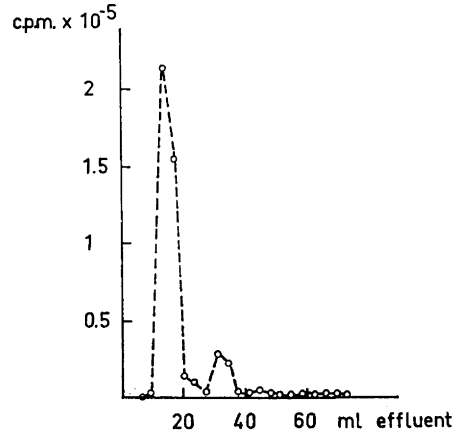


Fig. 1. Chromatogram of hydrolyzed bile collected 48 h after i. p. injection of 4-^{14}C-cholesterol. Column: 4.5 g Hostalene. Phase system F. Broken line: radioactivity.

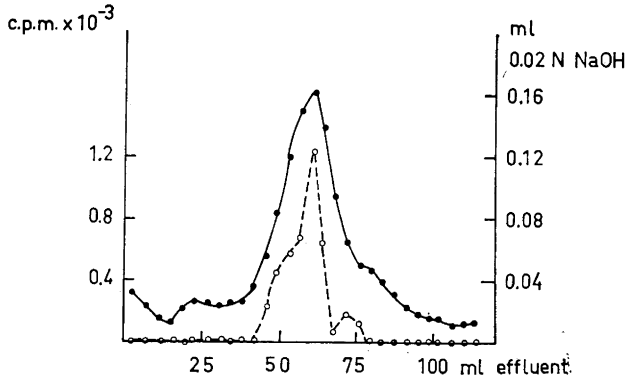


Fig. 2. Chromatogram of trihydroxycholanic acid fraction from bile collected 24 h after injection of 24-^{14}C-deoxycholic acid. Column: 4.5 g Hostalene. Phase system C. Solid line: titration. Broken line: radioactivity.

In order to provide further evidence for the similarity in the pattern of bile acid formation and metabolism in rat and in mouse, the metabolism of 24-^{14}C-chenodeoxycholic and 24-^{14}C-deoxycholic acid was studied in the mouse.

3 μC (0.3 mg) of 24-^{14}C-chenodeoxycholic acid were injected into each of 6 mice and another group of 6 mice received each 2 μC (0.3 mg) of 24-^{14}C-deoxycholic acid. 3, 24 and 72 h after injection, 2 animals in each group were sacrificed. The bile was hydrolyzed and chromatographed first on phase system F and then after addition of 5 mg unlabeled cholic acid on phase system C.

Bile collected 3 h after injection of either 24-^{14}C-deoxycholic or 24-^{14}C-chenodeoxycholic acid contained about 1/3 of the isotope in the trihydroxycholanic acid fraction, and the remainder was present as unchanged deoxy-

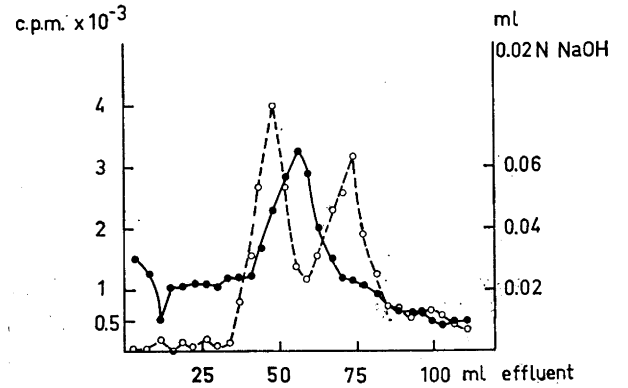


Fig. 3. Chromatogram of trihydroxycholanic acid fraction from bile collected 24 h after injection of 24-^{14}C-chenodeoxycholic acid. Column: 4.5 g Hostalene. Phase system C. Solid line: titration. Broken line: radioactivity.

cholic and chenodeoxycholic acid, respectively. The amount of isotope in the trihydroxycholanic acid band rose to 60—70 % in bile collected 24 h after injection and to 90—100 % after 72h. The trihydroxycholanic acid fractions were rechromatographed on phase system C. Fig. 2 shows the chromatogram of this fraction from animals injected 24 h before sacrifice with 24-^{14}C-deoxycholic acid. The radioactivity coincides with the titration peak of cholic acid. The identity of the isotope with cholic acid was confirmed by isotope dilution.

In the corresponding chromatogram of bile from animals injected with 24-^{14}C-chenodeoxycholic acid, the radioactivity is distributed in two peaks appearing before and after cholic acid, *cf.* Fig. 3. These two radioactive peaks have the same chromatographic mobility as the 6β-hydroxylated acids formed from chenodeoxycholic acid in the rat, 3α,6β,7α- and 3α,6β,7β-trihydroxycholanic acid [10,11].

The results obtained in this investigation indicate, that the bile acid formation and metabolism of the mouse is very similar to that of the rat.

Acknowledgments. This work is part of investigations supported by *Statens Medicinska Forskningsråd, Knut och Alice Wallenbergs Stiftelse,* Stockholm, and a research grant (H 2842) to Professor Sune Bergström from the *National Heart Institute, National Institutes of Health,* Bethesda, Maryland, USA.

REFERENCES

1. Lindstedt, S. *Arkiv Kemi* **11** (1957) 145.
2. Lindstedt, S. and Sjövall, J. *Acta Chem. Scand.* **11** (1957) 421.
3. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
4. Lindstedt, S. and Samuelsson, B. *J. Biol. Chem.* **234** (1959) 2026.
5. Bergström, S., Rottenberg, M. and Sjövall, J. *Z. physiol. Chem. Hoppe-Seyler* **295** (1953) 278.
6. Bergström, S., Danielsson, H. and Göransson, Å. *Acta Chem. Scand.* **13** (1959) 776.
7. Danielsson, H. and Kazuno, T. *Acta Chem. Scand.* **13** (1959) 1137.
8. Haslewood, G. A. D. and Wootton, V. *Biochem. J.* **47** (1950) 584.
9. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
10. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 573.
11. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **226** (1957) 667.

Received April 1, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1145–1148

α-Diethylamino-4-amino-2,6-dimethylacetanilide and Some Related Compounds

RICHARD DAHLBOM, CLAËS TEGNÉR and NILS-ERIK WILLMAN

Research Laboratories, AB Astra, Södertälje, Sweden

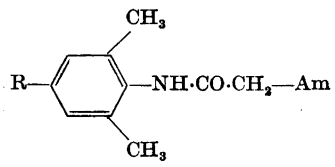
α-Diethylamino-4-amino-2,6-dimethylacetanilide and some alkyl and acyl derivatives have been prepared and tested for local anesthetic activity.

It has been stated by Fosdick and Rapp [1] that the introduction of an amino or a nitro group in the benzene nucleus of α-dialkylaminoacetanilides considerably lowered their anesthetic effect. The same observation was made by Löfgren and Fischer [2] who considered it to be a general rule in the α-dialkylaminoacetanilide series that the anesthetic activity was decreased by substituents with marked inductive or mesomeric effects. Beke, Lempert and and Gyermek [3] found, however, that the introduction of an amino group in the *para* position to the acylamido side chain of an α-diethylamino-2,6-dihalogenoacetanilide enhanced the anesthetic activity. These authors found that in general in this type of compound the introduction of an electron releasing group in the *para* position increased the activity whereas an electron attracting group decreased the activity independently of the mechanism of electron repulsion or attraction (inductive, mesomeric or both). β-Diethylaminobutyranilide and its *p*-amino derivative have been described by Hofstetter and Smith [4] but no definite conclusions can be drawn from their results.

In view of these facts we found it of great interest to study the *p*-amino derivative of α-diethylamino-2,6-dimethylacetanilide (Xylocaine ®). This paper describes the synthesis of this compound (III) and of some of its acyl and alkyl derivatives (IV—VIII). The *p*- amino derivative of α-*n*-propylamino-2,6-dimethylacetanilide (X) was also prepared.

Compound III was synthesised by chloroacetylation of 2,6-dimethyl-4-nitroaniline [5] followed by treatment with diethylamine to give α-diethylamino-4-nitro-2,6-dimethylacetanilide(II) and hydrogenation to the amino compound. This was then acylated with an acyl halide or alkylated by reductive alkylation.

Table 1.



Compound	R	Am	Anesthetic activity [a]	Toxicity [a] LD 50 g/kg
Xylocaine	H	$-N(C_2H_5)_2$	1	0.4
II	NO_2-	»	0.4	1
III	NH_2-	»	0.2	0.24
IV	$CH_3 \cdot CO \cdot NH-$	»	0	0.36
V	$C_2H_5 \cdot CO \cdot NH-$	»	0	0.38
VI	$n\text{-}C_3H_7 \cdot CO \cdot NH-$	»	0.5	0.39
VII	$n\text{-}C_5H_{11} \cdot CO \cdot NH-$	»	3	0.54
VIII	$n\text{-}C_4H_9 \cdot NH-$	»	2.6	0.042
X	NH_2-	$-NH\text{-}n\text{-}C_3H_7$	0	—
XIV	$NH_2 \cdot CO-$	$-N(C_2H_5)_2$	0.4	0.4
XV	$NH_2 \cdot NH \cdot CO-$	»	0	0.6

[a] See text for explanation.

In an attempt to obtain compound III *via* either the Hofmann or the Curtius reaction the 4-carbamoyl, 4-hydrazinocarbonyl, and 4-azidocarbonyl derivatives of Xylocaine were prepared and are described in this paper.

The new compounds were tested for local anesthetic action on rabbit cornea, using Xylocaine as standard. Toxicity tests were made by subcutaneous injection on white mice. The results of the biological tests * are summarised in Table 1.

It is evident from the table that both the electron releasing amino group and the electron attracting carbamoyl, hydrazinocarbonyl, and nitro groups decrease the anesthetic effect. Introduction of an acyl or alkyl residue of suitable size in the amino group restored the activity, but these compounds (VII, VIII) were very irritating and the butylamino compound VIII was extremely toxic.

EXPERIMENTAL

α-Chloro-4-nitro-2,6-dimethylacetanilide (I). Chloroacetyl chloride (6.0 ml, 0.079 mole) was added with vigorous stirring to a solution of 4-nitro-2,6-dimethylaniline [5] (12 g, 0.072 mole) and dry pyridine (5.8 ml, 0.072 mole) in benzene (500 ml). The precipitate formed was collected, washed with water and dried. Recrystallisation from benzene gave light yellow crystals (16.3 g, 93 %), m. p. 229–231°. (Found: 49.3; H 4.64; N 11.5. Calc. for $C_{10}H_{11}ClN_2O_3$: C 49.5; H 4.57; N 11.5.)

α-Diethylamino-4-nitro-2,6-dimethylacetanilide (II). The chloroacetyl derivative I (82 g, 0.34 mole) was refluxed for 10 h with diethylamine (87 ml, 0.85 mole) in dry benzene (400 ml). After cooling the reaction mixture was filtered and then extracted

* Acknowledgement is made to Dr. S. Wiedling for performing these tests.

thoroughly with 2 N hydrochloric acid. The extract was made alkaline with 4 N sodium hydroxide, and the precipitated base was collected and recrystallised from ethanol as light yellow crystals (82 g, 86 %), m. p. 98—99°. (Found: C 60.4; H 7.22; N 14.9. Calc. for $C_{14}H_{21}N_3O_3$: C 60.2; H 7.58; N 15.0.)

α-Diethylamino-4-amino-2,6-dimethylacetanilide (III). The nitro compound II (82 g, 0.29 mole), dissolved in ethanol (650 ml), was hydrogenated at 40° and normal pressure over Raney nickel (5 g, moist). The calculated amount of hydrogen was consumed after 2 h. The catalyst was filtered off, and the solvent was evaporated *in vacuo*. The residue was recrystallised from ether, m. p. 98—100° (70 g, 96 %). (Found: C 67.3; H 9.10; N 17.0. Calc. for $C_{14}H_{23}N_3O$: C 67.4; H 9.30; N 16.9.)

α-Diethylamino-4-acetamido-2,6-dimethylacetanilide (IV). A solution of α-diethylamino-4-amino-2,6-dimethylacetanilide (2.5 g, 0.01 mole) in acetone (30 ml), in which potassium carbonate (0.49 g, 0.005 mole) was suspended, was treated with acetyl chloride (0.71 ml, 0.01 mole) added dropwise with stirring. The reaction mixture was refluxed for half an hour and the solvent was then removed under vacuum. The residue was suspended in water and the suspension was made alkaline by the addition of sodium hydroxide. The precipitate was collected and recrystallised from benzene, m. p. 144—145° (2.4 g, 80 %). (Found: C 65.3; H 8.36; N 14.3. Calc. for $C_{16}H_{25}N_3O_2$: C 66.0; H 8.65; N 14.4.)

The following compounds were prepared by essentially the same procedure:

α-Diethylamino-4-propionamido-2,6-dimethylacetanilide (V). M. p. 152—153° (from benzene). Yield 62 %. (Found: C 67.0; H 8.86; N 13.8. Calc. for $C_{17}H_{27}N_3O_2$: C 66.9; H 8.91; N 13.8.)

α-Diethylamino-4-butyramido-2,6-dimethylacetanilide (VI). M. p. 142—143° (from benzene). Yield 62 %. (Found: C 68.0; H 9.09. Calc. for $C_{18}H_{29}N_3O_2$: C 67.7; H 9.15.)

α-Diethylamino-4-hexanoylamino-2,6-dimethylacetanilide (VII). This compound was prepared as described above with the difference that pyridine was used as proton acceptor instead of potassium carbonate. M. p. 118—120° (from benzene-ether). Yield 63 %. (Found: C 69.2; H 9.53; N 12.1. Calc. for $C_{20}H_{33}N_3O_2$: C 69.1; H 9.57; N 12.1.)

*α-Diethylamino-4-*n*-butylamino-2,6-dimethylacetanilide (VIII).* α-Diethylamino-4-amino-2,6-dimethylacetanilide (7.5 g, 0.03 mole) and *n*-butyraldehyde (4.3 g, 0.06 mole) were dissolved in ethanol (75 ml) in which anhydrous sodium acetate (0.3 g) was suspended. The mixture was hydrogenated at normal pressure and room temperature over Raney nickel for 40 min. The reaction mixture was then filtered, concentrated to dryness and the residue was dissolved in N hydrochloric acid. The acid solution was filtered, made alkaline with 4 N sodium hydroxide and the liberated base was extracted with ether. The ethereal solution was dried over sodium sulphate and the reaction product was precipitated as the hydrochloride by the addition of ethereal hydrochloric acid. Repeated recrystallisation from ethanol-benzene (1:1) gave colourless crystals (7.3, 71 %), m. p. 223—225°. The addition of sodium hydroxide to an aqueous solution of the hydrochloride gave the base, m. p. 42—44°. (Found: C 70.7; H 10.0; N 13.7. Calc. for $C_{18}H_{31}N_3O$: C 70.8; H 10.2; N 13.8.)

α-Propylamino-4-nitro-2,6-dimethylacetanilide (IX). The chloroacyl compound I (13 g, 0.054 mole) and *n*-propylamine (16 g, 0.268 mole) were refluxed in benzene solution as described for compound II. The base was crystallised from benzene-petroleum ether (b. p. 40—60°), m. p. 99—100° (11.5 g, 80 %). (Found: C 58.8; H 7.27; N 15.8. Calc. for $C_{13}H_{19}N_3O_3$: C 58.8; H 7.22; N 15.8.)

*α-*n*-Propylamino-4-amino-2,6-dimethylacetanilide (X).* The nitro compound IX (9 g, 0.034 mole) was hydrogenated over Raney nickel as described for compound III. The base was crystallised from benzene-petroleum ether (40—60°), m. p. 111—112° (5.5 g, 69 %). (Found: C 66.5; H 9.06; N 17.6. Calc. for $C_{13}H_{21}N_3O$: C 66.3; H 9.00; N 17.9.)

3,5-Dimethyl-4-nitrobenzamide (XI). An ice-cooled solution of 3,5-dimethyl-4-nitrobenzoyl chloride [6] (12 g, 0.056 mole) in benzene (150 ml) was treated with ammonia gas for 30 min. The temperature of the solution slowly rose to 30°. The solvent was evaporated and the residue was washed with water to remove ammonium chloride formed during the reaction. Recrystallisation from benzene gave colourless crystals, m. p. 169—171° (10.2 g, 93 %). (Found: C 55.4; H 5.36; N 14.3. Calc. for $C_9H_{10}N_2O_3$: C 55.7; H 5.19; N 14.5.)

4-Carbamoyl-2,6-dimethylaniline (XII). The nitro derivative XI (9.7 g, 0.05 mole) was dissolved in ethanol (200 ml) and hydrogenated over Raney nickel at room temperature and a pressure of 90 kg/cm². After 45 min the catalyst was filtered off and the solu-

tion was evaporated to dryness under reduced pressure. The residue was recrystallised from petroleum ether (40—60°)-ethanol, m. p. 181—182° (5.9 g, 72 %). (Found: C 65.9; H 7.43; N 17.0. Calc. for $C_9H_{12}N_2O$: C 65.8; H 7.37; N 17.1.)

α-Chloro-4-carbamoyl-2,6-dimethylacetanilide (XIII). 4-Carbamoyl-2,6-dimethylaniline was chloroacetylated in the same manner as described for compound I. The chloroacetyl derivative was obtained as colourless needles from ethanol, m. p. 241—243°. Yield 84 %. (Found: C 54.9; H 5.54; N 11.7. Calc. for $C_{11}H_{13}ClN_2O_2$: C 54.9; H 5.44; N 11.6.)

α-Diethylamino-4-carbamoyl-2,6-dimethylacetanilide (XIV). The chloroacetyl compound XIII (2.7 g, 0.011 mole) was refluxed with diethylamine (2.2 g, 0.03 mole) in benzene (50 ml) for 8 h at 70°. The base was isolated as described for compound II giving slightly yellow crystals, m. p. 175—177° (2.3 g, 74 %). On recrystallisation (charcoal) from ethanol-benzene (1:1) it gave colourless crystals, m. p. 176—178°. (Found: C 64.9; H 8.51; N 15.1. Calc. for $C_{15}H_{23}N_3O_2$: C 64.9; H 8.36; N 15.2.)

α-Diethylamino-4-hydrazinocarbonyl-2,6-dimethylacetanilide (XV). α-Diethylamino-4-methoxycarbonyl-2,6-dimethylacetanilide [6] (5.0 g, 0.017 mole) was refluxed with hydrazine hydrate (2.1 g, 0.043 mole) in propanol (5 ml) for 8 h and the reaction mixture was allowed to stand at room temperature over night. The hydrazide that separated was then recrystallised from ethanol, m. p. 185—186° (4.7 g, 94 %). (Found: C 61.4; H 8.35; N 19.0. Calc. for $C_{15}H_{24}N_4O_2$: C 61.6; H 8.27; N 19.2.)

α-Diethylamino-4-azidocarbonyl-2,6-dimethylacetanilide (XVI). The hydrazide XV (8.8 g, 0.03 mole) suspended in ice water (40 ml) containing hydrochloric acid (12.5 ml 5 N) was treated with a solution of sodium nitrite (2.2 g, 0.032 mole) in water (5 ml), added dropwise with stirring. The temperature of the reaction mixture was not allowed to rise above 10°. When all the nitrite had been added, the reaction mixture was neutralised by the addition of cold dilute ammonia and was then extracted thoroughly with ether. The extract was washed with water and dried over sodium sulphate. The solvent was removed and the residue was recrystallised from ether, m. p. 89—90° (7.2 g, 76 %). (Found: C 59.4; H 7.18; N 22.8. Calc. for $C_{15}H_{21}N_5O_2$: C 59.4; H 6.98; N 23.1.)

REFERENCES

1. Fosdick, L. S. and Rapp, G. W. *J. Am. Chem. Soc.* **65** (1943) 2307.
2. Löfgren, N. and Fischer, I. *Svensk Kem. Tidskr.* **58** (1946) 219.
3. Beke, D., Lempert, K. and Gyermek, L. *Acta Chim. Acad. Sci. Hung.* **5** (1954) 151.
4. Hofstetter, E. and Smith, A. E. W. *Helv. Chim. Acta* **36** (1953) 1698.
5. Dahlbom, R. and Misiorny, A. *Acta Chem. Scand.* **11** (1957) 1350.
6. Löfgren, N., Tegnér, C., Willman, N.-E. and Dahlbom, R. *To be published.*

Received April 2, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1149–1158

Immunologic Studies of the Old Yellow Enzyme. The Inhibition of Enzyme Activity by Antibodies

STEN KISTNER

Nobel Medical Institute, Biochemical Department, Stockholm, Sweden

The effect of precipitating antibodies against the Old Yellow Enzyme on the activity of the enzyme and its apoprotein has been investigated. Immediately after the addition of antiserum to the enzyme a partial inhibition of the activity occurred, evidently due to the reversible formation of enzyme-antibody complexes. The inhibition increased if enzyme and antiserum were incubated for some time prior to the activity determination. The antibodies and reduced triphosphopyridine nucleotide, which is oxidized by the enzyme, did not compete for the same binding sites on the enzyme molecule. No immediate effect of antiserum on the association reaction between apoprotein and coenzyme could be demonstrated. After incubation, the apoprotein was precipitated by the antibodies. The influence of the complexity of the enzyme-inhibitor system on the kinetics of the inhibition is discussed.

In a previous study [1] precipitating antibodies reacting with crystalline Old Yellow Enzyme (OYE) * were obtained after immunization of rabbits with impure enzyme preparations. The experimental results indicated the existence of specific antibodies against the pure OYE in the antisera. A comparison between the immune reactions of the holoenzyme and the apoprotein, using the agar-gel diffusion technique, showed an identical behaviour of the two proteins. It was concluded that the coenzyme group, FMN, was not involved in the antigenically active part of the enzyme molecule, or in its binding sites to antibody. This, however, is no obstacle for the inhibition of enzyme activity by antibody. Preliminary studies showed that inhibition did occur.

The precipitin reactions had thus provided some information concerning the relationship between active groups of the enzyme and its binding sites to antibody, suggesting that these had different localizations on the enzyme molecule. It then appeared of interest to further investigate the mechanism of inhibition of enzyme activity by antibodies.

* In this paper the following abbreviations are used: OYE = Old Yellow Enzyme; FMN = flavin mononucleotide; TPN = triphosphopyridine nucleotide; TPNH = reduced TPN.

MATERIALS AND METHODS

OYE was prepared by the method of Theorell and Åkeson [2], and the *apoprotein* obtained from the enzyme according to Warburg and Christian [3]. Evaluation of enzyme and apoprotein solutions with regard to purity, concentration, and equivalence was carried out as in the previous investigation [1]. OYE and apoprotein preparations used in the activity studies were of purity 0.54—0.70.

Glucose-6-phosphate (di-sodium salt), *glucose-6-phosphate dehydrogenase (Zwischenferment)*, *FMN*, *TPN* and *TPNH* used in the experiments were all commercial preparations (Sigma Chemical Company). The concentrations of the FMN, TPN and TPNH solutions were determined in a Beckman DU spectrophotometer at 445 mμ[2], 260 mμ[4], and 340 mμ[5], respectively. The activity of the Zwischenferment preparation was stated to be approximately 800 Kornberg units [6] per gram under optimal conditions. The reduction of TPN in the presence of Zwischenferment and excess glucose-6-phosphate in 0.13 M sodium phosphate buffer of pH 7.4 at +37°C was studied spectrophotometrically at 340 mμ[7]. At TPN concentrations from 1 to 20 × 10^{-6} M a fluorimetric technique [8] was employed by measuring the fluorescence of the TPNH formed and comparing it to that of a TPNH standard.

Immunization and testing of the antisera with precipitin reactions were performed as described before [1]. Two rabbits were used in these experiments. Each received 51.5 mg of an enzyme preparation of purity 0.54, mixed into an adjuvant, in the first injection, and 25.8 mg of the same preparation as a boosterdose. Control sera were collected from the same animals before immunization. Antisera used in the inhibition studies reacted with the crystalline enzyme, forming one single precipitin line, when tested with the agar-gel diffusion technique according to Ouchterlony [9]. Also, they showed identical reaction with the OYE and an equivalent apoprotein solution.

Activity studies of the OYE. The activity of the OYE was tested by the conventional Warburg manometric technique [10] in an oxidative system with glucose-6-phosphate, Zwischenferment, and TPN [11]. The activity of the test system is inhibited by the presence of phosphate [12,13] and other ions [14]. Nevertheless, in the present investigation a sodium phosphate buffer of pH 7.4 in a final concentration of 0.13 M was used, since it was considered appropriate to obtain a similar ionic atmosphere as in other OYE-antibody experiments. The reaction mixture also contained 0.2 ml 0.04 M KCN, and 0.2 ml 10 % KOH was added in the central well of each Warburg flask. In the different experiments the concentration of OYE or TPN varied. The addition of Zwischenferment was 1 mg, and glucose-6-phosphate was always added in excess. The volume of the reaction mixture was 3.0—4.0 ml.

In the inhibition studies with antiserum, the OYE was usually added after the serum and other reactants to the main compartment of the reaction vessel, and the reaction was started by the addition of Zwischenferment from the sidearm. When the effect of incubation time was studied, the mixing of the reactants followed a different procedure, which is described in the result section.

The experiments were performed at +37.0°C. The filled reaction vessels were equilibrated with oxygen for 15 min, then with stopcorks closed for another 15 min, before the reactions were started. The activity was expressed as μl oxygen consumed during the first 30 min of the reaction, when the oxygen uptake was constant.

Activity studies of the apoprotein. The activity of the apoprotein could be determined by the method used for the OYE, when FMN was also added to the reaction mixture. In order to estimate the influence of antiserum on the association between apoprotein and FMN, it was necessary to consider its effect on the reaction product OYE. The experiments were arranged in the following way (also illustrated in Table 1). OYE was formed in the presence of antiserum by incubating apoprotein and serum and, later, adding FMN equivalent to the apoprotein. The activity of this enzyme was compared to the activity of that formed in the presence of control serum. To eliminate the effect of the antibodies on the formed OYE, antiserum was also added in the second case. Besides the effect of a different incubation time, which could be controlled in separate experiments, a difference in activity could now be attributed to the fact that in the first case antiserum reacted with the apoprotein before the FMN was bound.

The reaction between apoprotein and FMN was also studied fluorimetrically [15] by adding small aliquots of a FMN solution to an apoprotein solution in 0.13 M sodium

Table 1. A scheme of the method for testing the influence of antiserum on the apoprotein, which later reacted with FMN to form OYE, the activity of which was measured.

Incubation, +22°C, 10 min.	Incubation, +22°C, 10 min.	Enzyme activity measured
Apoprotein Antiserum	+ FMN	+ Control serum
Apoprotein Control serum	+ FMN	+ Antiserum

phosphate buffer of pH 7.4 and observing the quenching of the fluorescence of the FMN. In the inhibition studies, antiserum was mixed with the apoprotein a few minutes before the FMN was added. The experiments were performed at +37°C.

RESULTS

The activity of the OYE in the Zwischenferment-OYE system. The oxygen consumption of the test system varied with the concentration of the OYE, when all other reactants were added in excess, *i.e.* further addition of anyone of these did not increase the activity. Using an enzyme solution of purity 0.64, 0.10 mg OYE * caused an uptake of 100 μl oxygen per 30 min. The turnover number corresponding to this value is 155, expressed as moles of oxygen per mole of OYE per minute **.

Keeping the OYE concentration constant, the activity varied with the concentration of TPN. Plotting the oxygen consumption against the negative logarithm of the TPN concentration resulted in a sigmoid curve (Fig. 1). Raising the TPN concentration to values higher than those shown in Fig. 1 did not cause increased activity. The same reaction is drawn as a double reciprocal plot [16] in Fig. 2, bottom line. The Michaelis constant determined from such a plot was 0.90×10^{-5} M. Spectrophotometric and fluorimetric studies of the reduction of TPN by the amount of Zwischenferment, used in each Warburg experiment, showed that the rate of reduction exceeded the rate of oxygen uptake in the Zwischenferment-OYE system at all TPN concentrations tested.

* In the following the amounts of OYE are always expressed as the amount of pure OYE, as calculated from the light absorption at 464 mμ.

** The corresponding value, expressed as moles of oxygen per mole of protein-bound FMN, is 77.5. This figure is higher than the previously reported values [3,11]. This may be explained by the fact that purer reagents, especially Zwischenferment, were used in the present investigation. Similar results were obtained with other OYE preparations.

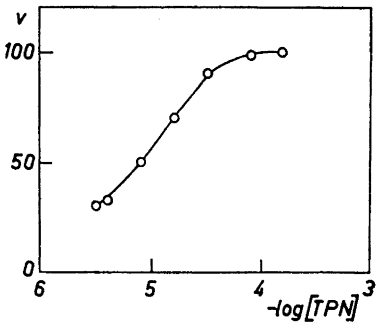


Fig. 1. The activity of 0.10 mg OYE at different concentrations of TPN, expressed as μl O_2 consumed per 30 min (v), and marked along the ordinate. Along the abscissa the negative logarithm of [TPN], expressed in moles per liter, is marked. Each reaction vessel also contained 1.75×10^{-2} M glucose-6-phosphate, KCN, and 0.13 M sodium phosphate buffer, pH 7.4. The reaction was started by adding 1 mg Zwischenferment. The total volume of the reaction mixture was 3.3 ml.

The results of the Warburg experiments were influenced by the ionic conditions. Thus it was found that lowering the phosphate concentration had no effect at high concentrations of TPN, but caused increased enzyme activity when less TPN was used, (*cf.* Refs.[12-13]).

The inhibition of the OYE by antiserum at different concentrations of TPN. The activity of 0.10 mg OYE (same preparation as in the preceding group of experiments) was tested at different TPN concentrations in the presence of antiserum of two different concentrations. The results are summarized in Fig. 2, where they are shown in a double reciprocal plot. The inhibition

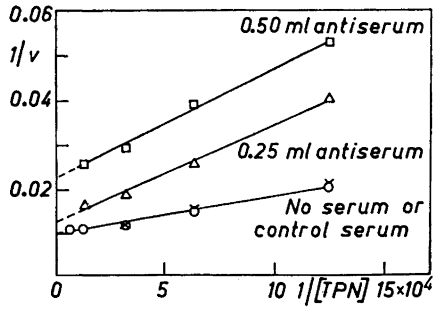


Fig. 2. The inhibition by antiserum of the activity of 0.10 mg OYE at different concentrations of TPN, shown in a Lineweaver-Burk plot. Along the ordinate $1/v$ is marked, along the abscissa 1/TPN. Antiserum and TPN were added before the OYE to the main compartment of the Warburg flask. Otherwise, the experimental conditions were as described under Fig. 1. Bottom curve O — O shows the activity without any serum added, × marks the activity in the presence of 0.50 ml control serum, □ — □ shows the activity in the presence of 0.50 ml antiserum, △ — △ the activity in the presence of 0.25 ml antiserum. Quantitive precipitin reaction between the OYE and antiserum used showed equivalence when 0.17 mg OYE was added to 1.00 ml antiserum.

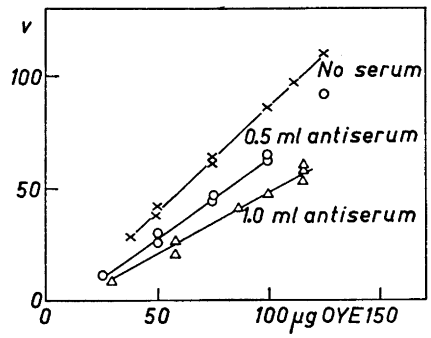


Fig. 3. The inhibition by antiserum at different concentrations of OYE. The activity (v) is marked along the ordinate, the amount of OYE added along the abscissa. Excess TPN was added in each reaction. Other experimental conditions essentially as described under Figs. 1 and 2. The top line × ——— × represents activity studies without the addition of antiserum, the other lines experiments where 0.5 ml (O — O) or 1.0 ml (△ — △) was added in each reaction. In quantitative precipitin reaction between the enzyme and serum used, 0.12 mg OYE was in equilibrium with 1.0 ml antiserum.

caused by the antiserum appeared to be non-competitive [16]. However, prolonging the lines, representing the inhibited and uninhibited reactions, to the left of the ordinate showed that it did not completely follow the kinetics of a non-competitive inhibition, but probably represented some intermediate form.

Control serum was practically without effect. In some experiments a slight inactivation was observed, perhaps due to an alteration of the ionic milieu. As can be seen in Figs. 2 and 5, the effect of control serum was by no means comparable to that of antiserum.

The inhibition of varying concentrations of OYE by antiserum. In these experiments different dilutions of an OYE preparation of purity 0.54 were used. TPN was added in excess. The results are shown in Fig. 3, where the top line shows the reaction without antiserum. The addition of 1 ml of control serum in each experiment gave essentially identical values. The prolongation of the line, representing the activity of the OYE without inhibitor, does not pass through the origin of the coordinate system. This is probably due to the fact that oxygen was released during the experiments, perhaps in connection with the tipping procedure. It might also be that a constant amount of enzyme was blocked by some impurity in the reaction mixture. There was a rectilinear relationship between the amount OYE added and the oxygen consumption. In the presence of antiserum there was a rather constant relative inhibition by the same amount of serum at the different OYE concentrations, at least at the low concentrations tested.

The inhibition by different concentrations of antiserum. The reactants were the same as in the preceding group of experiments, but the amount of OYE was 0.10 mg in each reaction, and the amount of antiserum added varied. Fig. 4 shows the relative activity of the OYE at the different serum concentrations. At the low concentrations the decrease in activity appeared to be pro-

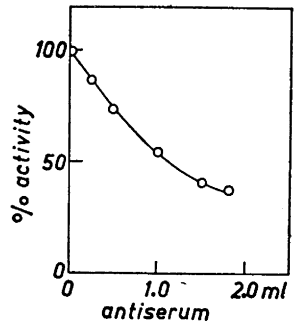


Fig. 4. The activity of 0.10 mg OYE at different concentrations of antiserum. Other experimental conditions were as described under Fig. 3, and the enzyme and antiserum were the same as those, used in the experiments illustrated in that figure. The relative activity is marked along the ordinate, where 100 % represents the activity in the absence of antiserum. (Addition of 1.0 ml control serum gave the same activity.) Along the ordinate the amount of antiserum, used in each experiment, is indicated.

portional to the amount serum added, but further increase in serum concentration was not as effective.

The effect of the incubation time on the inhibition of the OYE by antiserum. In all experiments described OYE was added to the main compartment of the Warburg flask, which contained glucose-6-phosphate, TPN, and antiserum, and Zwischenferment was added 30 min later from the sidearm. A series of experiments were performed in an identical way, except that the antiserum

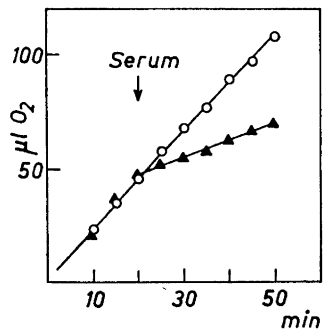


Fig. 5. The influence of antiserum on the "active" enzyme. 0.10 mg OYE was used in each reaction, the TPN concentration was 1.62×10^{-5} M. The reaction was started by the addition of Zwischenferment from one side-arm of the Warburg flask, the experimental conditions being the same as described under Fig. 1. After 20 minutes 0.50 ml serum was added from the other side-arm. Along the ordinate are marked consumed µl of O_2, along the abscissa the time in minutes from the start of the reaction. In the flask where antiserum was added (△ — △) there was a clear effect upon the reaction velocity. The addition of control serum (○ — ○) had some effect, although much less pronounced. Enzyme and antiserum were the same as those used in experiments illustrated in Fig. 2.

was not added at the beginning but kept in a second sidearm of the Warburg flask. After the reaction had been started, and the oxygen uptake studied for 20 min, the serum was added (Fig. 5). The inhibition caused by a given amount of antiserum under these conditions was of the same magnitude as when the antiserum was added at the same time as the TPN.

The inhibition was also studied when the OYE was added to the main compartment of the reaction vessel, containing glucose-6-phosphate, Zwischenferment, and antiserum, and the TPN was added 30 min later from the sidearm. This procedure did not cause any difference in the degree of inhibition except at low TPN concentrations, where a slight increase was noticed.

From these experiments it might appear that the inhibition was practically the same whether the antiserum was allowed to come in contact with the enzyme before, at the same time as, or after the TPN. However, when OYE and antiserum were first incubated for 30 min at + 37°C, then added to the Warburg flask, and the TPN was introduced 30 min later from the sidearm, the inhibiting capacity of the serum increased markedly. The antibodies could thus react with the OYE for 60 min before the appearance of the reduced TPN. Under the experimental conditions described in Fig. 4, 1.0 ml antiserum inhibited the activity of 0.10 mg OYE 45 %. Using the same reactants, but following the preincubation procedure described above, the inhibition was 71 %. This increase could not be due to the mere fact that OYE and antiserum had reacted for 60 min, because in the first case the inhibition was still approximately 45 % when OYE and serum had been mixed for a considerably longer time.

The effect of antiserum on the association reaction between apoprotein and FMN. No influence of the antiserum was observed when this reaction was studied by the manometric technique. These experiments were performed with concentrations of serum which had an immediate effect on an OYE solution, equivalent to the apoprotein used. Incubation with antiserum for a longer period of time, and removal of formed precipitate before the addition of FMN, decreased the activity of the apoprotein solution. When antiserum was added in excess, incubation performed overnight and the precipitate removed, no enzymatic activity remained.

The study of the reaction between apoprotein and FMN, using the fluorimetric technique, encountered certain difficulties, because the presence of serum caused a large background fluorescence, thus making the readings less accurate. Therefore, the experiments were carried out in excess antigen zone, but with serum concentrations that inhibited OYE solutions equivalent to the apoprotein used. The ability of the apoprotein to bind FMN was not impaired by the presence of antiserum. A typical experiment is shown in Table 2. Preincubation of apoprotein and antiserum before the addition of FMN resulted in the appearance of a precipitate, which made the fluorimetric technique unsuitable.

DISCUSSION

The special character of the enzyme-inhibitor system must be considered in interpreting kinetic data of the inhibition of enzyme activity by antibodies. Even if the antibodies possess a very specific affinity to the enzyme molecules,

Table 2. Results of a typical experiment for studying the influence of antiserum on the association reaction between apoprotein and FMN by the fluorimetric technique. The molarity of the FMN bound, and the association reaction velocity constant k_1 were determined as described in Ref.[15]. From the amount FMN bound in the sample of apoprotein, which did not contain serum, it was calculated that 0.19 mg apoprotein was added in each test. 0.17 mg of an equivalent OYE solution was needed to reach the equivalence zone in quantitative precipitin reaction with 1.0 ml of the antiserum used.

Reaction mixture to which FMN was added.	FMN coupled, M.	Association velocity constant, k_1, $M^{-1} \times sec^{-1}$.
3.0 ml apoprotein 0.5 ml buffer	1.058×10^{-6}	16.8×10^4
3.0 ml apoprotein 0.5 ml control serum	1.160×10^{-6}	17.6×10^4
3.0 ml apoprotein 0.5 ml antiserum	1.137×10^{-6}	15.9×10^4

there is no simple relationship in their reaction with the antigen. During the immunization antibodies of different reactivity may develop [17]. The inhibition may depend upon the number of antibody molecules bound to each enzyme molecule [18]. The enzyme-antibody interaction changes considerably with the time, and thus it is of importance at which stage in the reaction the enzyme is brought to act, that is to combine with the substrate [19,20]. The inhibition may be influenced by the fact that the inhibitor molecules are of equal magnitude as, or often larger than the enzyme [21]. Such characteristics emphasize the difficulties in arriving at definite conclusions from inhibition studies of enzyme-antibody reactions.

The OYE preparations used in the activity studies were not pure, and the antisera were obtained after immunization with impure OYE. Still it is reasonable to believe that the enzyme reacted with specific antibodies in the sera. Evidence in favour of this has been presented in a previous paper [1], and the antisera used in the activity studies reacted in an identical way when tested in precipitin reactions. The action of antiserum on Zwischenferment, which is also a yeast enzyme, was tested by measuring spectrophotometrically the ability of this enzyme to reduce TPN, and no effect was found.

In the system for testing the activity of the OYE this enzyme acts as an oxidant to the TPNH formed, when glucose-6-phosphate is oxidized in the presence of Zwischenferment and TPN. Observations have been made of the formation of a complex between the OYE and the reduced pyridine nucleotide [22,23], and it appeared likely that such a complex was formed in the present experiments. Confirmatory evidence seemed to be obtained, when the activity of the OYE was tested with different concentrations of TPN in the reaction mixture. Plotting the activity against the negative logarithm of the TPN

concentration resulted in a sigmoid curve (Fig. 1), similar to the dissociation curve typical of enzyme-substrate interactions [24]. This curve evidently described the activity of the OYE, since the activity of the Zwischenferment alone exceeded that of the Zwischenferment-OYE system. Also, further additions of TPN did not cause increased oxygen uptake when all reactants, except OYE, were in excess.

The inhibitory effect of antiserum on the OYE was immediate. From the experiments, illustrated in Fig. 3, where a constant amount of antiserum caused the same relative inhibition at different concentrations of OYE in the test system, it appeared that the enzyme-antibody association was reversible under the existing experimental conditions [25]. Also, the curvilinear relationship obtained, when the relative activity of a constant amount of OYE at different concentrations of antiserum was plotted against the amount antiserum added (Fig. 4), indicated a dissociation of the antigen-antibody complexes. When the OYE and antiserum were allowed to react for a long enough time before the enzymatic activity was tested, the inhibitory capacity of the antiserum increased. Since this occurred, when no changes were made in the experimental conditions except the preincubation of enzyme and serum, the effect must depend upon a firmer association between OYE and antibodies. Allowing the OYE to react with the TPNH seemed to protect the enzyme from the action of the antibodies. This observation is similar to the reduction of the inhibition, which has been found to occur in other enzyme-antibody systems, when the enzyme is incubated with the antiserum in the presence of substrate or coenzyme (see Ref.[21]), and which has been ascribed to the fact that the aggregation of enzyme-antibody complexes is prevented [19].

The studies of the inhibition with different concentrations of TPN in the reaction did not quite clarify, whether the antibodies and the pyridine nucleotide competed for the same binding sites. Since the enzyme-antibody reaction was reversible under the conditions of these experiments, and the K_M value in the OYE—TPNH system indicated a high affinity between enzyme and TPNH [24], the activity of the enzyme would have been completely dependent on the TPN concentration if the binding sites were identical. Therefore it seems more reasonable that the inhibition was non-competitive. That it nevertheless showed some trends of competitiveness could be due to the fact that the large inhibitor molecules caused a steric hindrance for the TPNH to reach its binding sites [21].

It appeared that concentrations of antiserum, having an immediately noticeable effect on the activity of the OYE, did not cause a corresponding inhibition of the ability of equivalent apoprotein solutions to bind FMN. In these experiments apoprotein and antiserum were mixed for a time, long enough to allow interaction, before the FMN was added. The affinity of the apoprotein to FMN is very high [15], and thus it is understandable that the FMN might have displaced the antibodies from the apoprotein, before the activity was tested in the Warburg experiments. However, the use of the fluorimetric technique, allowing direct registration of the association reaction and determination of the velocity constant, did not reveal any influence of the antiserum on the ability of the apoprotein to bind FMN, although this could only be tested at low serum concentrations. Such an effect has been demonstrated

after chemical modification of the apoprotein [26]. On the other hand, the apoprotein was precipitated by the antiserum. These results suggest that the antibodies were bound to other sites on the apoprotein molecule than the FMN, and thus they support the findings of previous precipitin reactions.

Besides the blocking of catalytic sites on the OYE, other factors might have contributed to explain the observed inhibition. The binding of large antibody molecules to the OYE must undoubtedly have altered the physical properties of the enzyme. It may also be that the association caused intramolecular changes in the enzyme, impairing its ability to function as an electron transmittor.

Acknowledgements. The author is indebted to Professor Hugo Theorell and Dr. A. D. Winer for helpful criticism and suggestions and to Dr. K. G. Paul and Dr. A. Ehrenberg for valuable discussions during the course of this work.

REFERENCES

1. Kistner, S. *Acta Chem. Scand.* **12** (1958) 2034.
2. Theorell, H. and Åkeson, Å. *Arch. Biochem. Biophys.* **65** (1956) 439.
3. Warburg, O. and Christian, W. *Biochem. Z.* **298** (1938) 368.
4. Kornberg, A. and Horecker, B. L. *Biochem. Preparations* **3** (1953) 24.
5. Horecker, B. L. and Kornberg, A. *J. Biol. Chem.* **175** (1948) 385.
6. Kornberg, A. *J. Biol. Chem.* **182** (1950) 805.
7. Warburg, O., Christian, W. and Griese, A. *Biochem. Z.* **282** (1935) 157.
8. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
9. Ouchterlony, Ö. *Arkiv Kemi, Mineral. Geol.* **26B** (1948) No. 14.
10. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. *Manometric Techniques and Tissue Metabolism.* Burgess Publ. Co. Minneapolis 1951.
11. Warburg, O. and Christian, W. *Biochem. Z.* **266** (1933) 377.
12. Theorell, H. *Biochem. Z.* **275** (1935) 416.
13. Negelein, E. and Haas, E. *Biochem. Z.* **282** (1935) 206.
14. Rutter, W. J. and Rolander, B. *Acta Chem. Scand.* **11** (1957) 1663.
15. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877.
16. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
17. Najjar, V. A. and Ficher, J. *Science* **122** (1955) 1272.
18. Marucci, A. A. and Mayer, M. M. *Arch. Biochem. Biophys.* **54** (1955) 330.
19. Cinader, B. *Biochem. Soc. Symposia (Cambridge, Engl.) No.* **10** (1953) 16.
20. Sevag, M. G., Newcomb, M. D. and Miller, R. E. *J. Immunol.* **72** (1954) 1.
21. Cinader, B. *Ann. Rev. Microbiol.* **11** (1957) 371.
22. Haas, E. *Biochem. Z.* **290** (1937) 291.
23. Ehrenberg, A. and Ludwig, G. D. *Science* **127** (1958) 1177.
24. Neilands, J. B. and Stumpf, P. K. *Outlines of Enzyme Chemistry* John Wiley and Sons, Inc. New York 1955.
25. Ackermann, W. W. and Potter, V. R. *Proc. Soc. Exptl. Biol. Med.* **72** (1949) 1.
26. Nygaard, A. P. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1587.

Received April 23, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1159–1166

A Spectroscopic Study of Molecular Interaction with Phosphoryl Chloride

PER-OLOF KINELL, INGVAR LINDQVIST and MARGARETA ZACKRISSON

Institute of Physical Chemistry and Institute of Chemistry, University of Uppsala, Uppsala, Sweden

Raman and infrared spectra of mixtures between $POCl_3$ and a number of other molecules have been studied. Two types of interaction have been found, one tentatively described as dipole-dipole, the other as electron-pair acceptor-donor interaction.

The phosphoryl (P—O) bond frequency in addition compounds with $POCl_3$ was first studied by Sheldon and Tyree[1,2] who correlated the decrease in frequency with the donor function of oxygen in the adduct formation with different acceptor molecules. In some cases, *e.g.* with $SnCl_4$ in $SnCl_4 \cdot 2POCl_3$, they obtained more than one infrared band in the expected phosphoryl frequency range, and explained this fact as due to the co-existence of two types of $POCl_3$, one bonded and one free. A recent crystal structure determination [3] proves, however, that both $POCl_3$ molecules in the solid compound are bonded in a very similar way, and another explanation must be sought. We therefore decided to make a more complete spectroscopic study of the molecular interaction with $POCl_3$, going from very weak to very strong interaction, covering a wider spectral range, using infrared and Raman spectroscopy. The present paper mainly deals with weak interaction.

EXPERIMENTAL

Chemicals. $POCl_3$, $AsCl_3$ and C_6H_5COCl were purified by distillation. (B. p. +105°, +129°, and +197°C.) CCl_4, CS_2, $CHCl_3$ and 1,2-$C_2H_4Cl_2$ were purified by fractional distillation (76°—77°, 46.5°, 61°—63° and 83°—84°C). $SnCl_4$ and $SbCl_5$ were purified according to Dodd and Robinson [4]. $SbCl_3$ was purified by sublimation.

Spectroscopic measurements. The Raman spectra, excited by Hg 4 358 Å, were photographed using an E 612 Hilger spectrograph equipped with two glass prisms and a camera having an aperture of f/5.7 (Exposure times 1—2 h). The illuminating system consisted of a Toronto type helical mercury lamp. A saturated solution of sodium nitrite in water was used as a filter. The temperature was 20°C. The spectroscopic arrangement is de-

scribed by Kinell [5]. Normally a Raman tube containing 20 ml was used. When the amount of solution was small, a 5 ml tube could be utilized. The tubes were filled very carefully in order to avoid as far as possible any contact between the liquids and moisture. For that purpose each tube had a ground joint by means of which it could be attached to the container holding the liquids.

The Ilford Zenith super sensitive plates used were processed with Kodak D-76 fine grain developer. The positions of the Raman lines were determined using Hartmann's disperson formula. As reference lines Hg 4 077, 4 339 and 4 916 Å were selected. Measurements were as far as possible made on several exposures for the same solution. The accuracy of the wave numbers is $\pm 1-3$ cm^{-1}, depending upon the shape of the line. In a few cases the lines were too weak to be measured in the comparator. The positions of such lines have been estimated approximately and are especially marked (with circles) in the figures. No measurements of the band width and the intensities were made. The spectral slit width was $5-10$ cm^{-1}.

The infrared spectra were recorded on a Perkin-Elmer Model 21 Spectrophotometer equipped with NaCl, KBr and CsBr prisms (in the Institute of Biochemistry). The spectra were run with the solutions in thin layers between two KBr plates. The spectral slit width was 3 cm^{-1}.

RAMAN SPECTRA OF $POCl_3$ MIXED WITH SOME OTHER MOLECULES

The following molecules, in order of increasing dipole moment and dielectric constant, have been studied: $CS_2 = CCl_4 < CHCl_3 < 1,2\text{-}C_2H_4Cl_2 < AsCl_3 = C_5H_5N < C_6H_5COCl < SbCl_3$. $POCl_3$ would come between C_5H_5N and C_6H_5COCl in this sequence and the spectrum of pure liquid $POCl_3$ has been placed accordingly in Fig. 1 showing the $POCl_3$ frequencies in the different mixtures. (A few lines could not be measured because of overlap with the spectrum of the other molecules.)

The very interesting molecules $SO(CH_3)_2$, $SeOCl_2$, CH_3CN and C_6H_5CN were also tried but gave complicating chemical reactions with $POCl_3$.

A brief summary and discussion of the results is given in the following.

1. The shifts in the four lowest frequencies are very small. The largest shift is obtained with $SbCl_3$ for the symmetrical P-Cl bond vibration frequency, 486 cm^{-1}, (+ 7 cm^{-1}).

2. The antisymmetrical P-Cl bond vibration frequency, 584 cm^{-1}, is shifted towards higher frequencies with all solvents except C_5H_5N, the largest shifts being found with $AsCl_3$ (13 cm^{-1}) and $SbCl_3$ (20 cm^{-1}).

3. The symmetrical phosphoryl bond vibration frequency, 1 297 cm^{-1}, shows both positive and negative shifts. The largest positive shift is obtained with the nonpolar CCl_4 (7 cm^{-1}), the largest negative shifts with $AsCl_3$ (13 cm^{-1}) and with $SbCl_3$ (29 cm^{-1}).

4. There is obviously no direct correlation between dipole moment and shift. It has been pointed out, however, that a dipole-dipole interaction depends also upon the charge distribution in the molecules [6].

5. Fig. 1 shows that $AsCl_3$ and $SbCl_3$ are exceptional compared with the other molecules. These two molecules differ in two respects from the others. Their molecular shapes offer good opportunities for approach to the center of the positive charge in the dipole, their charge distributions are thus favourable for dipole-dipole interaction. They are, however, also the only possible acceptor molecules (Lewis acids) among the compounds studied.

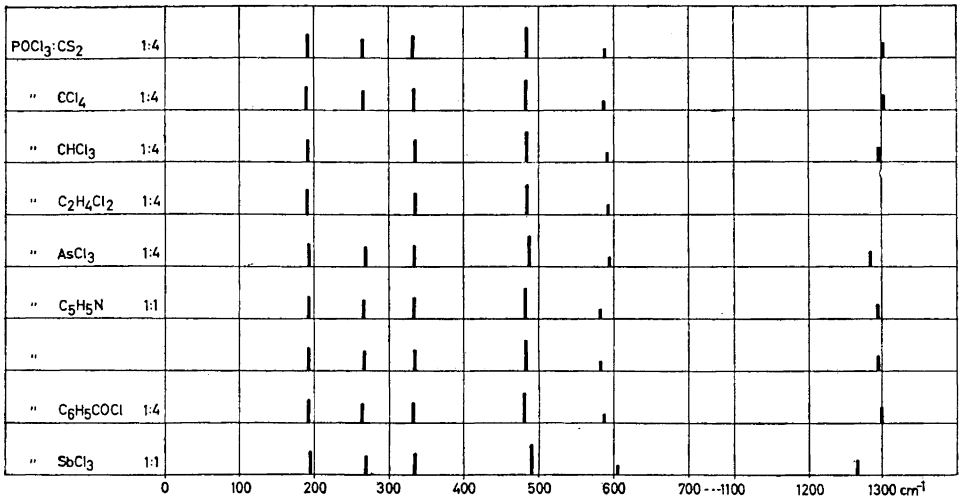


Fig. 1. Raman spectra of $POCl_3$ in different mixtures.

These two attributes are thus inseparable in this case. This is not the rule, however, as most Lewis acids are nonpolar molecules like $SnCl_4$ and $SbCl_5$.

6. The increase in the antisymmetrical P—Cl bond frequency has an analogy in the increases of the antisymmetrical C—C chain vibration frequency obtained by compound formation between acetone and acceptor molecules like BF_3 (52 cm^{-1}) or $AlBr_3$ (40 cm^{-1}) [7,8].

7. The large decreases in the symmetrical phosphoryl bond frequency by adduct formation were first measured by Sheldon and Tyree [1,2], who also found large negative shifts of the Se—O bond frequency in $TiCl_4 \cdot 2SeOCl_2$. Similar effects on the carbonyl bond frequency in ketones have also been reported [7,9]. In all these cases the shift has been caused by interaction with Lewis acids. This does not exclude the possibility, however, that the same effect can be obtained by favourable dipole-dipole interaction.

8. The spectrum with C_5H_5N is of particular interest because of the chloride ion transfer processes caused by C_5H_5N in $POCl_3$. Gutmann and Baaz [10] have recently studied the conductances in the similar system $(C_2H_5)_3N$-$POCl_3$ and conclude that the following equilibria exist:

$$POCl_3 + Et_3N = [POCl_2(Et_3N)^+Cl^-] \text{ (ion pair)} = POCl_2(Et_3N)^+ + Cl^-$$

The corresponding K values are 2.3×10^{-2} and 1.2×10^{-3}, and it is thus probable that the effect on the spectrum of $POCl_3$ should be small. (The ionic transfer process can in this case not be preceded by a compound formation $POCl_3 \cdot Et_3N$ because $POCl_3$ has no acceptor properties. The conditions are quite different with $SeOCl_2$ or $AsCl_3$ and amines [11].) The small shifts obtained with C_5H_5N in this case are thus easy to understand. The possibility cannot

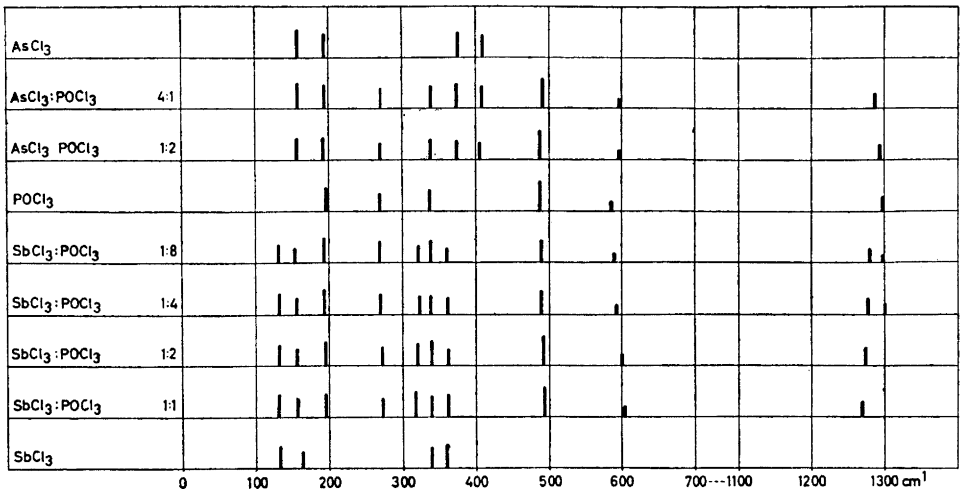


Fig. 2. Raman spectra of the systems $AsCl_3$—$POCl_3$ and $SbCl_3$—$POCl_3$.

be excluded, however, that the exceptional influence of C_5H_5N on the antisymmetrical P-Cl bond vibration (decrease in frequency instead of increase as obtained with all the other molecules) is associated with a tendency to loosen the P-Cl bond.

RAMAN SPECTRA OF THE SYSTEMS $AsCl_3$-$POCl_3$ AND $SbCl_3$-$POCl_3$

The Raman spectra of solutions with varying mole ratios $AsCl_3$:$POCl_3$ and $SbCl_3$:$POCl_3$ are given in Fig. 2. A brief summary and discussion of the results follows.

1. In both systems a *continuous* change in band positions is found.

2. This change is not connected with any obvious tendency to band broadening. The band profile of the antisymmetrical P-Cl band is slightly altered, however.

3. The continuous change would be expected for a dipole-dipole interaction without formation of stable molecules. The same effect has been reported for the system H_2O_2—$C_4H_8O_2$ where H_2O_2 has an exceptional influence upon the dioxane spectrum (over a range of 19 cm^{-1}). The authors [12] comment: "Die Wechselwirkung muss also dynamischer Art sein und von der ganzen Umgebung ausgehen".

4. The $AsCl_3$ spectrum is almost unchanged in the mixtures.

5. The $SbCl_3$ spectrum is changed more in the concentration range studied but not appreciably. The spectrum of liquid $SbCl_3$ (from Kohlrausch, *Hand- und Jahrbuch der chemischen Physik*, 9:IV, Leipzig 1943) is taken at + 250°C and cannot safely be used for comparisons.

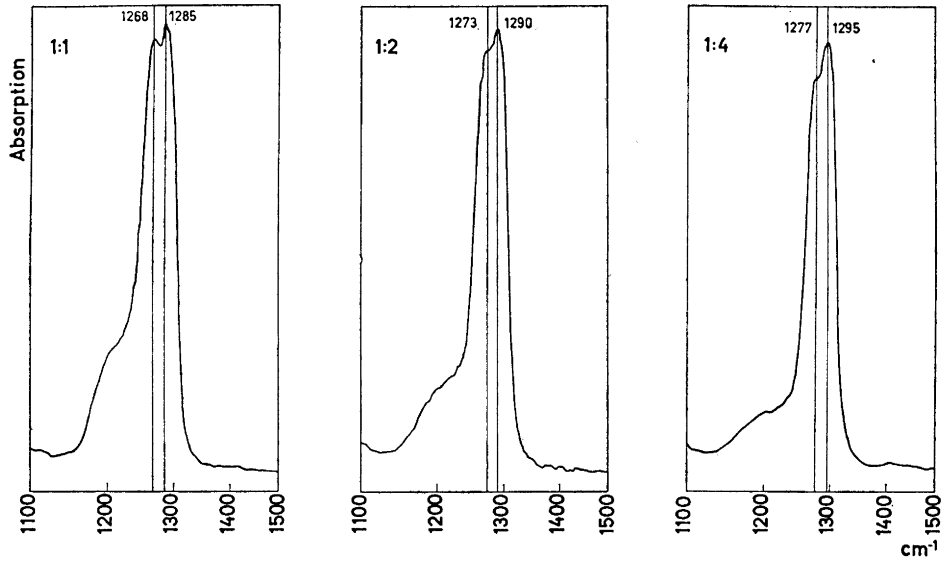


Fig. 3. Infrared spectra, in the phosphoryl band region, of the system $SbCl_3$—$POCl_3$. The shoulder near 1 200 cm^{-1} is a $POCl_3$ overtone band.

6. The typical Lewis acids usually change their structures by adduct formation with donor molecules like $POCl_3$ (*cf.* the structure of $SbCl_5 \cdot POCl_3$[13]). As a result the spectrum of the acceptor molecule disappears by the adduct formation.

7. In these cases therefore everything indicates dipole-dipole interaction. It would be very interesting to know the structure of the compound $AsCl_3 \cdot POCl_3$ [11] (M.p. —24°C) to find out whether the formation of the solid compound is also due to electrostatic interaction.

8. The only point of evidence indicating the existence of free and bonded molecules is the double phosphoryl band obtained with excess of $POCl_3$. One band is shifted towards lower frequencies, the other is almost fixed. The latter successively disappears with increasing concentration of $SbCl_3$ and cannot be seen at the composition $SbCl_3 \cdot 2POCl_3$. This evidence will be discussed in the next section.

INFRARED SPECTRA OF THE SYSTEM $SbCl_3$-$POCl_3$

Infrared spectra have been taken of all the systems discussed in the preceding section. They have confirmed the Raman spectroscopic results in all cases but one, and are therefore not reproduced here. The exception is the disappearance of the double phosphoryl band at mole ratios $POCl_3$:$SbCl_3$ smaller than 2:1 (Fig. 3). The trend in the intensity ratio is the same but the intensity of the high frequency band is not reduced as much as in the Raman spectrum. It is actually as strong as the low frequency band even at concentrations where

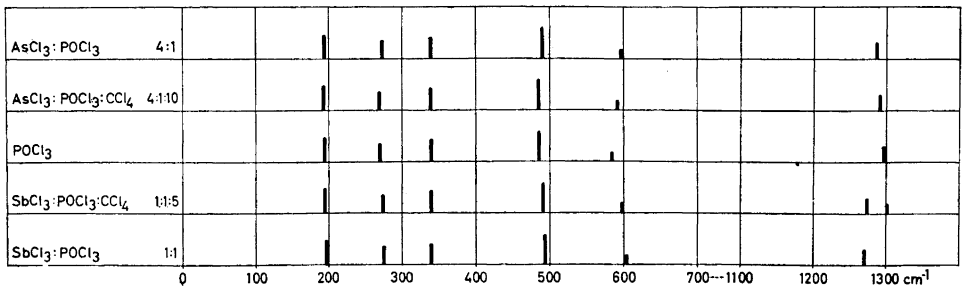


Fig. 4. Raman spectra of solutions in CCl_4.

it has disappeared in the Raman spectrum. This indicates that the disappearance in the latter at a composition $SbCl_3 \cdot 2POCl_3$ is purely incidental and is unrelated to compound formation. This result shows the importance of parallel work with Raman and infrared spectra. A study of the phosphoryl band only in the Raman spectrum would in this case have led to a completely erroneous interpretation.

RAMAN SPECTRA OF MIXTURES $POCl_3$-$AsCl_3$-CCl_4 AND $POCl_3$-$SbCl_3$-CCl_4

The Raman spectra of the solutions $POCl_3$:$AsCl_3$:CCl_4 = 1:4:10 and $POCl_3$:$SbCl_3$:CCl_4 = 1:1:5 have been studied. The $POCl_3$ bands are shown in Fig. 4 compared with the CCl_4-free mixtures.

The spectra indicate a diluting effect of CCl_4, reducing the shifts caused by $AsCl_3$ and $SbCl_3$ in agreement with the interpretation as dipole-dipole interaction.

RAMAN SPECTRA OF SOLUTIONS OF STRONG LEWIS ACIDS IN $POCl_3$

A preliminary study has been made of solutions of strong acceptor molecules in $POCl_3$ ($SbCl_5$:$POCl_3$ = 1:20 and $SnCl_4$:$POCl_3$ = 1:9). The results are shown in Fig. 5 together with the spectra of $SbCl_5$ (from Kohlrausch, *Hand- und Jahrbuch der chemischen Physik*, 9:IV, Leipzig 1943), $POCl_3$ and $SnCl_4$. A summary and discussion is given in the following.

1. The spectra of $SnCl_4$ and $SbCl_5$ have disappeared; as expected for strong Lewis acids.

2. The spectrum of free $POCl_3$ (in excess) is found in both cases as expected for strong compound formation of acceptor-donor type (in contrast to the continuous effect by electrostatic interaction).

3. New bands are formed. These have not been analysed but the knowledge of the exact crystal structure [13] has opened possibilities for a complete analysis.

4. Some of the free $POCl_3$ bands may also belong to the spectra of the adduct molecules $SbCl_5 \cdot POCl_3$ and $SnCl_4 \cdot 2POCl_3$ formed. Infrared spectra

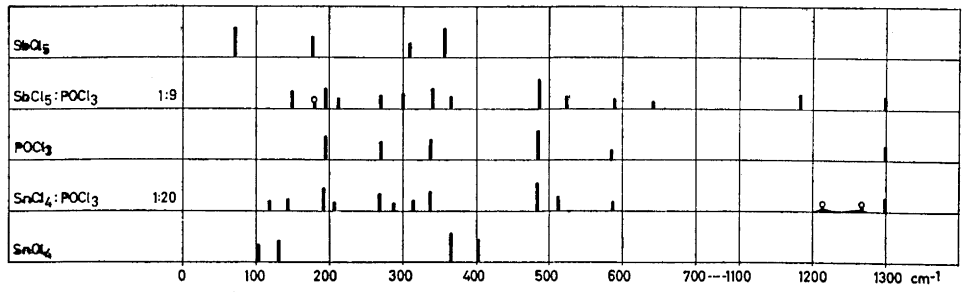


Fig. 5. Raman spectra of solutions of $SbCl_5$ and $SnCl_4$ in $POCl_3$.

of the solid compounds actually show that the phosphoryl band near 1 297 cm^{-1} is also present in the adduct spectra.

5. The phosphoryl band region thus shows two bands with $SbCl_5$ (the maximum shift is 117 cm^{-1}), and three bands with $SnCl_4$ (the maximum shift is 85 cm^{-1}).

6. Similar large negative shifts with strong Lewis acids have been mentioned earlier in this paper [1,2,7,9] and the splitting has also been found earlier. Examples of such splitting of carbonyl bands has been given by Susz and Chelandon [7,9]. It has also been reported for the Se—O band [2] in, *e.g.*, $SnCl_4 \cdot 2SeOCl_2$.

7. The opinion that the splitting is due to the existence of two types of donor molecules in the adduct has already been rejected in the introduction to this paper. We have no alternative explanation to offer at present but think that the common occurrence of the splitting should be emphasized.

8. A complicating fact is that the phosphoryl band splitting has also been found in some pure phosphoryl compounds [14]. In those cases the variation is largest in the high-frequency band which has therefore been considered as the typical phosphoryl band.

9. The magnitude of the shift for the lowest frequency phosphoryl band (obtained by the splitting by adduct formation) seems to be a rough measure of the acceptor strength of the acceptor molecule. An interesting survey of the shifts in the carbonyl band has been published recently [9]. If this relation has general validity a very simple method of determining relative acceptor strengths is available. It can, however, only be used if the acceptor molecules to be compared are under very similar conditions.

10. The donor strength of the oxygen atom is not reflected directly in the shifts. The weak donor molecule $SOCl_2$ does not show any shift [2], but $POCl_3$ gives a larger shift than POR_3[1] with the same acceptor molecule although the latter without doubt is a stronger donor (*cf.* the recent assessment of the relative donor strengths of $POCl_3$ and $PO(CH_3)_3$[15]).

CONCLUDING DISCUSSION

The preceding discussions can be summarized as follows: Negative shifts and splitting of the phosphoryl band can be caused by dipole-dipole interaction as well as by Lewis acid-base reactions. The former will give rise to a general medium effect (the shifts vary continuously with the mole ratios). The latter will lead to formation of kinetically stable adduct molecules (spectra of free and bonded molecules). The spectrum of the acceptor molecule will disappear if its structure is changed by the adduct formation. (This is not a necessary condition with molecules like $HgCl_2$).

If this description is correct (it can only be considered as a tentative suggestion) $AsCl_3$ and $SbCl_3$ give favourable dipole-dipole interaction with $POCl_3$ (in contrast to C_6H_5COCl), while $SnCl_4$ and $SbCl_5$ give adduct formation by acceptor-donor reactions. It is possible that there exist intermediate cases. We have here deliberately not discussed the nature of the acceptor-donor bond, pending the accumulation of more structural informations, but we intend to study these problems further by spectroscopic and X-ray methods.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.
Finally we wish to thank Professor S. Claesson, Professor G. Hägg and Professor A. Tiselius for all facilities put at our disposal and Mr. S. Bergvall for valuable assistance.

REFERENCES

1. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **80** (1958) 4775.
2. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **81** (1959) 2290.
3. Brändén, C. I. and Lindqvist, I. *To be published.*
4. Dodd, R. E. and Robinson, P. L. *Experimental Inorganic Chemistry*, Amsterdam 1954, p. 210, 216.
5. Kinell, P.-O. *Arkiv Kemi. In press.*
6. Bauer, E. and Magat, M. *J. phys. radium* **9** (1938) 319.
7. Chelandon, P. and Susz, B. P. *Helv. Chim. Acta* **41** (1958) 697.
8. Terenin, A., Filimonov, W. and Bystrov, D. *Z. Elektrochem.* **62** (1958) 181.
9. Susz, B. P. and Chelandon, P. *Helv. Chim. Acta* **41** (1958) 1332.
10. Gutmann, V. and Baaz, M. *To be published.*
11. Agerman, M., Andersson, L. H., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **12** (1958) 477.
12. Simon, A. and Fehér, F. *Z. Elektrochem.* **42** (1936) 688.
13. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134; *Acta Cryst. In press.*
14. Bell, J. V., Heisler, J., Tannenbaum, H. and Goldenson, J. *J. Am. Chem. Soc.* **76** (1954) 5185.
15. Kinell, P.-O., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **13** (1959) 190.

Received March 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1167–1170

Reappearance of Metachromasia after Digestion of Hyaluronate with Hyaluronidase

L. HANSEN, B. HEGNER and C. E. JENSEN

Københavns Universitets Fysisk-Kemiske Institut, Copenhagen, Denmark

The present investigation supports the recent theories on the metachromatic reaction. It is shown that the metachromatic colour can reappear after digestion of hyaluronate with testicular hyaluronidase. A difference exists between the absorption maxima of hyaluronate and heparin.

It has long been recognized that certain macromolecular substances present in animal tissues, *e.g.* acidic mucopolysaccharides, couple with basic dyes such as azure A, thionine and toluidine blue, forming coloured complexes. The term metachromasia is used to describe the phenomenon by which these tissue structures are stained by the dye solutions to a colour which is different from the dye solutions, the colours of the dye solution and of the stained tissue or solution being called orthochromatic and metachromatic, respectively. The metachromatic reaction combined with enzymic investigations is widely used for the detection and differentiation of substances such as hyaluronic acid, chrondroitin sulphuric acids and heparins.

MATERIAL AND EXPERIMENTS

The samples of the hyaluronate employed were prepared according to Jensen [1]. The preparation of toluidine blue used was a commercial one ("Toluidinblau nach Hoyer", Riedel de Haën A.-G., Seelze, Hannover, Germany). In some experiments this commercial product was recrystallized from water. The employed preparations of hyaluronidase were commercial Invasin "Lundbeck", Copenhagen, and "Penetrase" which was kindly supplied by Dr. Jørgen Ploug, Leo Pharmaceutical Products, Copenhagen. The sample of chondroitin sulphate used was prepared by Boström, Institute of Medical Chemistry, University of Uppsala, Sweden. The used sample of heparin was kindly placed at our disposal by Novo Terapeutisk Laboratorium, Copenhagen. All experiments were performed at pH 6.5.

The absorption of aqueous solutions of toluidine blue was measured in a Beckman Spectrophotometer in the interval of 200—800 mμ, the concentration of toluidine blue being 0.01 % (w/v). In Fig. 1 curve B shows two absorption

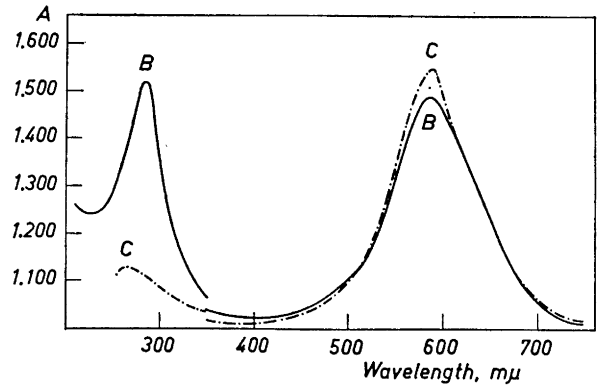


Fig. 1. Absorption spectra of commercial toluidine blue (B) and recrystallized toluidine blue (C). Ordinates are the absorbance $A = \log_{10} (1/T)$, where $T = I/I_0$. Length of cuvette 1 cm. Concentration of toluidine blue 0.1 g/l. Abscissae are the wavelengths in mμ.

maxima at 285 mμ and 590 mμ, respectively. The curve C (stippled) which is for recrystallized toluidine blue, likewise shows two maxima, at 265 and 590 mμ, respectively. It is seen that the first maximum has shifted towards a shorter wave-length after recrystallization.

Fig. 2 shows the absorption of a solution which was 0.03 % with regard to hyaluronate and 0.01 % with regard to toluidine blue, the solvent being a phosphate buffer (pH = 6.5). It is seen that the hyaluronate-toluidine blue complex has absorption maxima at 284 and 555 mμ.

It was found that the chondroitin sulphate-toluidine blue complex shows the same maxima as does hyaluronate-toluidine blue, whereas the heparin-toluidine blue complex shows a maximum at 540 mμ as seen in Fig. 3.

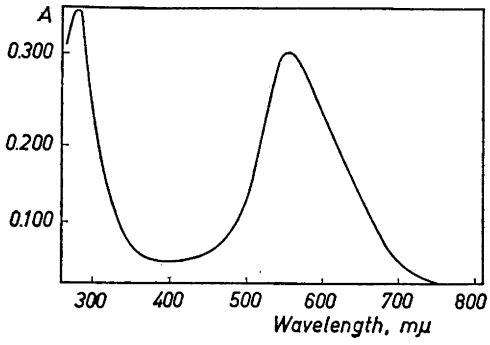


Fig. 2. Absorption spectrum of the hyaluronate-toluidine blue complex. Experimental conditions: *cf.* text to Fig. 1.

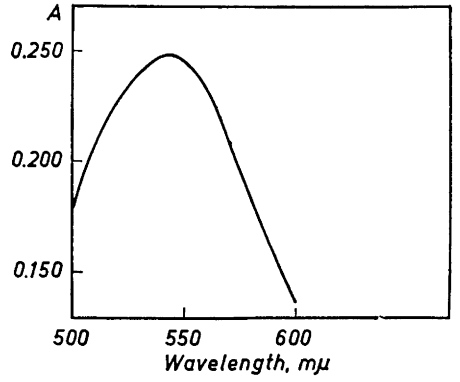


Fig. 3. Absorption spectrum of the heparin-toluidine blue complex. Experimental conditions: *cf.* text to Fig. 1.

Table 1. The changes in absorption spectrum of hyaluronate-toluidine blue complex caused by hyaluronidase digestion.

Expt. No.	Concn. of hyaluronate in %	Concn. of hyaluronidase in VRU	Time after incubation, min.	Absorption maxima, mμ	Type of enzyme
1	0.25	0	0	555	
2	0.25	10	5	585	Invasin
3	0.25	10	18	563	Invasin
4	0.25	10	5	585	Penetrase
5	0.25	2.5	2	580	Invasin
6	0.25	2.5	30	590	Invasin
7	0.25	2.5	1 290	563	Invasin
8	0.25	2.5 + 4	1 320	586	Invasin + Penetrase
9	0.25	2.5 + 4	1 410	585	Invasin + Penetrase
10	0.25	2.5 + 4	1 560	590	Invasin + Penetrase
11	0.30	7.5	9	560	Invasin
12	0.30	7.5	19	585	Invasin
13	0.30	7.5	30	585	Invasin
14	0.30	7.5	60	585	Invasin
15	0.30	7.5	90	560	Invasin
16	0.30	7.5	120	560	Invasin
17	0.30	7.5	240	560	Invasin
18	0.30	17.6	8	560	Penetrase
19	0.30	17.6	60	560	Penetrase
20	0.30	17.6	240	560	Penetrase
21	0.30	17.6	300	560	Penetrase
22	0.30	26.4	315	570	Penetrase
23	0.30	26.4	360	575	Penetrase
24	0.30	4.4	2	575	Penetrase
25	0.30	4.4	150	580	Penetrase
26	0.30	4.4	240	585	Penetrase
27	0.30	4.4	1 080	585	Penetrase

In the following experiments the hyaluronate solutions were incubated with testicular hyaluronidase. At different times aliquots were withdrawn and toluidine blue was added, whereafter the absorptions were recorded in order to follow the degradation of hyaluronate. Table 1 gives the results of these experiments. It is noted that toluidine blue has been added in all experiments, and that toluidine blue plus enzyme and buffer did not change the maximum of toluidine after 19 h.

It is evident from experiments 1,2,4 and 18—27 that the hyaluronate has been degraded by the hyaluronidase as the absorption maxima have shifted towards the maximum of pure toluidine blue during the incubation dependent upon concentration of enzyme and time. It is seen from the table that a difference exists between the two enzyme preparations. While penetrase always degrades hyalyronate at a certain (high) concentration, the invasin preparation causes only a temporary degradation (judged from the metachromatic reaction) as the absorption maximum starts moving towards the toluidine blue maximum, but later on the maximum shifts back to the hyaluronate-

toluidine blue complex maximum. (Expts. 11—17). Further it was noted that the solutions became more viscous from the time at which the maximum moved from 585 to 563 (Expts 1, 2 and 3 and 5,6 and 7 and 12—16 and 17).

DISCUSSION

According to recent theories (see, *e.g.*, McManus [2], Kinzel [3] and Larsen [4]) the reactions making the basis of the metachromatic reaction are the following: The positively charged dye-stuff ions are adsorbed to the negatively charged polyelectrolyte ions. Now, if the polyelectrolyte molecule has a sufficient size and electric surface charge density (Sylvén [5]) the dye ions will aggregate longitudinally, which means that a polymerization of the dye occurs. According to Michaelis and Granick [6] and others [7] the metachromatic colour of the dye depends on a polymerization of the dye itself.

The facts reported in the present paper seem to support the above theory. When a sample of high-molecular hyaluronate ($\overline{M}_{n\ (reduced)}$ = about 8×10^5) was used the metachromatic reaction was strong. With decreasing degree of polymerization caused by enzymic degradation the staining property or toluidine blue decreased markedly.

However, in some experiments the metachromatic colour reappeared after some time, and at the same time the viscosity of the solutions increased markedly. These observations suggest that a restructuration of hyaluronate has taken place resulting in a re-polymerization of the toluidine blue molecules.

Note added in proof: The observation that the viscosity of a solution of partly degraded hyaluronate can increase with time seems to agree with the results obtained by S. Zamenhof and E. Chargoff (*J. Biol. Chem.* **186** (1950) 207). They showed that considerably degraded DNA may have a tendency to repolymerize, attaining high viscosity. In contrast to this, undegraded DNA retains its η_{sp} constant during several hours.

We are indebted to Professor J. A. Christiansen, head of this institute, for his interest in this investigation. One of us (C.E.J.) wishes to thank Københavns Universitet for financial support.

REFERENCES

1. Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 603.
2. McManus, J. F. A. in Asboe-Hansen, G. *Connective Tissue in Health and Disease*, Munksgaard, Copenhagen 1954, p. 31.
3. Kinzel, H. *Protoplasma* **50** (1958) 1.
4. Larsen, B. *J. Histochem. Cytochem.* **6** (1958) 181.
5. Sylvén, B. *Quart. J. Microscop. Sci.* **95** (1954) 327.
6. Michaelis, L. and Granick, S. *J. Am. Chem. Soc.* **67** (1945) 1212.
7. Bergeron, J. A. and Singer, M. *J. Biophys. Biochem. Cytol.* **4** (1958) 433.

Received April 9, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1171–1175

A Phosphopeptide Isolated from Bovine α-Casein after Tryptic Hydrolysis

HANS BENNICH, BENGT JOHANSSON and RAGNAR ÖSTERBERG

Department of Medical Biochemistry, University of Gothenburg, Sweden

Bovine α-casein has been subjected to a short-time tryptic hydrolysis. From the mixture of phosphopeptides in the hydrolysate an apparently homogeneous phosphopeptide has been isolated through zone electrophoresis. From the amino acid composition and phosphorus analyses the empirical formula is suggested to be: Asp_3, Glu_9, Gly_1, Ala_2, Val_2, $Ileu_3$, Met_2, Ser_5, Thr_1, Pro_1, Lys_{1-2}, $(PO_4)_6$. The N-terminal position is occupied by an aspartic acid residue and a lysine residue is probably in the C-terminal position.

Enzymic degradation of cow's casein with pepsin, followed by pancreatic enzymes, yields a mixture of phosphorus-containing peptides [1,2], which has been prepared and studied by several authors; the literature has been reviewed by Mellander [3] and Perlmann [4]. Attempts to purify such a mixture at this institute resulted in the isolation of a phosphopeptide consisting of eleven amino acids and three phosphate groups [5].

Peterson *et al.*[6] diminished the number of possible phosphopeptides resulting from hydrolysis of casein by considering the inhomogeneity of this protein and by using one specific enzyme for the degradation. These authors thus found that only one phosphopeptide could be recovered after treatment of β-casein with trypsin. On the other hand, they found that tryptic digestion of α-casein furnished at least three different phosphopeptides. Later, Pantlitschko and Gründig [7,8] reported the preparation of "phosphopeptones" from α- and β-casein.

As part of a study of the structure and metabolic significance [9,10] of phosphopeptides from bovine casein this paper describes the preparation and main composition of an apparently homogeneous phosphopeptide from α-casein. The isolation of this phosphopeptide was undertaken through zone electrophoresis of a crude phosphopeptide fraction, obtained from a short-time tryptic hydrolysate of the α-casein.

MATERIAL

α-Casein was prepared from bovine casein according to the method described by Hipp *et al.*[11] The product obtained was homogeneous in moving boundary electrophoresis (0.1 M veronal buffer, pH 8.7). The preparation showed an atomic ratio N/P of 32.9.

The trypsin was a commercial preparation (Armour, twice crystallized, containing 50 % $MgSO_4$).

METHODS

Tryptic hydrolysis and preparation of a crude phosphopeptide fraction. 30 g of α-casein were suspended in 1 litre of distilled water, and the pH adjusted to 8.0 by addition of N NaOH. The solution was incubated in a water bath at 25°. Two hundred mg of trypsin, dissolved in a small volume of distilled water, were added to the substrate. The hydrolysis was carried out at a constant pH recorded by a pH-meter, titrator TTT 1a (Radiometer, Copenhagen). The course of hydrolysis was followed by measuring the amount of 0.5 N NaOH necessary for maintaining the pH at 8.0 in the reaction mixture. The reaction was almost complete after 50 min, and interrupted by boiling the solution for 2 min. The solution was cooled and the pH rapidly adjusted to 4.6 by addition of glacial acetic acid. Small amounts of precipitate was filtered off through a fluted paper (Munktell 95). From the slightly opalescent filtrate, a crude fraction containing phosphopeptides was precipitated by adding saturated lead acetate [8]. The precipitate was suspended in distilled water and the pH adjusted to 7.5 with NaOH. The lead was removed by a stream of hydrogen sulphide, the lead sulphide centrifuged off, and the supernatant precipitated by addition of several volumes of absolute ethanol. After lyophilization, the yield of substance was 2.2 g. Analyses of nitrogen and phosphorus gave an atomic ratio N/P of 6.2. In order to find out whether any carbohydrate from α-casein [12] could be recovered in this fraction, analyses of hexose [13], hexosamine [14] and sialic acid [15] were carried out. Only small amounts (0.12 %) of hexose could be detected.

Electrophoresis. Moving boundary electrophoresis of the crude fraction was run in 0.1 M sodium acetate buffer at pH 4.6. Three peaks appeared. Paper electrophoresis (apparatus manufactured by LKB, Stockholm; Schleicher and Schüll paper 2043 B, 4 × 41 cm) in 0.2 M formic acid, adjusted to pH 2.8 with triethyl amine, revealed at least six ninhydrin-positive zones (0.3 % ninhydrin dissolved in ethanol:glacial acetic acid, 99:1, v/v) (Fig. 1A). At least four of these gave positive reaction with the phosphorus reagent described by Hanes and Isherwood [16] (Fig. 1B).

Isolation of a zone electrophoretic component. Small amounts (20—30 mg) of the fastest moving component was eluted from cut out strips of 40 electropherograms with distilled water, after localization of zones in ultraviolet light. Up to 3 mg of crude phosphopeptide could be applicated on every paper. Re-electrophoresis under the same conditions of the eluted fraction showed an essentially homogeneous component (Fig. 1C). Isolation of other components in pure form by this method was not very successful.

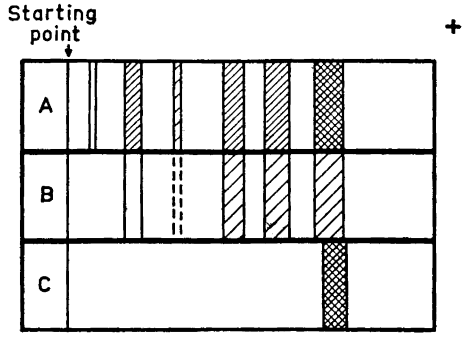


Fig. 1. Paper electrophoretic patterns. A. Crude phosphopeptide, ninhydrin color. B. Crude phosphopeptide treated with phosphorus reagent. C. Isolated component, ninhydrin color.

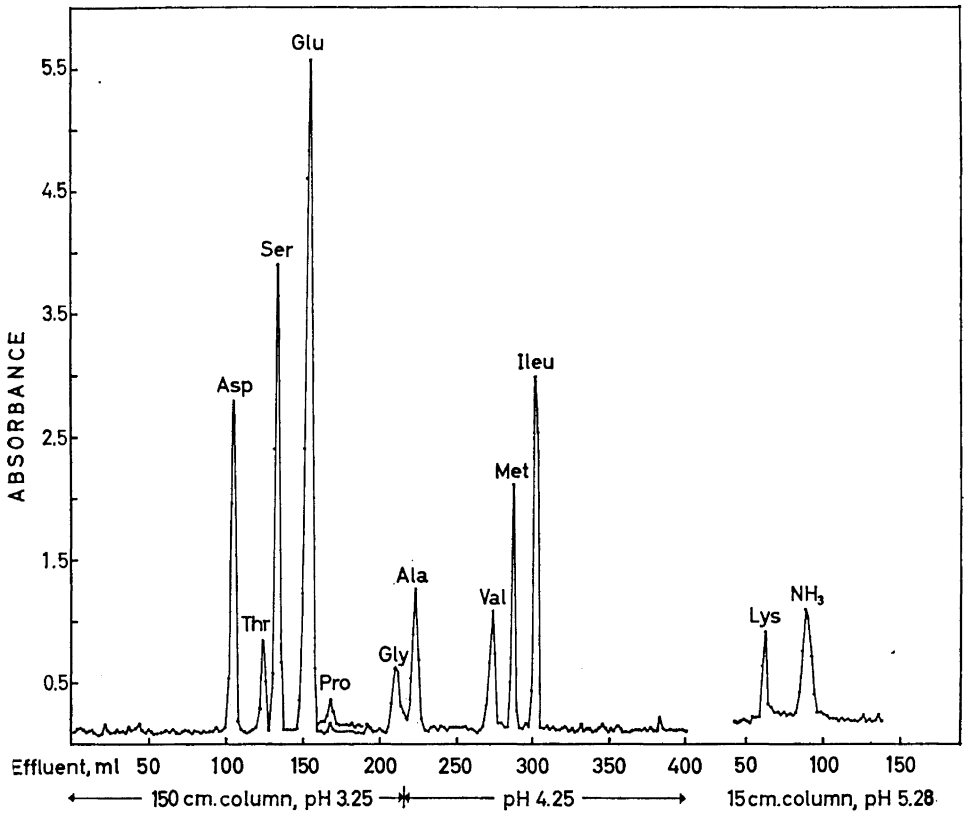


Fig. 2. Amino acid chromatogram according to Moore *et al.*[19] of the isolated component. The values of absorbance for lysine and ammonia should be multiplied with 2.6 to correspond to those of the other amino acids.

Analysis of the isolated component: N 8.56; P 3.20; S 1.1 % (dry weight, not corrected for ash content).* Atomic ratio N/P and N/S was calculated to 5.9 and 21.7, respectively.

N-Terminal amino acid residues were determined by Sanger's dinitrophenylation method[17]. The DNP-peptide was hydrolysed in 5.7 N HCl at 108° in sealed tubes for 10 and 16 h. Two-dimensional descending chromatography was carried out on Whatman No. 1 filter paper, using *tert*-amylalcohol: 0.1 M phthalate buffer, pH 6.0, in the first, and 1.5 M phosphate buffer, pH 6.0, in the second direction. This system was found to give satisfactory separation of DNP-amino acids. In the ether phase, DNP-aspartic acid only could be detected, whereas ε-DNP-lysine was present in the water phase.

Quantitative amino acid analysis. The amino acid composition of the isolated fraction was determined by the dinitrophenylation method as described by Koch and Weidel[18]. The chromatographic separation of the DNP-amino acids was, however, performed in the solvents mentioned above. Amino acid analysis was also performed by the chromatographic procedure on Amberlite IR-120, recently described by Moore *et al.*[19] A chrom-

* We are indebted to Analytiska Laboratoriet, Kemiska Institutionen, Lund, Sweden, for these analyses.

Table 1. Amino acid composition of the isolated phosphopeptide fraction expressed as molar ratios. The values are corrected for blank, and the molar ratios stated are based on the minimum glutamic acid content, taken as 9.00.

Method	Duration of hydrolysis, h	Molar ratios of amino acids											
		Asp	Glu	Gly	Ala	Val	Ileu	Met	Ser	Thr	Pro	Lys	NH_3
M and St *	22	3.40	9.00	1.07	1.86	1.57	2.91	1.56	4.70	0.93	1.12	2.45	7.73
K and W **	22	3.50	9.00	0.91	1.97	1.82	2.71	1.92	4.86	0.92	1.11	0.97	—
K and W	72	3.14	9.00	0.94	1.94	2.06	2.99	2.22	4.05	0.93	0.94	1.01	—
Probable minimum number of amino acid residues		3	9	1	2	2	3	2	5	1	1	1—2	—

* According to Moore and Stein [19].
** According to Koch and Weidel [18].

atographic run is shown in Fig. 2. In order to certify the identity of the three last peaks emerging from the 150 cm column, a known amount of leucine was added in one of the runs. This resulted in an extra peak, corresponding to the quantity of leucine added.

No tryptophan could be detected in the isolated fraction, using the method of Spies and Chambers [20].

RESULTS AND DISCUSSION

It is evident from the moving boundary electrophoresis and zone electrophoresis (Fig. 1) that a mixture of phosphopeptides is obtained by tryptic hydrolysis of α-casein. One of these zone electrophoretic components was isolated and its amino acid composition determined, the results of which are shown in Table 1. It is evident from this table that the same main amino acids, *i.e.* glutamic acid, serine, *iso*leucine, and valine are found in the present peptide, as earlier reported for phosphopeptides obtained from whole casein [5,10], as well as from α- and β-casein [6–8] after enzymic hydrolysis.

The results of paper electrophoresis, N-terminal amino acid analysis and quantitative amino acid analysis, indicate that the apparently homogeneous component may constitute a single peptide. If this is true, the minimum molecular composition of the phosphopeptide will be 30 amino acid residues and 6 phosphate groups, corresponding to a minimum molecular weight of about 4 000. The N-terminal position is occupied by aspartic acid and the C-terminal might be a lysine residue which would be in accordance with the specificity of trypsin.

The fact that the bovine α-casein fraction gives several phosphorus-containing peptides after tryptic hydrolysis has previously been pointed out by Peterson *et al.*[6], who found at least three peaks in boundary electrophoresis of the phosphopeptone fraction from a tryptic digest of α-casein. No data concerning these phosphopeptides were given by them. Boundary electrophoresis of our crude phosphopeptide preparation also revealed the existence of three or more components. Pantlitschko and Gründig [7] have recently reported the isolation of a "phosphopeptone" after tryptic digestion of α-casein.

It seems remarkable that no basic amino acids were found in their preparation and, furthermore, it contained a certain amount of acetylglucosamine. No certain criteria of homogeneity of the "phosphopeptone" are to be found in their communication. It seems also likely that α-casein, in accordance with our results, should furnish several phosphorus-containing peptides, as it is known that the α-casein fraction from cow's milk consists of at least two phosphoprotein components [21,22].

Further investigations concerning the separation of phosphopeptides from bovine α-casein by means of ion exchange chromatography and zone electrophoresis are in progress.

We are indebted to Professor O. Mellander for his kind interest and support of the work and also to Miss Anita Boman and Mrs. Birgit Johansson for skilful technical assistance.

REFERENCES

1. Mellander, O. *Acta Soc. Med. Upsaliensis* **55** (1950) 247.
2. Ågren, G. and Glomset, J. *Acta Chem. Scand.* **7** (1953) 1071.
3. Mellander, O. *Upsala Läkarfören. Förh.* **52** (1947) 107.
4. Perlmann, G. *Advances in Protein Chem.* **10** (1955) 1.
5. Österberg, R. *Arkiv. Kemi* **13** (1959) 409.
6. Peterson, R. F., Nauman, L. W. and McMeekin, T. L. *J. Am. Chem. Soc.* **80** (1958) 95.
7. Pantlitschko, M. and Gründig, E. *Monatsh.* **89** (1958) 274.
8. Pantlitschko, M. and Gründig, E. *Monatsh.* **89** (1958) 489.
9. Mellander, O. *Nutrition Rev.* **13** (1955) 161.
10. Mellander, O. and Vahlqvist, B. *Am. J. Clin. Nutrition* **5** (1957) 493.
11. Hipp, N. J., Groves, M. L., Custer, J. H. and McMeekin, T. L. *J. Dairy Sci.* **35** (1952 272.
12. Johansson, B. and Svennerholm, L. *Acta Physiol. Scand.* **37** (1956) 324.
13. Svennerholm, L. *J. Neurochem.* **1** (1956) 42.
14. Svennerholm, L. *Acta Soc. Med. Upsaliensis* **61** (1957) 287.
15. Svennerholm, L. *Arkiv. Kemi* **10** (1957) 577.
16. Hanes, C. S. and Isherwood, F. A. *Nature* **164** (1949) 1107.
17. Sanger, F. *Biochem. J.* **39** (1945) 507.
18. Koch, G. and Weidel, W. *Z. physiol. Chem. Hoppe-Seyler* **303** (1956) 213.
19. Moore, S., Spackman, D. H. and Stein, W. H. *Anal. Chem.* **30** (1958) 1185.
20. Spies, J. R. and Chambers, D. C. *Anal. Chem.* **21** (1949) 1249.
21. Waugh, D. F. and von Hippel, P. H. *J. Am. Chem. Soc.* **78** (1956) 4576.
22. Long, J., Van Winkle, Q. and Gould, J. A. *J. Dairy Sci.* **41** (1958) 317.

Received April 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1176–1184

Conversion of the Perkin-Elmer Infrared Microscope Attachment for Double-Beam Operation

CARL LAGERCRANTZ and MARGARETA YHLAND

Division of Medical Physics, University of Gothenburg, Sweden

The Perkin-Elmer Infrared Microscope Attachment, used together with model 13 spectrometer, has been converted to double-beam operation by removing the beam-chopper and inserting a shutter-disk in the image plane of the microscope. The shutter-disk, which is revolved synchronously with the spectrometer breakers, is equipped with two semi-circular slits so situated that the light is passed alternatively from the sampling and reference area to the detector. The arrangement allows for recording of spectra from microgram quantities with medium resolution.

The reflecting microscopes employed in infrared microspectrometers have been placed in two different positions in the spectrometer: (a) Between the source and the entrance slit of the monochromator. (b) Between the exit slit of the monochromator and the detector. Temperature and photochemical effects in the sample are generally considered to be of importance when the microscope is situated in position (a), where the sample is subjected to a concentrated beam of undispersed radiation. With the microscope in position (a) Ford *et al.*[1], however, found the rise to be only about 1°, when using a Nernst source. They consider also the ultraviolet radiation from this source to be of minor importance. On the other hand, the possible effects of concentrating an undispersed beam of radiation on the sample are certainly dependent on the material under investigation and the radiation source used. Since there is a general need of more intensive radiation sources in infrared microspectrometry than the Nernst and Globar sources, the disadvantage of position (a) will no doubt be more pronounced when, for instance, a carbon arc is employed. Therefore, the construction of microspectrometers which take advantage of both double-beam operation and of position (b) is of importance. As far as the present authors know, the only apparatus hitherto designed according to these specifications is that described by Haggis [2] which employs a rocking mirror to displace the optic axis through the microscope, so that the beam passes alternatively through the sample and an adjacent reference area.

The present paper describes the conversion of the Perkin-Elmer Infrared Microscope Attachment [3] for double-beam operation. The spectrometer used together with the microscope was a Perkin-Elmer model 13, equipped with a Nernst source and a sodium chloride prism.

GENERAL DESCRIPTION

In order to dispose the sample and the reference alternatively into the beam, experiments with a vibrating stage, placed in the sampling plane of the microscope, were performed by the present authors. However, neither a solenoid driver, nor a pure mechanical system synchronized with the beam-chopper and breakers of the filter circuits were found to give a reliable operation. The difficulties involved were no doubt connected with the rather high frequency of the oscillatory movement necessary to fit the 13-cps system of the spectrometer. Ford *et al.*[1], who employed such a technique successfully, used an oscillatory frequency of 4-cps only.

In consequence of these experiments, another method was developed, utilizing a rotating shutter-disk, placed in the image plane of the microscope. The shutter-disk is equipped with two semi-circular slits. Being of different radii, the two shutter-slits, termed I for sample and I_0 for reference, alternatively pass light from the sample and an adjacent clear area or reference. The relation between the rotating shutter-slits and the image of the spectrometer exit-slit is shown schematically in Fig. 1. The slits are situated in such a relation to each other, that the four operations described below take place when the shutter-slits cross the image of the spectrometer slit during one complete revolution of the shutter-disk:

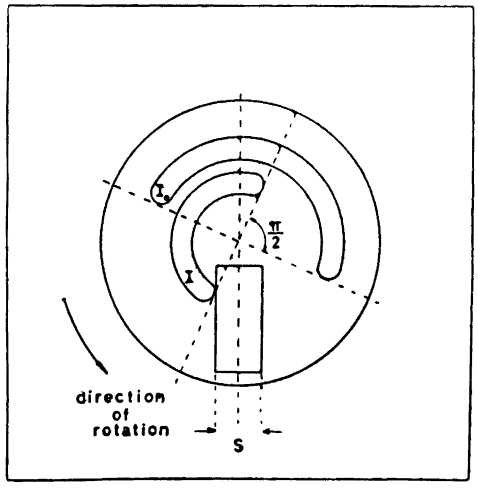


Fig. 1. Schematic diagram of the two semi-circular slits and the image of the spectrometer exit-slit of the width *S*.

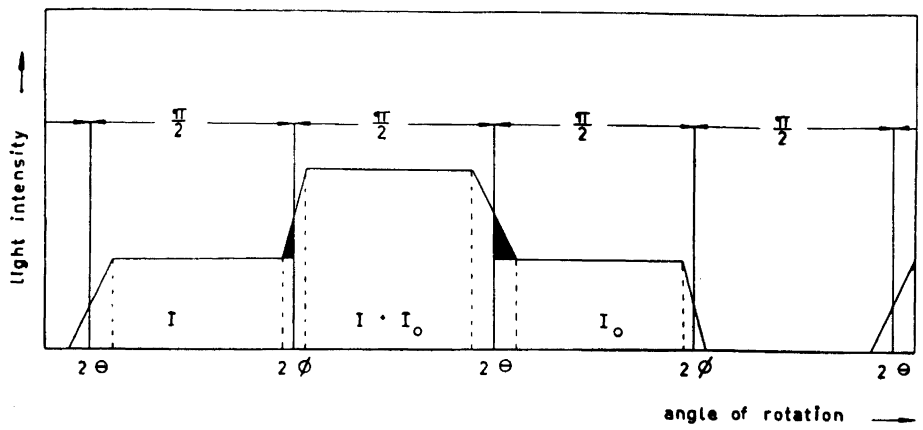


Fig. 2. Schematic diagram of the angular distribution and light transmission of the four spectrometer operations during one revolution of the shutter-disk. The solid vertical lines indicate the position in the cycle where the breakers are actuated. The dashed lines indicate the positions where the leading and trailing edges of the shutter-slits cross the lateral sides of the spectrometer slit image. The shaded areas correspond to the cross-talk. The diagram is constructed for a spectrometer slit-width of 500 μ.

(1) Light from the sample area passes through the I-slit to the detector.
(2) Light from both the sample and the reference areas passes simultaneously through the appropriate shutter-slit to the detector.
(3) Light from the reference area passes through the I_o-slit to the detector.
(4) No light passes through the shutter-disk.

By these means the cycle of operation of the model 13 ratio recording system is reproduced when the shutter-disk is revolved synchronously and in proper phase with the spectrometer breakers. In order to match the spectrometer system, the duration of each of the four operations listed above has to be equal to a quarter of a revolution. Such a condition, however, prevails only when the spectrometer slits are infinitely narrow. Obviously, the individual lengths of the four operations are functions of the actual slit-width, as seen from Fig. 1. These lengths, together with the distribution of light passed through the rotating shutter-disk are shown in Fig. 2. The solid vertical lines drawn in the diagram indicate the position in the cycle, where the spectrometer breakers are actuated. Under the conditions outlined in Fig. 2 it is evident that a certain amount of light from the I_o-channel is introduced into the I-channel and *vice versa* when the spectrometer slit is > 0. The cross-talk, $C(I)$ and $C(I_o)$, introduced into the sampling and reference channel, respectively, may be expressed as the ratio of the shaded area to the total area bordered by the solid vertical lines in Fig. 2 or, provided no sample is inserted into the beam, thus:

$$C(\mathrm{I}) = \frac{b \cdot \varphi}{\frac{1}{2}\pi - a \cdot \vartheta + b \cdot \varphi} \quad (1)$$

$$C(\mathrm{I_o}) = \frac{a \cdot \vartheta}{\frac{1}{2}\pi + a \cdot \vartheta - b \cdot \varphi} \quad (2)$$

where $\varphi = \sin^{-1} \frac{S}{2\,R_{\mathrm{I_o}}}$ and $\vartheta = \sin^{-1} \frac{S}{2\,R_{\mathrm{I}}}$

R_{I} denotes the radius of the sampling shutter-slit, and $R_{\mathrm{I_o}}$ that of the reference shutter-slit. S designates the width of the spectrometer slit in the image plane of the microscope objective. When the operating positions of the spectrometer breakers are in exact quadrature, and the centre of rotation of the shutter-disk is aligned symmetrically with the image of the spectrometer slit, the constants a and b are equal, and may be set approximately to 1/4. $C(\mathrm{I})$ and $C(\mathrm{I_o})$ are shown in Table 1 for some various slit-widths. The numerical values of R_{I} and $R_{\mathrm{I_o}}$ are those of the apparatus to be described, and are equal to 5.0 and 8.0 mm, respectively. The evaluation was performed under the assumption of an image reduction by the microscope condenser of 8.4 × and a magnification by the objective of 25 ×.

The percentage cross-talk will increase enormously in a region of low transmission, and the absorption is thus erroneously decreased. In a 100 % transmission run, the presence of cross-talk will cause a deviation of the recorded curve. Being of negligible magnitude in the short-wave region, the error will increase towards that of long waves. The error introduced by the cross-talk can be reduced by the following procedures:

(1) By selecting the channel of less cross-talk, see Table 1, as the sampling channel, *i.e.* the channel corresponding to the shutter-slit of minor radius.

(2) By increasing the energy output from the source in order to obtain a narrower slit, especially in the long wave range.

(3) By inserting a rectangular diaphragm into the image plane of the microscope objective, thus limiting the effective maximum slit-width of the spectrometer.

(4) By moving the trigger position of the spectrometer breakers, so that the shaded areas of Fig. 2 are reduced.

Of the four procedures outlined above, (1) and (4) have been applied in the present work. Since no light source other than the standard Nernst filament

Table 1. Percentage cross-talk introduced into the sampling and reference channels for some different spectrometer slit-widths, evaluated from eqns (1) and (2). No adjustment for reduction of cross-talk.

Slit-width, μ	$C(\mathrm{I})$	$C(\mathrm{I_I})$
0	0	0
500	1.7	2.5
1 000	3.3	5.1
1 500	5.3	7.7
2 000	6.9	10.5

has been employed, the procedures (2) and (3) were not tried. In order to reduce the error by procedure (4), the cross-talk of either channel can be reduced by readjusting the phase between the rotating shutter-disk and the breaker assembly, or by displacing the centre of rotation of the shutter-disk. It is thus possible to displace all the vertical lines, *i.e.* the trigger positions of the breakers in Fig. 2, to the left, resulting in reduced cross-talk in the I-channel. To reduce the cross-talk of the I_o-channel also, the solid vertical line between the $(I + I_o)$ — and the I_o-operations has to be moved to the right by adjusting the individual position of the appropriate spectrometer breaker. It is not possible, however, to displace the trigger position of the breakers over a certain limit from the position of quadrature without seriously disturbing the operation of the model 13 rectifier system.

MECHANICAL DESIGN AND CONSTRUCTION

A photograph of the modified model 13 Spectrometer and Microscope Attachment is given in Fig. 3. Fig. 4 shows the rotating shutter-disk with the semi-circular slits.

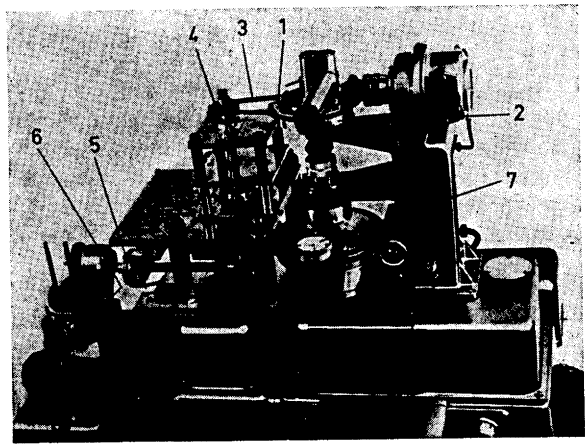


Fig. 3. Complete view of the apparatus. Refer to the text for numerical designations.

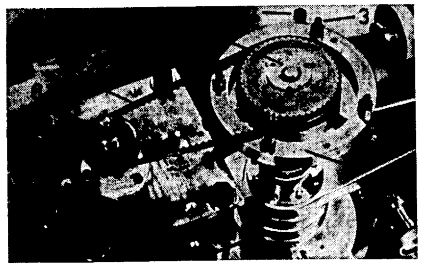


Fig. 4. The rotating shutter-disk with the semi-circular slits. The moulded housing containing the diaphragm field mirror has been removed. Refer to the text for numerical designations.

The diaphragm leaves of the microscope attachment, together with the leaf controls, were removed, and the standard diaphragm holder was replaced by a special holder (Fig. 3:1, 2, Fig. 4:2), intended to support the rotating shutter mechanism. The new holder was equipped with the appropriate spacers (Fig. 4:3) for supporting the moulded housing containing the diaphragm field mirror assembly, for the purpose of maintaining the accurate position of this mirror. The rotating shutter-disk with the two semi-circular slits (Fig. 4:1) was attached to the centre of a socket fitted into the inner ring of a ball bearing (SKF type 16007 X, 62 × 35 × 9 mm). The outer ring of the bearing was supported in a frame machined to the shape of a frustrum of a cone. This frame, together with the ball bearing and the rotating shutter-disk, was placed in a horizontal position on the special holder and was then accurately secured in position by three adjusting screws (Fig. 4:4).

The shutter-disk was caused to revolve by the aid of a Power Grip Timing Belt system. The driven pulley was attached to a flange on the socket supporting the shutter-disk. The timing belt (type 190 XL 025) (Fig. 3:3, Fig. 4:5) was led outside from the microscope assembly through the open space between the special holder and the diaphragm field mirror housing.

The driving pulley (Fig. 3:4, Fig. 4:6) was attached to the end of a vertical shaft supported by ball bearings in a heavy frame-work mounted on the lower of the two spectrometer source covers. After removal of the spectrometer beam-chopper, the pulley shaft was linked to the breaker shaft of the spectrometer by a gear and shaft train and a second timing belt system (Fig. 3:6) to provide direct transmission between the breaker shaft and the shutter-disk. As the standard chopper motor was not powered for driving this assembly, a synchronous motor (1500 rpm, 25 watts) was employed. This motor was placed beside the spectrometer. A pulley (Fig. 3:5) and belt system was used for transmission, so that the frequency of rotation of the breaker shaft was approximately 13-cps. This transmission system has been found to be very reliable and devoid of mechanical vibrations. The rotating shutter and the heavy frame-work were made of brass, the special holder and pulleys of duraluminium.

The two semi-circular slits are cut at a slightly larger angle than the theoretical value of π radians. After the spectrometer had been properly adjusted for the standard macro-operation, the angular values, to be used when cutting the slits, were obtained by reading the angular distribution of the four spectrometer operations from the position of a fixed index on a dial, engraved on the driven pulley (Fig. 3:5) of the spectrometer breaker assembly. The chopper shaft was rotated manually, and the positions at which the light from the source was obscured or just passed by the chopper-blade, were noted.

With shutter-slits of 2.0 mm width, the width of the sampling area amounts about 0.08 mm. The length of the sampling area depends upon the spectrometer slit-width and consequently varies with the wave-length. When the slit is fully opened, *i.e.* 2.00 mm, the sampling area becomes $0.08 \times 0.240 = = 1.92 \times 10^{-4}$ mm^2. In the sampling plane, the free space between the sampling and the reference areas is about 0.12 mm.

When adjusting the double-beam operated microscope to "Zero" and "100 % transmission", edge-formed shutters could be inserted into the appro-

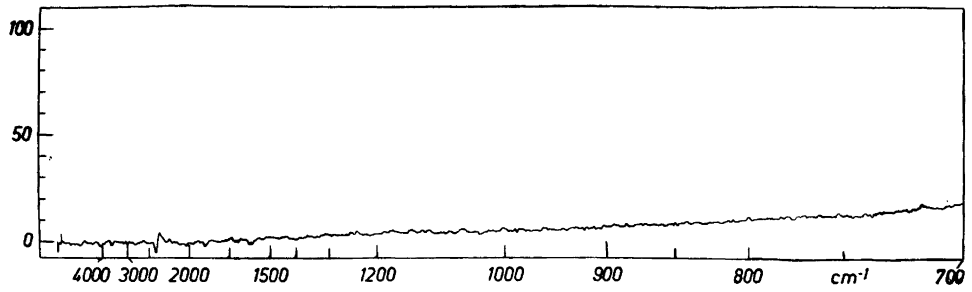


Fig. 5. Recorded curve of a 100 % transmission run. The y-coordinate gives the percentage absorption. Operating positions of the model 13 spectrometer controls: Ratio I/I_o, constant I_o, slit-servo 1.60, gain 18, full scale 6, response 3, speed 2 (1:1).

priate path in the sampling plane of the microscope. One of these shutters was also utilized for supporting the sample. The position of the shutters and the sample was controlled by a vernier screw and a dial displacement gauge (Fig. 3:7), mounted on the microscope stage. Precautions have to be taken to prevent the aperture of the microscope condenser and objective from being impaired when using these shutters.

ADJUSTMENTS

The apparatus was adjusted to minimum cross-talk by observing the position of the shutter-slits through the microscope viewer in visible light when the transmission system was manually revolved. The trigger position of each breaker was indicated by the lighting of a low-voltage lamp, connected in

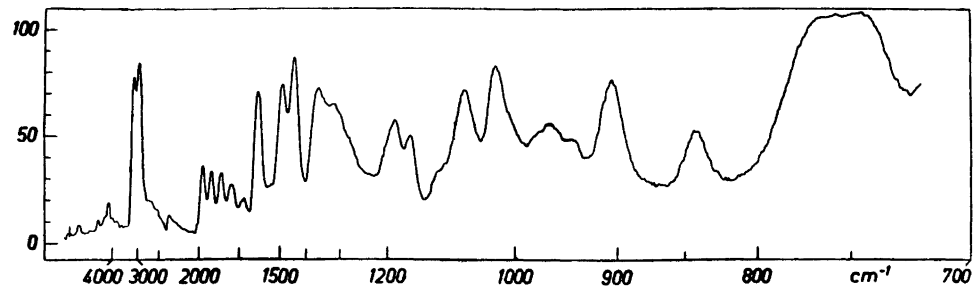


Fig. 6. Recorded spectrum of a polystyrene film. Maximum amount of substance in the sampling beam about 1.3 μg. The y-coordinate gives the percentage absorption. Operating positions of the spectrometer controls are the same as those given for Fig. 5. Reference channel: air.

series with the breaker and a small battery *. By these means it was possible to eliminate the cross-talk of the sampling channel. The cross-talk of the reference channel, however, could not be decreased much below the values given in Table 1 for the I_0-cross-talk without interfering with the spectrometer operation.

In order to make sure that the light, passed through both shutter-slits, falls well inside the sensitive area of the thermocouple, its position was aligned when observing the signals of the sampling and reference channels on a dual trace oscilloscope.

PERFORMANCE

The curve of a 100 % transmission record is shown in Fig. 5. The deviation from a constant level, increasing towards the long wave extreme, is a consequence of the light distribution already discussed above, and shown in Fig. 2. The compensation of atmospheric disturbances is found to be rather effective. The humidity of the air was about 80 % when the record was made.

The recorded spectrum of a polystyrene film of 0.07 mm thickness is shown in Fig. 6. Assuming the density of polystyrene to be equal to 1.0, the effective amount of substance present in the sampling beam is about $0.08 \times 0.240 \times 0.07 \times 1.0 = 1.3$ μg when the spectrometer slits are opened at their maximum width. Due to lack of sufficient energy, the record had to be interrupted at about 725 cm^{-1}, since the spectrometer slits had opened at maximum width at this wave-number. For the same reason the resolution is slightly lower than that generally obtained in macro-operation. The actual slit-width at 3 000 cm^{-1} was about 70 μ.

DISCUSSION

The problem associated with the presence of cross-talk might be simplified by the use of a breaker and filter system different from that of the model 13 spectrometer. However, the modification described above has the advantage of simplicity as it involves no changes either in the electronic system, or in the optics apart from the insertion of the shutter-disk instead of the beam-chopper. As compared with the system described by Haggis [2], the present arrangement has the advantage of avoiding abberations caused by the off-axis displacement inherent to this method.

The preparation of samples suitable for use with the present method will largely depend on the material to be investigated. Methods for preparation and handling of samples for infrared microspectroscopy have been described elsewhere [4]. With some forms of material, the problem of providing a clear space close to the sample will no doubt constitute a limiting factor of the present method.

* A similar technique is recommended by the makers when adjusting the breakers in the standard operation of model 13 spectrometer.

Acknowledgements. The authors are obliged to Mr. Elof Gustafsson for his excellent cooperation in the construction of the mechanical parts of the apparatus, and to Mr. Staffan Haeger for defraying the cost of the Microscope Attachment.

REFERENCES

1. Ford, M. A., Price, W. C., Seeds, W. E. and Wilkinson, G. R. *J. Opt. Soc. Am.* **48** (1958) 249.
2. Haggis, G. H. *J. Sci. Instr.* **33** (1956) 491.
3. Coates, V. J., Offner, A. and Siegler, Jr. E. H. *J. Opt. Soc. Am.* **43** (1953) 984.
4. Blout, E. R., Parrish, Jr. M., Bird, G. R. and Abbate, M. J. *J. Opt. Soc. Am.* **42** (1952) 966.

Received March 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1185–1188

Studies Related to Naturally Occurring Acetylene Compounds. Part XXV. The Occurrence of Methyl *cis*-β(5-Propynyl-2-thienyl)-acrylate in the Roots of Tansy

ERLING GUDDAL and NILS ANDREAS SÖRENSEN

Institutt for organisk kjemi, Norges tekniske högskole, Trondheim, Norway

The roots of tansy *(Chrysanthemum vulgare* Bernh.) have been investigated for their content of acetylenic compounds, and a sulphur containing compound identified as methyl *cis*-β(5-propynyl-2-thienyl)-acrylate has been isolated.

As briefly mentioned previously [1], the investigation of the highly unsaturated compounds in the roots of *Chrysanthemum vulgare* Bernh. has revealed the presence of an ester of a sulphur containing C_{10}-carboxylic acid. This compound is present in approximately the same amounts in acetone extracts and in the volatile oil obtained by steam distillation of the roots. The isolation, however, is best achieved by the latter method. From the oil of roots collected in the autumn or the late summer, the unsaturated ester crystallized on cooling to —10°C. This would indicate a certain increase in the content of this compound during the summer season, accompanied by distinctly increasing amounts of volatile oil during the same period. (Calculated on fresh roots, the yields of volatile oil range form 160 p.p.m. in June to 250 p.p.m. in August.)

When chromatographed on deactivated alumina, the unsaturated ester was eluted with light petroleum-benzene (2:1), and crystallized in nearly colourless prisms from hexane. It showed a melting point of 101°C in an evacuated tube, and elementary analysis agreed with $C_{11}H_{10}O_2S$. Its infrared spectrum is shown in Fig. 1 A. The infrared absorption bands at 1 435 cm^{-1} and 800 cm^{-1} indicate that the sulphur atom is present in a thiophene nucleus [2], and the band at 2 220 cm^{-1} shows the presence of a triple bond. A strong band at 1 715 cm^{-1} followed by another strong band at 1 618 cm^{-1} indicates an α,β-unsaturated carbonyl group. The very strong band in the ethylenic stretching region and the very moderate absorption in the acetylenic stretching region makes it probable that the double bond and not the triple bond is conjugated with the carbonyl group [3].

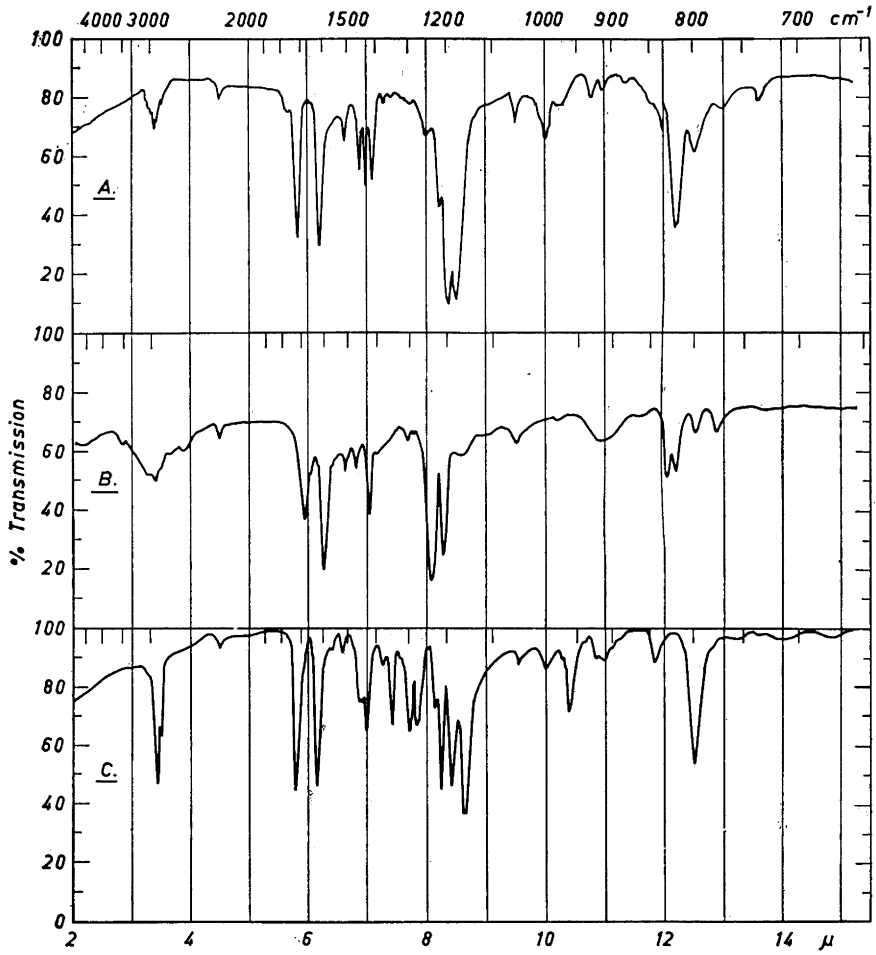
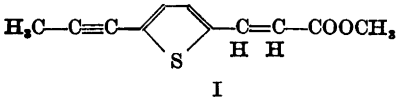


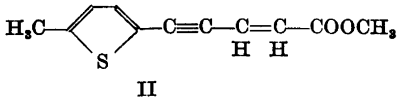
Fig. 1. Curve A: Methyl-*cis*-β(5-propynyl-2-thienyl)-acrylate. In $CHCl_3$ 2—12 μ, in CS_2 12—15 μ. Curve B: *Cis*-β(5-propynyl-2-thienyl)-acrylic acid. In KBr-disc. Curve C: Methyl-*trans*-β(5-propynyl-2-thienyl)-acrylate. In KBr-disc.

Hydrolysis under mild conditions gave an acid in poor yield which after recrystallisation from ether-hexane, was obtained as yellow needles melting at 148.5—149.5°C (decomp.), and the oxygen atoms thus obviously belongs to an ester grouping. The infrared spectrum of this acid is shown in Fig. 1 B.

The data leave only the two constitutional formulas I and II as reasonable structures.



I



II

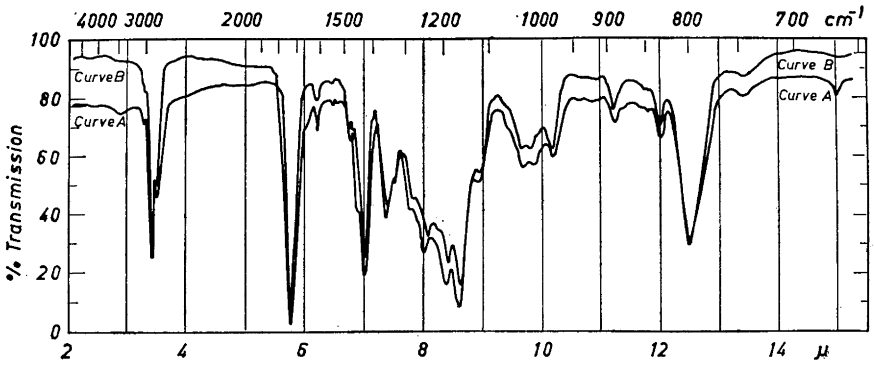


Fig. 2. Curve A: Hexahydro-methyl-β(5-propynyl-2-thienyl)-acrylate. Liq. Curve B: Synthetic Methyl-β(5-propyl-2-thienyl)-propionate. Liq.

The choice between I and II was made possible through a study of the hexahydro-compound. Catalytic hydrogenation of the unsaturated ester on $Pd/BaSO_4$ gave nearly colourless oil with a pleasant, sweetish odour, identical with synthetic methyl β(5-propyl-2-thienyl)-propionate, as shown by the identity of their infrared spectra which are reproduced in Fig. 2. The acid obtained upon saponification of this ester showed an infrared spectrum identical with that of synthetic β(5-propyl-2-thienyl)-propionic acid, and its melting point was not depressed on admixture of this compound. The synthetic specimens for these comparisons are readily obtained by hydrogenation of β(5-propyl-2-thienyl)-acrylic acid [4] with sodium amalgam according to Barger and Easson [5], and subsequent esterification of the acid. It thus follows that the new compound is methyl β(5-propynyl-2-thienyl)-acrylate (I).

When irradiated in the presence of iodine, the natural, unsaturated ester was partly converted to the *trans*-isomer which melted at 76°C and crystallized from hexane in thin leaflets. Its infrared spectrum, as shown in Fig. 1 C, contains a strong absorption band at 960 cm^{-1}, indicative of a *trans*-double bond. The *cis*-configuration of the natural ester could not conclusively be stated from its infrared spectrum because the *cis*-absorption band appeared at 820 cm^{-1}, very close to the band at 800 cm^{-1} characteristic of 2:5-disubstituted thiophenes [2]. Based on this evidence we conclude that the structure of the natural unsaturated ester is methyl *cis*-β(5-propynyl-2-thienyl)-acrylate (I).

Varying amounts of the *trans*-isomer have also been isolated from the plant material. An acetone extract worked up in the dark, however, contained only traces of this isomer, and it is most probably an artefact.

Methyl *trans*-β(5-propynyl-2-thienyl)-acrylate has recently been synthesized in this laboratory [6] and proved identical with the *trans*-isomer of the natural compound.

EXPERIMENTAL

Nine kg of fresh roots of *Chrysanthemum vulgare* Bernh. collected in August were steam distilled to give a yellowish brown volatile oil. On prolonged cooling at −10°C, slightly yellow prisms were deposited. The supernatant oil was submitted to chromatography on

deactivated alumina. The fraction eluted with light petroleum-benzene (2:1) afforded additional amounts of this crystalline compound. After two crystallisations from hexane, the compound melted at 101°C in an evacuated capillary. On heating in air, the compound showed destruction at 50—60°C, and was completely carbonized before melting occurred. The yield of this compound was 90 mg. (Found: C 63.5; H 5.05; S 15.1—15.4. Calc. for $C_{11}H_{10}O_2S$: C 64.0; H 4.88; S 15.5.) The ultraviolet spectrum has two broad maxima at 3 415 Å (log ε: 3.85) and 2 350 Å (log ε : 3.57). Infrared spectrum Fig. 1, curve A.

Saponification. 10 mg of the isolated compound was treated for one hour at room temperature with 5 % KOH in dilute EtOH (water : EtOH 2:1). From the reaction mixture 2 mg of an acidic compound was obtained. It crystallized from an ether-hexane mixture in beautiful, yellow needles, melting with destruction at 148.5—149.5°C. (Found: S 16.26. Calc. for $C_{10}H_8O_2S$: S 16.6.) The ultraviolet spectrum in hexane shows one maximum at 3 260 Å (log ε : 3.75). Infrared spectrum Fig. 1, curve B.

Hydrogenation. 26 mg of the isolated compound was hydrogenated with a 5 % $Pd/BaSO_4$ catalyst in ethanol solution. 8.6 ml hydrogen was consumed (755 mm Hg, 20°C), corresponding to 3.1 double bonds at a molecular weight of 206. The hydrogenation product was a nearly colourless oil with a pleasant, sweetish odour. It distilled at 40—45°C (bath temperature) at 3 × 10^{-3} mm Hg. n_D^{20} 1.5043. Infrared spectrum Fig. 2, curve A.

14 mg of the distilled hydrogenation product was saponified as above, and an acidic substance, which crystallized from hexane in ill-defined prisms, was isolated. It was obtained pure after distillation at 80—85°C (bath temperature) at 5 × 10^{-5} mm Hg, and subsequent crystallisation from hexane. The best preparation melted at 57—58°C, not depressed on admixture with synthetic β(5-propyl-2-thienyl)-propionic acid.

Isomerisation. To a solution of 10 mg of the natural, unsaturated ester in hexane was added a small amount of iodine, and the solution was irradiated with a mercury lamp for 45 min. Chromatography on deactivated alumina of the reaction mixture furnished 4 mg of unchanged material, and 4 mg of a new compound, eluted from the column with benzene-light petroleum (2:1). It crystallized from hexane in thin leaflets, melting at 76°C. Varying amounts of this compound also could be isolated from the volatile oil, the identity being proved by identical infrared spectra and mixed melting point determination. Its ultraviolet spectrum shows two broad maxima at 3 370 Å (log ε : 4.43) and 2 300 Å (log ε : 3.95). Infrared spectrum Fig. 1, curve C.

Preparation of β (5-propyl-2-thienyl)-propionic acid and its methyl ester. β(5-propyl-2-thienyl)-acrylic acid [4] was dissolved in saturated sodium bicarbonate solution and shaken with excess 3 % sodium amalgam at 10°C. Throughout the reaction, carbon dioxide was bubbled through the solution to avoid excessive rise in pH. After acidification, the acid obtained was taken up in ether and recrystallized from ether-hexane. M. p. 59.5—60°C. Yield 90 %. The acid was esterified by boiling with methanol and H_2SO_4, and the ester was distilled at 35—40°C (bath temperature) at 10^{-3} mm Hg. Yield 62 %. Infrared spectrum Fig. 2, curve B.

Acknowledgements. Kjemiingeniör Jörgine Stene Sörensen and Dr. Torger Bruun analysed the compounds for S and C—H, respectively. This work was carried out with a research grant from *Norges tekniske högskole.*

REFERENCES

1. Sörensen, J. S. and Sörensen, N. A. *Acta Chem. Scand.* **12** (1958) 771.
2. Sörensen, N. A. *Acta Chem. Scand. Forthcoming publication.*
3. Allan, J. L. H., Meakins, G. D. and Whiting, M. C. *J. Chem. Soc.* **1955** 1874.
4. King, W. J. and Nord, F. F. *J. Org. Chem.* **14** (1949) 405.
5. Barger, G. and Easson, A. P. T. *J. Chem. Soc.* **1938** 2100.
6. Skatteböl, L. *Acta Chem. Scand. Forthcoming publication.*

Received March 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1189–1192

Synthesis of Dialkylaminoalkyl Esters of Sterically Hindered 4-Alkoxybenzoic Acids

ERKKI HONKANEN

Lääketehdas Orion Oy, Helsinki, Finland

A series of dialkylaminoalkyl esters of 2,6-dimethyl-4-alkoxybenzoic acids was prepared for pharmacologic evaluation.

A number of dialkylaminoalkyl esters of *p*-alkoxybenzoic acids have been prepared previously [1,2]. They were found to have a greater local anesthetic activity than procaine, β-diethylaminoethyl *p*-aminobenzoate. These esters, however, hydrolyze in an organism more easily than the corresponding *p*-aminoesters, and this shortens the length of the anesthetic action.

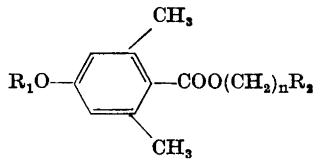
Rabjohn *et al.*[3] have synthesized some β-dialkylaminoethyl esters of sterically hindered benzoic acids which contained only alkyl substituents and reported that β-diethylaminoethyl 2,3,5,6-tetramethylbenzoate is twice as potent as procaine in infiltration anesthesia.

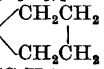
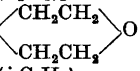
The Upjohn Co [4,5] has prepared dialkylamino alkyl esters of 2,6-dimethyl-4-aminobenzoic acid and reported that the *ortho* dialkyl substitution has an advantageous effect on the local anesthetic activity and that these esters do not hydrolyze readily. However, Lespagnol and Bar [6] claimed that that 1-(dimethylaminomethyl)-1-methylpropyl 2,6-dibromobenzoate hydrolyzes more readily than the unsubstituted benzoate. Childress *et al.*[7] reported that β-diethylaminoethyl 2,6-dichloro-4-aminobenzoate has an intradermal local anesthetic activity 3.5 times that of procaine.

As can be seen from the foregoing, the *ortho* dialkyl substitution not only increases the stability of the ester but also its local anesthetic activity. It was therefore also of interest to investigate the effect of the *ortho* dialkyl substitution on the esters of 4-alkoxybenzoic acids.

During the course of the present investigation two publications have appeared. In one, Reid [8] reported the synthesis of some pyrrolidylalkyl esters of 2,6-dimethyl-4-propoxybenzoic acid, and in the other Bernstein *et al.*[9] that of alkylaminoalkyl esters of 2,6-dimethyl-4-methoxy- and 4-ethoxybenzoic acids.

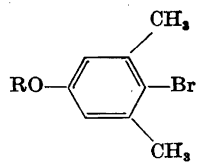
Table 1. Dialkylaminoalkyl esters of 2,6-dimethyl-4-alkoxybenzoic acids.



R_1	n	R_2	B.p., °C	M.p., °C	Analyses		
					Formula	Calc. N	Found N
C_2H_5	2	$N(C_2H_5)_2$	120—123/0.01	·HCl 149—150*	$C_{17}H_{27}NO_3$.HCl	4.24	4.36
C_2H_5	2		120—122/0.05	—	$C_{17}H_{25}NO_3$	4.81	4.94
n-C_3H_7	2	$N(C_2H_5)_2$	124—125/0.01	·$(COOH)_2$, 125—126	$C_{18}H_{29}NO_3$.$(COOH)_2$	3.52	3.58
i-C_3H_7	2	$N(C_2H_5)_2$	116—117/0.005	—	$C_{18}H_{29}NO_3$	4.56	4.43
n-C_4H_9	2	$N(CH_3)_2$	116—117/0.005	—	$C_{17}H_{27}NO_3$	4.77	4.53
n-C_4H_9	2	$N(C_2H_5)_2$	136—137/0.01	·$(COOH)_2$ 113—115	$C_{19}H_{31}NO_3$.$(COOH)_2$	3.40	3.59
n-C_4H_9	2		145—147/0.003	—	$C_{19}H_{29}NO_4$	4.18	4.10
n-C_4H_9	2	$N(i\text{-}C_3H_7)_2$	130—132/0.009	—	$C_{21}H_{35}NO_3$	4.01	4.05
n-C_4H_9	2	$N(n\text{-}C_3H_9)_2$	146—148/0.002	—	$C_{23}H_{39}NO_3$	3.71	3.58
n-C_4H_9	3	$N(n\text{-}C_3H_9)_2$	153—154/0.005	—	$C_{24}H_{41}NO_3$	3.58	3.34
i-C_4H_9	2	$N(C_2H_5)_2$	117—118/0.008	—	$C_{19}H_{31}NO_3$	4.36	4.17
n-C_5H_{11}	2	$N(C_2H_5)_2$	128—129/0.003	—	$C_{20}H_{33}NO_3$	4.18	4.12
i-C_5H_{11}	2	$N(C_2H_5)_2$	122—123/0.004	—	$C_{20}H_{33}NO_3$	4.18	4.03

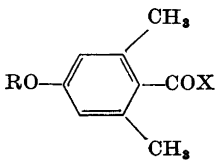
* Bernstein *et al.*[9] also reported the m. p. 149—150°.

Table 2. 2,6-Dimethyl-4-alkoxybromobenzenes.



R	B.p., °C	Yield %
C_2H_5	133—134/14	86
n-C_3H_7	145—146/15	90
i-C_3H_7	146—147/24	83
n-C_4H_9	156—157/15	94
i-C_4H_9	163—164/25	50
n-C_5H_{11}	182—184/24	90
i-C_5H_{11}	179—181/24	75

Table 3. 2,6-Dimethyl-4-alkoxybenzoic acids and 2,6-dimethyl-4-alkoxybenzoyl chlorides.



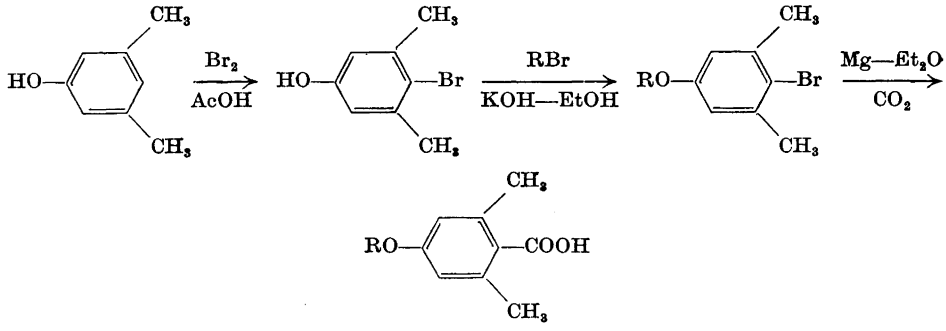
Acids X=OH								Acid chlorides X = Cl	
R	M.p., °C	Analyses					Yield %	B.p., °C	Yield %
		Formula	Carbon		Hydrogen				
			Calc.	Found	Calc.	Found			
C_2H_5	122—124	$C_{11}H_{14}O_3$	68.02	68.23	7.27	7.36	60	138—140/ 9	93
n-C_3H_7	88— 90 *	$C_{12}H_{16}O_3$	69.21	69.63	7.74	7.73	48	150—151/10	92
i-C_3H_7	123—124 *	$C_{12}H_{16}O_3$	69.21	69.37	7.74	7.51	54	158—160/26	85
n-C_4H_9	97— 98	$C_{13}H_{18}O_3$	70.24	70.43	8.16	8.02	57	163—165/13	92
i-C_4H_9	116—117	$C_{13}H_{18}O_3$	70.24	69.88	8.16	7.97	49	172—175/25	60
n-C_5H_{11}	93— 95	$C_{14}H_{20}O_3$	71.16	71.04	8.53	8.25	43	109—110/0.2	73
i-C_5H_{11}	104—105	$C_{14}H_{20}O_3$	71.16	71.01	8.53	8.22	40	99—100/0.07	79

* Reid [8] reported the melting points 90—91° and 121—122° for the *n*- and *i*-propoxy-2,6-dimethylbenzoic acids, respectively.

In the present paper the synthesis of a number of dialkylaminoalkyl esters of 2,6-dimethyl-4-alkoxybenzoic acids is described. These esters have a high local anesthetic activity. In addition they are very resistant to hydrolysis. With the exception of β-diethylaminoethyl 2,6-dimethyl-4-ethoxybenzoate, which Bernstein *et al.*[9] have prepared, all the esters here described are new.

The esters of 2,6-dimethyl-4-alkoxybenzoic acids, which were prepared by allowing the corresponding 2,6-dimethyl-4-alkoxybenzoyl chlorides to react with a dialkylaminoalkanol in boiling benzene solution, are recorded in Table 1.

The 2,6-dimethyl-4-alkoxybenzoic acids were synthesized from 3,5-dimethylphenol by a series of reactions, first described by Fuson *et al.*[10] in the case of 2,6-dimethyl-4-methoxybenzoic acid.



The acid chlorides were prepared in the usual way with thionyl chloride. The 2,6-dimethyl-4-alkoxybromobenzenes from which the 2,6-dimethyl-4-alkoxybenzoic acids were prepared by carbonation of the magnesium compounds are presented in Table 2, the corresponding benzoic acids and acid chlorides in Table 3.

EXPERIMENTAL

The melting points were determined on a Kofler microscope. All boiling points are uncorrected. Microanalyses were performed by the Microanalytisches Laboratorium in Max-Planck-Institut für Kohlenforschung, Mülheim, Germany.

Synthesis of dialkylaminoalkyl esters of 2,6-dimethyl-4-alkoxybenzoic acids. 0.01 Mole of the required 2,6-dimethyl-4-alkoxybenzoyl chloride was dissolved in 50 ml of dry benzene. To this solution was added with stirring 0.02 mole of dialkylaminoalkanol dissolved in 20 ml of benzene. The mixture was refluxed and stirred for 24 h. The benzene solution was washed, first with 2 N aqueous sodium hydroxide, then with water, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue distilled in a high vacuum. The yield varied from 70 to 95 %.

REFERENCES

1. Rohmann, C. and Scheurle, B. *Arch. Pharm.* **274** (1936) 110.
2. Lott, W., Harris, S. and Christiansen, W. *J. Am. Pharm. Assoc.* **27** (1938) 661.
3. Rabjohn, N., Fronabarger, J. and Linstromberg, W. *J. Org. Chem.* **20** (1955) 271.
4. The Upjohn Company, Brit. 768.770 (1957).
5. The Upjohn Company, Brit. 791.599 (1958).
6. Lespagnol, A. and Bar, D. *Bull. soc. pharm. Lille,* **No 1** (1948) 20; *Chem. Abstr.* **44** (1950) 10263.
7. Childress, S., Cordasco, M., Plekss, O. and Reiner, L. *J. Am. Chem. Soc.* **76** (1954) 3988.
8. Reid, W. US 2.719.851 (1949).
9. Bernstein, J. and Stearns, B. US 2.844.621 (1955).
10. Fuson, R., Weldon, F. and Corse, J. *J. Am. Chem. Soc.* **63** (1941) 2645.

Received March 18, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1193–1208

An X-Ray Investigation of the Nickel-Boron System

The Crystal Structures of Orthorhombic and Monoclinic Ni_4B_3

STIG RUNDQVIST

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The Ni-B system has been investigated with X-ray powder and single-crystal methods in the range Ni_3B-NiB. In addition to the previously known intermediate phases, Ni_3B, Ni_2B and NiB, the existence of two other phases, both with the ideal compositions Ni_4B_3 but one orthorhombic and the other monoclinic, has been established. The following two-phase regions have been found: Ni_3B + Ni_2B, Ni_2B + orthorhombic Ni_4B_3, orthorhombic Ni_4B_3 + monoclinic Ni_4B_3 and monoclinic Ni_4B_3 + NiB. The unit cell of orthorhombic Ni_4B_3 varies slightly with composition: the dimensions in two-phase Ni_2B + orthorhombic Ni_4B_3 alloys are the following: $a = 11.953$ Å, $b = 2.981$ Å, $c = 6.569$ Å and in two-phase orthorhombic Ni_4B_3 + monoclinic Ni_4B_3 alloys: $a = 11.973$ Å, $b = 2.985$ Å, $c = 6.584$ Å. The unit cell dimensions of monoclinic Ni_4B_3 are $a = 6.430$ Å, $b = 4.882$ Å, $c = 7.818$ Å, $\beta = 103°18'$. No unit cell variation of the latter phase has been found.

The crystal structures of the two new phases have been determined and refined from single-crystal intensity data. The space group of orthorhombic Ni_4B_3 is $P\,n\,m\,a$; the unit cell contains sixteen nickel atoms and twelve boron atoms situated in 4(*c*) positions. Two-thirds of the boron atoms form infinite zig-zag chains, while one-third have no close boron contacts. There are probably boron vacancies in the structure.

The space group of monoclinic Ni_4B_3 is $C\,2/c$. Sixteen nickel atoms are situated in two 8(*f*) positions, eight boron atoms in one 8(*f*) position and four boron atoms in one 4(*e*) position. All boron atoms are connected in infinite chains.

Among the borides of the iron group metals, the nickel borides have received comparatively little attention. The nickel-boron equilibrium diagram has not been accurately determined, but it appears that the Ni-B system is much more complex than either the Fe-B or the Co-B systems. A short summary of the previous work on the nickel borides is given below.

Giebelshausen [1] made a thermal and microscopic analysis in the range 0—60 atom-% boron and claimed the existence of the phases Ni_2B, Ni_3B_2, NiB and Ni_2B_3. Recently, Hoppin III [2] made a microscopic and thermal ana-

lysis in the range 0—17 atom-% boron and found a maximum solid solubility of boron in nickel of 3.7 atom-% at 1 082°C. Bjurström [3] studied the Ni-B system with X-ray powder methods and determined the crystal structure of Ni_2B to be that of the $CuAl_2$ (C 16) type. A phase analysis with X-ray powder methods by Andersson and Kiessling [4] showed the existence of at least four intermediate phases with compositions close to Ni_3B, Ni_2B, Ni_3B_2 and NiB. The crystal structure of NiB was determined by Blum [5] who found NiB to be isostructural with CrB [6]. The preparation of nickel borides by igneous electrolysis was studied by Marion [7], who prepared in addition to Ni_2B and NiB, two phases with compositions between 33 1/3 and 50 atom-% boron and one phase with a boron content larger than 50 atom-%.

At this Institute, the Ni-B system has been reinvestigated with X-ray powder and single-crystal methods, and in previous papers [8,9] the cementite type structure of Ni_3B has been reported. The structure of Ni_3B has been confirmed by Fruchart and Michel [10], and some physical and chemical properties of Ni_3B have been studied by Blok, Kozlova, Lashko and Shpunt [11], who also published X-ray powder data of Ni_3B. Their powder data are in agreement with the present author's findings [8,9].

EXPERIMENTAL

The samples were prepared by heating nickel powder (99.9 %, kindly donated by Mond Nickel Co., Ltd) and boron powder (99.7 % or better, kindly donated by Borax Consolidated Ltd) in evacuated, sealed silica tubes at temperatures between 750° and 950°C. It has been found that the silica tubes are attacked by Ni-B mixtures at elevated temperatures which results mainly in the formation of a ternary compound with the ideal composition Ni_6Si_3B [12]. Because of this the samples were protected from direct contact with the silica by placing them in small Al_2O_3 crucibles inside the silica tubes. No appreciable reaction between Al_2O_3 and the nickel borides was observed. However, the compositions of boron-rich alloys did not correspond to the weighed amounts of the starting materials, since varying amounts of boron were unavoidably lost during the reactions. It was, therefore, often necessary to make a large number of samples in order to obtain an alloy with a composition within a desired narrow range.

X-Ray powder photographs were taken with Guinier-type focusing cameras with Cu$K\alpha$ and Cr$K\alpha$ radiations. Each powder film was calibrated with CaF_2 as internal standard ($\lambda_{CuK\alpha} = 1.5418$ Å, $\lambda_{CrK\alpha} = 2.2909$ Å, CaF_2: $a = 5.4630$ Å). The accuracy of the lattice parameters given in this paper is greater than 0.05 %.

For the structure determinations, Weissenberg photographs were taken with MoK radiation of small single-crystal fragments selected from the crushed alloys. The multiple-film technique was used with thin iron foils between successive films, and the intensities were estimated visually by comparison with a standard intensity scale. No correction for absorption or anomalous scattering was applied. Fourier summations were made with the Hägg-Laurent machine and with Beevers-Lipson strips. Atomic scattering factors were interpolated from tables given by Thomas and Umeda [13] for nickel and by Ibers [14] for boron.

THE PHASE ANALYSIS

Since the main purpose of the present investigation was to study the crystal structures of the intermediate phases, the phase analysis was performed with X-ray powder methods only.

The existence of the previously reported phases Ni_3B, Ni_2B and NiB was confirmed. The unit cell dimensions were determined (see Table 1) and were

in agreement with the results of earlier measurements. No nickel boride with the composition Ni_3B_2, as reported by Giebelshausen [1], was found. Instead, the existence of two new phases, both with compositions near Ni_4B_3, was established. This is in agreement with the findings of Marion [7], who reported two phases of composition within the range Ni_2B-NiB. The X-ray measurements showed that the owo phases possessed orthorhombic and monoclinic symmetry, respectively, and the phases are therefore denoted in the following text as *o*-Ni_4B_3 and *m*-Ni_4B_3.

Since *o*-Ni_4B_3 and *m*-Ni_4B_3 appeared to have the same composition, the existence of a polymorphic transition was suspected. Samples of *o*-Ni_4B_3 and *m*-Ni_4B_3, the powder photographs of which were free from traces of Ni_2B or NiB, were subjected to various heat treatments and rapid quenchings, but no signs of transitions in the solid state were observed. For *m*-Ni_4B_3, no lattice parameter variations were found; but for *o*-Ni_4B_3, a small decrease of the unit cell was indicated in alloys quenched from higher temperatures.

The two-phase regions Ni_3B + Ni_2B, Ni_2B + *o*-Ni_4B_3 and *m*-Ni_4B_3 + NiB were found in the temperature range 750°—950°C, but no two-phase regions *o*-Ni_4B_3 + NiB or Ni_2B + *m*-Ni_4B_3 were observed. Attempts to prepare two-phase *o*-Ni_4B_3 + *m*-Ni_4B_3 alloys were successful in only a few cases. One alloy was heated for 14 days at 850°C, and the two phases still coexisted after the heat treatment. The lattice parameters of *o*-Ni_4B_3 were slightly larger in two-phase *o*-Ni_4B_3 + *m*-Ni_4B_3 alloys than in Ni_2B + *o*-Ni_4B_3 alloys (see Table 1); this indicates that the unit cell of *o*-Ni_4B_3 increases with increasing B/Ni ratio.

From the single-crystal structure determinations (*vide infra*) it was evident that the ideal composition of both *o*-Ni_4B_3 and *m*-Ni_4B_3 should be Ni_4B_3. A study of the available space in the structures showed that the lattices could hardly accommodate more boron or nickel atoms than the stoichiometrical number. Any deviation from stoichiometry must therefore be due either to the existence of lattice vacancies or to boron-nickel substitution.

It is quite clear that additional information, *e.g.*, from microscopic and thermal analytical investigations, must be available before any definite conclusions can be drawn about the phase relationships in the nickel-boron system. However, the following interpretation is suggested by the present observations.

Around the composition 40—43 atom-% boron, two discrete intermediate phases, *o*-Ni_4B_3 and *m*-Ni_4B_3, exist at least in the temperature range 750°—950°C. The two-phase range is probably very narrow, as indicated by the diffi-

Table 1. Crystallographic data of the nickel borides (unit cell edges in Å).

Boride	Structure type	Space group	*a*	*b*	*c*	*β*
Ni_3B	*D* O_{11}	*Pnma*	5.211	6.619	4.389	—
Ni_2B	*C* 16	*I4/mcm*	4.989	—	4.246	—
o-Ni_4B_3 (metal-rich)		*Pnma*	11.953	2.981	6.569	—
o-Ni_4B_3 (boron-rich)		*Pnma*	11.973	2.985	6.584	—
m-Ni_4B_3		*C2/c*	6.430	4.882	7.818	103°18′
NiB	CrB	*Cmcm*	2.928	7.391	2.964	—

Table 2. Powder data for metal-rich orthorhombic Ni_4B_3 (Cr*Ka* radiation).

h k l	$\sin^2\Theta_o \times 10^4$	$\sin^2\Theta_c \times 10^4$	I_o	I_c	*hkl*	$\sin^2\Theta_o \times 10^4$	$\sin^2\Theta_c \times 10^4$	I_o	I_c
200	368	367	v.w	10	410	2 944	2 945	st	484
101	—	396	—	0	212	3 059	3 060	v.st	714
201	672	671	v.w	3	203	3 104	3 104	v.w	9
301	—	1 131	—	0	411	3 248	3 249	m	145
002	1 217	1 216	v.w	12	600	3 305	3 306	w	19
102	1 308	1 308	w	26	502	} 3 517	3 512	} st	18
400	1 470	1 469	m	72	312		3 519		353
202	1 585	1 584	m	117	303	3 564	3 563	st	364
401	1 774	1 773	v.w	7	601	3 609	3 610	st	489
011	1 781	1 780	w	29	511	4 075	4 076	st	385
210	—	1 843	—	0	412	4 164	4 162	w	27
111	1 872	1 872	w	33	403	} 4 212	4 206	} st	211
302	2 041	2 043	m	110	013		4 213		244
211	2 147	2 147	st	295	113	4 307	4 305	m	107
501	} 2 606	2 600	} st	12	602	4 524	4 522	m	131
311		2 607		199	213	4 583	4 581	w	26
402	2 684	2 686	w	26	610	—	4 782	—	9
112	2 784	2 784	m	150	701	4 803	4 804	m	139
103	2 828	2 829	m	170					

culties of obtaining a two-phase *o*-Ni_4B_3 + *m*-Ni_4B_3 alloy (compare the experimental part of the paper). The monoclinic phase is believed to correspond closely to the stoichiometrical composition and may be assigned the formula Ni_4B_3. The crystal lattice of the orthorhombic phase probably contains a varying amount of boron vacancies, which are never completely filled. The formula of this phase may therefore be written Ni_4B_{3-x}, $x > 0$. The number of vacancies probably increases with increasing temperature.

Table 3. Powder data for monoclinic Ni_4B_3 (Cr*Ka* radiation).

hkl	$\sin^2\Theta_o \times 10^4$	$\sin^2\Theta_c \times 10^4$	I_o	I_c	*hkl*	$\sin^2\Theta_o \times 10^4$	$\sin^2\Theta_c \times 10^4$	I_o	I_c
110	886	886	w	49	$\bar{3}11$	3 412	3 414	st	431
002	907	907	v.w	11	$\bar{2}21$	3 515	3 516	w	89
$\bar{1}11$	—	985	—	2	220	3 543	3 543	v.st	684
111	1 240	1 240	w	38	310	3 567	3 567	m	177
200	—	1 340	—	1	004	3 625	3 626	st	385
$\bar{1}12$	1 539	1 538	m	171	$\bar{3}12$	3 713	3 713	m	246
$\bar{2}02$	1 739	1 740	st	319	$\bar{2}22$	3 942	3 942	w	87
112	2 047	2 046	v.w	10	$\bar{2}04$	3 952	3 953	w	68
020	2 202	2 202	v.w	12	$\bar{1}14$	4 005	4 005	m	221
021	2 431	2 429	m	160	221	4 023	4 023	m	171
$\bar{1}13$	2 544	2 546	st	298	311	4 177	4 174	w	74
202	2 753	2 754	v.st	579	023	4 244	4 242	w	121
022	3 108	3 109	v.st	737	$\bar{3}13$	4 467	4 466	w	99
113	—	3 306	—	3	$\bar{2}23$	4 823	4 822	w	36

Unit cell dimensions of the nickel borides are given in Table 1. For identification purposes, powder data for o-Ni_4B_3 and m-Ni_4B_3 are given in Tables 2 and 3. It may be noted that for o-Ni_4B_3, the ratio a/b is nearly equal to 4; this makes it somewhat difficult to index correctly the powder lines without knowledge of the intensities of the reflexions.

THE CRYSTAL STRUCTURE DETERMINATION OF ORTHORHOMBIC Ni_4B_3

It is evident from the foregoing text that a knowledge of the crystal structures of o-Ni_4B_3 and m-Ni_4B_3 is valuable from the phase-analytical point of view. Furthermore, the structures also seemed interesting for crystal-chemical reasons, and complete single-crystal structure determinations of both phases were therefore made.

From the measured density value, 7.4 g. cm^{-3} the unit cell content of o-Ni_4B_3 was calculated to be 16 nickel atoms and 12 boron atoms, the unit cell volume being 234—235 Å^3. The following conditions which limit possible reflexions were found: (h k 0) for $h = 2n$, (0 k l) for $k + l = 2n$. The two space groups $Pn2_1a$ and $Pnma$ where therefore possible. Since the b-axis was only 2.98 Å, the 8(d) position of nickel atoms in space group $Pnma$ was ruled out. A study of the intensity material from the layer lines 0—3 around the b axis showed that the corresponding (h 0 l) and (h 2 l) intensities were equal (apart from the normal decline), and the same was true for the (h 1 l) and (h 3 l) reflexions. The simplest interpretation of these facts was to assume that the space group is $Pnma$ with all nickel atoms (and possibly also the boron atoms) in 4(c) positions. The Patterson section $P(x\ 0\ z)$ was computed with the three-dimensional intensity material. The heights and positions of the maxima were in accordance with the above assumptions. Rough parameter values were obtained for the four sets of nickel atoms, and signs were calculated for the $F(h\ 0\ l)$ values. The electron density projection $\varrho\ (x\ z)$ was then calculated and, in addition to the expected nickel maxima, faint boron maxima corresponding to three 4(c) positions were discernible. The x and z parameters of the atoms were refined with successive difference syntheses. The final difference synthesis map, in which all nickel atoms were subtracted, showed well resolved boron maxima. The heights of the B_{II} and B_{III} maxima were almost equal, whereas the B_I maximum was lower and not symmetrical in shape. As measured on this map, the position of the B_I maximum corresponded to the parameters $x = 0.469$, $z = 0.428$, and resulted in a rather uneven distribution of Ni—B_I distances. The parameter values $x = 0.473$, $z = 0.430$ gave a more reasonable environment for the B_I atoms. Since this shift was permissible with regard to the accuracy of the experimental intensity material, these parameters were considered to be more reliable than those obtained directly from the electron density map. The difference in height between the B_I and the B_{II} and B_{III} maxima points to the possible existence of boron vacancies in the structure, but the experimental intensity data do not permit drawing any significant conclusion. After the last refinement, the R value 0.085 was obtained for the 117 observed $|F(h\ 0\ l)|$-values, when an empirical temperature factor with $B = 0.39$ Å^2 was applied. Observed and calculated $F(h\ 0\ l)$-values are given in Table 4.

Table 4. Observed and calculated $F(h0l)$-values for orthorhombic Ni_4B_3.

h 0 l	F_o	F_c	h 0 l	F_o	F_c
0 0 0	—	508	19 0 2	51.8	−52.2
2 0 0	21.3	−14.3	20 0 2	—	12.3
4 0 0	79.6	−82.2	21 0 2	29.3	35.1
6 0 0	61.9	53.5	1 0 3	101.6	113.4
8 0 0	—	−13.8	2 0 3	26.4	26.0
10 0 0	65.1	61.2	3 0 3	133.3	−163.5
12 0 0	121.0	−121.0	4 0 3	103.6	114.9
14 0 0	—	20.2	5 0 3	—	−13.0
16 0 0	—	25.5	6 0 3	—	13.6
18 0 0	—	−14.8	7 0 3	70.7	70.2
20 0 0	54.8	49.5	8 0 3	70.5	70.7
22 0 0	—	−5.3	9 0 3	28.7	20.5
24 0 0	—	12.3	10 0 3	—	4.3
26 0 0	—	19.3	11 0 3	45.9	−41.1
28 0 0	38.4	−29.8	12 0 3	52.7	−51.7
1 0 1	—	− 0.2	13 0 3	—	−19.3
2 0 1	—	− 8.2	14 0 3	—	− 2.7
3 0 1	—	− 3.7	15 0 3	62.7	57.6
4 0 1	26.0	−19.6	16 0 3	50.4	−50.2
5 0 1	34.6	−29.1	17 0 3	—	−23.4
6 0 1	149.5	−189.7	18 0 3	—	− 9.2
7 0 1	85.6	84.2	19 0 3	—	5.0
8 0 1	—	− 5.0	20 0 3	—	− 0.9
9 0 1	62.8	−60.9	0 0 4	41.8	36.2
10 0 1	50.9	47.0	1 0 4	69.3	69.4
11 0 1	28.2	−29.7	2 0 4	87.7	96.2
12 0 1	—	15.6	3 0 4	40.9	42.4
13 0 1	88.5	86.8	4 0 4	27.2	−20.3
14 0 1	27.0	28.9	5 0 4	37.3	−39.0
15 0 1	34.7	−34.9	6 0 4	—	10.6
16 0 1	—	11.7	7 0 4	27.9	−27.6
17 0 1	18.0	−25.7	8 0 4	—	− 9.2
18 0 1	56.2	50.2	9 0 4	47.3	45.8
19 0 1	—	11.8	10 0 4	135.1	−141.8
20 0 1	—	5.7	11 0 4	27.8	28.3
21 0 1	—	3.7	12 0 4	26.6	23.7
22 0 1	47.1	−45.0	13 0 4	—	−21.5
0 0 2	30.8	−30.9	14 0 4	—	9.3
1 0 2	32.2	33.0	15 0 4	—	−14.9
2 0 2	84.7	−76.6	16 0 4	43.0	−39.7
3 0 2	84.0	−82.8	17 0 4	—	14.5
4 0 2	49.7	−43.7	18 0 4	27.8	30.6
5 0 2	39.1	36.5	19 0 4	—	8.6
6 0 2	82.3	86.2	20 0 4	—	35.6
7 0 2	88.5	96.8	21 0 4	—	−10.9
8 0 2	38.2	33.8	22 0 4	33.7	33.2
9 0 2	80.8	−77.7	30 0 4	40.2	−33.2
10 0 2	—	− 3.0	1 0 5	20.4	15.0
11 0 2	16.6	16.2	2 0 5	46.1	−44.6
12 0 2	57.5	58.2	3 0 5	71.4	−77.2
13 0 2	—	− 2.7	4 0 5	87.4	−99.7
14 0 2	46.4	42.2	5 0 5	57.5	57.9
15 0 2	26.4	29.2	6 0 5	13.1	19.8
16 0 2	53.8	−59.4	7 0 5	—	− 3.9
17 0 2	—	0.9	8 0 5	31.4	−30.6
18 0 2	44.4	−45.5	9 0 5	23.1	−23.2

Table 4. Contd.

h 0 *l*	F_o	F_c	*h* 0 *l*	F_o	F_c
10 0 5	33.8	−30.8	3 0 9	27.9	−32.2
11 0 5	—	1.8	4 0 9	25.2	−29.0
12 0 5	—	21.8	5 0 9	49.2	−56.1
13 0 5	24.2	32.5	6 0 9	61.9	−62.7
14 0 5	48.3	50.0	7 0 9	69.3	70.1
15 0 5	—	21.3	8 0 9	—	0.1
16 0 5	58.3	54.1	9 0 9	—	2.6
17 0 5	49.8	−49.1	10 0 9	—	− 3.3
23 0 5	33.8	−35.4	11 0 9	24.3	−33.2
0 0 6	45.5	47.7	12 0 9	—	−11.0
1 0 6	35.9	−34.6	13 0 9	—	17.9
2 0 6	51.3	−56.6	14 0 9	41.7	43.2
3 0 6	42.4	41.0	0 0 10	—	−13.3
4 0 6	91.7	−100.4	1 0 10	66.4	65.8
5 0 6	—	17.7	2 0 10	40.0	−35.2
6 0 6	41.7	38.5	3 0 10	—	6.1
7 0 6	—	−13.4	4 0 10	—	−14.1
8 0 6	92.7	99.4	5 0 10	—	29.0
9 0 6	56.3	55.0	6 0 10	23.9	29.7
10 0 6	—	− 2.1	7 0 10	—	0.5
11 0 6	42.6	−42.2	8 0 10	—	13.2
12 0 6	—	4.6	9 0 10	—	5.6
13 0 6	—	14.4	10 0 10	—	8.0
1 0 7	—	− 5.8	11 0 10	—	25.7
2 0 7	98.3	102.0	1 0 11	—	11.3
3 0 7	—	− 8.2	2 0 11	—	− 6.2
4 0 7	—	− 1.6	3 0 11	—	−18.3
5 0 7	—	−16.6	4 0 11	28.5	30.0
6 0 7	—	16.5	5 0 11	—	24.6
7 0 7	46.5	39.4	6 0 11	—	10.0
8 0 7	—	16.8	7 0 11	—	− 3.5
9 0 7	23.7	−35.5	8 0 11	—	33.8
10 0 7	24.3	25.3	9 0 11	—	−15.1
11 0 7	—	−14.7	10 0 11	—	−20.5
12 0 7	—	−11.5	11 0 11	—	− 2.9
13 0 7	56.5	57.0	12 0 11	45.1	−41.4
14 0 7	74.7	−72.2	0 0 12	46.8	46.0
26 0 7	31.1	30.0	1 0 12	—	14.8
0 0 8	78.2	87.4	2 0 12	33.2	31.9
1 0 8	—	−17.6	3 0 12	—	1.4
2 0 8	—	− 7.5	4 0 12	21.0	−32.6
3 0 8	—	−11.7	5 0 12	—	−16.3
4 0 8	—	12.5	6 0 12	—	− 3.5
5 0 8	—	0.9	7 0 12	—	7.8
6 0 8	—	26.2	8 0 12	—	28.1
7 0 8	24.3	25.5	9 0 12	—	5.5
8 0 8	68.1	−65.5	10 0 12	52.2	−54.2
9 0 8	—	− 1.6	1 0 13	—	7.6
10 0 8	—	−26.5	2 0 13	—	1.8
11 0 8	—	−32.4	3 0 13	—	−19.8
12 0 8	23.6	−26.0	4 0 13	—	−24.7
13 0 8	—	11.5	5 0 13	—	23.9
20 0 8	72.0	65.2	6 0 13	30.2	−39.2
1 0 9	—	26.4	11 0 14	27.5	−30.7
2 0 9	—	−19.2			

The final structure of orthorhombic Ni_4B_3 is the following:

Space group $Pnma$—(D_{2h}^{16}), $Z = 4$

$a = 11.953$ Å, $b = 2.981$ Å, $c = 6.569$ Å, $U = 234.1$ Å^3.

(Unit cell dimensions were measured on powder photographs of the alloy, from which the actual single-crystal specimen was selected.)

All atoms in 4(*c*) positions:

	x	*z*
Ni_I	0.148_6	-0.009_7
Ni_{II}	0.449_8	0.751_1
Ni_{III}	0.199_7	0.378_0
Ni_{IV}	0.376_3	0.167_5
B_I	0.47_3	0.43_0
B_{II}	0.03_7	0.48_1
B_{III}	0.26_5	0.67_3

Interatomic distances are given in Table 5.

Table 5. Interatomic distances in orthorhombic Ni_4B_3 (Distances shorter than 3.0 Å listed).

	Ni_I	Ni_{II}	Ni_{III}	Ni_{IV}	B_I	B_{II}	B_{III}
Ni_I	2.98 (2)	2.56 (2) 2.86	2.46 (2) 2.63	2.76 (2) 2.97	2.1_2 (2) 2.1_7		2.1_8 (2) 2.5_1
Ni_{II}	2.56 (2) 2.86	2.98 (2)	2.48 (2)	2.62 (2) 2.88	2.1_2 (2) 2.1_3	2.0_5 (2) 2.1_3	2.2_7
Ni_{III}	2.46 (2) 2.63	2.48 (2)	2.98 (2)	2.53 2.64 (2)		2.0_6	2.0_6 (2) 2.0_9
Ni_{IV}	2.76 (2) 2.97	2.62 (2) 2.88	2.53 2.64 (2)	2.98 (2)	2.0_8	2.1_6 (2) 2.1_9	2.2_6 (2)
B_I	2.1_2 (2) 2.1_7	2.1_3 (2) 2.1_3		2.0_8	1.8_6 (2)		
B_{II}		2.0_5 (2) 2.1_3	2.0_6	2.1_6 (2) 2.1_9		1.7_5 (2)	
B_{III}	2.1_8 (2) 2.5_1	2.2_7	2.0_6 (2) 2.0_9	2.2_6 (2)			

DESCRIPTION OF THE ORTHORHOMBIC Ni_4B_3 STRUCTURE

A projection of the structure on the *ac*-plane is shown in Fig. 1. The nickel atom lattice can be described as built-up by interconnected trigonal prisms of nickel atoms. The boron atoms are situated in the centers of these prisms. The B_I and B_{II} atoms form two separate boron chains running zig-zag throughout the structure in the *b* direction, whereas the B_{III} atoms have no close boron contacts.

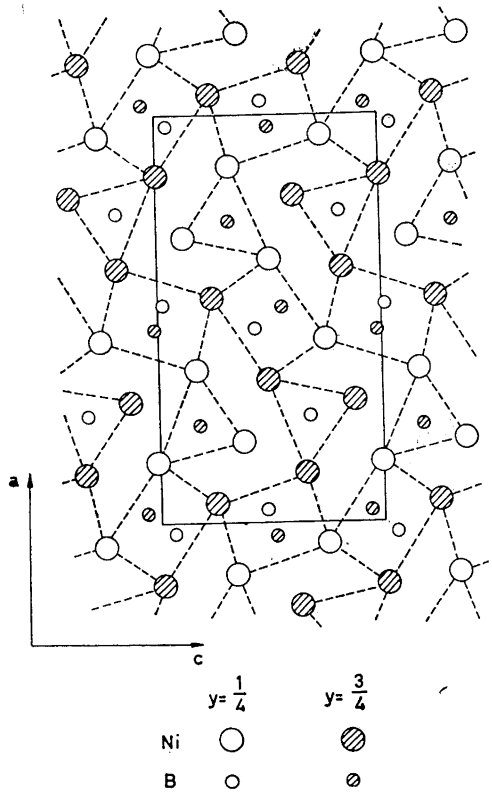


Fig. 1. The structure of orthorhombic Ni_4B_3 projected on (010).

From the crystal-chemical point of view, the *o*-Ni_4B_3 structure may thus be regarded as an intermediate between, *e.g.*, the Ni_3B (cementite type) structure [9], containing isolated boron atoms in the centers of trigonal metal atom prisms, and the FeB type structure [3] containing infinite chains of boron atoms, arranged in a way similar to *o*-Ni_4B_3. For comparison, the FeB structure (based on the same space group as *o*-Ni_4B_3) is shown in projection on the *ac*-plane in Fig. 2.

The three types of trigonal nickel atom prisms in *o*-Ni_4B_3 are shown in Fig. 3a, b and c, together with the environments of the B_I, B_{II} and B_{III} atoms. The triangular sides of the prisms are almost perpendicular to the rectangular sides; the deviations from 90° of the prisms I and II are at the limit of experimental error. The coordination number of the boron atoms is nine, the B_I and B_{II} atoms have seven nickel neighbours and two boron neighbours, and the B_{III} atoms have nine nickel neighbours.

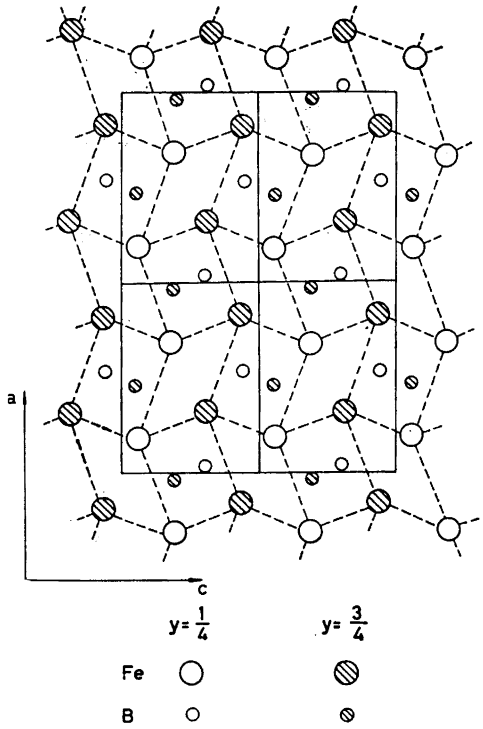


Fig. 2. The structure of FeB projected on (010).

THE STRUCTURE DETERMINATION OF MONOCLINIC Ni_4B_3

The unit cell volume of m-Ni_4B_3, 238.8 Å^3 is almost the same as that of o-Ni_4B_3, which shows that the unit cell content of m-Ni_4B_3 should be 16 nickel atoms and 12 boron atoms. The following conditions for possible reflexions were found: (h k l) only for $h + k = 2n$ and (h 0 l) only for $l = 2n$. This gives C c or $C/2c$ as possible space groups. The Patterson section $P(x$ 0 $z)$ was calculated with the threedimensional intensity material from the layer lines 0—6 around the b-axis. This section contained several large maxima, and indicated strongly that the space group is $C2/c$. Therefore, an attempt was made to interpret the Patterson section in accord with space group $C2/c$.

Large maxima were situated at the following approximate positions: (0, 0, 0) height 1; ± (0.08, 0, 0.46) height 1/4; ± (0.41, 0, 0.06) height 1/3 and (1/2, 0, 1/2) height 1/2. The maximum at ± (0.41, 0, 0.06) was unsymmetrically extended in the c direction. It was assumed that all these maxima corresponded mainly to Ni-Ni vectors. Since no maxima were found on either the a or the c axis, the positions 4(a) and 4(b) were ruled out. The 4(c) and 4(d) positions could not be occupied simultaneously, since in that case maxima at (1/2, 0, 0) and (0,0, 1/2) should exist. If the 4(e) position contained two four-fold sets of nickel atoms, the difference between their y parameters must be 1/2, because the b-axis is only 4.88 Å while the normal nickel atom radius is about 1.24 Å. The resulting maxi-

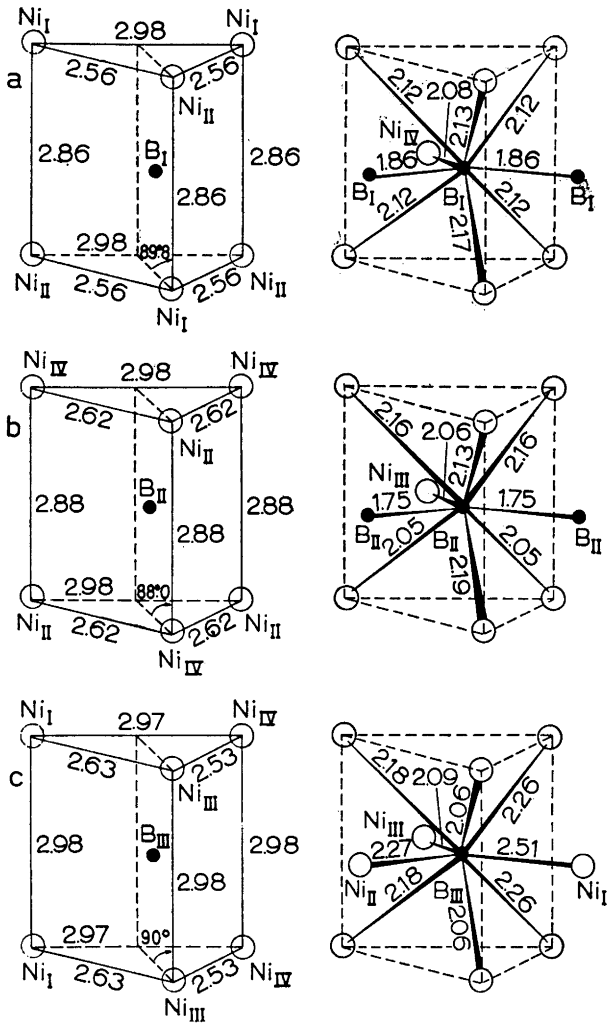


Fig. 3. The environment of the boron atoms in orthorhombic Ni_4B_3
a. the environment of the B_I atoms
b. the environment of the B_{II} atoms
c. the environment of the B_{III} atoms

mum at (0, 1/2, 0) is equivalent to a maximum at (1/2, 0, 0) and since no large maximum existed at (1/2, 0, 0), the 4(*e*) position cannot be occupied by more than four nickel atoms. The following possibilities thus remained: either sixteen nickel atoms in two 8(*f*) positions or eight nickel atoms in 8(*f*), four nickel atoms in 4(*e*) and four nickel atoms in either 4(*c*) or 4(*d*). An analysis of the last possibility did not lead to any acceptable solution, but a satisfactory explanation of the Patterson section $P(x, 0, z)$ was found by placing the nickel atoms in two 8(*f*) positions, one with $x_I = 0.04$; $z_I = 0.48$, and the other with $x_{II} = 0.20$; $z_{II} = 0.29$; this gives Harker maxima at $\pm$ (0.08, 0, 0.46) and $\pm$(0.40, 0, 0.08) with heights 1/4. If the Ni_I atoms have the y_I parameter $\pm$ 1/4, the observed maximum

Table 6. Observed and calculated $F(h0l)$- and $F(hk0)$-values for monoclinic Ni_4B_3.

h k l	F_o	F_c	h k l	F_o	F_c
0 0 0	—	508	12 0 12	—	13.0
2 0 0	—	— 8.0	0 0 14	72.0	64.9
4 0 0	146.9	137.0	2 0 14	—	—22.5
6 0 0	—	10.6	4 0 14	64.4	71.9
8 0 0	105.0	—94.0	6 0 14	—	34.6
10 0 0	—	4.0	8 0 14	—	6.8
12 0 0	101.3	—94.3	10 0 14	34.6	32.5
14 0 0	—	— 8.1	0 0 16	—	—30.9
16 0 0	—	— 9.0	2 0 16	70.6	61.5
0 0 2	25.4	23.0	4 0 16	—	6.4
2 0 2	223.2	291.7	6 0 16	45.6	50.0
4 0 2	—	—18.3	8 0 16	30.9	34.9
6 0 2	34.8	31.2	0 0 18	—	7.7
8 0 2	—	—12.0	2 0 18	—	—24.9
10 0 2	108.8	—103.8	4 0 18	54.6	58.0
12 0 2	—	— 5.6	6 0 18	—	3.9
14 0 2	73.7	—70.0	0 0 20	—	—12.2
16 0 2	—	— 7.0	2 0 20	—	22.0
0 0 4	178.0	236.5			
2 0 4	—	17.6	$\bar{2}$ 0 2	164.3	182.2
4 0 4	180.9	190.4	$\bar{4}$ 0 2	62.6	56.3
6 0 4	—	—18.8	$\bar{6}$ 0 2	122.1	—114.3
8 0 4	—	— 6.5	$\bar{8}$ 0 2	—	24.2
10 0 4	—	— 5.3	$\bar{10}$ 0 2	145.5	—142.4
12 0 4	94.9	—86.5	$\bar{12}$ 0 2	—	—13.9
14 0 4	—	7.7	$\bar{14}$ 0 2	—	—38.3
16 0 4	38.3	—46.9	$\bar{16}$ 0 2	—	—21.0
0 0 6	56.9	64.5	$\bar{2}$ 0 4	75.3	87.3
2 0 6	152.6	186.0	$\bar{4}$ 0 4	53.4	—45.7
4 0 6	33.8	—23.8	$\bar{6}$ 0 4	39.9	—46.7
6 0 6	120.7	115.5	$\bar{8}$ 0 4	147.7	—139.7
8 0 6	57.5	—49.0	$\bar{10}$ 0 4	—	—21.6
10 0 6	—	—22.6	$\bar{12}$ 0 4	58.1	—48.3
12 0 6	—	—22.1	$\bar{14}$ 0 4	—	—39.1
14 0 6	65.5	—65.5	$\bar{16}$ 0 4	—	37.7
0 0 8	26.4	30.0	$\bar{2}$ 0 6	—	— 1.8
2 0 8	71.0	78.0	$\bar{4}$ 0 6	64.0	69.3
4 0 8	131.2	129.6	$\bar{6}$ 0 6	151.6	—156.8
6 0 8	—	—10.2	$\bar{8}$ 0 6	—	—16.2
8 0 8	66.5	59.0	$\bar{10}$ 0 6	93.1	—90.0
10 0 8	—	—32.2	$\bar{12}$ 0 6	51.4	—53.2
12 0 8	—	—32.4	$\bar{14}$ 0 6	—	15.4
14 0 8	—	— 9.5	$\bar{2}$ 0 8	98.0	117.5
0 0 10	77.5	87.7	$\bar{4}$ 0 8	142.4	—129.5
2 0 10	52.0	50.8	$\bar{6}$ 0 8	—	21.6
4 0 10	—	47.5	$\bar{8}$ 0 8	100.1	—105.0
6 0 10	99.2	90.4	$\bar{10}$ 0 8	55.6	—56.4
8 0 10	—	—32.1	$\bar{12}$ 0 8	—	5.2
10 0 10	—	—31.4	$\bar{14}$ 0 8	—	—40.7
12 0 10	37.6	—35.1	$\bar{2}$ 0 10	69.4	—76.5
0 0 12	46.7	—35.2	$\bar{4}$ 0 10	—	18.2
2 0 12	78.0	89.0	$\bar{6}$ 0 10	104.5	—99.2
4 0 12	54.4	56.7	$\bar{8}$ 0 10	73.7	—78.4
6 0 12	—	20.0	$\bar{10}$ 0 10	—	—10.3
8 0 12	73.7	67.0	$\bar{12}$ 0 10	64.7	—72.0
10 0 12	—	—35.3	$\bar{2}$ 0 12	60.0	57.9

Table 6. Contd.

h k l	F_o	F_c	h k l	F_o	F_c
$\bar{4}$ 0 12	91.1	−86.0	0 4 0	81.4	88.0
$\bar{6}$ 0 12	—	−46.3	2 4 0	98.4	111.7
$\bar{8}$ 0 12	—	−29.3	4 4 0	—	29.5
$\bar{10}$ 0 12	73.2	−71.6	6 4 0	—	− 5.5
$\bar{2}$ 0 14	71.2	−70.5	8 4 0	47.8	−38.4
$\bar{4}$ 0 14	—	−33.8	10 4 0	63.1	−57.5
$\bar{6}$ 0 14	—	−39.0	1 5 0	—	8.9
$\bar{8}$ 0 14	80.6	89.0	3 5 0	39.0	−39.6
$\bar{10}$ 0 14	—	19.0	5 5 0	39.4	33.2
$\bar{2}$ 0 16	—	−14.1	7 5 0	—	−30.2
$\bar{4}$ 0 16	—	−22.6	9 5 0	—	5.3
$\bar{6}$ 0 16	72.4	−82.7	11 5 0	—	5.1
$\bar{8}$ 0 16	—	14.5	0 6 0	146.6	−168.7
$\bar{2}$ 0 18	—	−28.9	2 6 0	—	− 8.4
$\bar{4}$ 0 18	53.8	−64.8	4 6 0	69.5	−70.2
$\bar{6}$ 0 18	—	− 5.8	6 6 0	—	− 4.2
$\bar{2}$ 0 20	41.7	−47.0	8 6 0	62.9	57.4
			10 6 0	—	4.5
1 1 0	38.5	−36.6	12 6 0	63.3	64.5
3 1 0	123.2	110.0	1 7 0	—	16.7
5 1 0	100.2	−94.3	3 7 0	53.9	−53.3
7 1 0	73.0	70.4	5 7 0	48.4	55.0
9 1 0	—	−20.0	7 7 0	40.4	−46.5
11 1 0	—	− 8.2	9 7 0	—	16.1
13 1 0	—	32.3	0 8 0	—	0.2
0 2 0	43.5	−44.1	2 8 0	92.0	99.0
2 2 0	164.0	−216.0	4 8 0	—	3.5
4 2 0	—	−17.9	6 8 0	—	−13.7
6 2 0	—	18.4	8 8 0	—	3.7
8 2 0	—	13.1	10 8 0	72.2	−71.3
10 2 0	95.3	95.0	1 9 0	—	7.7
12 2 0	—	21.1	3 9 0	—	−29.8
1 3 0	—	−12.3	5 9 0	—	37.5
3 3 0	—	26.7	010 0	61.3	−58.5
5 3 0	—	29.7	210 0	—	−25.0
7 3 0	—	21.6	111 0	—	2.9
9 3 0	—	− 9.8	012 0	62.5	53.1
11 3 0	—	− 1.2			

at (1/2, 0, 1/2) is explained, and furthermore a maximum at ± (0.42, 0, 0.04), height 1/4, must exist. The large maximum observed at ± (0.41, 0, 0.06) is thus explained as resulting from an overlap of maxima at ± (0.40, 0, 0.08) and ± (0.42, 0, 0.04). From space considerations, the y_{II} parameter of the Ni_{II} atoms should have a value near 0.57, if the y_I parameter is taken to be 0.25.

The following approximate nickel positions were thus derived:

$$8\ Ni_I \text{ in } 8(f)\text{: } x_I = 0.04;\ y_I = 0.25;\ z_I = 0.48$$
$$8\ Ni_{II} \text{ in } 8(f)\text{: } x_{II} = 0.20;\ y_{II} = 0.57;\ z_{II} = 0.29$$

Signs of the $F(h\ 0\ l)$- and the $F(h\ k\ 0)$-values were computed from the model, and the electron density projections $\varrho\ (x\ z)$ and $\varrho\ (x\ y)$ were calculated, with the use of the intensity material from the zero layer lines around the b and the c axis. In addition to the nickel maxima, boron maxima corresponding to one 8(f) and one 4(e) position became visible. The two projections were refined

with successive difference syntheses. The final R-values obtained were 0.105 for the $|F(h\ 0\ l)|$-values and 0.103 for the $|F(h\ k\ 0)|$-values; an empirical temperature factor with $B = 0.20$ Å^2 was applied in both cases. The boron parameters obtained from the difference synthesis maps were not very accurate, but the following boron parameters were found: B_I in 8(f): $x_I = 0.234$; $y_I = -0.10_2$; $z_I = 0.437$ and B_{II} in 4(e): $y_{II} = -0.08_8$. From space considerations, the following B_I parameters seemed more reasonable: $x_I = 0.234$; $y_I = -0.091$; $z_I = 0.443$. These parameter values were wholly compatible with the experimental intensity data.

Observed and calculated $F(h\ 0\ l)$- and $F(h\ k\ 0)$-values are compared in Table 6.

The final structure of monoclinic Ni_4B_3 is the following:

Space group $C2/c$—(C_{2h}^6); $Z = 4$;

$a = 6.430$ Å; $b = 4.882$ Å; $c = 7.818$ Å; $\beta = 103°18'$; $U = 238.8$ Å^3; $D_x = 7.43$ g.cm^{-3}

		x	y	z
8 Ni_I	in 8(f):	0.043_5	0.250	0.483_9
8 Ni_{II}	in 8(f):	0.202_9	0.568	0.287_7
8 B_I	in 8(f):	0.23_4	0.09_1	0.44_3
8 B_{II}	in 8(e):	—	0.08_8	—

Interatomic distances are given in Table 7.

Table 7. Interatomic distances in monoclinic Ni_4B_3 (Distances shorter than 3.2 Å listed).

	Ni_I	Ni_{II}	B_I	B_{II}
Ni_I	2.53 (2), 2.61	2.52, 2.53, 2.56, 2.63 2.79, 2.81, 3.15	2.0_9, 2.1_3 2.1_4, 2.1_8	2.3_0 2.4_3
Ni_{II}	2.52, 2.53, 2.56, 2.63 2.79, 2.81, 3.15	2.62 (2), 2.54	2.0_4, 2.0_5 2.0_9	2.1_1 2.1_4
B_I	2.0_9, 2.1_3 2.1_4, 2.1_8	2.0_4, 2.0_5 2.0_9	1.8_3	1.8_7
B_{II}	2.3_0 (2) 2.4_3 (2)	2.1_1 (2) 2.1_4 (2)	1.8_7 (2)	

DESCRIPTION OF THE MONOCLINIC Ni_4B_3 STRUCTURE.

The m-Ni_4B_3 structure can be described as built-up by rather close-packed, slightly corrugated nickel atom layers with chains of boron atoms running through the channels in the metal atom lattice. The nickel atom layers are parallel with {202}, and the arrangement of the nickel atoms in one layer is shown schematically in Fig. 4. The {202} layers in m-Ni_4B_3 are closely related to the slightly corrugated {10$\bar{1}$1} layers in hexagonal close-packed structures, and are intermediates between the {103} layers in Ni_3B ($D\ O_{11}$ structure) and the {0 0 1} layers in Ni_2B (C 16 structure). (Compare Fig. 4 in the present paper

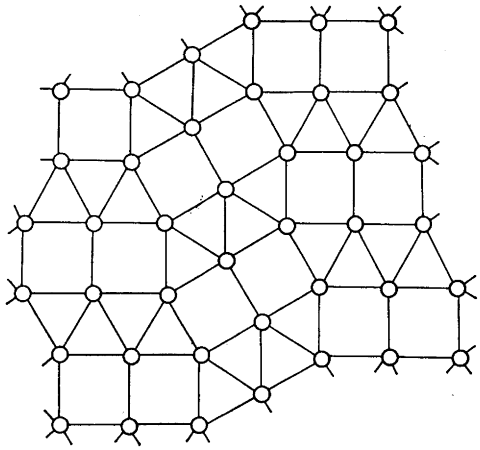


Fig. 4. Nickel atom layers parallel with {202} in monoclinic Ni_4B_3.

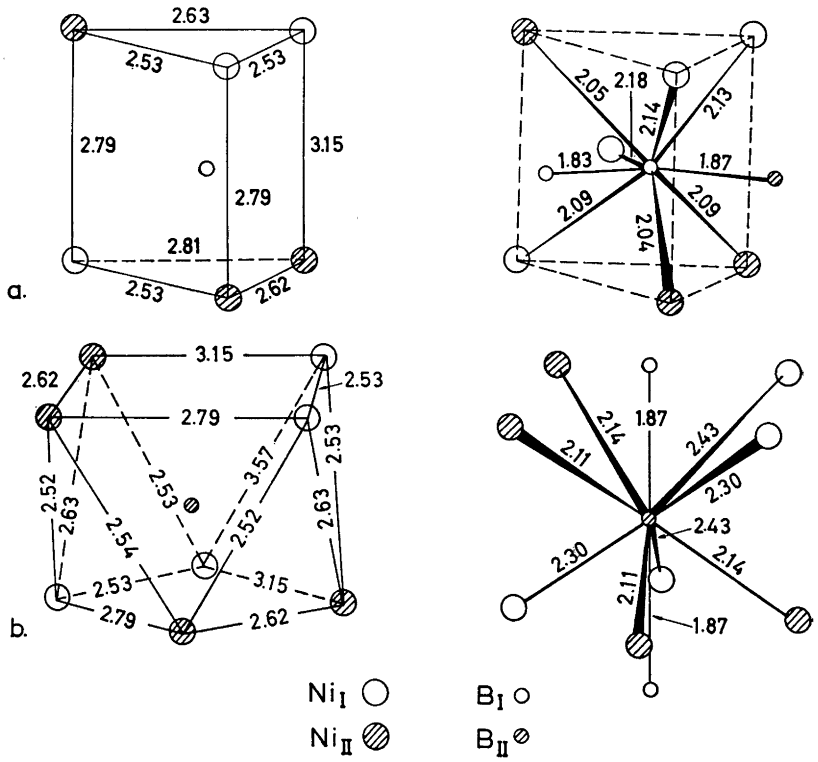


Fig. 5. The environment of the boron atoms in monoclinic Ni_4B_3
a. the environment of the B_I atoms
b. the environment of the B_{II} atoms

with Figs. 1, 2 and 3 in Ref.[9]). In Ni_3B, the boron atoms are situated in trigonal prismatic holes without forming any close B-B contacts. In Ni_2B, the boron atoms are situated in antiprismatic holes and form strings throughout the structure with a B-B distance of 2.12 Å. In *m*-Ni_4B_3, both types of holes exist; the B_I atoms occupy distorted trigonal prismatic holes, while the B_{II} atoms occupy distorted antiprismatic holes. The B_I environment is shown in Fig. 5a and the B_{II} environment in Fig. 5b. All of the boron atoms are connected in infinite chains, the sequence of which is $--B_{II}-B_I-B_I-B_{II}-B_I-B_I--$ with the B_I-B_{II} distance 1.8_7 Å and the B_I-B_I distance 1.8_3 Å. The mean Ni-B_{II} distance in the *m*-Ni_4B_3 antiprisms is 2.25 Å as compared with the Ni-B distance of 2.14 Å in the Ni_2B antiprisms.

GENERAL REMARKS

From the crystal-chemical point of view, the series of nickel borides Ni_3B, Ni_2B, *o*-Ni_4B_3, *m*-Ni_4B_3 and NiB provides a striking example of the gradually increasing B-B bond formation with increasing boron content in transition metal borides [15]. The structural similarities between the 'complicated carbides' and the nickel borides may also be noted; Ni_3B and Fe_3C are isostructural [9], while *o*-Ni_4B_3 and NiB are structurally related to Cr_3C_2 [16].

The present phase analysis has shown that the old equilibrium diagram given by Giebelshausen [1] is not reliable. The investigation of Hoppin III [2] provides accurate thermal data only up to 17 atom-% boron. It is desirable that the remaining part of the equilibrium diagram be investigated with modern metallographic and thermal analytical methods.

Acknowledgements. The author has performed this investigation during the tenure of a generous scholarship from the *Statens Tekniska Forskningsråd.* The work has been financially supported in part by the *Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force,* through its European Office under Contract No. AF 61(052)-40.

The author wishes to thank Professor G. Hägg for his kind interest. Thanks are also due Dr. B. Aronsson for many stimulating discussions.

REFERENCES

1. Giebelshausen, H. *Z. anorg. Chem.* **91** (1915) 257.
2. Hoppin III, G. S. *Welding J.* **36** (1957) 528.
3. Bjurström, T. *Arkiv Kemi, Mineral. Geol.* **11 A** (1933) No. 5.
4. Andersson, L.-H. and Kiessling, R. *Acta Chem. Scand.* **4** (1950) 160.
5. Blum, P. *J. phys. radium* **13** (1952) 430.
6. Kiessling, R. *Acta Chem. Scand.* **3** (1949) 595.
7. Marion, S. *Bull. soc. chim. France* **1957** 522.
8. Rundqvist, S. *Nature* **181** (1958) 259.
9. Rundqvist, S. *Acta Chem. Scand.* **12** (1958) 658.
10. Fruchart, R. and Michel, A. *Bull. soc. chim. France* **1958** 278.
11. Blok, N. J., Kozlova, M. N., Lashko, N. F. and Shpunt, K. Ya. *Doklady Akad. Nauk SSSR* **113** (1957) 811.
12. Rundqvist, S. and Jellinek, F. *Acta Chem. Scand.* **13** (1959) 425.
13. Thomas, L. H. and Umeda, K. *J. Chem. Phys.* **26** (1957) 293.
14. Ibers, J. A. *Acta Cryst.* **10** (1957) 86.
15. Kiessling, R. *Acta Chem. Scand.* **4** (1950) 209.
16. Hellbom, K. and Westgren, A. *Svensk Kem. Tidskr.* **45** (1933) 141.

Received March 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1209–1220

On the Effect of Ultrasonic Waves on Aqueous Solutions of 2-Chloro-Ethanol

JØRN THAMSEN*

Royal Danish School of Pharmacy, Copenhagen, Denmark

The present investigation deals with the influence of ultrasonic waves on the rate of hydrolysis of 2-chloro-ethanol (ethylene chlorohydrin) in pure aqueous solution at practically constant temperature. The experiments have been performed mainly with ultrasonic waves of frequency 0.8 Mcps.

The acoustic power from the transducer has been calculated from the radiation pressure, which is measured with a radiometer. It is established that the reaction is promoted in the ultrasonic field and the velocity of the reaction has been found dependent on certain experimental variables (the acoustic power, the concentration of 2-chloroethanol, and the ultrasonic field, *i. e.* the experimental arrangement).

The rate constant has been expressed by the experimental variables in a joint interpolation formula.

When certain alkyl halides, *e.g.* $C_2H_4Cl_2$, $CHCl_3$, $CHBr_3$, CCl_4 *etc.* are subjected to intense ultrasonic waves in aqueous solution (saturated with air)[1] the action of the ultrasonic waves is revealed by a partial liberation of halide from the alkyl halides. This effect has not been considered as being due to the oxidizing properties acquired by water containing air, when exposed to ultrasonic irradiation [2,3], and should thus be the result of a specific action of the ultrasonic waves on the alkyl halides. However, information in the literature about the action of the ultrasonic waves is often of a qualitative character and in particular there is not sufficient information about the sonic field.

The alkyl halides mentioned above are more or less insoluble in water, which reduces the experimental possibilities. Experiments with soluble chloro compounds such as chloroethanoic acid and 2-chloro-ethanol, however, have shown that these compounds in pure aqueous solution also partly liberate chloride ions, when exposed to ultrasonic waves. However, it should be noted that

* The investigation was carried out at The Laboratory of Technical Physics, Technical University of Denmark, Copenhagen, and supported by a grant from *Det teknisk-videnskabelige Forskningsraad.*

2-chloro-ethanol, without the influence of ultrasonic waves, is hydrolyzed very slowly in pure aqueous solution and that the reaction follows 1'-order kinetics to high degrees of conversion [4]. The reaction in the ultrasonic field could be due to such a hydrolysis, which is somehow accelerated by the ultrasonic waves. The present investigation deals with the rate by which chloride ion is formed in pure aqueous solutions of 2-chloro-ethanol subjected to ultrasonic waves under different experimental conditions, and with the dependence of the rate constant on certain experimental variables.

EXPERIMENTAL

In the present investigation the ultrasonic waves were generated by a Siemens sonostat universal piezoelectric generator, which is able to produce ultrasonic waves of the frequencies 0.8 and 2.4 Mcps corresponding to the 1′ and 3′ harmonic mode of vibration and deliver acoustic powers from 2 to 50 watts from a quartz with a radiating surface of 10 cm^2. A cross-section of the experimental arrangement is illustrated in Fig. 1. Since the ultrasonic field plays an important role in the experiments, it is necessary to be careful, in particular when transferring the ultrasonic waves to the test-samples. This transfer is carried out as follows. The transducer (1) is inserted into the bottom of a water thermostat (2). A cylinder made up of two parts, which forms a container in connection with the transducer (3), is mounted on top of the transducer. The lower narrow part, which fits on to the transducer, consists of a short cylinder provided with a packing groove, which is secured with a bayonet socket and a packing ring without causing injury to the transducer. The upper wider part is screwed on to the lower part, the former being provided with windows to ensure lighting inside the container. Another cylinder (6) of inner diameter 45 mm and with double walls so that the cylinder can be cooled by conducting a flow of water between the walls, is inserted inside this outer container. This reaction cylinder may be closed below by some sort of membrane ("acoustic window"), *e. g.* 0.05 mm triacetate foil * (5), which is distended between the cylinder and a screwed cap, and then constitutes a reservoir for the samples. This inside silverplated reaction cylinder is kept in a fixed position vertical to the transducer by a ring (4), which also provides for a practically constant distance between the transducer and the liquid surface of the sample (7) in the cylinder. The distance between the transducer and the membrane is about 50 mm. When the ultrasonic waves are transmitted vertically from the transducer through the "acoustic window" into the sample in the reaction cylinder, the space between the transducer and the membrane and around the reaction cylinder is filled up by water carefully freed from dissolved air. It is convenient to use such water, since the ultrasonic waves otherwise liberate small air bubbles from the water, which rise below the membrane and have a scattering effect.

When the sample in the reaction cylinder and the water between the transducer and the membrane are influenced by the ultrasonic waves the temperature immediately rises due to absorption. It is therefore necessary to use automatic cooling to keep the temperature practically constant in the sample during the experiment. This has been done by conducting cooling water through the reaction cylinder when the temperature in the sample rises. The flow of cooling water is directed by means of a Cu-constantan thermocouple with the junctions placed in the sample and in the water thermostat, respectively, (8), combined with a photo relay and a magnet valve. During the experiments the temperature in the sample is recorded by the voltage of a thermo-couple with the junctions placed in the sample and in crushed ice (9), respectively, on a "Speedomax"-recorder Type G. It is thus possible to keep the temperature practically constant to $25.0° \pm 0.8°$ C; otherwise the temperature would rise about 10 degrees.

In an experiment the sample, usually 50 ml, is treated for a definite time in the reaction cylinder, observing, before and after the treatment, the absence of air bubbles below the membrane. After the treatment the constancy of the volume of sample is also observ-

* The foil has been demonstrated to be impermeable to chloride ions.

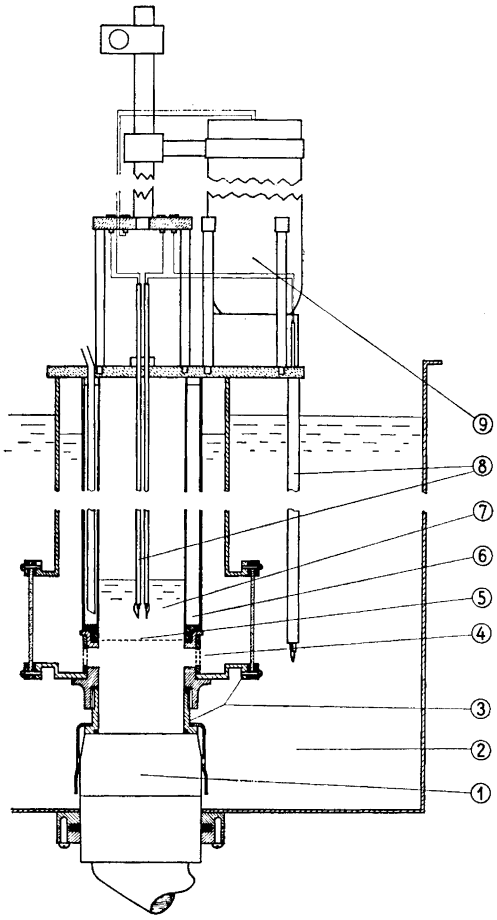


Fig. 1. Experimental arrangement in cross-section: 1, Transducer; 2, Water thermostat; 3, Outer container; 4, Distance-ring; 5, Membrane, "Acoustic window"; 6, Reaction cylinder; 7, Sample; 8, Thermo-couples; 9, Dewar flask for crushed ice.

ed to ensure that the sample has not been inadvertently diluted by water from the outer container, after which the chloride ions in a 20.0 ml sample are titrated with a 0.0028 N mercuric nitrate solution. Diphenylcarbazone is employed as indicator. This produces a red-purple color with excess of mercuric ions [5] in dilute acid solution. The color change during the end of the titration was followed by means of a Lange photoelectric colorimeter, which previously was adjusted against color-solutions for comparison prepared from the same quantities of reagents and an excess of 0.3 ml 0.0028 N mercuric nitrate. Since the initial solutions of 2-chloro-ethanol had a slight content of chloride ions, the data are corrected with regard to a blank value carried out with 20 ml solution. The blank values are about 0.4—3 ml 0.0028 N mercuric nitrate dependent on the concentration of 2-chloro-ethanol. The amounts of mercuric nitrate solution corresponding to the chloride ions formed during the experiments range from 1 to 40 ml 0.0028 N mercuric nitrate. In some of the long duration experiments the content of chloride ion was deter-

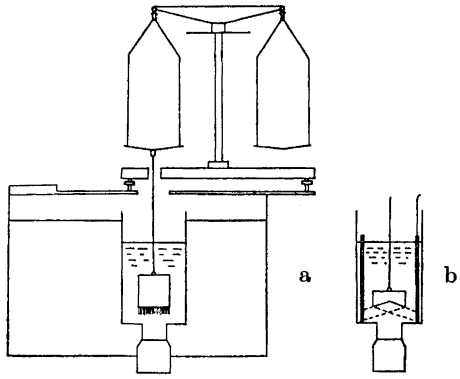


Fig. 2. Experimental arrangement for the measurement of the acoustic power.

mined by conductometric titration with 0.0028 N mercuric nitrate. The solutions of 2-chloro-ethanol were prepared from a fraction of 2-chloro-ethanol "Fluka" which distilled between 128.3° and 128.7°C at 753 mm Hg and had $n_D^{20} = 1.4422$. The boiling point of 2-chloro-ethanol is 128.6°C and $n_D^{20} = 1.4421$.

THE ACOUSTIC POWER FROM THE TRANSDUCER

When a beam of plane ultrasonic waves in a fluid encounters an obstacle, then the interaction of the obstacle with the plane wave motion results in a radiation pressure exerted upon the obstacle proportional to the mean energy density of the wave motion [6].

The power radiated in an ultrasonic field is closely related to the radiation pressure [7] and is usually determined by a measurement of the net force exerted on a proper "radiometer", which may be either a perfect reflector or a perfect absorber, placed in the path of the waves. For the present determinations of the total acoustic power emitted from the transducer two different radiometers have been used, one a nearly perfect absorbing nylon-brush, the other a nearly perfect reflecting hollow brass-radiometer. The experimental arrangement is shown in Fig. 2.

The brass-radiometer [8] which is provided with a conical turning out (135°) is filled with air. The air inside the radiometer ensures a practically complete reflection from the surface and the conical shape makes the radiometer self-centralizing and brings about a reflection parallel with the opposite surface. The reflected waves are absorbed in a cylinder of absorbing material to avoid standing waves acting back on the transducer (Fig. 2 b).

The radiometer is suspended below the pan of a balance in a thin nylon thread, and completely immersed in and surrounded by water freed from dissolved air, vertically above and as close as possible to the transducer. Air bubbles below the radiometer are carefully removed and the radiometer is tared. The resulting buoyancy when the ultrasonic waves interact with the

radiometer is finally counterbalanced with weights, q g. If the ultrasonic waves emitted from the transducer are assumed to be free plane waves in a lossless medium the alternating excess sound pressure p_e is related to the alternating particle velocity u by $p_e = u\varrho_o h_o$ and analogous $p_{e,rms} = u_{rms}\varrho_o h_o$ where ϱ_o is the static density of the medium, h_o the velocity of the waves and $\varrho_o h_o$ the characteristic impedance. The acoustic power W_P is

$$W_P = \frac{1}{T}\int_0^T up_e A\,dt = u_{rms}p_{e,rms}A = \frac{UPA}{2} = \frac{\varrho_o h_o U^2 A}{2} \text{ watts}$$

where T is the period, A the total area of the beam, and P and U are the respective amplitude values.

The radiation pressure Π is related to the particle velocity amplitude and the weights q g by

$$\Pi = \frac{\varrho_o U^2}{2} = q\ 9.81 \times 10^{-3} A^{-1} \text{ Newton m}^{-2}$$

With respect to the nylon-brush which is larger in section than the beam and as mentioned above acts as an absorber the counterbalanced weights q g corresponds thus, following the above expressions, with the acoustic power

$$W_P = \frac{\varrho_o U^2 h_o A}{2} = q\ 9.81 \times 10^{-3} h_o = 14.69\ q \text{ watts}$$

where the velocity of sound in water $h_o = 1\,497$ m sec^{-1} at 25°C.

In the case of the brass-radiometer, which is also larger in section than the beam and acts as a perfect reflector, the beam intersects the radiometer by an angle $\Theta = 22.5°$ and the acoustic power is then

$$W_P = \Pi\ h_o A = q\ 9.81 \times 10^{-3}\ h_o\ (2\cos^2\Theta)^{-1} = 8.61\ q \text{ watts}$$

Table 1. The acoustic power from the transducer at different primary voltages on the anode-transformer of the generator, as measured by a nylon-brush and a brass-radiometer.

V volt	0.8 Mcps				2.4 Mcps			
	Nylon-brush		Brass-radiometer		Nylon-brush		Brass-radiometer	
	q g	14.69 q watts	q g	8.61 q watts	q g	14.69 q watts	q g	8.61 q watts
50	0.15	2.2	0.26	2.2	0.12	1.8	0.20	1.7
75	0.36	5.3	0.60	5.2	0.34	5.0	0.44	3.8
100	0.61	9.0	1.05	9.0	0.51	7.5	0.80	6.9
125	1.04	15.3	1.75	15.0	0.83	12.2	1.30	11.2
150	1.44	21.2	2.63	22.6	1.23	18.1	2.00	17.2
175	2.01	29.5	3.65	31.4	1.73	25.4	2.85	24.5
200	2.69	39.5	4.93	42.4	2.27	33.3	3.80	32.7
225	3.30	48.5	6.35	54.7	2.81	41.3	4.70	40.5

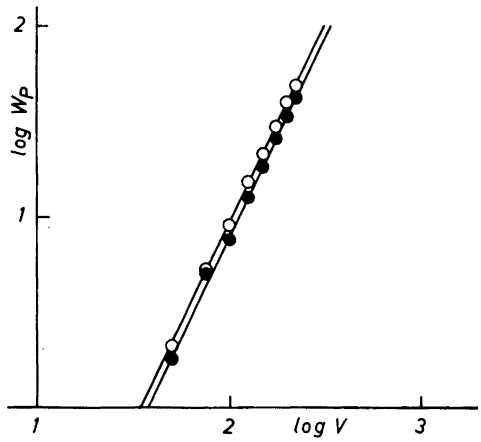


Fig. 3. A plot of log W_P against log V. W_P is the acoustic power as measured by the nylon-brush, V is the primary voltage on the anode-transformer of the generator. O, experiments performed at 0.8 Mcps; ●, experiments performed at 2.4 Mcps.

The results at different primary voltages on the anode transformer of the generator are shown in Table 1.

In Fig. 3 log W_P (as measured by the nylon-brush) is plotted against log V (primary voltage on the anode-transformer). From the figure it is inferred that approximately W_P = constant × V^2 in accordance with the expression for the acoustic power output from a piezoelectric quartz $W = V^2_{rms}/R_e$, where V_{rms} is the voltage applied to the quartz and R_e a motional resistance.

THE KINETIC OF THE REACTION IN THE ULTRASONIC FIELD

When 2-chloro-ethanol is hydrolyzed in dilute pure aqueous solution thus: $CH_2ClCH_2OH + HOH = CH_2OHCH_2OH + H^+ + Cl^-$, the reaction follows 1'-order kinetics, *i.e.* $-\frac{1}{c}\frac{dc}{dt} = k_1$, where $c = a-x$ is the concentration of 2-chloro-ethanol left at time t and a the initial concentration. When the reaction proceeds in the ultrasonic field the initial velocity $s = -\frac{dc}{dt}$ or $\frac{1}{c}s = -\frac{dc}{cdt}$ could be estimated from the experimental results.

1. The dependence on the acoustic power.

Experiments with samples of 0.05 litre at $\nu = 0.8$ Mcps and varying values of the acoustic power have shown that the initial velocity changes with the acoustic power.

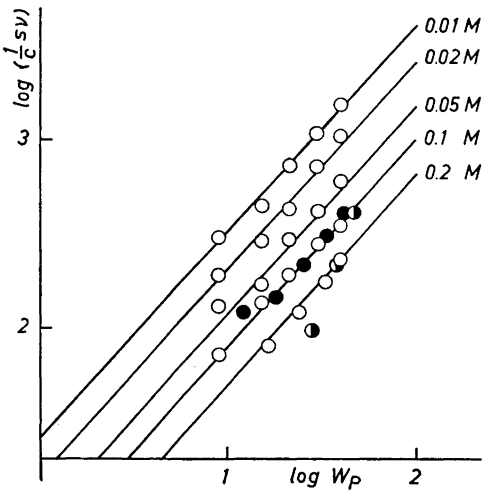


Fig. 4. A plot of $\log\left(\frac{1}{c}sv\right)$ against $\log W_P$. s is the initial velocity found in experiments with samples of 0.05 litre, c is the concentration of 2-chloro-ethanol, v the frequency and W_P the acoustic power. ○, experiments performed at 0.8 Mcps; ●, experiments with 0.1 M, and ◑ with 0.2 M, performed at 2.4 Mcps; 25°C.

In Fig. 4 for convenience $\log\left(\frac{1}{c}sv\right)$ is plotted against $\log W_P$. From the figure it could be inferred that $\frac{1}{c}sv = \text{constant} \times W_P^{1.1}$. In the figure are also indicated the results from experiments with 0.1 and 0.2 M solutions at $v = 2.4$ Mcps, whereof in all events the experiments with 0.1 M appear to follow the above relation fairly well. Similar experiments with a 0.01 M solution failed because the liberated amounts of chloride ion were too small for determining an initial velocity with any reasonable certainty.

2. The dependence on the concentration of 2-chloro-ethanol.

Experiments with samples of 0.05 litre at $v = 0.8$ Mcps, W_P kept constant and varying concentrations of 2-chloro-ethanol, have shown that the initial velocity also changes with the concentration. In Fig. 5 $\log\left(\frac{1}{c}sv\right)$ is plotted against $\log(10^2 c)$. From the figure it could be deduced that $\frac{1}{c}sv = \text{constant} \times c^{-0.62}$. By combining the results derived from Figs. 4 and 5 it could finally be inferred that the initial velocity in experiments with 0.05 litre sample, $v = 0.8$ Mcps, and the experimental conditions mentioned, follows the expression $s = 1.48\ v^{-1} W_P^{1.1} c^{0.38}$ mole litre^{-1} min^{-1}.

3. The dependence on the volume of sample.

Preliminary experiments with samples of 0.10 litre instead of 0.05 litre had shown that changes in the volume of sample had a marked effect on the initial velocity.

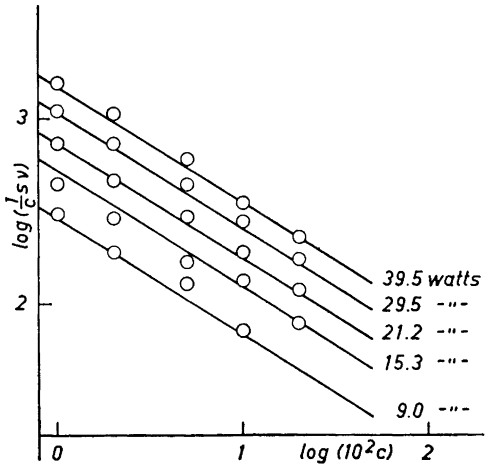


Fig. 5. A plot of $\log\left(\frac{1}{c}s\nu\right)$ against log (10^2c). Experiments with samples of 0.05 litre; $\nu = 0.8$ Mcps; 25°C.

Experiments with a 0.01 M solution of 2-chloro-ethanol at $\nu = 0.8$ Mcps, the acoustic power 43 watts and varying volume of sample have shown that this effect is closely connected with the dimension of the reaction cylinder (diameter 4.5 cm) and the ultrasonic field, *i.e.* the experimental arrangement.

In the present experimental arrangement the waves are radiated against the free liquid surface of the sample where the waves are almost completely reflected, which results in a field of standing waves. If we assume that the attenuation is small and the absorption coefficient nearly the same in the water below the membrane and in the sample, then we can say approximately that the acoustic energy absorbed in the sample is related to the part of the volume filled up by the ultrasonic waves, which is standing in the sample $\frac{V_1}{V_0 + V_1} = \frac{l_1}{l_0 + l_1}$, where l_0 is the distance from the surface of the transducer to the membrane in the reaction cylinder (about 5.0 cm), l_1 the height of sample in the reaction cylinder, and the radiating surface 10 cm^2.

When the absorbed acoustic energy finally is distributed in the sample, then the acoustic energy absorbed per unit volume of sample is related to $\frac{l_1}{(l_0 + l_1)V_s} = \frac{10^3}{(l_0 + l_1)A_R}$ where V_s litre is the volume of sample and A_R the area of the base of the reaction cylinder. In Fig. 6 $\log\left(\frac{1}{c}s\nu\right)$ is plotted against $\log\left(\frac{10^3}{(l_0 + l_1)A_R}\right)$. From this figure it could be inferred that $\frac{1}{c}s\nu = \text{constant} \times \left(\frac{10^3}{(l_0 + l_1)A_R}\right)^{2.0}$.

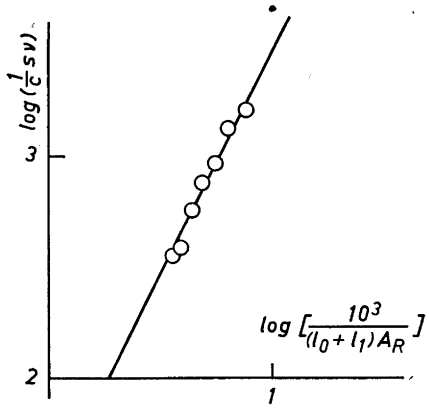


Fig. 6. A plot of $\log\left(\frac{1}{c}sv\right)$ against $\log\left(\frac{10^3}{(l_0+l_1)A_R}\right)$. Experiments with a 0.01 M solution of 2-chloro-ethanol and varying volume of sample; l_0 is the distance between the transducer and the "acoustic window", l_1 the height of sample in the reaction cylinder, A_R the area of the base of the reaction cylinder; $\nu = 0.8$ Mcps and the acoustic power $W_P = 43$ watts; 25°C.

By combination of this result with that previously obtained a joint interpolation formula could finally be deduced, thus

$$s = 2.48 \times 10^{-2}\left(\frac{10^3}{(l_0 + l_1)A_R}\right)^{2.0} W_P^{1.1}\nu^{-1}c^{0.38}$$

$= kc^{0.38}$ mole litre^{-1} min^{-1}, where $\nu = 0.8$ Mcps.

It is so established that

$$-\frac{d[CH_2ClCH_2OH]}{dt} = k[CH_2ClCH_2OH]^{0.38}$$

Table 2. Samples of 0.05 litre 0.01 M 2-chloro-ethanol subjected to ultrasonic waves of 0.8 Mcps and acoustic power 43 watts. 25°C. $t_{calc.} = \frac{1}{k_{graf.}} f(x)$; $k_{graf.} = 105 \times 10^{-6}$ and $k_{calc.} = 116 \times 10^{-6} \cdot (\text{mole/litre})^{0.62}\ \text{min}^{-1}$.

t min. obs.	x	$a - x$	$f(x)$	t min. calc.
60	0.00112	0.00888	0.00659	62.7
60	0.00115	0.00885	0.00677	64.5
60	0.00108	0.00892	0.00635	60.5
120	0.00214	0.00786	0.01287	122.6
120	0.00205	0.00795	0.01231	117.2
120	0.00203	0.00797	0.01218	116.0
120	0.00199	0.00801	0.01193	113.6
180	0.00277	0.00723	0.01691	161.0
180	0.00276	0.00724	0.01684	160.4
180	0.00282	0.00718	0.01723	164.1
240	0.00385	0.00615	0.02415	230.0
300	0.00483	0.00517	0.03116	296.8
360	0.00574	0.00426	0.03814	363.2

Table 3. The experimental values of the rate constant k found in experiments with samples of 0.05 litre and varying values of the acoustic power W_P and the concentration of 2-chloro-ethanol. 25°C. W_P^* is the acoustic power after a correction in the electric circuit of the generator.

0.8 Mcps. W_P^*	0.8 Mcps. W_P	0.2 M $k_{exp.}10^6$	0.1 M $k_{exp.}10^6$	0.05 M $k_{exp.}10^6$	0.02 M $k_{exp.}10^6$	0.01 M $k_{exp.}10^6$	$k_{calc.}10^6$	$k^*_{calc.}10^6$
43.0	39.5	106	104	118	117	108	105	—
33.0	29.5	83 *	82	85	79	78	76	86
24.0	21.2	55 *	56	58	52	52	53	61
16.6	15.3	38 *	41	32	32	30	37	41
10.1	9.0	—	21	24	22	21	21	—
2.4 Mcps. W_P^*	2.4 Mcps. W_P	0.2 M $k_{exp.}10^6$	0.1 M $k_{exp.}10^6$				$k_{calc.}10^6$	$k^*_{calc.}10^6$
47.2	41.3	68 *	45				37	43
37.6	33.3	36 *	35				29	33
28.2	25.4	17 *	25				22	24
20.2	18.1	—	14				15	—
14.3	12.2	—	12 *				—	11

which equation by integration gives

$$t = \frac{1}{k} f(x) = \frac{1}{k} \frac{1}{0.62} [(a)^{0.62} - (a-x)^{0.62}]$$

Table 2 gives the results from experiments with a 0.01 M solution of 2-chloro-ethanol, $\nu = 0.8$ Mcps, and the acoustic power 43 watts. From the table it appears that the reaction follows the above integrated form of the rate equation fairly well. For comparison of the rate of reaction in the ultrasonic field with that without ultrasonic waves a value of the rate constant at 25°C extrapolated from the data of Radulescu and Muresanu [9] should be stated $k_1 = 6 \times 10^{-8}$ min^{-1} and thus the rate of reaction is considerably increased

Table 4. The dependence of the rate constant k at 25°C on the volume of sample V_s in experiments with a 0.01 M solution of 2-chloro-ethanol at $\nu = 0.8$ Mcps and the acoustic power 43 watts. $l_0 = 5.0$ cm and $A_R = 15.9$ cm^2.

Sample V_s litre	Height of liquid column of sample l_1 cm	$\frac{10^3}{(l_0 + l_1) A_R}$ litre^{-1}	$k_{exp.}10^6$	$k_{calc.}10^6$
0.050	3.15	7.72	105	116
0.075	4.72	6.47	88	81
0.100	6.29	5.57	66	60
0.125	7.86	4.89	53	46
0.150	9.44	4.36	40	37
0.175	11.01	3.93	27	30
0.200	12.58	3.58	24	25

in the ultrasonic field. In Table 3 are the experimental values of the rate constant k found in experiments with 0.05 litre sample and with varying values of the acoustic power W_P and the concentration of 2-chloro-ethanol at $\nu = 0.8$ Mcps and extended up to 100 min.

For comparison the values calculated from

$$k = 2.48 \times 10^{-2} \left(\frac{10^3}{(l_0 \times l_1) A_R} \right)^{2.0} W_P^{1.1} \nu^{-1} \text{ (mole/litre)}^{0.62} \text{ min}^{-1}$$

are also given in Table 3.

It is remarkable that the experiments at 2.4 Mcps, which is indicated in Table 3, also look as if they comply with the expression for the rate constant at $\nu = 0.8$ Mcps, but this fact is for the present not sufficient to elucidate the dependence on frequency, the previously mentioned experiments with a 0.01 M solution also being taken into account.

In Table 4 are the experimental and calculated values of the rate constant k originating from experiments with a 0.01 M solution of 2-chloro-ethanol, $\nu = 0.8$ Mcps, $W_P = 43$ watts and varying volume of sample V_s extended up to 120 min.

From the results of the experiments it has been established that the ultrasonic waves promote the liberation of chloride ions from 2-chloro-ethanol in pure aqueous solution and that there is a connection between the rate of reaction in the ultrasonic field and the experimental conditions by which the experiments are performed, which in this instance means the acoustic energy dissipated per unit volume. When the chemical activation is connected with the acoustic energy dissipated in the sample, a subsequent investigation on the absorption coefficients of sound in solutions of 2-chloro-ethanol may possibly give important information, whether the activation is connected with the absorption and thus a relaxation mechanism alone or also other factors are of importance.

The experiments with varying volumes of sample have revealed a deficiency as to the present experimental arrangement. Because of the distance l_0 between the transducer and the "acoustic window" in the reaction cylinder only a part of the acoustic energy emitted has been available for the present purpose of activation in the sample, and thus l_0 has to be as small as possible.

When all the experiments are carried out with solutions containing air and with free access to the air, oxidation is to be expected in the samples exposed to the ultrasonic waves. This fact may have nothing to do with the liberation of chloride ions from the 2-chloro-ethanol, but it should be noted that aldehyde, possibly originating from 1,2-ethanediol (glycol), has been demonstrated in the samples, and may thus be regarded as a secondary phenomenon.

Acknowledgements. I am much indebted to professor R. E. H. Rasmussen for his hospitality, kindness and many interesting discussions, and to professor Carl Faurholt for his kind interest in the work. Finally I wish to thank Mr. S. J. Gjendal, electrical engineer, M. Sc., for technical support and Mr. C. Hartvig Andersen for promptitude in making requisite instruments.

REFERENCES

1. Kling, A. and Kling, R. *Compt. rend.* **223** (1946) 1131.
2. Kling, A. and Kling, R. *Ibid.* **223** (1946) 33.
3. Virtanen, A. and Ellfolk, N. *Acta Chem. Scand.* **4** (1950) 93.
4. Cowan, H., McCabe, C. and Warner, J. *J. Am. Chem. Soc.* **72** (1950) 1194.
5. Charlot, G. and Bézier, D. *Analyse Quantitative Minérale*, Masson et Cie, Paris 1955, p. 473.
6. Borgnis, F. *Revs. Modern Phys.* **25** (1953) 653.
7. Hueter, T. and Bolt, R. *Sonics*, Wiley and Sons, Inc., New York 1955, p. 41.
8. Oberst, H. and Rieckmann, P. *Amtsblatt der PTB, Braunschweig* **H.3** (1952) 106; **H.4** (1952) 143; Ref. Bergmann, L. *Der Ultraschall*, Hirzel Verlag, Stuttgart 1954, p. 216.
9. Radulescu, D. and Muresanu, P. *Bull. soc. sci. Cluj, Roumanie* **7** (1932) 129.

Received April 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1221–1225

Über Merkurierungen mit Quecksilbercyanat

ERIK SÖDERBÄCK

Chemisches Institut der Universität Uppsala, Schweden

Es wurden Cyanatomerkuriverbindungen von Pyrrol, Furan und Thiophen sowie von den 2-Methylderivaten der beiden letzteren durch Einwirkung von Merkuricyanat in Wasser auf die Wasserstoffverbindungen dargestellt. Von Pyrrol wird dabei in sehr glatter Reaktion ausschliesslich Dimerkuriverbindung gebildet, von Furan und Thiophen Mono- nebst Diderivat und von 2-Methylfuran (Silvan) und 2-Thiotolen lediglich 5-Monoderivat. Die Monoderivate sind gut kristallisierte Verbindungen mit scharfen Schmelzpunkten, die Diderivate sind nicht ohne Zersetzung schmelzbar. Die Monoderivate konnten auch in glatt verlaufenden Reaktionen durch Einwirkung von Merkuricyanat auf die entsprechenden Merkuridiaryle in Aceton gewonnen werden; es zeigte sich, dass auch stark komplexe Merkurisaltze wie Cyanid und $Hg(SCN)_4Cl_2$ einer solchen Umsetzung fähig sind. Bei Behandlung von Monomerkuricyanate mit Natriumjodid in Aceton werden sie in Merkuridiaryle zurückverwandelt, wobei die Cyanatgruppe als Natriumcyanat quantitativ ausgeschieden wird.

Das Quecksilberchlorid gibt bekanntlich Addukte mit einer Menge von Stoffen, die als Elektrondonatoren fungieren können. Wegen der grossen Ähnlichkeit zwischen Chlorid und Cyanat kann vermutet werden, dass das Cyanat zu analoger Adduktbildung im Stande ist. Da die Merkurierung mit Quecksilbercyanat wenigstens nicht schneller als diejenige mit dem Chlorid verläuft, sind die Aussichten zur Isolierung von Cyanataddukten, z.B. mit in Petroleum vorkommenden Schwefelverbindungen, gut.

In einer früheren Mitteilung [1] ist über die Darstellung und Eigenschaften einiger Metallcyanate berichtet worden. Besonderes Interesse wurde dabei dem Merkuricyanat $Hg(OCN)_2$ gewidmet, namentlich wegen gewisser hervorstechenden Ähnlichkeiten mit Quecksilberchlorid. Von diesen wurde der merkurierende Effekt von $Hg(OCN)_2$ auf gewisse heterocyklische Kerne kurz gestreift. Diese Reaktion ist nun etwas ausführlicher studiert worden, und die dabei gewonnenen Resultate sollen hier besprochen werden.

Auf Benzol und Benzolhomologen ist $Hg(OCN)_2$ eben wie $HgCl_2$, wenigstens bei Zimmertemperatur, ohne Einwirkung; mit Pyrrol, Furan und Thiophen sowie deren Homologen treten in Wasser Merkurierungen ein und die dabei entstehenden Produkte sind mit denjenigen von $HgCl_2$ als merkurierendes Agens durchaus analog. Besonders lebhaft ist in beiden Fällen die Reaktion mit Pyrrol; es wird sofortiges Ausfallen einer weissen Substanz beobachtet, wenn eine alkoholische Lösung von Pyrrol zu einer wässrigen gesättigten

Lösung von $HgCl_2$ bezw. $Hg(OCN)_2$ getropft wird. Die bei der Reaktion mit $Hg(OCN)_2$ entstehende Cyansäure tritt in so grossen Konzentrationen auf, dass sie trotz ihrer grossen Hydrolysengeschwindigkeit leicht durch den stechenden Geruch und andere Eigenschaften nachgewiesen werden kann. Das Reaktionsprodukt ist in diesem Fall Dicyanatomerkuripyrrol $C_4H_2NH(HgOCN)_2$ über dessen Struktur gegenwärtig nichts bestimmtes ausgesagt werden kann.

Die Reaktion von $Hg(OCN)_2$ mit Furan und Thiophen hat einen wesentlich langsameren Verlauf als mit Pyrrol und in diesem Fall entstehen eben wie mit $HgCl_2$ teils Mono- und teils Dimerkurierungsprodukte, von denen erstere als 2- und letztere als 2,5-Derivate zu betrachten sind. Sie können durch die Löslichkeit der Monoderivate in Äther leicht separiert werden; die Reindarstellung der Diderivate kann durch Extraktion mit Aceton bewirkt werden. Das Produkt aus Pyrrol ist in Aceton reichlich löslich, dasjenige aus Furan weniger und dasjenige aus Thiophen am schwersten löslich. Wenn die 2- oder 5-Stellung in Furan oder Thiophen durch Methyl besetzt ist, werden ausschliesslich Monoderivate gebildet, wodurch die Annahme, dass die Dimerkurierung in 2- und 5-Stellung angreift, erhärtet wird.

Die bisher dargestellten Monoarylmerkuricyanate sind gut kristallisierbare, in Wasser unlösliche Verbindungen von bestimmten Schmelzpunkten, die wesentlich niedriger als diejenigen der entsprechenden Chloride liegen. Sie können auch nach einer zweiten Methode erhalten werden; es ist lange bekannt, dass Quecksilberverbindungen vom Typus R_2Hg, wo R ein aliphatisches oder aromatisches Radikal bedeutet, mit Merkurisaltzen leicht nach der Gleichung

$$R_2Hg + HgX_2 \longrightarrow 2\ RHgX$$

disproportioniert werden. Als Lösungsmittel werden meistens Alkohol oder Aceton verwendet. Die Reaktion mit $Hg(OCN)_2$ wurde in Aceton studiert und als Merkuride diejenige von Phenyl, 2-Furyl, 2-Thienyl sowie die 5-Methylderivate der beiden letzteren benutzt. Die daraus gebildeten Arylmerkuricyanate waren mit den durch direkte Merkurierung von den entsprechenden Wasserstoffverbindungen gewonnenen identisch. Der Reaktionsverlauf war schon bei Zimmertemperatur ein sehr glatter und Steigerung der Temperatur wurde deshalb nur zwecks Abschliessen der Reaktion zugegriffen.

Die obige Reaktion kann in gewissen Fällen auch mit stark komplexen Hg^{2+}-Salzen wie Cyaniden durchgeführt werden. So konnten mit der Verbindung $Hg(SCN)_4Cl_2$, also dem Hg^{2+}-Salz des Chlor-merkaptothiodiazols [3], und Merkuridiphenyl sowie Merkuri-2-di-thienyl gut kristallisierte Verbindungen von scharfen Schmelzpunkten, $C_6H_5Hg(SCN)_2Cl$ bzw. $C_4H_3S{\cdot}Hg(SCN)_2Cl$, dargestellt werden. Der Umstand dass auch stark komplexe Merkurisalze reagieren lässt vermuten, dass auch das mit Merkuricyanat isomere Fulminat sich mit gewissen Hg-Dialkylen oder Arylen umsetzen lassen wird, wodurch vielleicht Initialzünder mit variabler Explosivität gewinnbar wären.

Mono-chlormerkuriverbindungen von Thiophen und Furan setzen sich in acetonischer Lösung glatt mit einem Mol Jod- bezw, Rhodannatrium zu Jod- bzw. Rhodan-merkurithiophen oder Furan um. Ein weiteres Mol NaJ bewirkt aber Bildung von HgJ_2 als komplexes Jodid und danebst Merkuridiaryl. Da

das komplexe $NaHgJ_3$ in Aceton löslich ist, kann das gebildete Diarylquecksilber durch Ausfällen mit Wasser isoliert werden. Phenyl und *p*-Tolyl und α-Naphthylmerkurichlorid geben nur die erste Stufe der Reaktion [4]. Furyl- und Thienylmerkuricyanat reagieren mit NaJ in Aceton genau wie die Chloride, wobei die Cyanatgruppe als NaOCN quantitativ abgeschieden wird, das nach Waschen mit Aceton von grosser Reinheit, namentlich ganz frei von Karbonat, isoliert werden kann.

EXPERIMENTELLER TEIL

Das für die folgenden Versuche benutzte Merkuricyanat war aus Quecksilberchlorid und Silbercyanat in Äther nach dem in einer früheren Mitteilung gegebenen Verfahren dargestellt.

Merkurierung von Pyrrol. Zu einer Lösung von 5,54 g $Hg(OCN)_2$ in 100 ml Wasser wurden langsam und unter regem Umschütteln 0,8 (ber. 0,67) ml Pyrrol in 10 ml Alkohol gelöst, getropft. Ein weisser Niederschlag entstand sofort, und der Geruch von HOCN wurde beobachtet. Das Reaktionsgemisch blieb über Nacht stehen und der Niederschlag dann auf ein Glasfilter G4 gesammelt, mehrmals mit Wasser gewaschen und in Vakuum über H_2SO_4 getrocknet. Gewicht 5,24 g statt 5,22. Das wässrige Filtrat enthielt nur geringe Mengen von Hg-Salz. Das Präparat wurde mit 500 ml Aceton extrahiert, wobei 0,75 g ungelöst blieben. Durch Abdestillieren des Acetons konnten 4,33 g des Merkurierungsproduktes als rein weisses undeutlich kristallines Pulver isoliert werden. Die Substanz wird von Salzsäure glatt unter CO_2-Entwicklung aufgelöst, was bei der Hg-Analyse (gravimetrisch als HgS) ausgenutzt wurde. (Gef. Hg 73,10; N 7,68 (Kjeldahl). Ber. f. $C_4H_3N(HgOCN)_2$ (550,3): Hg 72,91; N 7,69).

Merkurierung von Thiophen. 26,9 g $Hg(OCN)_2$ wurden in 450 ml Wasser gelöst und zu der Lösung eine solche von 31 g NaAc (Anhydrid) in 140 ml Wasser nebst 10 ml Thiophen gegossen. Das Gefäss, eine Flasche von 1 Liter, wurde mit einem Kork verschlossen. Das Reaktionsgemisch blieb während 10 Tagen bei Zimmertemperatur stehen; dann und wann wurde umgeschüttelt und die gebildete Kohlensäure ausgelassen. Der Niederschlag von Merkurierungsprodukten löste sich anfänglich im Thiophenüberschuss auf, fiel aber in dem Masse als dieses verbraucht wurde wieder aus. Wenn die Reaktion abgeschlossen war, wurde der Niederschlag auf ein Glasfilter G4 gesammelt, mehrmals mit Wasser gewaschen und in Vakuum über H_2SO_4 getrocknet. Ausbeute 26,6 g. Das Präparat wurde mit 1 500 ml Äther extrahiert, der unlösliche Rückstand mit Äther gewaschen und getrocknet; Gewicht 8,8 g. Die Ätherlösung wurde auf dem Wasserbade konzentriert, wonach 17 g Monomerkurierungsprodukt isoliert und durch nochmaliges Umlösen aus Äther gereinigt wurden. Prismen von Schmelzp. 104—105°. Für die Hg-Bestimmung wurde mit konz. HCl zersetzt und in der verdünnten Lösung Hg als HgS bestimmt. Stickstoff nach Kjeldahl. (Gef. Hg 61,56; N 4,27. Ber.f. $C_4H_3SHgOCN$ (325,63): Hg 61,61; N 4,30). Löslichkeit in Äther bei 20° 1,75 g pro 100 ml.

Der in Äther unlösliche Rückstand von 8,8 g wurde mit heissem Aceton extrahiert, was ziemlich zeitraubend war. Es konnten dabei 6,2 g Substanz ausgelöst und durch Einengen der Lösung isoliert werden.

Für die Hg-Analyse wurde die Substanz mit Salzsäure zersetzt und aus der verdünnten Lösung HgS ausgefällt. Stickstoff nach Kjeldahl. (Gef. Hg 70,55; N 4,77. Ber.f. $C_4H_2S(HgOCN)_2$ (567,35): Hg 70,72; N 4,93). Löslichkeit in Aceton bei 20° 0,08 g pro 100 ml. Molverhältnis Mono:Dimerkuriprodukt betrug bei diesen Versuch 4,7. Merkurierungen von Thiophenen mit $HgCl_2$ sind in grosser Anzahl von Steinkopf [2] und Mitarbeitern ausgeführt worden.

Merkurierung von Furan. 14,23 g $Hg(OCN)_2$ wurden in 220 ml H_2O gelöst und diese Lösung mit einer solchen von 16 g NaAc in 75 ml H_2O gemischt. Es wurden nun 5 ml Furan zugestzt, wonach das Reaktionsgemisch bei Zimmertemperatur aufbewahrt wurde. Ab und zu wurde umgeschüttelt. Nach einem Tage war eine beträchtliche Quantität eines gelbweissen Niederschlags ausgefallen. Nach zwei Tagen wurde das gebildete CO_2 ausgelassen und dann weiter alle zwei Tage. Nach 14 Tagen konnte die Reaktion als beendet angesehen werden; der Niederschlag wurde abfiltriert, mit Wasser gewaschen und getrocknet. Ausbeute 13,22 g. Bei Extraktion mit 800 ml Äther wurde ein in Äther unlöslicher Rückstand von 4,80 g erhalten. Durch Einengen der Ätherlösung

auf dem Wasserbade konnten 7,9 g einer in glänzenden Prismen kristallisierenden Substanz isoliert werden. Die Substanz wurde durch Umlösen aus Äther gereinigt und schmolz dann bei 122—123°. (Gef. Hg 65,05; N 4,52. Ber. $C_4H_3O.HgOCN$ (309.69): Hg 64.78; N 4,53). Löslichkeit in Äther bei 20° 1,04 g in 100 ml.

Das in Äther nicht lösliche Reaktionsprodukt, 4,8 g, wurde mit 500 ml Aceton extrahiert, wobei 0,5 g ungelöst blieben. Die etwas braun gefärbte Lösung wurde durch Abdestillieren des Acetons konzentriert, wobei zuletzt eine weisse feinkristallinische Substanz ausfiel. Totalausbeute 3,91 g. (Gef. Hg 72,62; N 5,01; Ber. f. $C_4H_2O(HgOCN)_2$ (551,31): Hg 72,78; N 5,08). Löslichkeit in Aceton bei 20° 0,75 g pro 100 ml.

Die Verbindung ist wie die analoge Thiophenverbindung nicht ohne Zersetzung schmelzbar. Das Molverhältnis Mono:Diverbindung beträgt bei diesem Versuch 3,2.

Chlormerkurifurane sind von Gilman und Wright [5] dargestellt worden.

Merkurierung von 2-Methylthiophen. Zu einer Lösung von 25,5 g $Hg(OCN)_2$ in 400 ml H_2O wurde eine solche von 33 g NaAc in 110 ml H_2O gesetzt und ferner 9,5 ml 2-Thiotolen. Schon nach einigen Minuten Kristallausfällung. Das Reaktionsgemisch wurde bei Zimmertemperatur aufbewahrt und dann und wann umgeschüttelt; jeden Tag wurde die Flasche zwecks Druckausgleich geöffnet. Nach 6 Tagen konnte keine weitere Drucksteigerung beobachtet werden, und der Niederschlag wurde abfiltriert, mit Wasser mehrmals gewaschen und in Vakuum über Schwefelsäure getrocknet. Ausbeute 29,9 g. Teor. f $CH_3C_4H_2S.HgOCN$ 30,43 g. Die Substanz wurde mit 4 Liter Äther extrahiert, wobei 0,09 g ungelöst blieben. Der Äther wurde zum grössten Teil abdestilliert, wobei 28,4 g rein weisser Kristalle erhalten wurden. Schmelzp. 146—146,5°. Umlösen aus Äther bewirkte keine Änderung des Schmelzpunktes. Da die Verbindung durch konzentrierte Salzsäure recht träge zersetzt wird, wurde die Substanz zwecks Hg-Analyse mit Brom in Wasser zersetzt, das überschüssige Brom nach Filtrieren mit Hydroxylaminhydrochlorid reduziert und dann Hg als HgS mit H_2S ausgefällt. (Gef. Hg 59,50; N 4,05 Ber.f. $CH_3C_4H_2S.HgOCN$ (339,76): Hg 59,05; N 4,12).

Merkurierung von 2-Methylfuran (Silvan). Eine Lösung von 24,9 g $Hg(OCN)_2$ in 440 ml Wasser wurde mit einer solchen von 33 g NaAc in 150 ml Wasser gemischt und zu der Lösung 10,0 ml Silvan gegossen. Nach kurzer Zeit Ausfällung eines gelblich weissen Körpers. Das Reaktionsgefäss wurde jeden Tag geöffnet. Nach 5 Tagen konnte keine weitere Drucksteigerung beobachtet werden, und nach 8 Tagen wurde der Niederschlag mit Wasser gewaschen und in Vakuum getrocknet. Gewicht 26,0 g; teor. 28,3 g. Präparat wurde mit 1 200 ml Äther extrahiert, wobei 2,3 g einer graubraunen Substanz ungelöst blieb. Die fast farblose Ätherlösung wurde auf dem Wasserbade auf ein Volumen von 50 ml konzentriert, wobei reichliche Kristallisation in Tafeln eintrat. Nach Kühlen in Kälteschrank wurde abfiltriert und in Vakuum getrocknet. Gewicht 21,3 g Schmelzp. 114—115°. (Gef. Hg 62,35; N 4,27 Ber. f. $CH_3C_4H_2O.HgOCN$(323,73): Hg 61,98 N 4,32).

Darstellung von C_6H_5HgOCN aus $(C_6H_5)_2Hg$ und $Hg(OCN)_2$. 3,54 g Quecksilberdiphenyl wurden in 25 ml Aceton gelöst und dazu eine Lösung von 2,84 g $Hg(OCN)_2$ in 15 ml Aceton gegeben. Reaktion unter schwacher Wärmeentwicklung. Nach einiger Zeit wurde die Lösung filtriert und das Aceton fast vollständig abdestilliert, wobei zuletzt plötsliche Kristallisation eintrat. Gewicht der Kristalle nach Trocknen in Vakuum 6,13 g; teor. 6,38 g. Löslich mit einem kleinen Rückstand in 400 ml Äther. Nach Filtrieren wurde der Äther bis auf 50 ml abdestilliert, wobei Kristallisation in glänzenden Schuppen eintrat. Gewicht nach Trocknen 4,85 g. Schmp. 124—125°. (Gef. Hg 62,81. Ber. f. C_6H_5HgOCN (319,73): Hg 62,74).

Das entsprechende Cyanid C_6H_5HgCN kann in ganz analoger Weise aus $Hg(CN)_2$ dargestellt werden. Es schmilz bei 204—205° und ist in Äther sehr schwerlöslich. Kristallisiert aus Aceton in Prismen. Die Thiophen- und Furancyanide sind auch dargestellt worden. Sie schmelzen erheblich niedriger, ersteres bei 120—121° und letzteres bei 123—123,5°.

Einwirkung von $(C_6H_5)_2Hg$ auf $Hg(SCN)_4Cl_2$. 1,77 g $(C_6H_5)_2Hg$ wurden in 25 ml Aceton gelöst und zu dieser Lösung 2,52 g $Hg(SCN)_4Cl_2$ gesetzt. Nach wenigen Augenblicken war der Kolbeninhalt zu einem Kristallblei erstarrt. Durch Zusatz von weiteren 50 ml Aceton und Wärmen konnte praktisch vollständige Auflösung erreicht werden. Durch Abkühlen der heiss filtrierten Lösung konnte Kristallisation bewirkt werden. Abfiltrieren und Waschen mit kleinen Acetonmengen und Trocknen in Vakuum. Gewicht 2,65 g. Die Substanz wurde aus 50 ml siedendem Aceton umkristallisiert und schmolz dann bei 129,5—130°; Prismen. (Gef. Hg 46,42. Ber. f. $C_6H_5Hg(SCN)_2Cl$ (429,3): Hg 46,73).

Die aus Hg-2-Dithienyl in analoger Weise dargestellte Verbindung C_4H_3S-Hg(SCN)Cl_2 hat denselben Schmelzpunkt, 129—130°, was wahrscheinlich in der Grösse des Chlordithiocyanations seine Erklärung findet.

Darstellung von C_4H_3S-HgOCN aus $Hg(C_4H_3S)_2$ und $Hg(OCN)_2$. Quecksilber 2-Dithienyl wurde nach Steinkopf [4] dargestellt. 3,66 davon und 2,84 g $Hg(OCN)_2$ wurden in 40 ml Aceton zu einer etwas trüben Lösung aufgelöst. Nach etwa 12-stündigem Stehen bei Zimmertemperatur wurde die Lösung durch ein Glasfilter G4 filtriert und das Aceton auf dem Wasserbad vollständig abdestilliert. Der flüssige Rückstand erstarrte sofort bei Kühlen mit kaltem Wasser. Gewicht nach Trocknen in Vakuum 6,46 statt 6,51 g; löste sich vollständig in 370 ml Äther. Nacht Filtrieren durch Glasfilter G4 wurde der Äther bis auf ein Volumen von 50 ml abdestilliert, wobei Kristallisation eintrat. Abfiltrieren und Waschen mit etwas Äther und Trocknen in Vakuum. Gewicht 4,46 g. Schmelzp. 104—105°. Identisch mit der durch direkte Merkurierung von Thiophen mit $Hg(OCN)_2$ dargestellten Substanz.

Darstellung von C_4H_3O.HgOCN aus 2,2'Quecksilberdifuryl und $Hg(OCN)_2$. 2,2'Quecksilberdifuryl wurde nach Gilman und Wright [5] aus 2-Chlormerkurifuran und NaJ in Aceton dargestellt, wobei auf die etwas abenteuerliche Reinigung durch Vakuumdestillation verzichtet wurde. 1,20 g der Verbindung wurden in 10 ml Aceton gelöst und dazu eine Lösung von 1,04 g $Hg(OCN)_2$ in 5 ml Aceton gegeben. Nach einigem Stehen bei Zimmertemperatur wurde das Aceton abdestilliert. Der flüssige Rückstand erstarrte sofort bei Kühlen mit kaltem Wasser. Er wurde pulverisiert, mit 20 ml Wasser gewaschen und in Vakuum getrocknet. Gewicht 1,85 g; löste sich vollständig in 200 ml Äther. Nach Abdestillieren des Äthers konnten 1,7 g Substanz als glänzende farblose Prismen isoliert werden. Schmelzp. 122—122,5°. Identisch mit dem durch direkte Merkurierung von Furan dargestellten Cyanat.

Darstellung von Quecksilber-5,5'Dithiotolyl. 16 g $CH_3C_4H_2S$.HgOCN wurden glatt in 200 ml Aceton gelöst und zu dieser Lösung eine solche von 16 g NaJ in 75 ml Aceton gesetzt. Sofortiges Ausfällen einer weissen feinkristallinischen Substanz. Nach Stehen über Nacht wurde abfiltriert, mehrmals mit Aceton gewaschen und getrocknet. Gewicht 2,83 g. Die Substanz war reines NaOCN. Nach Konzentrieren der Acetonlösung auf ein Volumen von 50 ml wurden 400 ml Wasser zugestzt, wobei eine weisse Substanz ausfiel. Nachdem sich die Mutterlauge geklärt hatte, wurde der Niederschlag auf einem Glasfilter G4 gesammelt, mit Wasser gewaschen und in Vakuum getrocknet. Gewicht 11,97 g. Umlösen aus 700 ml Äther und Konzentrieren der Lösung auf dem Wasserbade bis auf 35 ml. Nach Kühlen in Kühlschrank wurden die Kristalle abfiltriert und getrocknet. Gewicht 9,03 g. Nach Reinigung durch Umkristallisation aus siedendem Alkohol schmolz die Substanz bei 158—160°. Nach Steinkopf schmilz die Substanz bei 162—162,5°.

Überführung von Quecksilber 5,5'Dithiotolyl in 5-Merkuricyanato-2-Thiotolen mit $Hg(OCN)_2$. 1,97 g $(CH_3C_4H_2S)_2Hg$ wurden in 50 ml Aceton suspendiert wobei partielle Auflösung eintrat. Es wurden nun 1,42 g $Hg(OCN)_2$ zugestzt, was schnelle Auflösung des Merkurids bewirkte. Nach Stehen über Nacht wurde die Flüssigkeit filtriert und das Aceton bis zum Eintreten von Kristallisation abdestilliert. Kühlen mit kaltem Wasser bewirkte reichliche Kristallisation und nach einigem Stehen wurden die Kristalle abfiltriert und getrocknet. Gewicht 2,07 statt 3,39 g. Reinigung durch Umlösen aus 280 ml Äther. Blättchen von Schmelzp. 145—146°. Aus Aceton konnte die Substanz als grosse Tafeln von rhombischem Habitus erhalten werden.

Für die Ausführung von Kjeldahlanalysen danke ich dem hiesigen Analytischen Centrallaboratorium, Vorstand Dr. F. Nydahl.

LITTERATUR

1. Söderbäck, E. *Acta Chem. Scand.* **11** (1957) 1622.
2. Steinkopf, W. *Die Chemie des Thiophens*, Dresden und Leipzig 1941.
3. Söderbäck, E. *Ann.* **465** (1928) 187.
4. Steinkopf, W. *Ann.* **413** (1917) 310.
5. Gilman, H. und Wright, G. *J. Am. Chem. Soc.* **55** (1933) 3302.

Eingegangen am 30. April 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1226–1230

Amino-deoxy- and Deoxy-sugars from Methyl 3-Oxo-β-D-glucopyranoside

BENGT LINDBERG and OLOF THEANDER

Träkemiska avdelningen, Svenska Träforskningsinstitutet, Stockholm, Sweden

Methyl 3-oxo-β-D-glucopyranoside has been converted to the two corresponding 3-amino-3-deoxy-glucosides and to the 3-deoxy-glucoside.

Oxo-glucosides should be useful intermediates for syntheses in the carbohydrate field, where the recent isolation of a number of "non-classical" sugars from natural sources has created new synthetic problems. For example methyl β-D-glucopyranoside has been transformed into the D-alloside *via* the 3-oxo-derivative [1], thus proving the position of the oxo-group in the latter compound. A further example is the synthesis of a branched sugar, related to noviose by a sequence of reactions involving oxidation to an oxo-derivative followed by a Grignard reaction [2]. The present paper describes studies of some reaction routes, starting from methyl 3-oxo-β-D-glucopyranoside [3](I).

The oxo-glucoside (I) on treatment with hydroxylamine hydrochloride in aqueous solution was transformed into the oxime (II) in a yield of 95 % or better. The pH was kept at 4.0 by addition of sodium hydroxide, and this was also used to follow the oximation analytically [4]. The oxime proved to be quite labile and decomposed easily into the oxo-glucoside. Thus on paper chromatography in acid solvent systems or on attempted deionisation by carbon column chromatography, the oxo-glucoside was formed almost quantitatively. By careful concentration of the aqueous solution to dryness, after adjustment of the pH to 7.0, and extraction of the solid residue with butanol, the oxime was finally obtained reasonably pure, and this butanolic solution was used for further reactions.

Catalytic reduction of inososes [5] and oxo-glucopyranosides [6] with platinum catalysts is known to yield preferentially the isomer, in which the new hydroxyl is axial in the most stable chair form. Similar results have been obtained by catalytic reduction of oximes or hydrazones in the cyclohexane series [7,8]. Reduction with sodium amalgam, on the other hand, yields comparable amounts of equatorial and axial substituents or sometimes even higher proportions of the former, particularly in the case of oximes and hydrazones [8].

Catalytic reduction of the oxime (II) using Adams' catalyst, gave a crystalline compound, m.p. 199—200°, $[\alpha]_D$ —54° (water) in 85 % yield; this should be methyl 3-amino-3-deoxy-β-D-allopyranoside (III). A study of the optical rotations (Table 1) supports this conclusion. On reduction of II with sodium amalgam, a different compound, m.p. 205—207°, $[\alpha]_D$ —38° (water) was obtained in a yield of 45%. Peat and Wiggins [9] prepared methyl 3-amino-3-deoxy-β-D-glucopyranoside (IV) by another route and report the same m.p. but a lower optical rotation, —47°. The tetra-acetates of the two products, however, have identical constants, m.p. 159—160°, $[\alpha]_D$ —21.4° and —22°, resp. (chloroform). Our value for the optical rotation of the unacetylated product agrees somewhat better with the corresponding rotation for methyl β-D-glucopyranoside (—34°), which should be similar. The presence of the amino-alloside (III) in the mother liquors was demonstrated by paper chromatography and paper electrophoresis.

It is of interest that 3-amino-3-deoxy-D-glucose is identical with kanosamine, a component of the antibiotic kanamycin [10]. Lemieux and Chu [11] have recently demonstrated that some substances prepared by Freudenberg *et al.*[12] and claimed to be 3-amino-3-deoxy-D-glucose derivatives are actually the corresponding allose derivatives. The same seems to be true of some compounds recently prepared by Wolfrom *et al.*[13] by routes, analogous to those

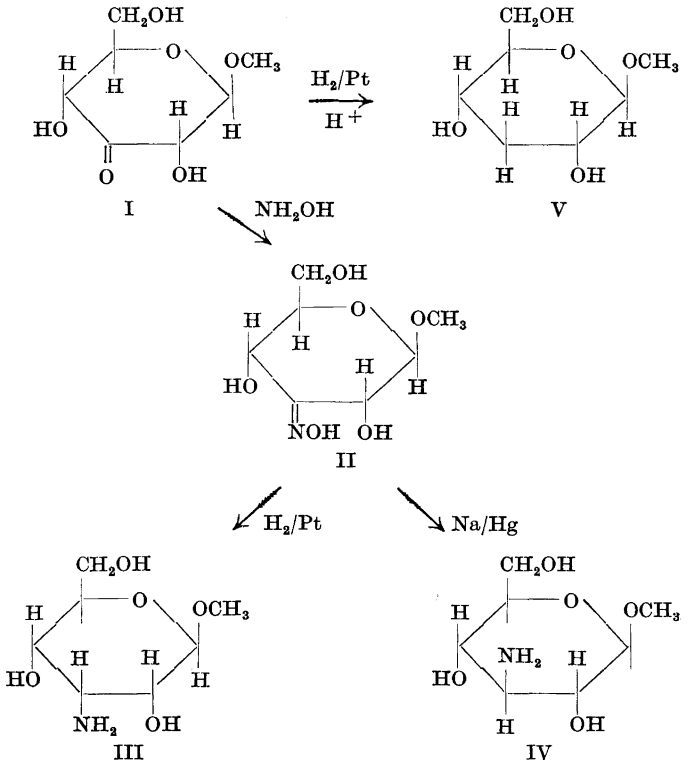


Table 1. Optical rotations of some methyl β-glycosides.

Substance		$[\alpha]_D$	$[M]_D$
Methyl β-D-glucopyranoside		−34°	6 600°
Methyl β-D-allopyranoside		−53°	10 300°
Methyl 3-amino-3-deoxy-β-D-glucopyranoside	(a)	−47°	9 100°
	(b)	−38°	7 300°
Methyl 3-amino-3-deoxy-β-D-allopyranoside		−54°	10 400°
Methyl 3-deoxy-β-D-glucopyranoside		−46°	8 200°
Methyl 3-oxo-β-D-glucopyranoside		−63°	12 100°

a. Ref.[9]
b. Present investigation.

used by Freudenberg *et al.* A new synthesis of 3-amino-sugars has recently been described by Baer and Fisher [14].

Posternak [15] has shown that the oxo-group in *myo*-inosose-2 is reduced to a methylene group on catalytic reduction with Adams' catalyst in acid solution. An analogous reduction of I yielded 59 % of methyl 3-deoxy-β-D-glucoside (V), m.p. 94—96°, $[\alpha]_D$—46° (water) together with smaller amounts of unchanged starting material and a mixture of methyl β-D-glucopyranoside and methyl β-D-allopyranoside. The glucoside was transformed into the β-tetraacetate of the free sugars, m.p. 130—131°, $[\alpha]_D$ −19° (chloroform). Richtmyer and Pratt [16] report m.p. 129—130°, $[\alpha]_D$ −14°, Černý and Pacák [17] m.p. 129—130°, $[\alpha]_D$ −20° for this substance. The free sugar, which was not isolated, had an electrophoretic mobility of 0.85, relative to glucose, in borate buffer. The corresponding value for 3-*O*-methyl-D-glucose is 0.76; close agreement between these values is to be expected [18].

Glycosides of deoxy-sugars hydrolyse faster than the corresponding glycosides of the parent sugars (*cf.* Ref.[19]). Thus Richards [20] reports rate constants of 1.7×10^{-2} and 2.3×10^{-3} for the hydrolysis of methyl 3-deoxy-α-D-glucopyranoside and methyl α-D-glucopyranoside, respectively, in N sulphuric acid at 100°. Under the same conditions, the values 6×10^{-2} and 7.8×10^{-3} (Briggs' logarithms, time in min) were now obtained for the corresponding β-glucosides. The first of these values is rather inaccurate, as the hydrolysis, which was followed polarimetrically, is accompanied by formation of the 1,6-anhydrosugar [16] and also because the reaction was inconveniently fast under the conditions used.

EXPERIMENTAL

Melting points are corrected. Solutions were concentrated under reduced pressure at a bath temp. of about 40°.

Paper chromatography was done on Whatman No. 1 and No. 3 MM filter papers using the solvent systems (v/v):

a. Ethyl acetate-acetic acid-water, 3:1:3 (upper phase)
b. Butanol-ethanol-water, 10:3:5

Table 2. Chromatographic and electrophoretic data for some methyl glycosides.

Substance	$R_{Glucose}$ (solvent a)	$M_{Glucose}$ (borate buffer)
Methyl β-D-glucopyranoside	1.94	0.16
Methyl β-D-allopyranoside	2.23	0.49
Methyl 3-deoxy-β-D-glucopyranoside	3.44	0.14
Methyl 3-oxo-β-D-glucopyranoside	3.57	0.60
Methyl 3-amino-3-deoxy-β-D-glucopyranoside	1.80	0.13
Methyl 3-amino-3-deoxy-β-D-allopyranoside	1.80	0.09

Paper electrophoreses were done on Whatman No. 1 filter paper in 0.1 M borate buffer of pH 10 and in 0.1 M acetate buffer at pH 4.

The following spray reagents were used: silver nitrate in acetone-ethanolic sodium hydroxide, *p*-anisidine hydrogen chloride in butanol, resorcinol-hydrochloric acid in ethanol and ninhydrin in ethanol-acetic acid. Chromatographic and electrophoretic data are summarised in Table 2.

Oximation of methyl 3-oxo-β-D-glucopyranoside (I). In a typical experiment, I (0.43 g) was dissolved in a solution of hydroxylamine hydrochloride (1.38 g) in water (40 ml) which had previously been adjusted to pH 4.0. The temperature was kept at 22° by external cooling and 0.1 N sodium hydroxide was added through an automatic titrator to keep the pH at 4.0. The reaction was followed by the consumption of sodium hydroxide; it was complete in about 8 h when the sodium hydroxide consumption corresponded to 97 % conversion. The aqueous solution was adjusted to pH 7, concentrated until the salts precipitated, and then further dried in a vacuum desiccator over silica gel. The solid residue was extracted with butanol (5 × 10 ml) and the filtered solution was concentrated to about 15 ml.

Methyl 3-amino-3-deoxy-β-D-allopyranoside. A solution of the oxime from 750 mg I in butanol (25 ml) was treated with hydrogen in the presence of Adams' catalyst (100 mg) for 20 h. The solution was filtered and concentrated to dryness. The crystalline residue on recrystallisation from ethanol gave pure III (435 mg), m. p. 199–200°, $[\alpha]_D^{20}$ −54° (water, c = 2). Fractionation of the material in the mother liquors on thick filter paper in solvent system a gave a further quantity of III (200 mg) together with smaller amounts of unidentified compounds, none of which seemed to be identical with IV. (Found: C 43.9; H 8.03; N 6.78. Calc. for $C_7H_{15}NO_5$: C 43.5; H 7.83; N 7.25.)

Acetylation with acetic anhydride in pyridine gave the tetraacetate, which was crystallised from butanone, m. p. 179–180°, $[\alpha]_D^{23}$ −43° (chloroform, c = 1). (Found: C 50.5; H 6.33. Calc. for $C_{15}H_{23}NO_9$: C 49.9; H 6·42.)

Methyl 3-amino-3-deoxy-β-D-glucopyranoside. A solution of the oxime from 450 mg I in butanol (15 ml) was diluted with ethanol (25 ml) and 2.5 % sodium amalgam (20 g) was added in portions with vigorous stirring over 4 h. The pH was kept at 6 by addition of 5 % aqueous acetic acid. The product was then put on a carbon-Celite column and the salts were eluted with water and the glycosides with 5 % aqueous acetic acid. The glycoside fraction was concentrated to dryness, dissolved in water, filtered through a column of Dowex 2 (OH^-) and concentrated to dryness. The crystalline residue (362 mg) on crystallisation from ethanol yielded almost pure IV (202 mg), m. p. 198–200°. After two further crystallisations the substance was pure, m. p. 205–207°, $[\alpha]_D^{20}$ −38 (in water, c = 2). The mother liquors contained IV, smaller amounts of III and minor amounts of other unknown substances.

Treatment of IV with acetic anhydride and pyridine yielded the tetraacetate, m. p. 159–160°, $[\alpha]_D^{2}$ −22° (chloroform, c = 2).

Methyl 3-deoxy-β-D-glucopyranoside (V). Hydrogen was bubbled through a solution of I (450 mg) in 5 % aqueous sulphuric acid (25 ml) containing Adams' catalyst (from

200 mg of platinum dioxide) for 4 h with the addition of a second portion of catalyst (100 mg) after 2 h. The catalyst was filtered off, the acid was neutralised with barium carbonate, the barium salts were filtered off and the solution was concentrated to a thick syrup (440 mg). The product on fractionation on a cellulose column (45 × 3.5 cm) using butanol saturated with water as eluent, yielded V (293 mg), unchanged starting material (27 mg) and a mixture of methyl β-D-glucopyranoside and -allopyranoside (92 mg). V crystallised from methyl ethyl ketone on standing and was recrystallised from the same solvent, m. p. 94—96°, $[\alpha]_D^{20}$ −46° (water, c = 2). (Found: OCH_3 16.6. Calc. for $C_7H_{14}O_5$: OCH_3 17.4.)

Part of the product was converted into the β-tetraacetate of 3-deoxy-D-glucose, m. p. 130—131°, $[\alpha]_D^{20}$ −19° (in chloroform, c = 2).

REFERENCES

1. Lindberg, B. and Theander, O. *Acta Chem. Scand.* **8** (1954) 1870.
2. Walton, E., Rodin, J. O., Stammer, C. H., Holly, F. W. and Folkers, K. *J. Am. Chem. Soc.* **80** (1958) 5168.
3. Assarson, A. and Theander, O. *Acta Chem. Scand.* **12** (1958) 1507.
4. Eitel, A. *J. prakt. Chem.* **159** (1942) 292.
5. Posternak, T. *Helv. Chim. Acta* **24** (1941) 1045.
6. Theander, O. *Acta Chem. Scand.* **12** (1958) 1883.
7. Posternak, T. *Helv. Chim. Acta* **33** (1950) 1597.
8. Anderson, L. and Lardy, H. A. *J. Am. Chem. Soc.* **72** (1950) 3141.
9. Peat, S. and Wiggins, L. F. *J. Chem. Soc.* **1938** 1810.
10. Cron, M. J., Evans, D. L., Palermiti, F. M., Whitehead, D. F., Hooper, I. R., Chu, P. and Lemieux, R. O. *J. Am. Chem. Soc.* **80** (1958) 4741.
11. Lemieux, R. O. and Chu, P. *J. Am. Chem. Soc.* **80** 4745.
12. Freudenberg, K., Barkhart, O. and Brauns, E. *Ber.* **59** (1926) 714.
13. Wolfrom, M. L., Shafizadeh, F. and Armstrong, R. K. *J. Am. Chem. Soc.* **80** (1958) 4885.
14. Baer, H. H. and Fisher, H. O. L. *Proc. Natl. Acad. Sci U.S.* **44** (1958) 991.
15. Posternak, T. *Helv. Chim. Acta* **33** (1950) 350.
16. Richtmyer, N. K. and Pratt, J. W. *J. Am. Chem. Soc.* **79** (1957) 2597.
17. Cerný, M. and Pacák, J. *Chem. Listy* **49** (1955) 1848.
18. Foster, A. B. *Advances in Carbohydrate Chem.* **12** (1957) 81.
19. Shafizadeh, F. *Advances in Carbohydrate Chem.* **13** (1958) 9.
20. Richards, G. N. *Chem. & Ind. London* **1955** 228.

Received April 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1231–1234

Studies on Mechanically Degraded Cellulose

ANDERS ASSARSSON, BENGT LINDBERG
and OLOF THEANDER

Träkemiska avdelningen, Svenska Träforskningsinstitutet, Stockholm, Sweden

Mechanically degraded cellulose was investigated by reduction with borohydride, hydrolysis and isolation of the glycitols in the hydrolysate. The major component was D-glucitol, but D-arabitol, erythritol and traces of glycerol were also found. These results show that carbon-carbon as well as carbon-oxygen linkages are ruptured and thus strongly indicate that the degradation is entirely a mechanical effect.

Mechanical treatment of cellulose by grinding in a ball mill and in other ways produces severe degradation. Previous studies, which have been summarised by McBurney [1], have shown that the DP is decreased during milling. The carboxyl content increases only slightly, and it has been definitely proved that the degradation is not due to oxidation by atmospheric oxygen. As the degradation is rather independent of temperature, a hydrolytic mechanism has also been considered less likely. The most probable explanation is that the degradation is entirely mechanical. Hess and Steurer [2] have shown that the kinetic energy available from the impact of the balls on the cellulose fibre should be sufficient if converted directly to molecular vibrational energy to rupture covalent bonds. The energy requirements for the rupture of carbon-carbon and carbon-oxygen bonds are of the same order, 80—90 kcal., and it could be expected that both types would be broken. The present paper describes a study of the linkages broken when cellulose is ground in a ball mill.

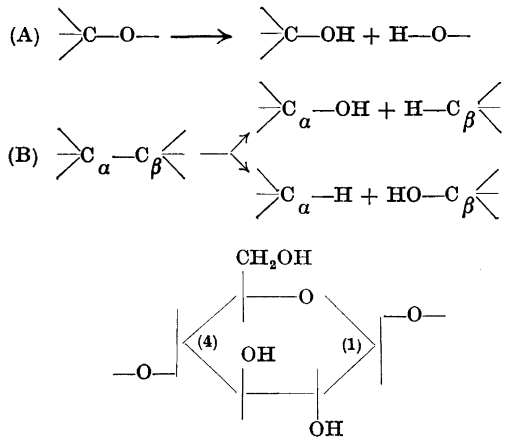
Cotton cellulose was ground in an agate ball mill and the copper number was determined at intervals (according to Hägglund). It rose from 0.2 to 6.5 in five days. A sample treated for 4 days was subjected to chemical investigation. A carbonyl determination by the borohydride method [3] indicated 2.7 carbonyl-groups per 100 glucose residues. According to a recent investigation [4], this value is somewhat too low and the real value should be about 35 % higher. The milled cellulose was treated with borohydride, filtered off and washed. The material that went into solution was recovered (0.5 %). Paper chromatographic investigation of this fraction indicated the presence of glucitol, cellobiitol and higher oligosaccharides together with glycitols with 3, 4 and 5

carbon atoms. The modified cellulose was subjected to a heterogeneous hydrolysis with N sulphuric acid. Most of the reducing sugars and any other aldehydes which might have been present were oxidised to acids by treatment with chlorite at pH 4 and removed by ion exchange. The last traces of sugars were removed by treatment with 0.5 N sodium hydroxide at 100°, followed by ion exchange. The neutral residue (0.6 %) contained components chromatographically indistinguishable from glucitol, arabitol, erythritol and glycerol. Their acetates were indistinguishable from the corresponding authentic acetates on chromatograms on dimethyl sulphoxide-impregnated paper [5], which gives good separation of the fully acetylated glycitols. Fractionation of the neutral mixture gave erythritol, D-arabitol and D-glucitol which were isolated and characterised as crystalline derivatives.

The relative proportions of glucitol, arabitol and erythritol were about 10:3:2, as estimated from the weights of the amorphous, not quite pure fractions obtained. 1,6-Anhydro-D-glucopyranose, which is known to be formed by the acid treatment of glucose [6], was also isolated. In many solvent systems it has the same R_F value as glycerol but the two substances can be separated by elution with butanone, saturated with water; traces of glycerol were detected using this solvent system.

Part of the residue from partial hydrolysis was hydrolysed completely and then worked up as above giving a small amount of neutral material (0.2 %) with the same chromatographic pattern as the material from the heterogeneous hydrolysis.

From these results it is obvious that both C—O and C—C linkages are broken. Rupture of these linkages could be either homolytic or heterolytic. A discussion of this and the subsequent reactions, leading ultimately to stable products, would be highly speculative, but for simplicity it may be assumed that the radicals or ions formed by rupture are stabilised by addition of the elements of water (A and B).



I

If a $C_{(1)}$—O or O—$C_{(4)}$ linkage in a glucose residue (I) of the cellulose molecule is broken, the final result should be equivalent to an acid hydrolysis, possibly accompanied in the second case by some inversion at $C_{(4)}$. Rupture of the linkage between the ring oxygen and either $C_{(1)}$ or $C_{(5)}$ would probably have a similar effect. Reduction and hydrolysis would then yield D-glucitol and possibly D-galactitol.

Rupture of a C—C linkage and subsequent reduction and hydrolysis should yield a glucitol with n carbon atoms together with an aldehyde of (6—n) carbon atoms, which would be removed by oxidation and ion exchange. It is easily seen that the glycitols which could be expected are D-arabitol, erythritol, glycerol and ethylene glycol. The first two were isolated and characterised and indications were found of the presence of glycerol, although in quite small quantities. The presence of ethylene glycol and of any methanol formed as a consequence of $C_{(5)}$—$C_{(6)}$ rupture might easily have been overlooked. D-Glucitol is, however, the major product, indicating that the C—O linkages are somewhat weaker than the C—C linkages. More detailed conclusions cannot be drawn from the results of this investigations, since a high percentage of the oxo-groups in the degraded cellulose is not accounted for and the determination of the relative proportions of the glycitols isolated is at best only semi-quantitative. In addition the course of the reactions leading to stabilisation of the primary products of the rupture of a linkage is quite unknown. As stated already the main result is the strong evidence of an entirely mechanical rupture of covalent linkages by mechanical treatment of cellulose, in agreement with the theory of Steurer and Hess [2].

EXPERIMENTAL

Melting points are corrected. Concentrations were done under reduced pressure at a bath temperature of about 40°.

Paper chromatograms were run on Whatman No. 1 and 3 MM filter papers, using the solvent systems (v/v):

a. Ethyl acetate-acetic acid-water, 3:1:3 (upper phase)
b. Butanone, saturated with water
c. Light petroleum. Paper impregnated with dimethylsulphoxide.

Silver nitrate in acetone-ethanolic sodium hydroxide was used as spray reagent for the detection of glycitols.

Preparation of degraded cellulose. Cellulose (cotton linters) was disintegrated in a Wiley mill and then milled in an agate ball mill for 4 days. During the milling the copper number, determined according to Hägglund [7], increased from 0.21 to 4.62. A borohydride analysis indicated the presence of 2.7 oxo-groups per hundred glucose residues.

Isolation of glycitols from the degraded cellulose. Mechanically degraded cellulose (25.0 g) was suspended in a solution of sodium borohydride (8.0 g) in water (250 ml) for 2 days at room temperature. It was then filtered off and washed with water. The filtrate and washings were concentrated, filtered through a column of Amberlite IR 120 (H^+), concentrated further and boric acid was removed by distillations with anhydrous methanol. A neutral residue (139 mg) was obtained.

This cellulose residue, which had a copper number of 0.37, was refluxed with N sulphuric acid for 4 h and then the undissolved residue (21.3 g) was removed by filtration. The filtrate was neutralised with barium carbonate and concentrated to a syrup (3.8 g). This syrup, dissolved in 0.1 M acetate buffer of pH 3.5, was treated with sodium chlorite (35 g) for 2 days at room temperature. Excess oxidant remaining was reacted with sulphur dioxide and the resultant solution was neutralised with barium carbonate, deionised by filtering through columns of IR 120 (H^+) and IR 4B (free base) and concentrated.

Table 1. Fractionation of the neutral products.

	Directly dissolved		Obtained after partial hydrolysis	
	I	II	I	II
Weight of sample, mg	139	230	150	331
D-Glucitol, mg	12	28	62	95
D-Arabitol, mg	5	10	15	27
Erythritol, mg	6	8	7	22
1,6-Anhydro-D-glucose + Glycerol, mg	Traces	Traces	42	59

The resulting syrup (430 mg) contained glycitols and 1,6-anhydro-D-glucose, together with reducing sugars, glucose, mannose and xylose, as shown by paper chromatography. It was therefore dissolved in 0.5 N sodium hydroxide and kept at 100° for 2 h and then deionised and concentrated, yielding a syrup (150 mg). An analogous experiment, starting from 42 g of degraded cellulose, yielded 230 and 331 mg of neutral material, obtained by direct solution and after partial hydrolysis, respectively.

Part of the residual cellulose (500 mg) was subjected to total hydrolysis in the usual manner and worked up as above, giving a small residue (1.3 mg). Chromatographic investigation showed the presence of 1,6-anhydro-D-glucose (60 %), D-glucitol (30 %) and smaller amounts of D-arabitol and erythritol.

Fractionation of the neutral products. The material that dissolved during the borohydride reduction was fractionated by chromatography on thick filter paper, using solvent a. Only glucitol and the components of higher R_F were recovered. The products obtained after partial hydrolysis were fractionated on a cellulose column, using butanol saturated with water, as eluant. The results of the fractionations are summarised in Table 1.

Characterisation of the components. The components found were chromatographically indistinguishable from authentic samples of D-glucitol, D-arabitol, erythritol, glycerol and 1,6-anhydro-D-glucose respectively, and the same was true for the corresponding acetates.

D-Glucitol was characterised as the hexaacetate, m. p. 99—100°, alone or mixed with an authentic sample.

D-Arabitol was obtained crystalline, m. p. 101—102°, undepressed on admixture with an authentic sample.

Erythritol was characterised as the tetrabenzoate, m. p. 189—191°, alone or mixed with an authentic sample.

1,6-Anhydro-D-glucose was characterised as the tribenzoate, m. p. 201—202°, alone or mixed with an authentic sample.

Glycerol was present in insufficient amount for a definite characterisation.

REFERENCES

1. McBurney, L. F. in Ott, E., Spurlin, H. M. and Grafflin, M. W. *Cellulose and Cellulose Derivatives* I, New York 1954, p. 191.
2. Steurer, E. and Hess, K. *Z. physik. Chem.* **193** (1944) 248.
3. Lindberg, B. and Misiorny, A. *Svensk Papperstidn.* **55** (1952) 13.
4. Lidman-Safvat, S. and Theander, O. *Svensk Papperstidn.* **61** (1958) 42.
5. Wickberg, B. *Acta Chem. Scand.* **12** (1958) 615.
6. Thompson, A., Anno, K., Wolfrom, M. L. and Inatome, M. *J. Am. Chem. Soc.* **76** (1954) 1309.
7. *Central Laboratory of the Swedish Cellulose Industry standards.* **CCA 3** (1940).

Received April 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1235–1238

Partial Methylation of some Glucose Derivatives

INGEMAR CROON

Wood Chemistry Department, Swedish Forest Products Research Laboratory, Stockholm, Sweden

Methyl 4,6-*O*-ethylidene-β-D-glucopyranoside, methyl 4,6-*O*-benzylidene-β-D-glucopyranoside and amylose have been partially methylated with dimethyl sulphate in alkaline solution and the distribution of the methoxyl groups has been determined by hydrolysis and resolution of the hydrolysate by chromatographic methods. In the first two derivatives the hydroxyl groups at C_2 and C_3 had approximately the same reactivity ($k_2:k_3 = 1:1$ and $k_2:k_3 = 1.3:1$, respectively); for amylose $k_2:k_3:k_6$ equalled 6:1:7. This investigation supports the idea that intrachain hydrogen bonds play an important role in the reactivity of the hydroxyl groups at positions C_2, C_3 and C_6 in cellulose and amylose, but other factors which can influence the results are also pointed out.

Studies on the distribution of substituents in various ethers of cellulose have been reported in previous communications from this Department [1-4]. In these studies, the cellulose ether was hydrolysed to a mixture of glucose and glucose ethers. This mixture was fractionated by chromatographic methods into pure components which were characterized and identified. The technique also allowed for the quantitative determination of all the expected components, except in the case of hydroxyethyl cellulose from which, however, ten separate components were isolated and identified [1]. This method of studying the distribution of substituents should be more accurate than the indirect methods previously used, which were based upon group analyses, such as the determination of glycol groups by periodate oxidation and of primary hydroxyl groups by tosylation-iodination.

For methyl cellulose, prepared with methyl sulphate in a homogeneous medium (triethylbenzylammonium hydroxide as solvent) the results agreed fairly well with those which could be calculated statistically, using the following assumptions [2]:

1. All glucose residues are equally accessible.
2. The relative rate constants for methylation at carbon atoms $C_{(2)}$, $C_{(3)}$ and $C_{(6)}$ of the glucose unit are constant during the reaction.
3. The effect of end groups is negligible.

The values of $k_2 = 3.5$, $k_3 = 1$ and $k_6 = 2$ for the relative rate constants in methylation reactions with methyl sulphate at $C_{(2)}$, $C_{(3)}$ and $C_{(6)}$ gave the best agreement, but there were deviations which could not be explained as experimental errors. A better agreement was obtained if it was assumed that the reactivity at $C_{(3)}$ was doubled when the hydroxyl at $C_{(2)}$ was methylated. This should not be unreasonable, as the $C_{(2)}$-hydroxyl is the most acidic in the glucose residue and, when dissociated, should considerably reduce the acidity of the $C_{(3)}$-hydroxyl.

In order to study this effect and learn more about the factors which influence the relative reactivities of different hydroxyl groups in a glucose residue, the course of the partial methylation of methyl 4,6-*O*-ethylidene-β-D-glucopyranoside, methyl 4,6-*O*-benzylidene-β-D-glucopyranoside and of amylose has now been investigated, using the same technique as used for the cellulose ethers. The relative reactivities of hydroxyl groups of carbohydrates has been summarised by Sugihara [5]. Most of the results discussed by him were, however, obtained before the development of modern chromatographic technique and are probably rather uncertain.

Table 1. Composition of the hydrolysates of the partially methylated ethylidene and benzylidene glucose derivatives

	Ethylidene derivative						Benzylidene derivative	
	Sample I		Sample II		Sample III			
Substance [a]	Found	Calc.[b]	Found	Calc.[b]	Found	Calc.[b]	Found	Calc.[c]
s_0	66.0	65.3	45.0	42.2	28.2	28.6	54.0	51.2
s_2	14.7	15.5	20.4	22.8	23.0	24.9	20.2	23.5
s_3	15.1	15.5	20.4	22.8	25.6	24.9	15.3	17.2
s_{23}	4.1	3.7	14.4	12.2	23.2	21.6	10.4	8.0

[a] s_0 = unsubstituted glucose; s_2 = 2-*O*-methyl-D-glucose, *etc.*
[b] Calculated for homogeneous reaction in alkaline solution, $k_2:k_3 = 1:1$.
[c] Calculated for homogeneous reaction in alkaline solution, $k_2:k_3 = 1.3:1$.

The two cyclic acetals were methylated with methyl sulphate in sodium hydroxide; ethanol was added to the system to keep the products in solution. The results given in Table 1 were quite unexpected and contrary to those from all cellulose ethers studied, the reactivities at $C_{(2)}$ and $C_{(3)}$ being approximately the same. The percentage of 2,3-di-*O*-methyl-D-glucose (s_{23}), however, was in all cases higher than the calculated value, thus supporting the assumption that the reactivity in one of the positions is increased when the other is methylated. The low reactivity at $C_{(3)}$ in cellulose has been attributed to a hydrogen linkage between this hydroxyl and the ring oxygen of the neighbouring glucose residue [6,7]. On the other hand, the higher reactivity at $C_{(2)}$ has been explained as a result of the higher acidity of the hydroxyl at this position [5,8], due to the inductive effect of the two oxygen atoms at $C_{(1)}$. It must, however, be borne in mind that the observed effect, a change in relative reactivity from 3.5:1 to 1:1, corresponds to a quite small difference in the energy of activation, which might equally well have other explanations, *e.g.* a different magnitude of steric hindrance from the bulky groups in the 4-position, or a slight deviation from the chair conformation of the pyranose ring in the cyclic acetals.

Table 2. Composition of the hydrolysate of the partially methylated amylose

Substance	Sample I (D.S. 0.76)			Sample II (D.S. 1.30)		
	Found	Calc.		Found	Calc.	
		a)*	b)**		a)*	b)**
s_0	43.0	40.1	41.4	18.5	15.1	16.3
s_2	16.5	19.2	18.4	16.7	18.8	17.8
s_3	2.6	2.8	2.7	1.6	2.2	2.2
s_6	21.6	23.2	22.9	20.7	23.8	24.0
s_{26}	11.3	11.1	10.2	28.6	29.7	26.5
s_{36}	1.3	1.6	1.5	2.5	3.5	3.3
s_{23}	2.7	1.3	1.8	5.1	2.7	4.0
s_{236}	1.1	0.8	1.0	6.3	4.3	5.9

* Calculated for a homogeneous reaction in alkaline solution $k_2:k_3:k_6 = 6:1:7$. (See the text.)
** As above, but with the further assumption that l_3 is doubled when the position at $C_{(2)}$ becomes methylated.

Amylose was dissolved in 18.9 % sodium hydroxide and methylated with methyl sulphate and the distribution of the methoxyl groups in the methylated product was studied as above. The results are summarised in Table 2. Here also, the percentage of s_{23} is higher than expected according to the direct statistical calculation, and a better agreement between observed and calculated values is obtained if it is assumed that the reactivity at $C_{(3)}$ is doubled when the position at $C_{(2)}$ is methylated [2]. The most interesting result is that the primary hydroxyl group showed the highest reactivity. The relative reactivity at $C_{(3)}$ is even lower than in cellulose. The conformation of the glucose residues in the amylose chain is not definitely known, but in a recent paper Reeves [9] assumed that there is an equilibrium between B1 and 3B (Fig. 1) and that in strongly alkaline solution a conformation approximating that of 3B predominates.

An inspection of a model of the amylose molecule in the 3B-conformation, using the Courtauld atomic models, shows that as in cellulose the atomic distances are favourable for the formation of a strong hydrogen bridge between the $C_{(3)}$-hydroxyl and the oxygen atom in the adjacent glucopyranoside ring. In the cellulose molecule a hydrogen bridge between the hydroxyl group at $C_{(6)}$ and a glycosidic oxygen atom is possible, but in the starch model this is not the case. The results thus favour the opinion that intramolecular hydrogen bridges play an important role in the relative reactivities in the methylation reaction, but for reasons discussed above great care must be used when trying to interpret these rather small effects. A careful study of a greater number of model substances might throw more light upon these questions.

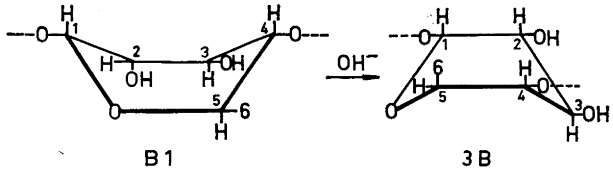


Fig. 1.

EXPERIMENTAL

Methyl 4,6-O-ethylidene-β-D-glucopyranoside (600 mg), prepared according to Helferich [10], was dissolved in 18.9 % sodium hydroxide (60 ml) and methyl sulphate (2 ml) was added during 15 min. After 30 min, traces of the dimethylated compound began to precipitate and, in order to keep the reaction medium homogeneous, ethanol (10 ml) was added. A further quantity (2 ml) of methyl sulphate was added during 30 min. The reaction was carried out for a total time of 4 h. The solution was neutralised with 6 N sulphuric acid and most of the sodium sulphate formed was precipitated with ethanol. The filtered solution was evaporated to 37 ml, and 72 % sulphuric acid was added until the concentration was 8 %. This solution was kept at 100°C for 4 h to remove the ethylidene and the glycosidic methyl groups. The sulphate ions were then removed by treatment with barium carbonate and the solution deionised by ion exchange with IR-120 and IR-4B exchange resins. Evaporation of the deionised solution yielded a light yellow syrup (370 mg) (sample 1 in Table 1). Two other samples were obtained by varying the amount of methyl sulphate and the time of reaction.

Methyl 4,6-O-benzylidene-D-β-glucopyranoside (500 mg), prepared according to Freudenberg, Toepffler and Anderson [11], was dissolved in 18.9 % sodium hydroxide (60 ml) and methyl sulphate (2 ml) was added during 15 min. After addition of ethanol (20 ml), additional methyl sulphate (2 ml) was added dropwise during 30 min. Ethanol (10 ml) was then added and the reaction was allowed to proceed for a total time of 4 h. Neutralisation, precipitation and hydrolysis were performed as above. The hydrolysate (50 ml) was shaken with ether (3 ml) to remove benzaldehyde. The neutralised and deionised solution on evaporation yielded a syrup (295 mg).

Amylose. The pure amylose (1.3 g) was dissolved in 18.9 % sodium hydroxide (60 ml) and methyl sulphate (8 ml) was added during 1 h. After a further 4 h, the solution was neutralised with 6 N sulphuric acid and was dialysed against tap water for a period of 14 days. The partially methylated amylose was recovered by freeze-drying, when a white, fluffy product (1.28 g) was obtained (sample 1 in Table 2). A higher methylated derivative was obtained by methylation of amylose (2 g) with methyl sulphate (20 ml) during 17 h (sample 2 in Table 2).

The amylose derivatives were hydrolysed exactly as previously described for the cellulose ethers [2] by treatment with 72 % sulphuric acid (16.7 ml) for 30 min, and dilution with water to 150 ml, after which the solution was kept at 100°C for 4 h.

All the methylations described above were carried out in an atmosphere of nitrogen.

Analysis of the hydrolysates. The hydrolysates were analysed by separation into pure components by carbon column chromatography, paper electrophoresis and paper chromatography as described in a previous paper [2].

Acknowledgement. The author is indebted to Docent Bengt Lindberg for his interest in this investigation and to Dr. Gunnar Neumüller for a gift of pure amylose.

REFERENCES

1. Croon, I. and Lindberg, B. *Svensk Papperstidn.* **59** (1956) 794.
2. Croon, I. and Lindberg, B. *Svensk Papperstidn.* **60** (1957) 843.
3. Croon, I. *Svensk Papperstidn.* **61** (1958) 919.
4. Croon, I. and Flamm, E. *Svensk Papperstidn.* **61** (1958) 963.
5. Sugihara, J. M. *Advances in Carbohydrate Chem.* **8** (1953) 1.
6. Hermans, P. H. *Physics and Chemistry of Cellulose Fibers*, Elsevier, New York and Amsterdam 1949.
7. Moore, W. R. and Russell, G. *J. Colloid Sci.* **9** (1954) 338.
8. Derewitskaya, V. *et al: Zhur. Obshchei Khim.* **26** (1956) 1466; *Chem. Abstr.* **50** (1956) 16103a.
9. Reeves, R. E. *J. Am. Chem. Soc.* **76** (1954) 4595.
10. Helferich, B. *Ber.* **64** (1931) 1841.
11. Freudenberg, K., Toepffer, H. and Anderson, C. C. *Ber.* **61** (1928) 1750.

Received April 29, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1239–1262

Short Communications

Artificial Peroxidases

K. G. PAUL

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

A reinvestigation of the activities of some artificial peroxidases has given results somewhat at variance with those previously [1-3] reported. The reasons are at present unknown, and the results are presented without comment.

The horse radish peroxidase was prepared, split, and combined with various hemins as previously described [4,5]. The activities were measured by the mesidine method [6] which is well fitted even for the determination of weak activities since no appreciable decolourization occurs. For convenience the activities were expressed as the increase in absorbancy (A) at 490 mμ per min for a micromolar protein concentra-

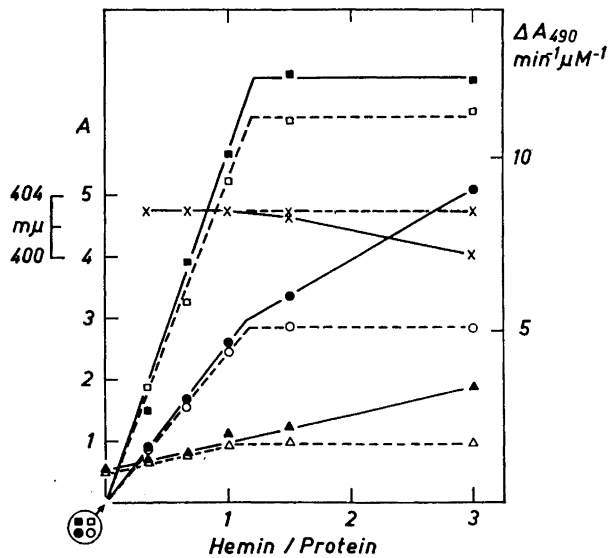


Fig. 1. Effect of the ratio between protohemin and apoprotein on enzymatic activity and light absorption in recombination experiments. All solutions were 28 μM in apo-HRP with 50 mM phosphate of pH 7.3 as buffer. Activities and spectra were determined after 40 h at 4°C (full drawn curves and filled symbols). Aliquots were chromatographed on Dowex 2, previously washed with the buffer until A< 0.01. Five retention volumes were collected and examined (dashed lines and open symbols). The next fifteen volumes gave no light absorption. Right scale and squares: Enzymatic activity of apoprotein with varying proportions of hemin. Left scale: Absorbances at the top of the Soret band (circles) and at 280 mμ (triangles) for 28 μM protein. Short scale and crosses: Wavelength for the maximum of the Soret band.

tion. The ratio of two for hemin to protein was used, thus ensuring that the native apoprotein was saturated with the prosthetic group. All protein concentrations were based upon micro-Kjeldahl determinations.

The activity of unsplit HRP has been determined for four preparations and ΔA_{490} min^{-1} μM^{-1} was found to be 10.6, 11.4, 12.1, and 11.8, average 11.5.

Fig. 1 confirms the observation that the hemin that is nonspecifically attached to the apoprotein is removed by an anion exchanger. The hemin bound as the prosthetic group of HRP and the hemin present in excess of the protein moiety differ in their effects on the light absorption in the Soret band region. At 280 mμ, however, no such difference can be seen. The activity of the recombined HRP in Fig. 1 was found to be 12.4 min^{-1} μM^{-1}.

Table 1. Enzymatic activities of some artifical peroxidases.

Hemin	Positions of the carboxyl groups in the prophyrin ring	Activity ΔA_{490} min^{-1} μM^{-1}	Positions of the α-band of pyridine hemochromogen, mμ
Protohemin IX	6,7	11.5	557
Hematohemin IX	6,7	15.5	548
Mesohemin IX	6,7	15.7	547
Deuterohemin IX	6,7	6.1	545
Diacetyl-deuterohemin IX	6,7	0.3	582
Mesohemin I	6,8	0.4	547
Coprohemin I	2,4,6,8	0.1	548
Coprohemin III	2,4,6,7	0.1	548

When seven different preparations of protohemin were used in recombination experiments with the same batch of apoprotein, the activities of the holoenzymes were found as 9.9 ± 0.8 (S.D.). Since all activity values are based on nitrogen determinations, the somewhat low value may be caused by a partial inactivation of the apoprotein.

Some results with various hemins are given in Table 1. Mesohemin IX peroxidase and hematohemin IX peroxidase were 1.3 times more active than the unsplit peroxidase, whereas deuterohemin IX peroxidase was only 0.5 times as active. Diacetyldeuterohemin IX does combine with the apoenzyme, as reported previously [5], but the resulting hemoprotein is inactive in this assay. The hemins with propionic acid residues in the six and eight positions are inactive, probably because of inability to react in an adequate way with the apoprotein. In the case of the four-carboxylic hemins the lack of activity may depend upon the nature of the hemin polymer.

There seems to be some correlation between the spectrum and the enzymatic activities of the meso-, hemato-, proto-, and diacetyldeuterohemin IX peroxidases.

A full report will be published.

1. Theorell, H., Bergström, S. and Åkeson, Å. *Arkiv Kemi Mineral. Geol.* **16A** (1942) No 13.
2. Gjessing, E. C. and Sumner, J. B. *Arch. Biochem.* **1** (1943) 1.
3. Theorell, H. and Maehly, A. C. *Acta Chem. Scand.* **4** (1950) 422.
4. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1312.
5. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1611.
6. Paul, K. G. *Acta Chem. Scand.* **8** (1954) 649.

Received June 18, 1959.

Deuterohäminperoxydase

K. G. PAUL

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Schweden

H. S. GEWITZ und W. VÖLKER

Max Planck-Institut für Zellphysiologie, Berlin-Dahlem, Deutschland

In früheren Untersuchungen wurde festgestellt, dass Meerrettichperoxydase — Apoeiweiss (Apo-MRP) sich mit gewissen Häminen kuppeln lässt, wobei peroxydatisch aktive Verbindungen entstehen [1]. So geben Proto-, Meso- und Hämatohämin IX, die alle Propionsäuren in den Stellungen 6 und 7 besitzen, aktive Peroxydasen, während Diacetyldeuterohämin IX zwar gekuppelt wird, die Verbindung ist aber peroxydatisch inaktiv [2]. Deuterohä-

tion. The ratio of two for hemin to protein was used, thus ensuring that the native apoprotein was saturated with the prosthetic group. All protein concentrations were based upon micro-Kjeldahl determinations.

The activity of unsplit HRP has been determined for four preparations and ΔA_{490} min^{-1} μM^{-1} was found to be 10.6, 11.4, 12.1, and 11.8, average 11.5.

Fig. 1 confirms the observation that the hemin that is nonspecifically attached to the apoprotein is removed by an anion exchanger. The hemin bound as the prosthetic group of HRP and the hemin present in excess of the protein moiety differ in their effects on the light absorption in the Soret band region. At 280 mμ, however, no such difference can be seen. The activity of the recombined HRP in Fig. 1 was found to be 12.4 min^{-1} μM^{-1}.

Table 1. Enzymatic activities of some artifical peroxidases.

Hemin	Positions of the carboxyl groups in the prophyrin ring	Activity ΔA_{490} min^{-1} μM^{-1}	Positions of the α-band of pyridine hemochromogen, mμ
Protohemin IX	6,7	11.5	557
Hematohemin IX	6,7	15.5	548
Mesohemin IX	6,7	15.7	547
Deuterohemin IX	6,7	6.1	545
Diacetyl-deuterohemin IX	6,7	0.3	582
Mesohemin I	6,8	0.4	547
Coprohemin I	2,4,6,8	0.1	548
Coprohemin III	2,4,6,7	0.1	548

When seven different preparations of protohemin were used in recombination experiments with the same batch of apoprotein, the activities of the holoenzymes were found as 9.9 ± 0.8 (S.D.). Since all activity values are based on nitrogen determinations, the somewhat low value may be caused by a partial inactivation of the apoprotein.

Some results with various hemins are given in Table 1. Mesohemin IX peroxidase and hematohemin IX peroxidase were 1.3 times more active than the unsplit peroxidase, whereas deuterohemin IX peroxidase was only 0.5 times as active. Diacetyldeuterohemin IX does combine with the apoenzyme, as reported previously [5], but the resulting hemoprotein is inactive in this assay. The hemins with propionic acid residues in the six and eight positions are inactive, probably because of inability to react in an adequate way with the apoprotein. In the case of the four-carboxylic hemins the lack of activity may depend upon the nature of the hemin polymer.

There seems to be some correlation between the spectrum and the enzymatic activities of the meso-, hemato-, proto-, and diacetyldeuterohemin IX peroxidases.

A full report will be published.

1. Theorell, H., Bergström, S. and Åkeson, Å. *Arkiv Kemi Mineral. Geol.* **16A** (1942) No 13.
2. Gjessing, E. C. and Sumner, J. B. *Arch. Biochem.* **1** (1943) 1.
3. Theorell, H. and Maehly, A. C. *Acta Chem. Scand.* **4** (1950) 422.
4. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1312.
5. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1611.
6. Paul, K. G. *Acta Chem. Scand.* **8** (1954) 649.

Received June 18, 1959.

Deuterohäminperoxydase

K. G. PAUL

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Schweden

H. S. GEWITZ und W. VÖLKER

Max Planck-Institut für Zellphysiologie, Berlin-Dahlem, Deutschland

In früheren Untersuchungen wurde festgestellt, dass Meerrettichperoxydase — Apoeiweiss (Apo-MRP) sich mit gewissen Häminen kuppeln lässt, wobei peroxydatisch aktive Verbindungen entstehen [1]. So geben Proto-, Meso- und Hämatohämin IX, die alle Propionsäuren in den Stellungen 6 und 7 besitzen, aktive Peroxydasen, während Diacetyldeuterohämin IX zwar gekuppelt wird, die Verbindung ist aber peroxydatisch inaktiv [2]. Deuterohä-

min IX-Peroxydase ist nun etwa halb so aktiv wie Protohäminperoxydase. Mesohämin I, das Propionsäuren an den Stellungen 6 und 8 besitzt, bildet keine Peroxydase [2].

In der vorliegenden Arbeit wurden einige Deuterohäminisomere bzw.-homologe sowie Cytodeuterohämin *, die als Porphyrinester aus früheren Arbeiten [4,5] zur Verfügung standen, in ihrem Verhältnis zu Apo-MRP untersucht.

Die Porphyrinester wurden mit 7 M HCl im Eisschrank verseift und in Hämine mittels Ferroacetat in Eisessig überführt. Die Einheitlichkeit der Hämine wurde papierchromatographisch mit silikonierten Papieren (Whatman Nr. 1), *n*-Propanol-Wasser-Pyridin 1:55:4 und aufsteigender Front geprüft [6]. Cytohämin teilte sich dabei so auf, dass der grösste Teil überhaupt nicht wanderte, der kleinere Teil mit R_F etwa 0,1. Ein Streifen mit abnehmender Intensität war aber bis an R_F 0,8 sichtbar. 2,3,4-H-6,7-Propionsäurehämin zeigte einen schwachen Schwanz, die übrigen Hämine aber wanderten als gut begrenzte Flecke.

Für jede Kuppelung wurde 1,0 ml Puffer (0,03 M Phosphat, pH 7,1), 0,5 ml Apo-MRP und 0,16 μMole des betreffenden Hämins in 0,1 ml 0,05 M NaOH abgemessen. Die Lösung von Apo-MRP war 160 μM (Kjeldahlbestimmung), und davon waren nach der Bestimmung mit Protohämin etwa 70 % nativ. Das Verhältnis Gesamthämin/Gesamtprotein war also 2,0. Kuppelung geschah während 40 Std. im Kühlschrank. Für die Aktivitätsmessung, die nach der Mesidinmethode [8] ausgeführt wurde, wurden immer 0,03 ml einer 15-fachen Verdünnung des Gemisches verwendet. In der Tabelle sind die Aktivitäten als Extinktionszuwachs pro Minute für eine mikromolare Enzymkonzentration im Rohr mit Mesidin und Peroxyd (2 + a ml [8]) ausgedrückt (ΔA).

Die Ergebnisse, im Tabelle 1 gesammelt, zeigen:

1. Die beiden Deuterohämin IX-Präparate geben die gleiche Aktivität und zwar etwa die Hälfte davon was mit Protohämin erreicht wird. Auch zeigen sie dieselben R_F-Werte. Die verwendeten Methoden scheinen also zuverlässig zu sein. Da inaktivierter Eiweiss sich mit Häminen nicht spezifisch kuppelt [7] kann die teilweise

* Cytodeuterohämin bezeichnet die Verbindung, die aus Cytohämin (die Wirkungsgruppe des sauerstoffübertragenden Ferments, der Cytochromoxydase) durch Schmelzen mit Resorcinol erhalten wird [3].

Tabelle 1. Ergebnisse der Papierchromatographie und der Aktivitätsbestimmung. Alle Hämine besassen Methylgruppen in den Stellungen 1 und 5, übrige Substituenten gehen aus der Tabelle hervor.

V = Vinyl, Et = Äthyl, Me = Methyl, S = Propionsäure

Substituent in Stellung 2	3	4	6	7	8	R_F	Aktivität in ΔA_{490} mμ min^{-1} μM^{-1}	% von Protohämin-Peroxydase
V	Me	V	V	S	Me	0.40	7.5	100
H	Me	H	S	S	Me [1])	0,49	3,9	52
H	Me	H	S	S	Me [2])	0,50	3,8	51
H	H	Et	S	S	Me	0,46	1,2	16
H	H	H	S	S	Me	0,56	0,3	4
H	Me	H	S	Me	S	0,68	0,04	0,5
H	H	Me	S	Me	S	0,66	0,08	1
Cytohämin						0,1?	1,2	16 (0) [3])
Cytodeuterohämin						0,52	0,4	5

1) Durch Eiseneinführung in Deuteroporphyrin IX erhalten.
2) Aus Protohämin IX durch Resorcinolschmelze erhalten.
3) Vgl. Text.

Inaktivierung des Apoeiweisses die relativen Aktivitäten der artifiziellen Peroxydasen nicht beeinflussen.

2. Die R_F-Werte der Deuterohäminisomere mit Propionsäuren in den 6- und 8-Stellungen sind höher als der des Deuterohämins IX, wo die Säuregruppen in 6- und 7-Stellung stehen. Dasselbe wurde auch bei den Mesohäminen beobachtet, wo die 6,7- und 6,8-Isomere die R_F-Werte 0,55 bzw. 0,70 gaben. Weil mit umgekehrten Phasen chromatographiert wurde bedeutet dies, dass die beiden Karboxylgruppen der 6,7-Isomere einander stärker beeinflussen bei ihrer Ionisation als die der 6,8-Isomere. pH der Eluierungsflüssigkeit ist etwa 7,5.

3. Die beiden Deuterohäminisomeren mit Propionsäuren in den 6- und 8-Stellungen geben keine Aktivität. Die Deuterohäminhomologen, die in 4-Stellung äthyl- bzw. wasserstoffsubstituiert sind, geben beide geringere Aktivität als Deuterohämin selbst. Das Homolog mit drei freien β-Stellungen erwies die schwächste Peroxydaseaktivität.

4. Das Chromatogram des zur Verfügung stehenden Cytohäminpreparates wurde von unbekannten Faktoren beeinflusst sodass sich keine gut abbegrenzte Flecken zeigten; es enthüllte aber, dass das Präparat nicht einheitlich war. Cytohämin ergab 16 % der Aktivität die mit Protohämin gefunden wurde. Lichtabsorptionsmessungen des Pyridinhämochromogens zeigten aber einen Gehalt von 7 % Protohämin in dem Cytohäminpreparat (D_{587} = 0,860, D_{557} = 0,309 und D_{540} = 0,255. $\beta_{587\ \text{Cytoh.}}$ = 6,4, $\beta_{557\ \text{Protoh.}}$ = 8,0, $\beta_{540\ \text{Protoh.}}$ = 2,3, alle β-Werte $\times$ $10^7 cm^2 Mole^{-1}$. Die Absorption bei 587 mμ des Protohämochromogens ist zu vernachlässigen.) Spektroskopisch war eine schwache Bande bei 557 mμ unterschiedbar. Da ein zweifacher Überschuss von Cytohämin im Verhältnis zum Eiweiss eingesetzt wurde, lässt sich die für Cytohämin beobachtete Aktivität vollständig durch den Protohämingehalt erklären. Cytohämin gibt also mit Apo-MRP keine peroxydatisch aktive Verbindung.

5. Der R_F-Wert des Cytodeuterohämins, der die Gegenwart von zwei Karboxylgruppen im Molekül bestätigt [3], war von denen des Deuterohämins IX, des 2,3,4-H-Hämins und des 2,3-H-4-Et-Hämins klar abweichend. Diese Beobachtung unterstützt den früheren Schluss, dass Cytodeuterohämin mit keiner dieser Verbindungen identisch sei. Es ist von Interesse, dass Cytodeuterohämin, das nach der Bromierung zu beurteilen drei freie β-Stellungen besitzt [3], dieselbe Peroxydaseaktivität wie das 2,3,4-H-6,7-Propionsäure-Hämin hervorrufen kann. Die Tatsache, dass Cytodeuterohämin eine zwar schwache aber doch gut messbare Peroxydase-aktivität erregte, deutet an, dass es Propionsäuren in den Stellungen 6 und 7 oder in zwei Stellungen in demselben Abstand von einander besitzt. Das verwendete Cytodeuterohämin stammte aus Cytohämin mit 7 % Protohämin, war aber über seinen mehrmals chromatographierten und krystallisierten Porphyrinester gereinigt und enthielt deshalb wahrscheinlich kein Deuterohämin IX.

Professor Otto Warburg sei für seine Interesse gedankt. K.G.P. dankt auch für Gastfreundschaft.

K. G. Paul dankt *Statens naturvetenskapliga forskningsråd* für ein Reisestipendium.

1. Theorell, H., Bergström S. und Åkeson Å. *Arkiv Kemi Mineral. Geol.* **16A** (1942) Nr. 3.
2. Paul, K. G. *Acta Chem. Scand.* **13** (1959) 1239.
3. Warburg, O. und Gewitz H. S. *Z.physiol. Chem. Hoppe-Seyler* **288** (1951) 1; **292** (1953) 174.
4. Warburg, O., Gewitz, H. S. und Völker, W. *Z. Naturforsch.* **10 b** (1955) 541.
5. Gewitz, H. S. und Völker, W. *Z. physiol. Chem. Hoppe-Seyler* **302** (1955) 119.
6. Chu, T. C. und Chu, E. J.-H. *J. Biol. Chem.* **212** (1955) 1.
7. Paul, K. G. *Acta Chem. Scand.* **12** (1958) 1611.
8. Paul, K. G. und Avi-Dor, Y. *Acta Chem. Scand.* **8** (1954) 649.

Eingegangen am 19. Juni 1959.

Liberation of an Amino Acid Derivative from its Copper Complex by means of Hydrogen Sulphide Generated *in situ* from Thioacetamide

GEORG FÖLSCH and
KLAUS SERCK-HANSSEN

Department of Medical Biochemistry, University of Gothenburg, Sweden

Thioacetamide, CH_3CSNH_2, may be smoothly hydrolysed to produce hydrogen sulphide[1], and generally allows easy domination of problems caused by the objectionable properties of the gas.

The use of thioacetamide for the detection of certain heavy metals was reported [2] in 1934, and during the last decade the reagent has been extensively used in inorganic analysis [3]. Compared with hydrogen sulphide [4], thioacetamide usually provides sulphide precipitates of superior coagulative properties.

When amino acids and derivatives are prepared or isolated *via* heavy metal compounds, the metal has hitherto been removed by means of hydrogen sulphide [5,6]. Heating is often necessary, and even in strongly acidic solution the reaction may take several hours, and frequently results in precipitates difficult to filter. It therefore appeared attractive to try thioacetamide in this field. The copper complex [7] of ε-benzoxycarbonyl-L-lysine was chosen as an example. In boiling water containing a little acetic acid, the precipitation of copper sulphide by means of thioacetamide was completed in a few minutes, and the precipitate was easily removed by filtration, with no visible colloidal sulphide passing the filter. The use of thioacetamide in similar cases is therefore indicated.

Experimental. Pulverized copper complex [7] (15.0 g) of ε-benzoxycarbonyl-L-lysine, prepared from 0.05 mole of L-lysine monohydrochloride with $[\alpha]_D^{25}$ + 24.5° ± 1.0° (6 N HCl, *c* 2, calc. as free base, *l* 1), was suspended in 350 ml of boiling water, and 20 ml of acetic acid added, immediately followed by 7.5 g (0.10 mole) of thioacetamide. The lysine derivative dissolved completely, and copper sulphide began to precipitate. After boiling 5 min the precipitate was filtered off on a hot Büchner funnel. ε-Benzoxycarbonyl-L-lysine crystallized immediately from the filtrate. After some hours at +4°, the crystals were collected, washed with a little cold water and dried *in vacuo* over potassium hydroxide. Yield 10.2 g (73 % calc. from L-lysine monohydrochloride), m.p. 252–254° (decomp.); $[\alpha]_D^{20}$ + 6.4° (2 N NaOH, *c* 6.6 *l* 1); reported [8] m.p. near 255°. (Found: N 9.8. Calc. for $C_{14}H_{20}O_4N_2$ (280.3): N 10.0).

To test the optical purity of the compound, 1.4 g (5 mmoles) were debenzoxycarbonylated in 5 ml of 12 N HCl (steam bath, 5 min.). Benzyl chloride separated, and when the reaction mixture had attained room temperature, 25 ml of ethanol and 50 ml of ether were added, causing 0.90 g (91 %) of L-lysine dihydrochloride to crystallize; $[\alpha]_D^{20}$ + 25.9° ± 1.5° (6 N HCl, *c* 1.3 calc. as free base, *l* 1). (Found: N 12.7. Calc. for $C_6H_{14}O_2N_2$, 2 HCl (219.1): N 12.8). Racemization is therefore not apparent.

The use of thiourea [9] or ethylenediaminotetraacetic acid (EDTA) for the removal of copper from the above complex has also been tried, with good preliminary results.

The hospitality of *AB Hässle*, Gothenburg, is gratefully acknowledged.

1. Butler, E. A., Peters, D. G. and Swift, E. H. *Anal. Chem.* **30** (1958) 1379.
2. Iwanow, F. W. *Chem. Zentr.* II (1935) 883: *Sovet. Farm.* **5** No. 12 (1934) 16.
3. Flaschka, H. *Chemist-Analyst* **44** (1955) 2.
4. Lehrmen, L. and Schneider, P. *J. Chem. Educ.* **32** (1955) 474.
5. Kurtz, A. C. *J. Biol. Chem.* **122** (1938) 477.
6. Wünsch, E., Fries, G. and Zwick, A. *Chem. Ber.* **91** (1958) 542.
7. Neuberger, A. and Sanger, F. *Biochem. J.* **37** (1943) 515.
8. Bergmann, M., Zervas, L. and Ross, W. F. *J. Biol. Chem.* **111** (1935) 245.
9. Linton, H. *AB Sunco*, Gothenburg; *Personal communication.*

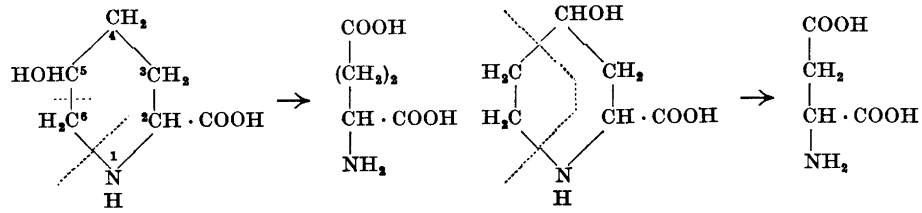
Received June 18, 1959.

On the Structure of 4-Hydroxypipecolic Acid Isolated from Green Plants

ARTTURI I. VIRTANEN and ROLF GMELIN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Some years ago Virtanen and Kari [1] isolated from some *Acacia*-species a new cyclic imino acid and identified it as 5-hydroxy-piperidine-2-carboxylic acid (5-hydroxypipecolic acid). From the same plants also another cyclic imino acid could be isolated [2]. This was characterized as 4-hydroxypipecolic acid because it was reduced with hydrogen iodide and red phosphorus to pipecolic acid and was oxidized with potassium permanganate to aspartic acid. Glycine and two other amino acids, not nearer characterized, were also formed then, but no glutamic acid which was the main or only oxidation product of 5-hydroxypipecolic acid. The positions of the hydroxyl group in the isolated hydroxypipecolic acids were concluded from the oxidation products



5-hydroxypipecolic acid glutamic acid 4-hydroxypipecolic acid aspartic acid

Witkop and Foltz [3] later confirmed the structure of our 5-hydroxy compound through synthesis and presented evidence for the *trans* arrangement of the two functional groups.

Last year Fowden [4] described the isolation of an imino acid from *Armeria maritima* which he identified with the 4-hydroxypipecolic acid isolated by Virtanen and Kari. As oxidation products with $KMnO_4$ he could identify β-alanine, glycine, γ-aminobutyric acid, and aspartic acid. Comparisons with synthetic 3- and 4-hydroxypipecolic acids indicated that the amino acid may not be 4-hydroxypipecolic acid, but 3-hydroxypipecolic acid. A strictly identical behaviour of natural and synthetic 3-hydroxypipecolic acid could not be demonstrated by Fowden, since the two substances differed in their stereoisomeric composition.

Because the formation of aspartic acid as an oxidation product of a hydroxypipecolic acid is difficult to understand if the hydroxy group is not in 4-position, we have reinvestigated the structure of the imino acid which according to Virtanen and Kari would be 4-hydroxypipecolic acid. The acid was now isolated from the seeds of *Acacia willardiana* and *Lysiloma bahamense*. Analysis of the preparation. Found: C 49.91; H 7.48; N 9.35; Calc. for $C_6H_{11}O_3N$: C 49.65; H 7.46; N 9.65. On the basis of IR-spectra, paper chromatographical behaviour, and colour reactions, it was identical with the 4-hydroxypipecolic acid isolated by Virtanen and Kari from fresh plants of *Acacia pentadena* and *Albizzia lophantha*. The acid could be found also in different *Armeria*-species.

Optical rotation of 4-hydroxypipecolic acid:

$[\alpha]_D^{21} = -12.5°$ in H_2O
» $= +0.34°$ in 1 N HCl
» $= -18.5°$ in 1 N NaOH

As principal products of acidic (H_2SO_4) and alkaline (NaOH) permanganate oxidation of the imino acid β-alanine and aspartic acid were identified (Fig. 1). In addition small amounts of glycine and some unidentified substances, coloured by ninhydrin, which possibly were intermediate products of the oxidation, were found on the paper chromatogram. γ-Aminobutyric acid was

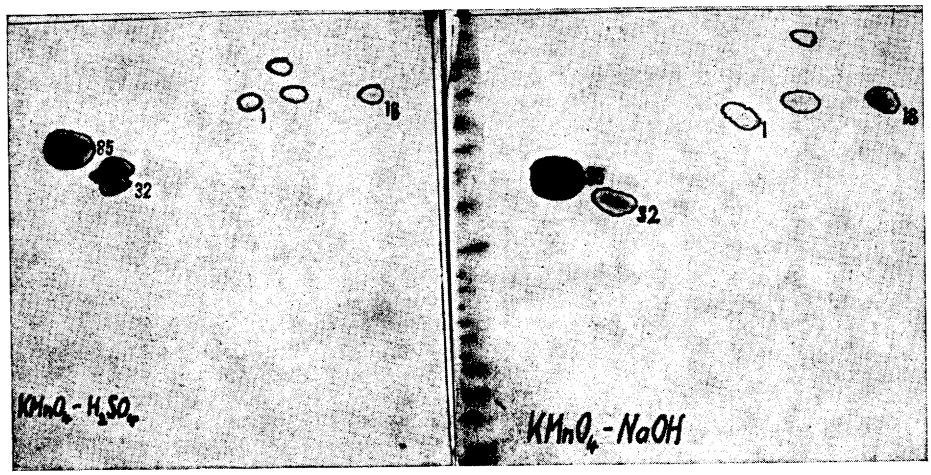


Fig. 1. Two-dimensional paper chromatogram of oxidation products of 4-hydroxypipecolic acid. Left: oxidation with $KMnO_4$ in 20 % H_2SO_4 solution; right: oxidation with $KMnO_4$ in NaOH solution. 1 gly, 16 asp, 32 β-ala, 85 unchanged 4-hydroxypipecolic acid. Solvents: butanol-acetic acid-water and phenol-NH_3.

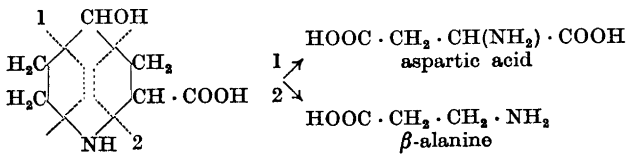
never found as an oxidation product. Excluding this important difference, the oxidation products were qualitatively the same as Fowden's. On the chromatogram of Virtanen and Kari [2] β-alanine was present as a "not nearer characterized" spot (*cf.* Fig. 3 in their paper).

The formation of aspartic acid and also of β-alanine as oxidation products from 4-hydroxypipecolic acid is reasonable, but not from 3-hydroxypipecolic acid.

Because γ-aminobutyric acid was not found as an oxidation product, there was no evidence for the 3-hydroxypipecolic acid.

In order to elucidate the properties of 3-hydroxypipecolic acid we have prepared this acid in the following way. The new antibiotic, etamycin, isolated by Heinemann *et al.*[5] and Bartz *et al.*[6] is according to Sheehan *et al.*[7] a *cyclo*peptide which contains 3-hydroxypyridine-2-carboxylic acid (3-hydroxypipecolic acid) as one member. 2.1 g of etamycin was hydrolyzed with 6 N HCl at 110°C for 24 h. The concentrated hydrolysate was dissolved in 1 l of water and passed through a column containing 100 ml of Dowex 50 (200 mesh) in the H-form. On elution with 1 % ammonia 3-hydroxypicolic acid emerged before the amino acids. The fraction was evaporated *in vacuo* and 3-hydroxypicolic acid was crystallized three times from methanol-ether. Yield 134 mg. M.p. 214—215°C. (Found: C 51.70; H 3.83; N 10.41. Calc. for $C_6H_5O_3N$: C 51.81; H 3.63; N 10.08).

100 mg of 3-hydroxypicolic acid was dissolved in 20 ml of water after addition of some drops of diluted HCl and was 48 h catalytically hydrogenated (50 mg PtO_2 as



4-hydroxypipecolic acid

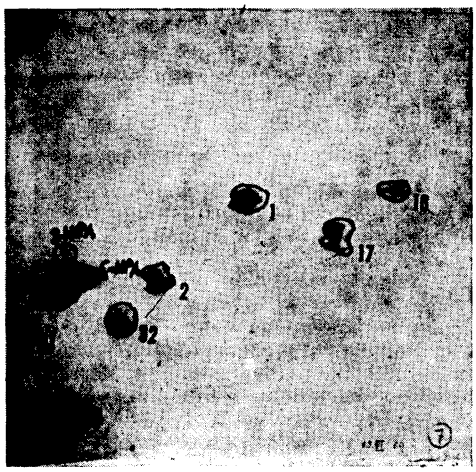


Fig. 2. Two-dimensional paper chromatogram of different amino acids including 3-, 4-, and 5-hydroxypipecolic acids. 1 gly, 2 ala, 11 pro, 16 asp, 17 glu, 29 γ-aminobut, 32 β-ala, 60 pipecol, 3-HPA, 4-HPA, and 5-HPA the corresponding hydroxypipecolic acids. Solvents as in Fig. 1.

catalyst). About seven ninhydrin-positive spots could be found on the paper chromatogram. The amino acids formed through ring splitting were removed with HNO_2 and the imino acids regenerated with HCl. After running through an Amberlite IR-120 column three compounds could be found: 3-hydroxypipecolic acid, pipecolic acid, and an unknown substance which on the basis of its R_F-values was possibly tetrahydro-3-hydroxypipecolic acid. The 3-hydroxypipecolic acid could be obtained in crystalline form by addition of acetone to a water solution. After recrystallization from water-acetone the substance was paper chromatographically pure. Yield 8.5 mg. Decomposition from about 250°C. (Found: N 9.96. Calc. for $C_6H_{11}O_3N$: N 9.66).

The position of the spots of 3-, 4-, and 5-hydroxypipecolic acid on a two-dimensional paper chromatogram can be seen in Fig. 2. As appears from the figure 3-, 4-, and 5-hydroxypipecolic acids can be separated from each other by paper chromatography using butanol-acetic acid-water and phenol-water-NH_3 as solvents. These imino acids also give different colours with ninhydrin, isatin, and naphthoquinonesulphonic acid (*e.g.* 3-hydroxy compound with isatin no colour, 4-hydroxy compound a very weak green colour, and 5-hydroxy compound a strong greenblue colour).

On the basis of optical rotation it is probable that the natural 4-hydroxypipecolic acid belongs to the L-series.

All known facts are in accordance with the structure Virtanen and Kari gave to the imino acid (4-hydroxypipecolic acid) they isolated from *Albizzia lophantha* and *Acacia pentadena*.

We are grateful to Dr. J. C. Sheehan for a smaller sample of etamycin and to Dr. B. Heinemann for a larger one.

1. Virtanen, A. I. and Kari, S. *Acta Chem. Scand.* **8** (1954) 1290.
2. Virtanen, A. I. and Kari, S. *Ibid.* **9** (1955) 170.
3. Witkop, B. and Foltz, C. M. *J. Am. Chem. Soc.* **79** (1957) 192.
4. Fowden, L. *Biochem. J.* **70** (1958) 629.
5. Heinemann, B., Gourevitch, A., Lein, J., Johnson, D. L., Kaplan, M. A., Vanas, D. and Hooper, I. R. *Antibiotics Annual* **2** (1954–1955) 728.
6. Bartz, Q. R., Standiford, J., Mold, J. D., Johannessen, D. W., Ryder, A., Maretzki, A. and Haskell, T. H. *Ibid.* **2** (1954–1955) 777, 784.
7. Sheehan, J. C., Zachau, H. G. and Lawson, W. B. *J. Am. Chem. Soc.* **80** (1958) 3349.

Received June 15, 1959.

Chromatography of Secretin on Carboxymethyl Cellulose

VIKTOR MUTT

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

In 1957 Ward and Guillemin [1] described a method for the purification of vasopressin in which the hormone is adsorbed on carboxymethyl cellulose from a solution of the crude material in 0.02 M ammonium acetate and selectively eluted by developing the column with a gradient to an ammonium acetate solution of higher concentration and pH. We decided to try a similar technique for the purification of secretin. Carboxymethyl cellulose has been used for the purification of secretin by Newton, Love, Heatley and Abraham [2] who adsorbed the activity from crude preparations on columns of carboxymethyl cellulose buffered with barium acetate at pH 4, eluted impurities with 0.02 N acetic acid and then a highly purified secretin with 0.1 N acetic acid.

We found, working with highly purified preparations, that secretin may be adsorbed on carboxymethyl cellulose * from solution in 0.02 M ammonium bicarbonate and eluted by increasing the concentration of the latter to 0.04 M. Further work showed, however, that it was unnecessary to change the concentration of the eluting solution. If elution was continued for a sufficiently long time with the 0.02 M bicarbonate the secretin emerged from the column in good yield and in a high state of purity. Large quantities of impurities were both eluted from the column before and left on it after the secretin. The impurities left on the column could be eluted by increasing the concentration of the ammonium bicarbonate to 0.2 M.

On lyophilizing the most active fractions of the eluated material with 150 000 Hammarsten cat units ** per mg has been obtained. The purity of the preparations is thus at least as good as that of preparations recently obtained on electrophoretic fractionation of highly purified material [3].

Experimental. Starting material. Crude secretin was prepared as described by Jorpes and Mutt [5] in 1956. For preliminary purification of this the technique outlined by Mutt [3] was followed until the extraction of the activity into methanol. The methanolic solution

* Prepared according to Peterson and Sober [4], obtained from the Brown company, New Hampshire, USA.

** One Hammarsten cat unit (HCU) is that amount of secretin activity which on intravenous injection stimulates the pancreas of the cat to the secretion of 0.1 ml 0.1 N alkali.

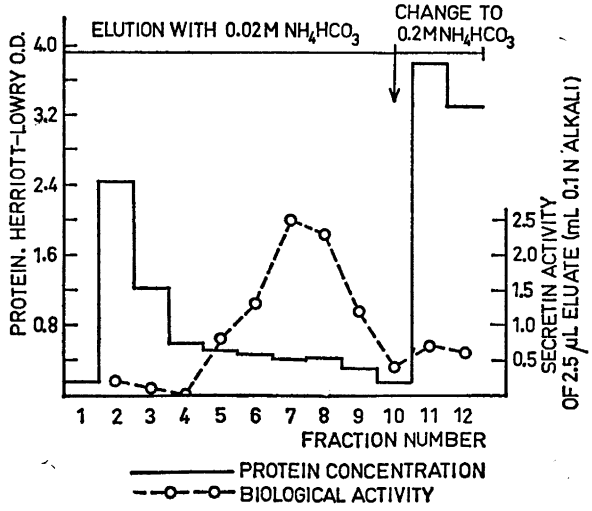


Fig. 1.

was brought to pH 7.5 with NaOH (glass electrode). Precipitated impurities were filtered off and discarded. The clear filtrate was acidified to pH 5.5 with HCl and the secretin precipitated with ether. After reprecipitation from water with sodium chloride at saturation it was collected on a suction filter and sucked as dry as possible. In repeated preparations the moist material had an activity of about 7 000 HCU per mg. This material was dissolved in water and the pH of the solution brought to 7.2. Three volumes of acetone were then added to the solution. The precipitate that formed was collected by centrifugation and discarded. The secretin was thrown out of solution by adding nine more volumes of acetone to the clear supernatant. The precipitate was collected by centrifugation and dissolved in water. The pH of the solution was adjusted to 5.5 with acetic acid and the secretin lyophilized from it. The lyophilized material had an activity varying in different preparations from 10 000 to 15 000 HCU per mg. The yield was 0.05—0.1 mg per hog.

Chromatography. 45 mg of a preparation with 12 000 HCU per mg was dissolved in 9 ml of 0.02 M ammonium bicarbonate and the solution allowed to sink into a column (7.5 × 1.4 cm) of 3 g of carboxymethyl cellulose which had been equilibrated with 0.02 M ammonium bicarbonate. Elution was carried out with 0.02 M ammonium bicarbonate. Fractions of 10 ml were collected. The flow rate was 40 ml/h. Collection of the fractions was started from the time when the secretin solution was applied to the column. Between the tenth and the eleventh fraction the eluting solution was changed to 0.2 M ammonium bicarbonate.

The approximate relative concentration of proteinaceous material in the fractions was determined by the Herriott reaction [6] as adapted by Lowry *et al.* [7], except that the copper salt containing stock solution of the latter authors was made up in 0.1 N NaOH instead of in water. The secretin activity was determined by injecting appropriate aliquots of the fractions intravenously into cats and titrating the amount of alkali secreted through a cannula in the main pancreatic duct in response to the injection.

The results are shown in Fig. 1.

The author wishes to thank the Head of the department Professor Erik Jorpes, for his advice and encouragement.

This work has been supported by research grants from *Olin Mathieson Chemical Corporation*, New York, from the *Swedish Cancer Society* and from *Magnus Bergvalls Stiftelse*, Stockholm.

1. Ward, D. N. and Guillemin, R. *Proc. Soc. Exptl. Biol. Med.* **96** (1957) 568.
2. Newton, G. G. F., Love, J. W., Heatley, N. G. and Abraham, E. P. *Biochem. J.* **71** (1959) 6P.
3. Mutt, V. *Arkiv Kemi* **14** (1959) 275.
4. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
5. Swed. pat. 156013/1956.
6. Herriott, R. M. *Proc. Soc. Exptl. Biol. Med.* **46** (1941) 642.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.

Received June 23, 1959.

Polymorphism in Calcium Tantalum(V) Oxide $CaTa_2O_6$

LENA JAHNBERG, STEN ANDERSSON and ARNE MAGNÉLI

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

It was recently reported by Gasperin [1] that an equimolecular mixture of tantalum(V)oxide and calcium carbonate upon heating at about 1 700°C gives a product possessing the composition $CaTaO_3$ with a multiple-cell, deformed perovskite structure, a of the pseudocubic subcell unit being 3.88 Å. The formation of the new compound should evidently be accompanied by a reduction of tantalum to the tetravalent state. Such a process however, seems rather unlikely in view of the high reluctance to reduction shown by tantalum(V)oxide and of the white colour of the specimen which rather suggests the presence of the state of maximum valency. The density reported for the product (7 $g.cm^{-3}$) likewise is not in good agreement with that calculated for the formula $CaTaO_3$ (7.6 $g.cm^{-3}$) but is in fair accordance with that of a compound such as $Ca_{0.5}Ta^{V}O_3$ (7.1 $g.cm^{-3}$). The validity of the latter formula was actually demonstrated by Gasperin in a subsequent analytical investigation of the

was brought to pH 7.5 with NaOH (glass electrode). Precipitated impurities were filtered off and discarded. The clear filtrate was acidified to pH 5.5 with HCl and the secretin precipitated with ether. After reprecipitation from water with sodium chloride at saturation it was collected on a suction filter and sucked as dry as possible. In repeated preparations the moist material had an activity of about 7 000 HCU per mg. This material was dissolved in water and the pH of the solution brought to 7.2. Three volumes of acetone were then added to the solution. The precipitate that formed was collected by centrifugation and discarded. The secretin was thrown out of solution by adding nine more volumes of acetone to the clear supernatant. The precipitate was collected by centrifugation and dissolved in water. The pH of the solution was adjusted to 5.5 with acetic acid and the secretin lyophilized from it. The lyophilized material had an activity varying in different preparations from 10 000 to 15 000 HCU per mg. The yield was 0.05—0.1 mg per hog.

Chromatography. 45 mg of a preparation with 12 000 HCU per mg was dissolved in 9 ml of 0.02 M ammonium bicarbonate and the solution allowed to sink into a column (7.5 × 1.4 cm) of 3 g of carboxymethyl cellulose which had been equilibrated with 0.02 M ammonium bicarbonate. Elution was carried out with 0.02 M ammonium bicarbonate. Fractions of 10 ml were collected. The flow rate was 40 ml/h. Collection of the fractions was started from the time when the secretin solution was applied to the column. Between the tenth and the eleventh fraction the eluting solution was changed to 0.2 M ammonium bicarbonate.

The approximate relative concentration of proteinaceous material in the fractions was determined by the Herriott reaction [6] as adapted by Lowry *et al.* [7], except that the copper salt containing stock solution of the latter authors was made up in 0.1 N NaOH instead of in water. The secretin activity was determined by injecting appropriate aliquots of the fractions intravenously into cats and titrating the amount of alkali secreted through a cannula in the main pancreatic duct in response to the injection.

The results are shown in Fig. 1.

The author wishes to thank the Head of the department Professor Erik Jorpes, for his advice and encouragement.

This work has been supported by research grants from *Olin Mathieson Chemical Corporation*, New York, from the *Swedish Cancer Society* and from *Magnus Bergvalls Stiftelse*, Stockholm.

1. Ward, D. N. and Guillemin, R. *Proc. Soc. Exptl. Biol. Med.* **96** (1957) 568.
2. Newton, G. G. F., Love, J. W., Heatley, N. G. and Abraham, E. P. *Biochem. J.* **71** (1959) 6P.
3. Mutt, V. *Arkiv Kemi* **14** (1959) 275.
4. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
5. Swed. pat. 156013/1956.
6. Herriott, R. M. *Proc. Soc. Exptl. Biol. Med.* **46** (1941) 642.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.

Received June 23, 1959.

Polymorphism in Calcium Tantalum(V) Oxide $CaTa_2O_6$

LENA JAHNBERG, STEN ANDERSSON and ARNE MAGNÉLI

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

It was recently reported by Gasperin [1] that an equimolecular mixture of tantalum(V)oxide and calcium carbonate upon heating at about 1 700°C gives a product possessing the composition $CaTaO_3$ with a multiple-cell, deformed perovskite structure, a of the pseudocubic subcell unit being 3.88 Å. The formation of the new compound should evidently be accompanied by a reduction of tantalum to the tetravalent state. Such a process however, seems rather unlikely in view of the high reluctance to reduction shown by tantalum(V)oxide and of the white colour of the specimen which rather suggests the presence of the state of maximum valency. The density reported for the product (7 $g.cm^{-3}$) likewise is not in good agreement with that calculated for the formula $CaTaO_3$ (7.6 $g.cm^{-3}$) but is in fair accordance with that of a compound such as $Ca_{0.5}Ta^{V}O_3$ (7.1 $g.cm^{-3}$). The validity of the latter formula was actually demonstrated by Gasperin in a subsequent analytical investigation of the

substance [2]. A compound of the composition $CaTa_2O_6$ was reported by Ismailzade [3] to have a complex structure.

In the present study mixtures of tantalum(V)oxide and calcium carbonate were heated at 1 200°C in air for several hours and then fused in an electric arc furnace in an argon atmosphere. The specimen rested on the water-cooled copper base of the furnace which caused rapid cooling upon discontinuing the arc. The products were characterized by their X-ray powder patterns, registered in a Guinier camera with strictly monochromatized CuKa_1 radiation and using potassium chloride (a = 6.2919 Å at 20°C) as an internal standard. The following results were thus obtained.

Starting composition	Colour of product	X-ray evidence
$CaCO_3 + Ta_2O_5$	greyish white	non-cubic perovskite-type pattern
$CaCO_3 + 0.5\ Ta_2O_5$	»	complicated pattern
$CaCO_3 + 0.4\ Ta_2O_5$	»	»

Obviously the first specimen, corresponding to a calcium to tantalum ratio of 1:2 is a monophasic, perovskite-type sample. In order to establish the valency of tantalum in this product it was heated for several hours in a stream of oxygen at 800°C. The weight of the sample was not affected by this treatment which shows the tantalum to be pentavalent and suggests the formula of the compound to be $Ca_{0.5}TaO_3$. The colour showed only a slight shift upon heating, from the original greyish to brownish white but the X-ray powder pattern displayed a considerable number of extra reflexions in addition to the lines corresponding to a perovskite-type phase.

In order to study the transformation thus found to occur at 800°C samples of the original preparation ($Ca_{0.5}TaO_3$) were heated for several hours at 1 300°C and 650°C, respectively. The following observations were made:

Heating temperature	Density	X-ray evidence
1 300°C	7.02	complicated pattern
650°C	6.75	cubic, perovskite-type

Upon melting both samples were reconverted to give the non-cubic perovskite-type pattern. The cubic perovskite, upon heating at 1 300°C, gave the complicated pattern mentioned above but this transformation could not be reversed.

The X-ray pattern of the sample which had been quenched from the melting temperature was not indexed. It showed a splitting up of several of the perovskite-type reflexions.

The pattern of the sample heat-treated at 1 300°C, was quite different from the perovskite type. It could be interpreted in terms of an orthorhombic unit cell with

$$a = 11.067\ \text{Å},\quad b = 5.380\ \text{Å},\quad c = 7.505\ \text{Å}$$

indicating a cell content of four formula units of $CaTa_2O_6$ (calculated density = 7.40). Studies on the crystal structure of this modification are in progress.

The unit cell edge of the cubic perovskite structure obtained at 650°C was found to be 3.886 Å. No lines indicating a multiple-cell structure were observed. The arrangement of the calcium atoms should thus be a random one and comparable with that of the alkali atoms in the cubic sodium [4] and lithium [5,6] wolfram bronzes of perovskite type.

This investigation forms part of a research programme on metal oxides and related compounds financiaily supported by the *Swedish Natural Science Research Council.*

1. Gasperin, M. *Acta Cryst.* **11** (1958) 739.
2. Editorial note. *Acta Cryst.* **12** (1959) 422.
3. Ismailzade, I. G. *Izvest. Akad. Nauk S.S.S.R., Ser. Fiz.* **22** (1958) 1485. (Quoted according to *C.A.* **53** (1959) 5803.)
4. Hägg, G. *Z. physik. Chem.* **B29** (1935) 192.
5. Magnéli, A. and Nilsson, R. *Acta Chem. Scand.* **4** (1950) 398.
6. Straumanis, M. E. and Hsu, S. S. *J. Am. Chem. Soc.* **72** (1950) 4027.

Received June 26, 1959.

Ion Exchange Properties of Alginate Fractions

ARNE HAUG

Norwegian Institute of Seaweed Research, N.T.H., Trondheim, Norway

Recently, two methods for the separation of alginates into chemically different fractions have been published. McDowell [1] separated alginate from *Laminaria hyperborea* (= *L. Cloustoni*) and *Ascophyllum nodosum* into fractions with different ion exchange properties, by precipitation with manganous salts. Haug [2] fractionated alginates from *Laminaria digitata* f. *stenophylla* by means of precipitation with potassium chloride, and the fractions differed in uronic acid composition. The present note reports an investigation of the ion exchange properties and uronic acid composition of the fractions obtained by the two methods.

Materials and methods. The sampling of seaweed and the preparation of alginate is described earlier [2]. The precipitation with potassium chloride was carried out by mixing equal volumes of saturated aqueous potassium chloride and a solution of 0.3 % sodium alginate in water. The amounts of alginate precipitated with manganous salts were found to depend on the pH and the concentration of potassium and sodium ions present. In this investigation, one part of a solution of 0.09 M manganous sulphate and 0.3 M potassium chloride was added to two parts of a solution containing approximately 0.3 % sodium alginate, adjusted to pH 5. After one hour the gellike precipitate was separated from the solution by centrifugation. The alginate was converted to the sodium salt by washing with a dilute solution of sulphur dioxide in water and dissolving in dilute sodium carbonate solution.

A procedure similar to that of Mongar and Wassermann [3] was used for the determination of ion exchange properties. Alginic acid fibers were made by forcing a viscous sodium alginate solution from a pipette with a narrow opening (0.5 mm) into 1 N hydrochloric acid, stirred with a magnetic stirrer. The alginic acid fibers were transferred to a 0.1 M calcium acetate solution. After thorough washing with water, the calcium alginate fibers were placed in 20—35 ml 0.2 M potassium chloride solution and allowed to stand overnight. The calcium concentration in the solution was determined by oxalate precipitation and titration. The calcium bound to the alginate was liberated by 1 N hydrochloric acid and determined.

The methods used for hydrolysis of alginates and chromatography of uronic acids were those given by Fischer and Dörfel [4].

Alginates from *Laminaria digitata* f. *stenophylla*, collected at Tarva, outside the Trondheimsfjord, 3/7 1957, and from *L. hyperborea*, stipes, collected at Munkholmen in the Trondheimsfjord, 26/8 1957, were investigated. The alginates were fractionated according to the two methods, and the ion exchange properties and uronic acid composition of the fractions investigated. The results are shown in Table 1. No attempts have been made to determine the amounts of uronic acids quantitatively, and the results given in the table are estimates based on the size and strength of the spots. The ion exchange properties are characterized by the selectivity coefficient

$$k = \frac{[Ca_g]\,[Na_s]^2}{[Na_g]^2[Ca_s]}$$

where subscripts g and s refer to gel and solution and the concentrations are given as equivalent fractions and normalities, respectively. No attempt has been made to determine the true equilibrium constant involving activities. All figures given in the table are an average of several determinations, and the conditions were chosen so that the equivalent fraction of calcium in the gel in all cases was between 0.4 and 0.7.

The results show that there is a marked difference in ion exchange properties and uronic acid composition between alginate from *Laminaria digitata* and *L.hyperborea* stipes. Preliminary experiments have shown that alginate from *L.hyperborea* fronds resembles that from *L.digitata*. In the latter plant, no large difference between alginate from stipes and fronds have been observed.

The results also show that for both alginates the soluble fraction contains more guluronic acid and has a higher selectivity coefficient than the precipitated fraction when fractionated with potassium chloride, while the opposite is the case when manganous ions are used as precipitating agent. This result indicates that the same type of fractions are produced by both methods, but that the fraction precipitated by potassium chloride corresponds to the fraction that is not precipitated by manganous sulphate. Combining the two methods

Table 1. Uronic acid composition and selectivity coefficients of alginate fractions.

Fractionation method	Fraction	*Laminaria digitata*			*L. hyperborea*, stipes		
		k	Mannuronic acid	Guluronic acid	k	Mannuronic acid	Guluronic acid
No fractionation		7.5	++	+	21.1	+	+++
Potassium chloride	Insoluble (K_i)	6.7	+++	+	15.5	+	++
» »	Soluble (K_s)	9.2	++	++	20.5	(+)	+++
Manganous sulphate	Insoluble (Mn_i)	8.4	++	++	22.9	(+)	+++
» »	Soluble (Mn_s)	7.4	+++	+	10.2	+	++
	Mn_sK_i	6.5	+++	(+)	8.6	++	++
	Mn_iK_s	11.2	+	++	20.3	(+)	+++
	$Mn_sK_iMn_sK_i$	5.1	+++	traces			
	$Mn_iK_sMn_iK_s$	13.1	+	++			

The number of +'s indicates approximately the proportion between the uronic acids in each fraction.

(+): One fifth or less of the total amount of uronic acids.

Traces: One tenth or less of the total amount of uronic acids.

should therefore offer a good opportunity for purifying the fractions. The fractions from the precipitation with manganous sulphate were subjected to fractionation with potassium chloride, and the selectivity coefficient and uronic acid composition were determined for the fraction soluble in manganous sulphate and insoluble in potassium chloride (Mn_sK_i) and the fraction insoluble in manganous sulphate and soluble in potassium chloride (Mn_iK_s). To obtain a further purification the procedure was repeated on the two purified fractions of *L.digitata* alginate. The results are given in Table 1.

The results confirm that alginate from *L.digitata* and *L.hyperborea* stipes is a mixture of macromolecules with different uronic acid composition and different ion exchange properties. Molecules containing a large proportion of guluronic acid residues are accumulated in the fractions which are soluble in potassium chloride solution and precipitated by manganous ions, while molecules rich in mannuronic acid residues are accumulated in the opposite fractions. The fractions containing molecules rich in mannuronic acid residues have a lower affinity for calcium in a calcium-potassium ion exchange reaction than fractions with molecules with predominately guluronic acid residues. Alginates from different sources may have different uronic acid composition, and, consequently, different ion exchange properties, a fact which stresses the necessity of defining the source of the alginate in investigations of these polyuronides.

Further investigations of the alginate fractions are in progress.

1. McDowell, R. H. *Chem. & Ind. London* **1958** 1401.
2. Haug, A. *Acta Chem. Scand.* **13** (1959) 601.
3. Mongar, I. L. and Wassermann, A. *J. Chem. Soc.* **1952** 492.
4. Fischer, F. G. and Dörfel, H. *Z.physiol. Chem. Hoppe-Seyler* **302** (1955) 186.

Received June 9, 1959.

Complexes of Divalent Tellurium

OLAV FOSS and SVERRE HAUGE

Chemical Institute, University of Bergen, Bergen, Norway

Tellurium dioxide dissolved in hydrochloric or hydrobromic acid has been found to react with four moles of aqueous thiourea as follows:

$$Te^{IV} + 4\ SC(NH_2)_2 + 2\ X^- \rightarrow Te[SC(NH_2)_2]_2X_2 + [(H_2N)_2C\text{-}S\text{-}S\text{-}C(NH_2)_2]^{++}$$

where Te^{IV} represents tetravalent tellurium and X the halide. Tellurium is reduced to the divalent state, by two moles of thiourea which themselves become oxidized to formamidinium disulphide. The divalent tellurium compound crystallizes out on mixing of the tellurium dioxide and thiourea solutions.

The compounds, $Te(tu)_2Cl_2$ and $Te(tu)_2Br_2$ (tu = thiourea), dissolve in aqueous thiourea to give yellow solutions from which salts containing the cation $Te(tu)_4^{++}$ crystallize out. They also dissolve in aqueous potassium thiocyanate to give, similarly, the thiocyanate $Te(tu)_2(SCN)_2$.

X-Ray crystallographic data have been obtained from oscillation and Weissenberg photographs taken with copper radiation, $\lambda(\alpha) = 1.542$ Å.

Dichloro-dithiourea-tellurium(II), $Te(tu)_2Cl_2$. Monoclinic prismatic, $a = 9.90$ Å, $b = 7.62$ Å, $c = 14.10$ Å, $\beta = 100\frac{1}{2}°$. There are four molecules per unit cell; density, calc. 2.23, found 2.26 g/cm³. Systematic absences, hkl when $h + k + l$ is odd, $h0l$ when h is odd or l is odd.

From Fourier projections along the b and a axes [1], the space group is the centrosymmetric one, C_{2h}^6-I2/c. The tellurium atom lies on a twofold axis, with the other atoms in general positions. Sulphur and chlorine are bonded to tellurium in a planar or approximately planar, square arrangement, with equal atoms in *cis* positions.

Dibromo-dithiourea-tellurium(II), $Te(tu)_2Br_2$, is isomorphous with the dichloro compound. The unit cell dimensions are, $a = 10.10$ Å, $b = 7.74$ Å, $c = 14.67$ Å, $\beta = 100°$, and the density, calc. 2.59, found 2.58 g/cm³.

Dithiocyanato - dithiourea - tellurium (II), $Te(tu)_2(SCN)_2$. Orthorhombic pyramidal, $a = 18.48$ Å, $b = 18.64$ Å, $c = 7.90$ Å. There are eight molecules per unit cell; density, calc. 1.93, found 1.94 g/cm³. The space group, from systematic absences, is $C_{2v}^{19} - Fdd2$, which requires that the tellurium atom lies on a twofold axis.

Presumably the molecular structure is analogous to that of the parent dichloro and dibromo compounds. The slight increase in the length of the c axis relative to the twofold axis of the monoclinic analogs is indicative in this respect. The length, 7.90 Å, excludes the possibility that the atoms of the thiocyanate groups lie on the twofold axis.

The compound was obtained as well developed prisms {110} terminated by {111}, or when prepared from the dibromo compound, as plates {010} elongated in the direction of the c axis; the two forms gave identical X-ray photographs.

Tetrathiourea-tellurium(II) dichloride, $Te(tu)_4Cl_2 \cdot 2H_2O$. Monoclinic prismatic, $a = 6.01$ Å, $b = 16.49$ Å, $c = 9.95$ Å, $\beta = 98\frac{1}{2}°$. There are two formula units per unit cell; density, calc. 1.84, found 1.86 g/cm³. The space group, from systematic absences, is $C_{2h}^5-P2_1/c$. The tellurium atom is thereby required to lie in a centre of symmetry, which is also consistent with the intensity distribution of the $0kl$ reflections, those with $k + l$ even being in most cases markedly stronger than those with $k + l$ odd. It follows from the centrosymmetry that the TeS_4 grouping is planar.

The analytical data and the density point to the presence of two moles of crystal water per mole of salt. The crystals occur as six-sided prisms, bounded by {011} and {010} and terminated by {100}.

Tetrathiourea-tellurium(II) dibromide, $Te(tu)_4Br_2$. The crystals are triclinic, and form thin plates {001} normally elongated in the direction of the a axis. From oscillation and Weissenberg zero-layer photographs about the a and b axes and the shorter ab diagonal, $a = 5.95$ Å, $b = 7.16$ Å, $\gamma = 107°$ or $73°$, $d_{100} = 5.69$ Å, $d_{010} = 6.82$ Å, $d_{001} = 11.05$ Å, $a^* = 84°$, $\beta^* = 88°$. This gives a cell volume of 450 Å³ and one formula unit per unit cell; density, calc. 2.18, found 2.21 g/cm³. If the triclinic space group is the centrosymmetric one, the tellurium atom lies in a centre of symmetry also in this salt.

These are apparently the first instances of complexes of divalent tellurium, apart from the compound $(NH_4)_2TeCl_4$ reported by Lindner and Apolant [2] in 1924 which may possibly contain the ion $TeCl_4^{--}$. In the four-co-ordinated state, divalent tellur-

ium has six electron pairs in its valency shell; a square-planar arrangement of the four bonding pairs is in accordance with the two lone pairs occupying *trans* octahedral positions. The square-planar ICl_4^- ion represents an analogous case.

The compounds have a yellow colour, and are quite stable in the solid state. They blacken on contact with water, probably because of hydrolysis and subsequent rearrangement to zero- and tetravalent tellurium, in analogy with the alkaline hydrolysis of other derivatives of divalent tellurium as for example the telluropentathionate ion.

The $Te(tu)_4^{++}$ ion is probably the yellow species which forms the basis for the photometric determination [3-6] of tellurium with thiourea. The compounds $Te(tu)_2Cl_2$ and $Te(tu)_2Br_2$ may be the same as those isolated recently from thiourea and the tellurium tetrahalides by Aynsley and Campbell [7], and by these authors formulated with two hydrogen atoms less, as amidinothio derivatives of tetravalent tellurium.

Further preparative andstructural work is being made.

1. Foss, O. and Johnsen, K. *Work in progress.*
2. Lindner, K. and Apolant, L. *Z. anorg. u. allgem. Chem.* **136** (1924) 381.
3. Falciola, P. *Ann. chim. appl.* **17** (1927) 359.
4. Töpelmann, H. *Z. anal. Chem.* **82** (1930) 284.
5. Nielsch, W. and Böltz, G. *Z. Metallk.* **45** (1954) 380.
6. Nielsch, W. and Giefer, L. *Z. anal. Chem.* **145** (1955) 347.
7. Aynsley, E. E. and Campbell, W. A. *J. Chem. Soc.* **1958** 3290.

Received June 28, 1959.

Gas Chromatographic Separation of 3- and 4-Methyl-1-pentene

BENGT SMITH and RAGNAR OHLSON

Institutionen för organisk kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

The separation of 3- and 4-methyl-1-pentene by gas chromatography has, according to our knowledge, not been reported yet. Even on very long columns containing various polar or nonpolar liquids as stationary phases, no resolution of the pair was obtained [1]. This is due to the similarity in type and the close boiling points of the two compounds, the difference being only 0.2°C. It is obvious that this separation problem calls for a stationary phase with a highly selective action on the molecules of the two hexenes.

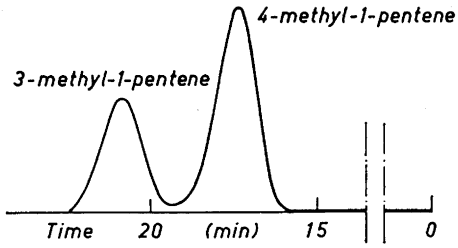


Fig. 1. Mixture of 3- and 4-methyl-1-pentene. Silver nitrate-glycol column, 4 m. Temperature 25°C. Flow of He 50 ml/min.

Such a phase is available in the form of silver nitrate in a suitable solvent, *e.g.* glycol [2,3]. In this case, there exist forces of attraction between the silver ions and the unsaturated hydrocarbons. Thus it was found that the pair in question could be resolved on a 4 m silver nitrate — glycol column containing 17 % of silver nitrate. It should be pointed out, however, that if the content of silver nitrate is appreciably diminished or increased the separation is impaired. The separation of these and other alkenes on a silver nitrate — glycol column will be more fully discussed in a forthcoming paper.

The column was prepared in the following way. Ethylene glycol was saturated with powdered silver nitrate under slight warming and agitation. The solution was decanted from undissolved silver nitrate and mixed with Chromosorb (30—60 mesh). Two parts of Chromosorb were taken to one part of the solution. The mixture was rotated in a round-bottomed flask to ensure thorough mixing of the carrier and the stationary phase. The mixture obtained was packed in two aluminium tubes each 2 m long and with a 4 mm internal diameter.

1. Knight, H. S. *Anal. Chem.* **30** (1958) 9.
2. Bradford, B. W., Harwey, D. and Chalkley, D. E. *J. Inst. Petroleum* **41** (1955) 80.
3. Bednas, M. E. and Russel, D. S. *Can. J. Chem.* **36** (1958) 1272.

Received July 1, 1959.

ium has six electron pairs in its valency shell; a square-planar arrangement of the four bonding pairs is in accordance with the two lone pairs occupying *trans* octahedral positions. The square-planar ICl_4^- ion represents an analogous case.

The compounds have a yellow colour, and are quite stable in the solid state. They blacken on contact with water, probably because of hydrolysis and subsequent rearrangement to zero- and tetravalent tellurium, in analogy with the alkaline hydrolysis of other derivatives of divalent tellurium as for example the telluropentathionate ion.

The $Te(tu)_4^{++}$ ion is probably the yellow species which forms the basis for the photometric determination [3-6] of tellurium with thiourea. The compounds $Te(tu)_2Cl_2$ and $Te(tu)_2Br_2$ may be the same as those isolated recently from thiourea and the tellurium tetrahalides by Aynsley and Campbell [7], and by these authors formulated with two hydrogen atoms less, as amidinothio derivatives of tetravalent tellurium.

Further preparative andstructural work is being made.

1. Foss, O. and Johnsen, K. *Work in progress.*
2. Lindner, K. and Apolant, L. *Z. anorg. u. allgem. Chem.* **136** (1924) 381.
3. Falciola, P. *Ann. chim. appl.* **17** (1927) 359.
4. Töpelmann, H. *Z. anal. Chem.* **82** (1930) 284.
5. Nielsch, W. and Böltz, G. *Z. Metallk.* **45** (1954) 380.
6. Nielsch, W. and Giefer, L. *Z. anal. Chem.* **145** (1955) 347.
7. Aynsley, E. E. and Campbell, W. A. *J. Chem. Soc.* **1958** 3290.

Received June 28, 1959.

Gas Chromatographic Separation of 3- and 4-Methyl-1-pentene

BENGT SMITH and RAGNAR OHLSON

Institutionen för organisk kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

The separation of 3- and 4-methyl-1-pentene by gas chromatography has, according to our knowledge, not been reported yet. Even on very long columns containing various polar or nonpolar liquids as stationary phases, no resolution of the pair was obtained [1]. This is due to the similarity in type and the close boiling points of the two compounds, the difference being only 0.2°C. It is obvious that this separation problem calls for a stationary phase with a highly selective action on the molecules of the two hexenes.

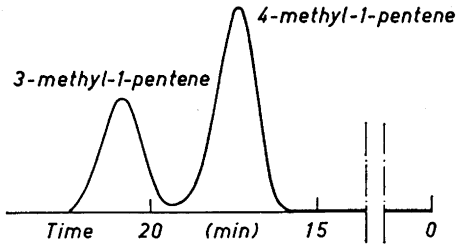


Fig. 1. Mixture of 3- and 4-methyl-1-pentene. Silver nitrate-glycol column, 4 m. Temperature 25°C. Flow of He 50 ml/min.

Such a phase is available in the form of silver nitrate in a suitable solvent, *e.g.* glycol [2,3]. In this case, there exist forces of attraction between the silver ions and the unsaturated hydrocarbons. Thus it was found that the pair in question could be resolved on a 4 m silver nitrate — glycol column containing 17 % of silver nitrate. It should be pointed out, however, that if the content of silver nitrate is appreciably diminished or increased the separation is impaired. The separation of these and other alkenes on a silver nitrate — glycol column will be more fully discussed in a forthcoming paper.

The column was prepared in the following way. Ethylene glycol was saturated with powdered silver nitrate under slight warming and agitation. The solution was decanted from undissolved silver nitrate and mixed with Chromosorb (30—60 mesh). Two parts of Chromosorb were taken to one part of the solution. The mixture was rotated in a round-bottomed flask to ensure thorough mixing of the carrier and the stationary phase. The mixture obtained was packed in two aluminium tubes each 2 m long and with a 4 mm internal diameter.

1. Knight, H. S. *Anal. Chem.* **30** (1958) 9.
2. Bradford, B. W., Harwey, D. and Chalkley, D. E. *J. Inst. Petroleum* **41** (1955) 80.
3. Bednas, M. E. and Russel, D. S. *Can. J. Chem.* **36** (1958) 1272.

Received July 1, 1959.

A Halogen Exchange Reaction in a Diazonium Compound

BO LAMM

Nobel Institute of Chemistry, Stockholm 50, Sweden

Some 60 years ago, Hantzsch [1,2] found that under suitable conditions, a halogen exchange reaction takes place in 2,4- and 2,6-dibromobenzenediazonium chloride, leading to mixtures of bromochlorobenzenediazonium bromides. An aromatic halogen atom, activated by electron-attracting groups in the positions *ortho* and *para* to the halogen, is known to be particularly reactive towards nucleophilic reagents [3]. 2,4-Dinitrohalobenzenes are well-known compounds of this kind. One might think that the diazonium group, $-N_2^+$, should have a considerably activating influence upon an *ortho*- or *para*-substituted halogen atom. In virtue of its formal positive charge, the diazonium group might be expected to attract the mobile π-electron cloud of the benzene ring even more than a nitro group would do. This idea has been emphasized by Bunnett and Zahler [3]. In a recent paper [4], Lewis and Johnson also give strong support to this concept.

Sihlbom [5,6] made a kinetic investigation of a rather unusual type of reaction occurring in some dinitroanilines, yielding corresponding dichloronitrobenzenes. The reaction was found to proceed with diazonium ions as intermediates.

Sihlbom's results suggested the present work, in which it has been found that 4-bromo-3-nitrobenzenediazonium chloride in dilute acetic acid containing excess hydrochloric acid exchanges its bromine atom for chlorine, thereby yielding 4-chloro-3-nitrobenzenediazonium chloride. This was shown by replacing the diazonium group with a chlorine atom in a Sandmeyer reaction with cuprous chloride. That the halogen exchange reaction did not occur during the Sandmeyer reaction was checked as described in the experimental part.

The reaction product from the Sandmeyer reaction would be 1,4-dichloro-2-nitrobenzene if the abovementioned halogen exchange had taken place, otherwise 1-bromo-4-chloro-2-nitrobenzene. In order to assay a mixture of these two compounds, IR-spectra (2—15 μ) of the pure components were run both in carbon disulphide and carbon tetrachloride solutions. The spectra, however, were too alike to make any quantitative analysis feasible. Therefore, a chemical method, previously used by Holleman [7], was employed. This involved a nucleophilic reaction with sodium methoxide converting the halogen atoms *ortho* to the nitro group into ionic form, thereby making it possible to precipitate them as silver salts after separation from the resulting 4-chloro-2-nitroanisole.

Experimental. 2.17 g (0.01 mole) of 4-bromo-3-nitroaniline, synthesized according to Blanksma [8], were dissolved in 25 ml of glacial acetic acid. This solution was carefully added to a solution of 0.7 g (0.01 mole) of sodium nitrite in 5 ml of conc. sulphuric acid while cooling in an ice-bath. The temperature of the reaction mixture was kept between + 10° and + 20°. 10 g of ice and 50 ml of conc. hydrochloric acid were added, and the resulting diazonium solution was left at + 25° for one week. It was then filtered from some brown sludge and poured into a cuprous chloride solution, prepared by dissolving 9.9 g of cuprous chloride in 35 ml of conc. hydrochloric acid (making 0.1 mole of cuprous chloride.) Nitrogen was evolved, and a yellowish substance was precipitated. By steam distillation, which was continued until no more organic material was carried over, this substance was purified and obtained as an almost colourless, crystalline mass. This, after washing and drying, was finally purified by sublimation *in vacuo*. Yield 1.15 g of colourless crystals, m.p. 53°, reported for 1,4-dichloro-2-nitrobenzene 53° [9]. Mixed with an authentic sample, m.p. found 53°. Hardly any material was left in the sublimation residue. Assuming the reaction product to be a mixture of 1,4-dichloro-2-nitrobenzene and 1-bromo-4-chloro-2-nitrobenzene, a halogen analysis was performed according to Holleman [7]. By this method, no bromine compound could be detected. The yield of 1,4-dichloro-2-nitrobenzene in the reaction series could then be calculated, being 60 %.

In order to prove that the halogen exchange had not taken place during the Sandmeyer reaction, the exchange experiment was repeated exactly as above, except that immediately after the conc. hydrochloric acid had been added to the diazonium solution, the Sandmeyer reaction was carried out. Steam distillation followed by vacuum sublimation yielded 1.8 g (76 %) of a colourless product melting at 68°, undepressed upon admixture with a sample of 1-bromo-4-chloro-2-nitrobenzene, synthesized according to Holleman [10], m.p. 69—70°. Thus it was concluded that halogen

exchange must have taken place in the diazonium compound at + 25°. The kinetics of this and similar reactions will be investigated.

All melting points were determined on the Kofler bank.

Financial support from *Knut and Alice Wallenbergs Stiftelse* is gratefully acknowledged.

1. Hantzsch, A. *Ber.* **30** (1897) 2334.
2. Hantzsch, A. and Smythe, J. S. *Ber.* **33** (1900) 505.
3. Bunnett, J. F. and Zahler, R. E. *Chem. Revs.* **49** (1951) 273.
4. Lewis, E. S. and Johnson, M. D. *J. Am. Chem. Soc.* **81** (1959) 2070.
5. Sihlbom, L. *Acta Chem. Scand.* **5** (1951) 872.
6. Sihlbom, L. *Acta Chem. Scand.* **7** (1953) 1197.
7. Holleman, A. F. *Rec. trav. chim.* **34** (1915) 204, p. 214.
8. Blanksma, J. J. *Rec. trav. chim.* **28** (1909) 97.
9. Booy, J. and Dienske, J. W. *Rec. trav. chim.* **45** (1926) 449.
10. Holleman, A. F. *Rec. trav. chim.* **34** (1915) 204, p. 208.

Received July 2, 1959.

Butyrylcholine Esterase: Influence of pH on Enzyme Activity and Irreversible Denaturation

EDITH HEILBRONN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

There is much confusion in the literature concerning the shape of the pH-activity curves of choline esterases. It is obvious that there are several factors which together form the shape of these curves. Assuming that the substrate used does not change its charge in the examined pH-area, the following factors have to be considered. Concerning the substrate: The concentration of the substrate is important, as the substrate may compete with hydrogen or hydroxyl ions for an active group in the enzyme. Concerning the medium: In using buffers for maintenance of different pH-values from, *e.g.*, 5—10, both the charge of the ions (*e.g.* phosphate), and thus the ionic strength, and the kind of substance present has to be changed. Concerning the protein: A change in enzyme activity from one pH to another may depend on true reversible changes in one or more of the active centres in the enzyme. On the other hand, the irreversible denaturation of the protein has to be considered. This depends on the temperature used in the experiment, the incubation time, the buffer ions, the ionic strength, the kind of enzyme and the state of purity of the enzyme used. Also, the procedure used during the experiment influences the enzyme activity; in this laboratory (unpublished work) it has thus been observed that the shaking rate in a Warburg apparatus influences the activity. Vigorous shaking causes a lowering of the activity.

During experiments on the purification of choline esterases we had reason to look into the stability of serum fraction IV-6-3 from human retroplacentar serum (BuChE). The studies were performed by means of an automatic recording titrator [1]. The sample containing the enzyme was kept, under stirring, in 0.10 M potassium chloride at 25°C during 5 — 120 min and at pH-values between 6.00 and 10.00. These pH-values were obtained by the addition of minute amounts of sodium hydroxide from the syringe of an automatic titrator. Afterwards, the pH was adjusted to 8.00 and the enzyme activity was determined. The substrate was a 10^{-2} M solution of bu-

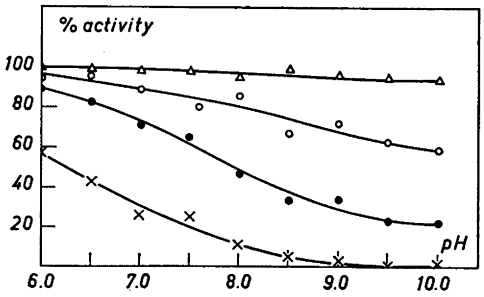


Fig. 1. Butyrylcholine esterase activity measured at pH 8.00 after incubation at different pH-values, expressed in percent of activity obtained at pH 8.00 with neglectible incubation time.
Incubation times: △ = 5 minutes, ○ = 30 minutes, ● = 60 minutes, × = 120 minutes. Temperature 25°C.

exchange must have taken place in the diazonium compound at + 25°. The kinetics of this and similar reactions will be investigated.

All melting points were determined on the Kofler bank.

Financial support from *Knut and Alice Wallenbergs Stiftelse* is gratefully acknowledged.

1. Hantzsch, A. *Ber.* **30** (1897) 2334.
2. Hantzsch, A. and Smythe, J. S. *Ber.* **33** (1900) 505.
3. Bunnett, J. F. and Zahler, R. E. *Chem. Revs.* **49** (1951) 273.
4. Lewis, E. S. and Johnson, M. D. *J. Am. Chem. Soc.* **81** (1959) 2070.
5. Sihlbom, L. *Acta Chem. Scand.* **5** (1951) 872.
6. Sihlbom, L. *Acta Chem. Scand.* **7** (1953) 1197.
7. Holleman, A. F. *Rec. trav. chim.* **34** (1915) 204, p. 214.
8. Blanksma, J. J. *Rec. trav. chim.* **28** (1909) 97.
9. Booy, J. and Dienske, J. W. *Rec. trav. chim.* **45** (1926) 449.
10. Holleman, A. F. *Rec. trav. chim.* **34** (1915) 204, p. 208.

Received July 2, 1959.

Butyrylcholine Esterase: Influence of pH on Enzyme Activity and Irreversible Denaturation

EDITH HEILBRONN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

There is much confusion in the literature concerning the shape of the pH-activity curves of choline esterases. It is obvious that there are several factors which together form the shape of these curves. Assuming that the substrate used does not change its charge in the examined pH-area, the following factors have to be considered. Concerning the substrate: The concentration of the substrate is important, as the substrate may compete with hydrogen or hydroxyl ions for an active group in the enzyme. Concerning the medium: In using buffers for maintenance of different pH-values from, *e.g.*, 5—10, both the charge of the ions (*e.g.* phosphate), and thus the ionic strength, and the kind of substance present has to be changed. Concerning the protein: A change in enzyme activity from one pH to another may depend on true reversible changes in one or more of the active centres in the enzyme. On the other hand, the irreversible denaturation of the protein has to be considered. This depends on the temperature used in the experiment, the incubation time, the buffer ions, the ionic strength, the kind of enzyme and the state of purity of the enzyme used. Also, the procedure used during the experiment influences the enzyme activity; in this laboratory (unpublished work) it has thus been observed that the shaking rate in a Warburg apparatus influences the activity. Vigorous shaking causes a lowering of the activity.

During experiments on the purification of choline esterases we had reason to look into the stability of serum fraction IV-6-3 from human retroplacentar serum (BuChE). The studies were performed by means of an automatic recording titrator [1]. The sample containing the enzyme was kept, under stirring, in 0.10 M potassium chloride at 25°C during 5 — 120 min and at pH-values between 6.00 and 10.00. These pH-values were obtained by the addition of minute amounts of sodium hydroxide from the syringe of an automatic titrator. Afterwards, the pH was adjusted to 8.00 and the enzyme activity was determined. The substrate was a 10^{-2} M solution of bu-

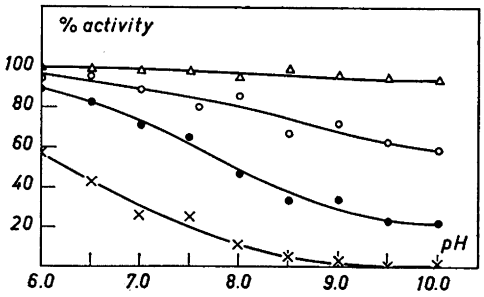


Fig. 1. Butyrylcholine esterase activity measured at pH 8.00 after incubation at different pH-values, expressed in percent of activity obtained at pH 8.00 with neglectible incubation time.
Incubation times: △ = 5 minutes, ○ = 30 minutes, ● = 60 minutes, × = 120 minutes. Temperature 25°C.

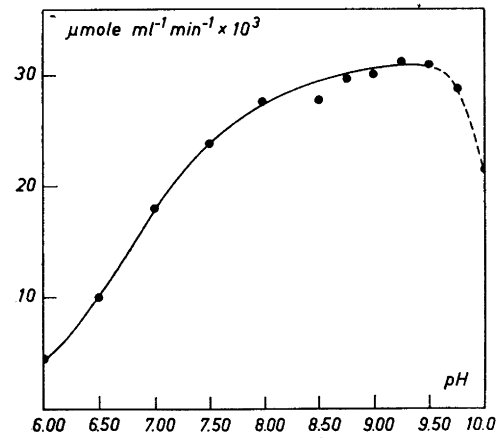


Fig. 2. pH-activity curve for butyrylcholine esterase at 25°C. Substrate: 10^{-2} M acetylcholine iodide.

tyrylcholine iodide (final concentration). The activity was recorded during a 5 min period immediately after addition of the substrate and after a small correction of the pH had been made. (This correction was necessary since the substrate was somewhat acid.) Fig. 1 shows the result. The activities are expressed in per cent of an initial enzyme activity obtained at pH 8.00. Irreversible denaturation was found to be slowest around pH 6.00 and increases with the pH and with the incubation time.

The automatic recording titrator was then used to reinvestigate the pH-activity curve for BuChE. A 10^{-2} M solution of acetylcholine iodide was used as substrate. The obtained values of the activity were corrected for spontaneous hydrolysis as described earlier [2]. Fig. 2 shows the curve, which agrees well with the experimental data published earlier by Wilson [3] and a curve published by Hase [4]. (Hase, however, used equine serum as source for the enzyme). The curve obtained in this work is, however, different from curves published by many others and is also different from two curves obtained in this laboratory. [5,6] The present curve and those published by Wilson and Hase were obtained with an automatic titrator, thus without buffer solutions, and in two cases without incubation times for the enzyme (Hase seems to have incubated for 15 min). The curves were determined with a 10^{-2} M or $10^{-2.4}$ M (Hase) concentration of the substrate. Wilson, furthermore, used a 0.1 % solution of gelatine to protect the enzyme. An earlier published curve by Heilbronn [5] was obtained in buffer solutions and at a lower substrate concentration. The different buffer ions may have caused denaturation of the enzyme during the incubation time of 30 min and may also have enhanced hydrolysis in different ways. Tammelin [6] used unpurified human serum, and also a lower substrate concentration. Obviously, the shape of the pH-activity curve depends on the method used.

1. Larsson, L. and Hansen, B. *Svensk Kem. Tidskr.* **68** (1956) 521.
2. Heilbronn, E. *Acta Chem. Scand. In press.*
3. Wilson, J. B. *J. Biol. Chem.* **208** (1954) 123.
4. Hase, E. *J. Biochem. (Japan)* **39** (1952) 259.
5. Heilbronn, E. *Acta Chem. Scand.* **8** (1954) 1368.
6. Tammelin, L. E. *Arkiv Kemi* **12** (1958) 287.

Received July 3, 1959.

Fractionation of Snake Venom by the Gel-Filtration Method

WALTER BJÖRK and JERKER PORATH

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

The use of gel-filtration as a fractionation method has recently been reported [1]. Attempts to apply the procedure to the fractionation of crude biological extracts have been successful [2,3], but in these cases only for separating proteins and protein-complexes from low-molecular weight material. It was shown, however [2], that proteins of low molecular weight like ribonuclease and cytochrome c could be retarded on a gel of suitable crosslinking, and Gelotte has recently obtained a separation of enzymes from pancreatic juice (private communication). In earlier work carried out at this institute [4] most of the UV-absorbing components of the venom from the ringhals cobra, *Hemachatus haemachates*, including lecithinase A, had been found to have a rather low molecular weight, and thus we thought it would be of great value to in-

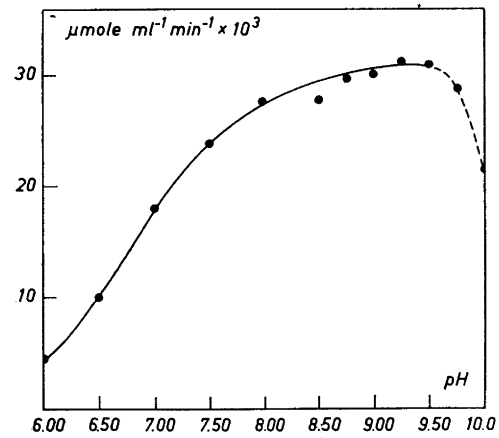


Fig. 2. pH-activity curve for butyrylcholine esterase at 25°C. Substrate: 10^{-2} M acetylcholine iodide.

tyrylcholine iodide (final concentration). The activity was recorded during a 5 min period immediately after addition of the substrate and after a small correction of the pH had been made. (This correction was necessary since the substrate was somewhat acid.) Fig. 1 shows the result. The activities are expressed in per cent of an initial enzyme activity obtained at pH 8.00. Irreversible denaturation was found to be slowest around pH 6.00 and increases with the pH and with the incubation time.

The automatic recording titrator was then used to reinvestigate the pH-activity curve for BuChE. A 10^{-2} M solution of acetylcholine iodide was used as substrate. The obtained values of the activity were corrected for spontaneous hydrolysis as described earlier [2]. Fig. 2 shows the curve, which agrees well with the experimental data published earlier by Wilson [3] and a curve published by Hase [4]. (Hase, however, used equine serum as source for the enzyme). The curve obtained in this work is, however, different from curves published by many others and is also different from two curves obtained in this laboratory. [5,6] The present curve and those published by Wilson and Hase were obtained with an automatic titrator, thus without buffer solutions, and in two cases without incubation times for the enzyme (Hase seems to have incubated for 15 min). The curves were determined with a 10^{-2} M or $10^{-2.4}$ M (Hase) concentration of the substrate. Wilson, furthermore, used a 0.1 % solution of gelatine to protect the enzyme. An earlier published curve by Heilbronn [5] was obtained in buffer solutions and at a lower substrate concentration. The different buffer ions may have caused denaturation of the enzyme during the incubation time of 30 min and may also have enhanced hydrolysis in different ways. Tammelin [6] used unpurified human serum, and also a lower substrate concentration. Obviously, the shape of the pH-activity curve depends on the method used.

1. Larsson, L. and Hansen, B. *Svensk Kem. Tidskr.* **68** (1956) 521.
2. Heilbronn, E. *Acta Chem. Scand. In press.*
3. Wilson, J. B. *J. Biol. Chem.* **208** (1954) 123.
4. Hase, E. *J. Biochem. (Japan)* **39** (1952) 259.
5. Heilbronn, E. *Acta Chem. Scand.* **8** (1954) 1368.
6. Tammelin, L. E. *Arkiv Kemi* **12** (1958) 287.

Received July 3, 1959.

Fractionation of Snake Venom by the Gel-Filtration Method

WALTER BJÖRK and JERKER PORATH

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

The use of gel-filtration as a fractionation method has recently been reported [1]. Attempts to apply the procedure to the fractionation of crude biological extracts have been successful [2,3], but in these cases only for separating proteins and protein-complexes from low-molecular weight material. It was shown, however [2], that proteins of low molecular weight like ribonuclease and cytochrome c could be retarded on a gel of suitable crosslinking, and Gelotte has recently obtained a separation of enzymes from pancreatic juice (private communication). In earlier work carried out at this institute [4] most of the UV-absorbing components of the venom from the ringhals cobra, *Hemachatus haemachates*, including lecithinase A, had been found to have a rather low molecular weight, and thus we thought it would be of great value to in-

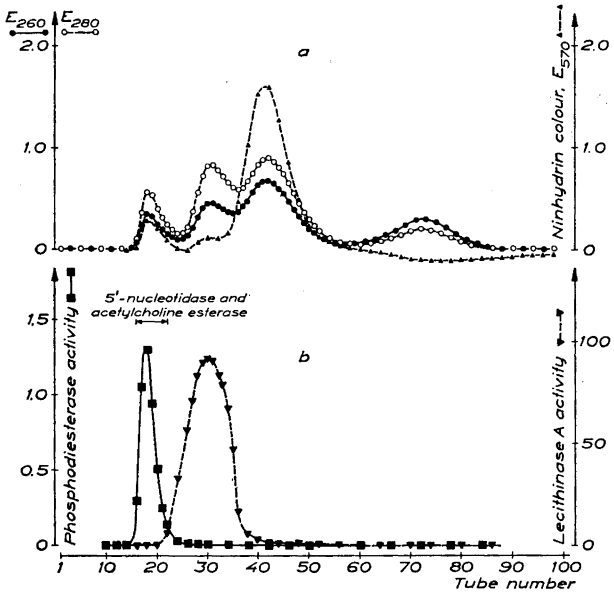


Fig. 1. Gel-filtration of 30 mg of freeze-dried ringhals cobra venom on a 60 ml column (1.9 × 21 cm) of dextran gel. The water regain of the gel was about 8 g/g. The venom was dissolved in 1 ml 0.05 M THAM-HCl of pH 7.6, and elution was performed with the same buffer. Fraction volumes about 1.0 ml. For the ninhydrin tests (▲ — — — ▲) 0.2 ml aliquots were taken. Phosphodiesterase activity (■——■) is given in mmoles of substrate split in 30 min per litre of incubation mixture, and lecithinase A activity (▼ — — — ▼) as increased extinction in 30 min at 540 mμ (hemoglobin).

vestigate the behaviour of this venom on gels of a low degree of crosslinking.

Freeze-dried ringhals venom was obtained from The South African Institute for Medical Research, Johannesburg. In order to compare it with another type of snake venom, we also used the venom of a rattle-snake, *Crotalus adamanteus;* this venom was bought from Ross Allen's Reptile Institute, Silver Springs, Florida.

Starch gel with a water regain of 5.1 g/g and dextran gel with a water regain of about 8 g/g were supplied by A.B. Pharmacia, Uppsala, Sweden. The buffer system used was tris(hydroxymethyl)aminomethane-hydrochloric acid (THAM-HCl). Before packing a column the gel was allowed to swell in the buffer overnight, and then it was deaerated in a suction flask. All the experiments were performed in a cold room at 4°.

The extinctions at 280 and 260 mμ were measured in a Beckman DU spectrophotometer using a 1-cm semi-micro cell. Phosphodiesterase, lecithinase A, L-amino acid oxidase and acetylcholine esterase activities were determined as described earlier [4,5]. For the estimation of 5'-nucleotidase activity a substrate solution of the following composition was used: 0.01 M adenosine-5'-phosphate, 0.01 M magnesium sulphate and 0.05 M THAM-HCl of pH 8.9. Of this solution 0.5 ml was incubated for 30 min at 37° with 0.05 ml of each fraction to be tested. The reaction was stopped with 1 ml of 2.5 % H_2SO_4, and the inorganic phosphate liberated was determined by a modification of King's method [6].

The ninhydrin curve was obtained according to Moore and Stein [7]. In the figures the colour given by the filtration buffer has been subtracted.

Fig. 1 shows the result of a gel-filtration of 30 mg of ringhals venom on a 60 ml column (1.9 × 21 cm) of dextran gel. The venom had been dissolved in 1 ml THAM-HCl of pH 7.6, and after applying the solution to the

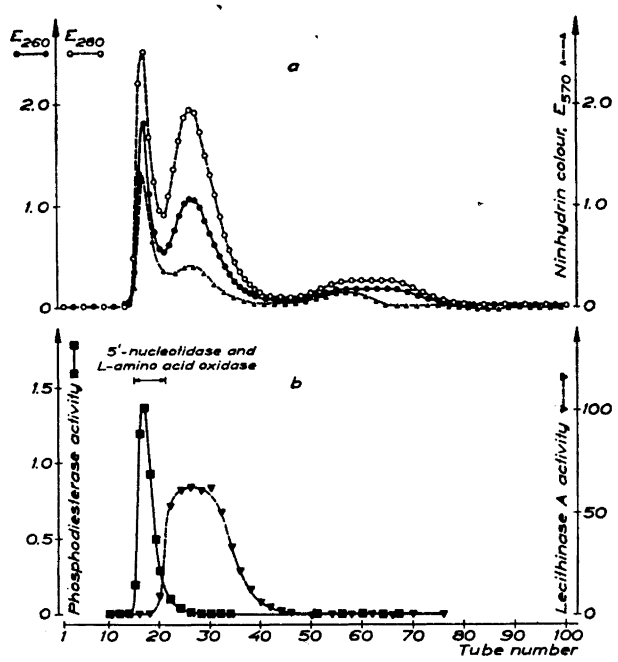


Fig. 2. Gel-filtration of 30 mg of rattle-snake venom on the same column and under the same conditions as in Fig. 1.

column, elution was performed with the same buffer. The rate of elution was about 6 ml/h, and 1 ml fractions were collected. In the upper part of the figure it can be seen that the venom was fractionated into four different zones, and that 90 % of it was retarded on the column. Three of the enzymes tested for, phosphodiesterase, acetylcholine esterase and 5'-nucleotidase, appeared together in the first peak (lower part of the figure), whereas the lecithinase A was eluted in the second peak, well separated from the other enzymes. In all the experiments performed the recovery of UV-absorbing material was about 100 %.

By chromatography on diethylaminoethyl (DEAE) cellulose the phosphodiesterase has been separated from cholinesterase and lecithinase A[4], but then the latter two enzymes left the column in a single zone. Gel-filtration would therefore be a useful step in the purification of the three enzymes, especially as a transference to another buffer could be achieved at the same time. Unfortunately, the recovery of cholinesterase from the dextran gel was very low, 10—20 %, probably depending on impurities in the gel. Washing with *ortho*-phenanthroline in order to remove traces of heavy metals had no significant effect, but after treating the gel with 10 % hydrochloric acid the recovery of cholinesterase was increased considerably. The inactivation could be ascribed neither to removal of a cofactor nor to reversible adsorption.

The recoveries of phosphodiesterase and lecithinase A were about 90 % and 100 %, respectively. In the experiments made on starch gel we also got a very high recovery of cholinesterase activity, but in that case the fractionation was not so good. One third of the UV-absorbing material was eluted in the first peak and more than half of it in the second peak, while the third peak had disappeared completely. However, with a more weakly cross-linked starch gel the separation properties would certainly be improved.

The quotient E_{260}/E_{280} was found to be higher than unity in the most retarded zone (tubes 60—85 in Fig. 1), which may be taken as an indication of a large proportion of nucleotides in this zone. It should also

be observed that the corresponding ninhydrin values are below zero. This might be caused by the formation of complexes between the substances eluted and the THAM buffer, and such a binding of THAM by some components of snake venom has already been suggested as an explanation of a phenomenon encountered in ion exchange chromatography of venom [4,5]. By gel-filtration of the chromatographic fractions in question we have indeed shown that they are partly identical with the fourth zone in Fig. 1.

Gel-filtration of the rattle-snake venom (Fig. 2) was performed on the same dextran-gel column and under the same experimental conditions as described above. This venom does not contain any cholinesterase but has a much higher concentration of L-amino acid oxidase and a considerably stronger UV-absorption than the cobra venom. As in the former case lecithinase A was the only enzyme retarded to any appreciable degree. The ninhydrin and UV-extinction patterns, however, were quite different from those obtained with the ringhals venom. About 85 % of the material applied appeared in the first two peaks, and the large ninhydrin zone corresponding to the tubes 35—50 in Fig. 1 was missing. The most retarded zone had a very flat and elongated shape, and the quotient E_{260}/E_{280} was lower than unity in all fractions. As the ringhals and the rattle-snake belong to different families (*Elapidae* and *Crotalidae*, respectively), it would be of great interest to know to what extent these differences are typical. In the general mapping of the components of snake venoms gel-filtration may prove to be a valuable tool.

We have also compared gel-filtration experiments carried out at different pH values and with buffers of varying concentrations. The only deviation found was a slightly more pronounced tailing of the lecithinase A at low ionic strengths. This might be interpreted as a weak adsorption tendency, but at present no definite opinion can be formed on this question.

We are indebted to professor A. Tiselius for his stimulating interest and to Dr. J. H. Mason, Johannesburg, for the supply of ringhals venom. The work has been financially supported by grants from the *Rockefeller* and *Wallenberg Foundations*.

1. Porath, J. and Flodin, P. *Nature* **183** (1959) 1657.
2. Porath, J. *To be published.*
3. Lindner, E. B., Elmquist, A. and Porath, J. *To be published.*
4. Björk, W. and Boman, H. G. *Biochim. et Biophys. Acta* **34** (1959) 503.
5. Boman, H. G. and Kaletta, U. *Biochim. et Biophys. Acta* **24** (1957) 619.
6. King, E. J. *Biochem. J.* **26** (1932) 292.
7. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.

Received July 19, 1959.

Quantitative Determination of Carotene by Paper Chromatography

ARNE JENSEN

Norwegian Institute of Seaweed Research, N.T.H., Trondheim, Norway

It has been shown previously [1] that carotenes can be quantitatively separated from the other chloroplast pigments by chromatography on a filter paper with a kieselguhr filler. On the basis of this observation a method for the quantitative determination of carotene in dried grass and seaweed meal has been worked out.

Procedure. Approximately 1 g of the finely ground sample is moistened * with 2 ml of water in a 100 ml Erlenmeyer flask. After standing for 15 min (to allow swelling), acetone (25 ml) is added and the sample left over night under nitrogen in the dark. The solution is then decanted through a sintered glass funnel, and the residue is extracted with another 25 ml of acetone. The extraction is repeated until the filtrate remains colourless. Then the combined extracts are concentrated *in vacuo* to a small volume, transferred quantitatively to a volumetric flask (10 or 25 ml according to the content of carotene) and made up to the mark with acetone.

An aliquot of the extract (0.2—0.8 ml) is applied onto the centre of a circular filter paper; (Schleicher & Schüll, No. 287, Kieselguhr-filter, 18 cm diameter) the solvent being continuously evaporated by a stream of nitrogen. When the spot is dry, the paper is placed between similar halves of 15 cm petri dishes, and the chromatogram is developed with pure petroleum ether (b.p. 60—80°C) according to the technique of Rutter [2]. As soon as the carotenes, which migrate close to the solvent front, are separated from the other pigments,

* Essential for quantitative extraction of carotene from the seaweed meal.

be observed that the corresponding ninhydrin values are below zero. This might be caused by the formation of complexes between the substances eluted and the THAM buffer, and such a binding of THAM by some components of snake venom has already been suggested as an explanation of a phenomenon encountered in ion exchange chromatography of venom [4,5]. By gel-filtration of the chromatographic fractions in question we have indeed shown that they are partly identical with the fourth zone in Fig. 1.

Gel-filtration of the rattle-snake venom (Fig. 2) was performed on the same dextran-gel column and under the same experimental conditions as described above. This venom does not contain any cholinesterase but has a much higher concentration of L-amino acid oxidase and a considerably stronger UV-absorption than the cobra venom. As in the former case lecithinase A was the only enzyme retarded to any appreciable degree. The ninhydrin and UV-extinction patterns, however, were quite different from those obtained with the ringhals venom. About 85 % of the material applied appeared in the first two peaks, and the large ninhydrin zone corresponding to the tubes 35—50 in Fig. 1 was missing. The most retarded zone had a very flat and elongated shape, and the quotient E_{260}/E_{280} was lower than unity in all fractions. As the ringhals and the rattle-snake belong to different families (*Elapidae* and *Crotalidae*, respectively), it would be of great interest to know to what extent these differences are typical. In the general mapping of the components of snake venoms gel-filtration may prove to be a valuable tool.

We have also compared gel-filtration experiments carried out at different pH values and with buffers of varying concentrations. The only deviation found was a slightly more pronounced tailing of the lecithinase A at low ionic strengths. This might be interpreted as a weak adsorption tendency, but at present no definite opinion can be formed on this question.

We are indebted to professor A. Tiselius for his stimulating interest and to Dr. J. H. Mason, Johannesburg, for the supply of ringhals venom. The work has been financially supported by grants from the *Rockefeller* and *Wallenberg Foundations*.

1. Porath, J. and Flodin, P. *Nature* **183** (1959) 1657.
2. Porath, J. *To be published.*
3. Lindner, E. B., Elmquist, A. and Porath, J. *To be published.*
4. Björk, W. and Boman, H. G. *Biochim. et Biophys. Acta* **34** (1959) 503.
5. Boman, H. G. and Kaletta, U. *Biochim. et Biophys. Acta* **24** (1957) 619.
6. King, E. J. *Biochem. J.* **26** (1932) 292.
7. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.

Received July 19, 1959.

Quantitative Determination of Carotene by Paper Chromatography

ARNE JENSEN

Norwegian Institute of Seaweed Research, N.T.H., Trondheim, Norway

It has been shown previously [1] that carotenes can be quantitatively separated from the other chloroplast pigments by chromatography on a filter paper with a kieselguhr filler. On the basis of this observation a method for the quantitative determination of carotene in dried grass and seaweed meal has been worked out.

Procedure. Approximately 1 g of the finely ground sample is moistened * with 2 ml of water in a 100 ml Erlenmeyer flask. After standing for 15 min (to allow swelling), acetone (25 ml) is added and the sample left over night under nitrogen in the dark. The solution is then decanted through a sintered glass funnel, and the residue is extracted with another 25 ml of acetone. The extraction is repeated until the filtrate remains colourless. Then the combined extracts are concentrated *in vacuo* to a small volume, transferred quantitatively to a volumetric flask (10 or 25 ml according to the content of carotene) and made up to the mark with acetone.

An aliquot of the extract (0.2—0.8 ml) is applied onto the centre of a circular filter paper; (Schleicher & Schüll, No. 287, Kieselguhr-filter, 18 cm diameter) the solvent being continuously evaporated by a stream of nitrogen. When the spot is dry, the paper is placed between similar halves of 15 cm petri dishes, and the chromatogram is developed with pure petroleum ether (b.p. 60—80°C) according to the technique of Rutter [2]. As soon as the carotenes, which migrate close to the solvent front, are separated from the other pigments,

* Essential for quantitative extraction of carotene from the seaweed meal.

Table 1. Carotene content of dried grass and seaweed meal, mg/kg.

Material	Method: Paper chromatography	Column chromatography
Dried grass	274, 277, 284, 284	250
» »	248, 249, 252	240
Seaweed meal	101, 103	95

the development is interrupted, and the carotene zone is cut out immediately. The strip is then packed tightly in a glass tube (of approximately 8 mm inner diameter) which has a capillary end. The elution is carried out with 5 ml of acetone applied in several small portions and the eluate is made up to precisely 5 ml. The optical density is read in a spectrophotometer at 456 mμ and the carotene content calculated from the extinction coefficient of β-carotene ($E^{1\,\%}_{1\,cm}$ = 2 490 in acetone). No interference with the absorption at 456 mμ was obtained with the eluates from a blank chromatogram.

The method described above has certain advantages over the conventional method, mainly because it avoids time consuming transference of the carotenes from acetone to petroleum ether. It is simple, requires very little equipment, and may, with a few alterations, be used for microdetermination of carotenes. From Table 1 it is seen that the accuracy of the method was satisfactory. The results obtained with the above procedure were comparable with, although somewhat higher than, those obtained by the conventional analysis [3]. This is believed to be caused by the higher over all recoveries obtained with the new method.

1. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand. In press.*
2. Rutter, L. *Nature* **161** (1948) 435.
3. Haug, A. and Larsen, B. *Norwegian Institute of Seaweed Research*, Report No. 16, 1957.

Received July 20, 1959.

The Structure of the Benzene Diazonium Ion

CHR. RØMMING

Universitetets Kjemiske institutt, Blindern-Oslo, Norway

The structure of benzene diazonium chloride ($C_6H_5N_2Cl$) has been examined by X-ray methods. As the general features of the diazonium group is settled after *one* Fourier projection owing to the high crystal symmetry, a preliminary report of the first part of the structure determination is given.

Benzene diazonium chloride single crystals were grown from an ethanol solution by slow precipitation with ether at 0°C. The space group is $C222_1$ and the unit cell dimensions are a = 15.16 Å, b = 4.95 Å, c = 9.05 Å as determined from oscillation and Weissenberg photographs taken at −90°C. With four molecules in the unit cell (calculated density 1.374) this space group demands the molecules to be in special positions, *i.e.* the molecules have twofold axes of symmetry parallel to the x or y axis.

Integrated Weissenberg photographs were taken at −90°C with rotation about the b axis and the intensities measured with a microphotometer. Approximate x and z parameters were determined from a Patterson projection and refined on the basis of successive Fourier syntheses. Finally, a few cycles of least squares refinements brought the reliability factor R down to 6.5 %. Absorption and secondary extinction corrections were not applied, and the thermal vibrations were regarded as isotropic.

A part of the Fourier projection (plane group *pmg*; $c' = c$, $a' = a/2$) along the b axis is shown in Fig. 1. The chloride ion is situated at the origin and the twofold axis of the diazonium ion has the coordinates (x, 1/4). The interatomic distances within the diazonium ion to be found in true lengths in this projection are as follows: N_1-N_2 = 1.11 Å, N_1-C_1 = 1.42 Å and C_1-C_4 = 2.70 Å. Supposing the line through C_2-C_3 to be parallel to the x axis

Table 1. Carotene content of dried grass and seaweed meal, mg/kg.

Material	Method: Paper chromatography	Column chromatography
Dried grass	274, 277, 284, 284	250
» »	248, 249, 252	240
Seaweed meal	101, 103	95

the development is interrupted, and the carotene zone is cut out immediately. The strip is then packed tightly in a glass tube (of approximately 8 mm inner diameter) which has a capillary end. The elution is carried out with 5 ml of acetone applied in several small portions and the eluate is made up to precisely 5 ml. The optical density is read in a spectrophotometer at 456 mμ and the carotene content calculated from the extinction coefficient of β-carotene ($E^{1\,\%}_{1\,cm}$ = 2 490 in acetone). No interference with the absorption at 456 mμ was obtained with the eluates from a blank chromatogram.

The method described above has certain advantages over the conventional method, mainly because it avoids time consuming transference of the carotenes from acetone to petroleum ether. It is simple, requires very little equipment, and may, with a few alterations, be used for microdetermination of carotenes. From Table 1 it is seen that the accuracy of the method was satisfactory. The results obtained with the above procedure were comparable with, although somewhat higher than, those obtained by the conventional analysis [3]. This is believed to be caused by the higher over all recoveries obtained with the new method.

1. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand. In press.*
2. Rutter, L. *Nature* **161** (1948) 435.
3. Haug, A. and Larsen, B. *Norwegian Institute of Seaweed Research*, Report No. 16, 1957.

Received July 20, 1959.

The Structure of the Benzene Diazonium Ion

CHR. RØMMING

Universitetets Kjemiske institutt, Blindern-Oslo, Norway

The structure of benzene diazonium chloride ($C_6H_5N_2Cl$) has been examined by X-ray methods. As the general features of the diazonium group is settled after *one* Fourier projection owing to the high crystal symmetry, a preliminary report of the first part of the structure determination is given.

Benzene diazonium chloride single crystals were grown from an ethanol solution by slow precipitation with ether at 0°C. The space group is $C222_1$ and the unit cell dimensions are a = 15.16 Å, b = 4.95 Å, c = 9.05 Å as determined from oscillation and Weissenberg photographs taken at −90°C. With four molecules in the unit cell (calculated density 1.374) this space group demands the molecules to be in special positions, *i.e.* the molecules have twofold axes of symmetry parallel to the x or y axis.

Integrated Weissenberg photographs were taken at −90°C with rotation about the b axis and the intensities measured with a microphotometer. Approximate x and z parameters were determined from a Patterson projection and refined on the basis of successive Fourier syntheses. Finally, a few cycles of least squares refinements brought the reliability factor R down to 6.5 %. Absorption and secondary extinction corrections were not applied, and the thermal vibrations were regarded as isotropic.

A part of the Fourier projection (plane group *pmg*; $c' = c$, $a' = a/2$) along the b axis is shown in Fig. 1. The chloride ion is situated at the origin and the twofold axis of the diazonium ion has the coordinates (x, 1/4). The interatomic distances within the diazonium ion to be found in true lengths in this projection are as follows: N_1-N_2 = 1.11 Å, N_1-C_1 = 1.42 Å and C_1-C_4 = 2.70 Å. Supposing the line through C_2-C_3 to be parallel to the x axis

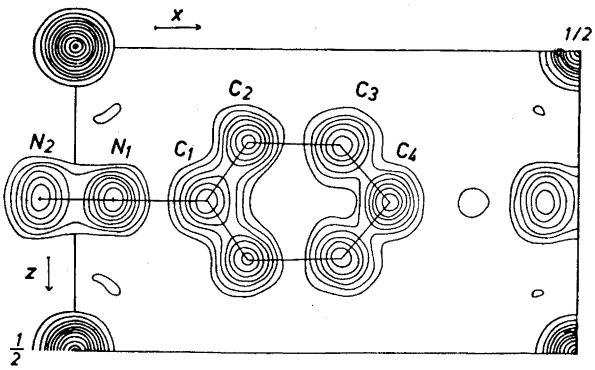


Fig. 1. Electron density projection along the *b* axis. Contour intervals: 2 e.Å^{-2} for chloride ions and 1 e.Å^{-2} for nitrogen and carbon atoms. The first contour is the 2-electron line.

the distance between these atoms is 1.40 Å. The angle between the benzene plane and the plane of projection is about 45°.

The conclusion of this investigation is that solid benzene diazonium chloride has a typical ionic structure, the N-N distance corresponds to that of a normal triple bond, and the N_1-C_1 distance is shortened relative to a single C—N bond by an amount to be expected from similar systems where a multiple bond is conjugated to a benzene ring.

Received June 30, 1959.

Charge Transfer Compounds with a Halogen Atom linked to Carbon as the Electron Acceptor

T. BJORVATTEN and O. HASSEL

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The crystal structures of several addition compounds have recently been determined in which halogen molecules act as electron acceptors. Structures of compounds in which a halogen atom is linked to carbon in the acceptor molecule have not been investigated, however. Compounds like those formed by iodoform with three molecules of quinoline or S_8 therefore appeared to be of considerable interest. The crystal structure of the former has now been determined and the investigation of the sulphur compound has been started.

The unit cell of the quinoline compound is rhombohedral with the hexagonal axes $a = 22.40$ Å and $c = 4.59$ Å. The rhombohedral unit cell contains *one* molecule of iodoform which is therefore situated on the trigonal axis (the space group is $R3$) whereas the three quinoline molecules occupy general positions. It was not difficult to localize the former, but the lack of symmetry elements others than a threefold axis made the localization of the quinoline molecules more cumbersome.

Fig. 1 shows a difference Fourier map (projection along the trigonal axis) with subtraction of the contribution from iodine atoms. The position of one iodine atom is indicated by an asterisk and the three axes indicated are the projections of the rhombohedral axes into the (111) plane. Already from this projection a linear arrangement N-I-C might be expected. This expectation is confirmed by the analysis. The N-I distance is found equal to 2.99 Å, the C-I distance to 2.12 Å.

These findings also confirm the suggestion that the iodine atoms act as electron acceptors towards the nitrogen atoms and

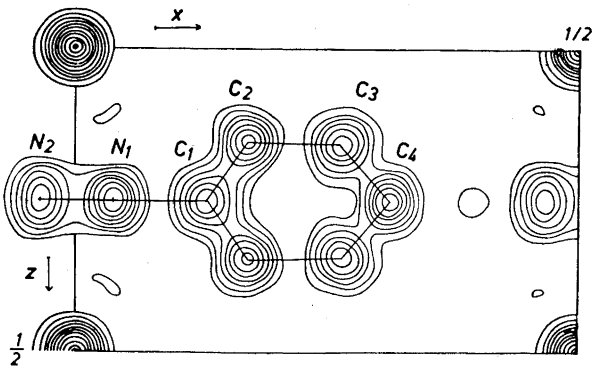


Fig. 1. Electron density projection along the *b* axis. Contour intervals: 2 e.Å^{-2} for chloride ions and 1 e.Å^{-2} for nitrogen and carbon atoms. The first contour is the 2-electron line.

the distance between these atoms is 1.40 Å. The angle between the benzene plane and the plane of projection is about 45°.

The conclusion of this investigation is that solid benzene diazonium chloride has a typical ionic structure, the N-N distance corresponds to that of a normal triple bond, and the N_1-C_1 distance is shortened relative to a single C—N bond by an amount to be expected from similar systems where a multiple bond is conjugated to a benzene ring.

Received June 30, 1959.

Charge Transfer Compounds with a Halogen Atom linked to Carbon as the Electron Acceptor

T. BJORVATTEN and O. HASSEL

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The crystal structures of several addition compounds have recently been determined in which halogen molecules act as electron acceptors. Structures of compounds in which a halogen atom is linked to carbon in the acceptor molecule have not been investigated, however. Compounds like those formed by iodoform with three molecules of quinoline or S_8 therefore appeared to be of considerable interest. The crystal structure of the former has now been determined and the investigation of the sulphur compound has been started.

The unit cell of the quinoline compound is rhombohedral with the hexagonal axes $a = 22.40$ Å and $c = 4.59$ Å. The rhombohedral unit cell contains *one* molecule of iodoform which is therefore situated on the trigonal axis (the space group is $R3$) whereas the three quinoline molecules occupy general positions. It was not difficult to localize the former, but the lack of symmetry elements others than a threefold axis made the localization of the quinoline molecules more cumbersome.

Fig. 1 shows a difference Fourier map (projection along the trigonal axis) with subtraction of the contribution from iodine atoms. The position of one iodine atom is indicated by an asterisk and the three axes indicated are the projections of the rhombohedral axes into the (111) plane. Already from this projection a linear arrangement N-I-C might be expected. This expectation is confirmed by the analysis. The N-I distance is found equal to 2.99 Å, the C-I distance to 2.12 Å.

These findings also confirm the suggestion that the iodine atoms act as electron acceptors towards the nitrogen atoms and

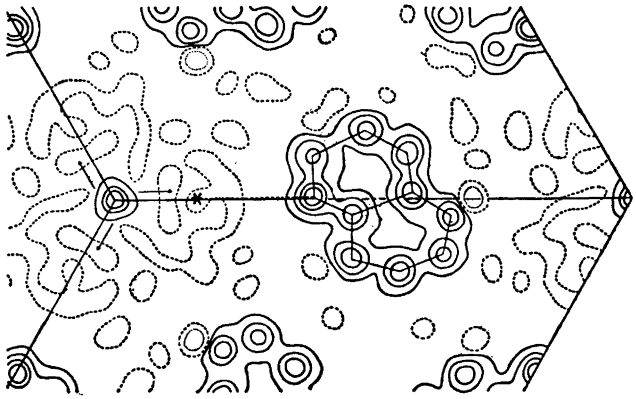


Fig. 1.

makes it appear probable that the iodine atoms have acquired ten electron systems resembling those of the "first" halogen atom in addition compounds formed by halogen molecules.

Apparently, addition compounds in which halogen atoms linked to carbon act as electron acceptors are not numerous. The 1:1 compounds, however, formed by oxalyl halides and dioxan are probably of this kind. X-Ray work on these oxalyl chloride and bromide compounds has therefore been started.

Received July 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1263–1273

Fractionation of Polymers by Liquid-Liquid Distribution

II. Selection of Experimental Conditions

KARL ERIK ALMIN

Swedish Forest Products Research Laboratory, Paper Technology Department, Stockholm, Sweden

Polyglycols have been distributed between selected solvents in order to establish proper experimental conditions for countercurrent experiments. The concentration dependence of the partition coefficient has been discussed and the resolving power of a Craig apparatus has been discussed from theoretical point of view.

In previous communications [1,2] the fractionation of polymers by means of countercurrent methods was discussed from a theoretical point of view. In this paper, experiments will be reported which give information about the most suitable experimental conditions. The polymer used in these experiments was polyglycol

$$CH_2OH - [CH_2 - O - CH_2]_n - CH_2OH$$

SOLVENT SYSTEMS

The choice of solvents for these experiments must be made with due consideration to several factors. The mean partition coefficient of the polymer must not deviate too much from unity. This ensures that we do not have to use a volume ratio for the two phases which differs too much from one in order to get suitable amounts of the polymer in the different phases. A system composed of two immiscible or partly immiscible pure liquids cannot be expected to fulfil, at a fixed temperature, the requirements with respect to the partition coefficient since the polymer will most probably pass completely into one of the phases. A two phase liquid system composed of at least three pure liquids would therefore appear necessary. In a three component system, changes in the composition of the phases can be made in order to make it possible to obtain a suitable mean partition coefficient for the polymer. It is important from a practical point of view that the phases should separate easily, thus avoiding loss of time or the use of a centrifuge.

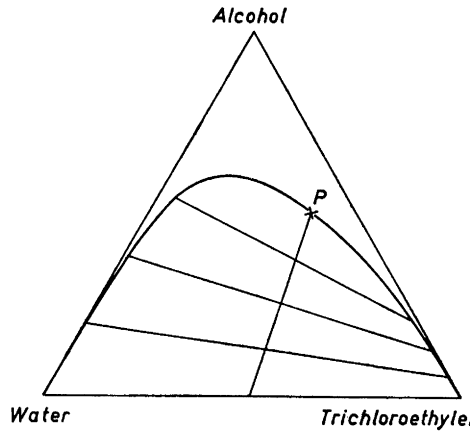


Fig. 1. Approximate phase diagram of the liquid system, trichloroethylene, alcohol (95 %) and water. P = plait-point.

Schulz and Nordt [3] have reported that the liquid system, water-chloroform-benzene, is not satisfactory for the fractionation of polyglycols because of the ease of formation of emulsions. For this reason, some other three component liquid systems have been chosen. The three component system, trichloroethylene-alcohol-water (TAW), proved to be very satisfactory in this respect. The three component system, trichloroethylene-chloroform-water (TCW), was nearly as good in this respect and had — as will be shown in the experiments — very good properties with respect to the resolving power.

In order to examine the possibility of using the liquid system TAW, the ternary phase diagram was first determined. The binodal curve was obtained by mixing suitable amounts of trichloroethylene and alcohol or water and alcohol and titrating with water or trichloroethylene, respectively, to the point of first appearance of turbidity. Approximate tie-lines were determined in the following way. The three components were mixed in suitable amounts in a graduated vessel and the volume ratio of the two phases was determined. The tie-lines were then considered to be divided into this ratio by the point which gives the composition of the system. The plait-point was obtained as the point where the binodal curve and the curve joining the middle points of the approximate tie-lines intersect. The phase diagram obtained, see Fig. 1, is thus not theoretically correct. On the other hand, it gave all the information necessary for the subsequent experiments. One can see how the three components should be mixed in order to obtain a two phase system with a predicted composition and volume ratio for the two phases. The author only dealt with equal volumes for the two phases and since the plait-point corresponds to the volume ratio 0.40: 0.51: 0.09 for trichloroethylene, alcohol (95 %) and water, it can be calculated that the three liquids should be mixed in the volume ratio

$$(0.5-0.20\ v) : v : (0.5-0.80\ v) \text{ with } v < 0.51.$$

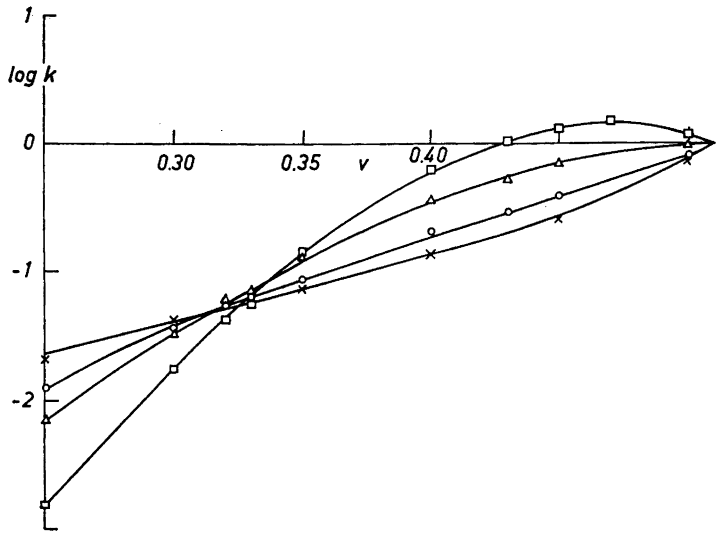


Fig. 2. Apparent partition coefficient for polyglycol in the liquid system TAW as a function of the composition. × A 300, O A 1 500, △ A 4 000, □ A 6 000.

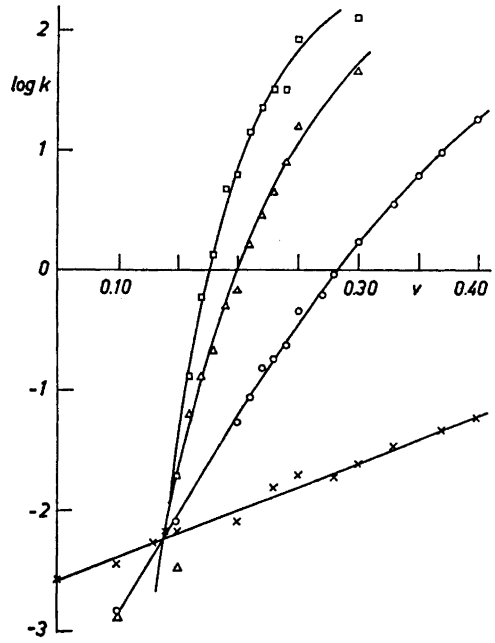


Fig. 3. Apparent partition coefficient for polyglycol in the liquid system TCW as a function of the composition (for notation see Fig. 2).

Both trichloroethylene and chloroform are practically immiscible with water. Equal volumes of the two phases of the liquid system TCW are thus obtained when the three liquids are mixed in the volume ratio $(0.5 - v) : v : 0.5$.

An analysis of this type is necessary in order to facilitate the planning of a systematic investigation of these liquid systems. Furthermore, any undesirable waste of solvents can be avoided.

APPARENT PARTITION COEFFICIENTS

In Fig. 2, the apparent partition coefficients for four commercial polyglycols in the liquid system TAW are plotted as a function of the composition parameter v. Since the upper phases will be used as the stationary phases in the forthcoming countercurrent experiments, the partition coefficient is, in accordance with the definition given in Ref.[1], the ratio between the weights of polyglycol in the lower and the upper phases. In Fig. 3, the apparent partition coefficients for the polyglycol preparations in the liquid system TCW are plotted as a function of the composition parameter v.

From Figs. 2 and 3, it can be seen that both solvent systems are suitable for fractionation purposes. The system TCW, in particular, shows large changes in partition coefficient for a change in molecular weight. At the same time it is very sensitive to small changes in the composition. For this reason, it was considered convenient to study the system TAW first.

However, a few comments of general interest must be made. The curves in Figs. 2 and 3 intersect each other. Owing to the experimental inaccuracy, it is impossible to decide whether the curves have a common point of intersection. The same observation has been reported by Schulz [4]. However, he has not investigated more than two polyglycol preparations in the solvent system chloroform-benzene-water. According to his theories, the point of intersection should occur for $k = 1$. Neither in his own experiments nor in those reported in this paper does this occur. Schulz explained this as being due to the difference in temperature which existed between the two series of experiments performed by him. In the present work, however, all experiments at a specified composition of the solvent system were carried out simultaneously. Thus, there is no doubt that the curves cut in a point or points at which the partition coefficient differs appreciably from unity. The reason for this deviation is that the Brønsted equation [5] for the relation between partition coefficient and molecular weight, on which Schulz' theories are based, is too approximate. In addition, the linear relation between the logarithm of the partition coefficient and the composition of the solvent system, proposed by Schulz [4], cannot be regarded as satisfactory.

EQUILIBRIUM CONDITIONS

Preliminary experiments with a conventional 20 tube allglass countercurrent apparatus, constructed according to Craig, showed that the amount of polyglycol in the different fractions was not independent of the shaking time unless at least a 30 min shaking time was used at each step.

Table 1. Countercurrent distribution of polyglycol A 6 000. Solvent system: TAW (v = 0.43).

Time of shaking at each step	30 min	240 min
Weight of sample (w_0)	2.0002 g	2.0002 g
p	$w(p)$ mg	$w(p)$ mg
0	2.1	2.9
1	3.3	3.1
2	5.4	6.2
3	13.8	13.2
4	32.8	30.2
5	64.3	63.4
6	108.5	112.1
7	171.4	162.5
8	216.8	226.9
9	259.0	250.5
10	256.6	249.2
11	238.0	234.5
12	203.0	204.3
13	156.3	152.5
14	113.4	111.0
15	73.9	71.3
16	43.3	41.5
17	23.4	19.7
18	9.5	9.7
19	4.3	3.9
$\sum w(p)$	1.9991 g	1.9686 g

Table 1 contains the results from two series of experiments in which polyglycol was distributed according to the fundamental countercurrent process, separatory funnels being used as contacting vessels. The two series only differ in the time of shaking at each step. In one case, the shaking time was 30 min, in the other 120 min. The differences in the results of the two series must be ascribed to random errors. For this reason, a shaking time of 30 min was used at each step in the subsequent countercurrent experiments.

In order to investigate the reversibility of the distribution process, two series of experiments were carried out, one in which the sample was dissolved in the upper phase before the lower phase was added, and one in which the sample was first dissolved in the lower phase. The results are shown in Fig. 4. It can be seen that the value of k is independent of the direction in which the distribution process proceeds. This means that the process is reversible and that no obstacles exist for its treatment as a real equilibrium process.

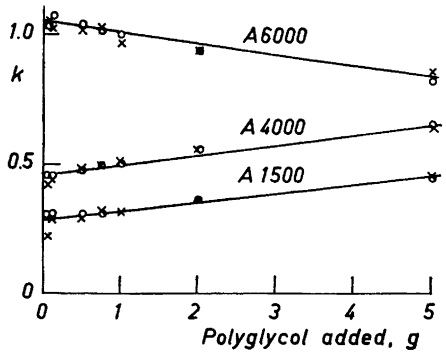


Fig. 4. Apparent partition coefficient for polyglycol at different sample weights. Solvent system: TAW ($v = 0.43$). Volume of each phase = 50 ml. × sample first dissolved in the upper phase. O sample first dissolved in the lower phase.

CONCENTRATION DEPENDENCE

The problem is, as can be seen from Fig. 4, complicated by the appreciable concentration dependence of the partition coefficient. This effect can be quite considerable at high concentrations. The slopes of the lines drawn in Fig. 4 have different signs, implying that reverse distribution conditions at high and low concentrations are conceivable. However, the matter is further complicated in the distribution experiments reported in this paper. The solvent system used was a three component system. Upon adding an appreciable quantity of a fourth component, polyglycol, the composition of the two solvents might have been changed. This change may have been different for different molecular weights of the polyglycol. No attempts have been made to determine whether such a change takes place. Corrections were introduced which were thought to make this effect negligible in the author's results.

In a multiple-contact-countercurrent experiment, the concentration of the substance to be distributed decreases appreciably as the experiment proceeds. This is especially striking when the partition coefficient is close to unity. The errors introduced due to the concentration dependence of the partition coefficient must therefore be most serious during the first stages of the countercurrent process. However, the results obtained are, at least qualitatively, comparable with the results obtained in a countercurrent experiment where the partion coefficient is independent of the concentration but where the sample has been added to more than one tube at the start of the process. As shown by Craig [6], no appreciable errors are introduced in this way. However, a mathematical analysis of the problem will be made.

As can be seen from Fig. 4, the apparent partition coefficient can be expressed by

$$k = k_o \ (1 + \varepsilon \ m) \tag{1}$$

where k_o is the partition coefficient at infinite dilution, m the amount of substance distributed between the two phases and ε a constant. (Indications of a slight curvature, at least for preparation A 4 000, are left out of consideration).

We assume that a pure compound containing molecules of only one size is distributed between two solvents according to the fundamental Craig distribution procedure. The partition coefficient is assumed to obey eqn. 1. The concentration is chosen so that $\varepsilon\, m \ll 1$. Using the notations given in Ref.[2], we obtain

$$w_n(p) = \frac{k_o[1+\varepsilon w_{n-1}(p-1)]}{1+k_o[1+\varepsilon w_{n-1}(p-1)]} w_{n-1}(p-1) + \frac{1}{1+k_o[1+\varepsilon w_{n-1}(p)]} w_{n-1}(p) =$$

$$= \frac{k_o}{1+k_o} w_{n-1}(p-1) + \frac{1}{1+k_o} w_{n-1}(p) + \Delta_n(p) \qquad (2)$$

where $\Delta n(p)$ is a correction term which has to be calculated. Rearrangement gives

$$\Delta_n(p) = \frac{\varepsilon k_o}{1+k_o}\left[\frac{[w_{n-1}(p-1)]^2}{1+k_o[1+\varepsilon w_{n-1}(p-1)]} - \frac{[w_{n-1}(p)]^2}{1+k_o[1+\varepsilon w_{n-1}(p)]}\right]$$

Approximating this expression by neglecting the terms in the denominators containing ε and exchanging the w's for their idealized values (*i.e.* ε is considered to be 0) gives

$$\Delta_n(p) = \frac{\varepsilon}{k_o} \cdot \frac{p^2 - k_o^2 \cdot (n-p)^2}{n^2} \cdot [w_n^*(p)]^2 \qquad (3)$$

where the asterisk indicates idealized values. From eqn. 2, we obtain by successive additions

$$w_n(p) = \binom{n}{p} \cdot \frac{k_o^p}{(1+k_o)^n} w_o(0) + \sum_{i=0}^{n-1} \sum_{r=0}^{i} \binom{i}{i-r} \cdot \frac{k_o^{i-r}}{(1+k_o)^i} \cdot \Delta_{n-i}(p-i+r)$$

Insertion of the Δ values from eqn. 3 and division with $w_o = w_o(0)$ give

$$W_n(p) - W_n^*(p) =$$

$$\frac{\varepsilon w_o}{k_o} \cdot \sum_{i=0}^{n-1} \sum_{r=0}^{i} \frac{(p-i+r)^2 - k_o^2(n-p-r)^2}{(n-i)^2} \cdot W_i^*(i-r) \cdot [W^*_{n-i}(p-i+r)]^2 \qquad (4)$$

This expression thus gives the difference between the experimental and the idealized relative amounts of the sample in each fraction as a function of the total amount of sample distributed, the partition coefficient of the sample at infinite dilution and ε. In Fig. 5, the expression $\frac{W_n(p) - W_n^*(p)}{\varepsilon w_o}$ is plotted as a function of the fraction number p for $k_o = 1$ and $n = 19$ (full-drawn curve). This curve is compared with the curve obtained when it is assumed that the partition coefficient of an ideal substance is changed by a small amount Δk_o. The expression for $W_n(p)$ (eqn. 2 in Ref.[2]) is differentiated with respect to k. Then

$$\Delta W_n(p) = W_n(p) \cdot \left(\frac{p}{k_o} - \frac{n}{1+k_o}\right) \cdot \Delta k_o \qquad (5)$$

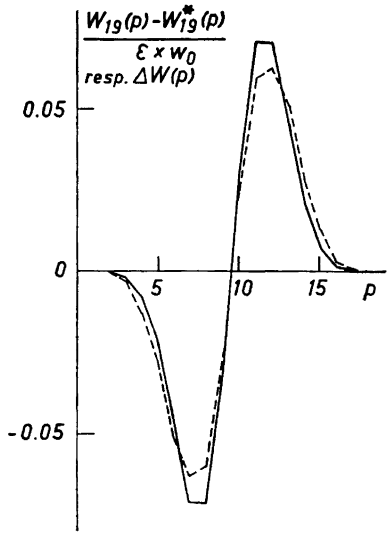


Fig. 5. Full-drawn curve: Graphical representation of eqn. 4 for $k = 1$ and $n = 19$. Dotted curve: Graphical representation of eqn. 5 for $k = 1$, $n = 19$ and $\Delta k = 0.28$.

The dotted curve in Fig. 5 gives this expression calculated for $k_0 = 1$, $n = 19$ and $\Delta k_0 = 0.28$.

The agreement between the two curves is good. The concentration dependence of the partition coefficient thus results in an effect comparable to an overall change in the partition coefficient rather than a broadening of the $W(p)$ *vs.* p curve. From eqn. 4, it can be seen that the $W(p)$ values at infinite dilution can be obtained by determining the $W(p)$ values at different values of w_0 and extrapolating them as a function of w_0 to the value $w_0 = 0$. Because of the initial assumptions, this extrapolating method is only applicable to substances containing molecules of the same size. The method will, however, be applied to common polymers as a first approximation. It is assumed that such a method, in addition, automatically corrects for small changes in the composition of the two liquid phases which might occur due to the addition of the polymer.

The reason for the concentration dependence is that there probably exists an interaction between molecules of the same size. The question now arises as to what extent molecules of different sizes interact in the distribution experiments. It is necessary to know whether micelles or complexes of some other type between molecules of different sizes occur, resulting in an effect closely related to the coprecipitation found in polymer precipitation fractionation. This problem will be treated in a separate paper. In the subsequent parts of this paper, this effect will be considered to be negligible.

RESOLVING POWER

As was pointed out by Craig [7], the peak of the curve representing the relation between the amount in each fraction and the fraction number in the funda-

mental Craig countercurrent distribution procedure can be approximately expressed by

$$p_{max} = \frac{nk}{1+k} \tag{6}$$

if molecules of only one size are present and if the volumes of the two phases are equal. If the ratio between the volumes of the lower and upper phases is φ, then the relation can be written

$$p_{max} = \frac{n\varphi k}{1+\varphi k} \tag{7}$$

When a polymer is distributed in a Craig apparatus, the derivative $|\partial p_{max}/\partial M|$ can be regarded as a measure of the resolving power of the apparatus. This derivative should of course be as large as possible, *i.e.* the positions of the peaks caused by two samples differing in molecular weight by an infinitesimal amount should differ as much as possible. By differentiation of eqn. 7 we obtain

$$\left|\frac{\partial p_{max}}{\partial M}\right| = \frac{\partial p_{max}}{\partial k} \cdot k \cdot \left|\frac{\mathrm{d} \ln k}{\mathrm{d}M}\right| = n \cdot \frac{\varphi k}{(1+\varphi k)^2} \cdot \left|\frac{\mathrm{d} \ln k}{\mathrm{d}M}\right| \tag{8}$$

The expression $\frac{\varphi k}{(1+\varphi k)^2}$ attains its highest value, *viz.* 1/4 for $\varphi k = 1$ and the derivate $\mathrm{d} \ln k/\mathrm{d}M$ is independent of φ. The highest resolving power is thus obtained if the distribution experiment is carried out employing a φ value equal to the inverse of the apparent partition coefficient of the substance. However, φ k can differ appreciably from unity without the above expression and thus the resolving power being too far below the maximum value.

It now remains to find the maximum value of $|\mathrm{d} \ln k/\mathrm{d}M|$. No current theory about the activity of polymer solutions is able to provide an acceptable expression for this derivative. The expressions provided are either too approximate or too unwieldy for the present purpose. An empirical method must therefore be used. The author has chosen to regard the curves in Figs. 2 and 3 as representative for polyglycols containing molecules of only one size, the molecular weight being identical with the one given by the manufacturer. By means of graphical methods, it was found that, for the solvent system TAW, the derivative $|\mathrm{d} \ln k/\mathrm{d}M|$ attains its maximum value for $v = 0.43$ independent of the molecular weight. For the solvent system TCW, the derivative attains its maximum value for each preparation when k is about unity, the latter condition being far from critical.

As can be predicted by the theory of Schulz, it is possible that, with the solvent system TAW, an extremely good resolving power can be obtained at compositions corresponding to $v < 0.32$ and, with the system TCW, at $v < 0.13$ or at v values rather close to 0.50. It is however probable that the gain is not sufficient to compensate for the enormous increase in experimental difficulty. Furthermore, when the experiments are carried out at a volume ratio for the two phases which differs appreciably from unity, the concentration of

polymer in the phase of less volume must be kept very high if sufficient quantities of polymer are to be obtained in the final fractions. In view of the above discussion of the concentration dependence of the partition coefficient, such experimental conditions should be avoided.

An exact analysis of the resolving power problem cannot be carried out until a more accurate relation between molecular weight and the partition coefficient is known. The above analysis was, however, regarded as sufficient for planning the subsequent countercurrent distribution experiments.

EXPERIMENTAL

All experiments reported in this paper were carried out at room temperature (20—21°C). Variations in temperature have been avoided.

Chemicals used. Trichloroethylene: Technical grade quality, distilled before use. Chloroform: pro analysi quality, stabilized with 1 % alcohol. Alcohol: 95 % ethyl alcohol ("Extra finsprit"). In the countercurrent experiments, spectroscopic grade quality alcohol was used. All solvents were checked with respect to evaporation residue before use.

Four different polyglycol preparations, *viz.* A 6 000, A 4 000, A 1 500, A 300, manufactured by Mo och Domsjö AB were used in the experiments — the figures indicating their approximate, number-average molecular weights. The first three samples were purified by dissolving them in trichloroethylene, shaking with water, shaking the water phase with chloroform and evaporating the chloroform. The preparations were finally dried in vacuum at 50°C.

Fisher & Porter Co 250 ml separatory funnels equipped with glass stoppers and teflon valve taps were used — they permitted an almost perfect separation of the two phases.

The one-stage extraction experiments were carried out by shaking a weighed amount of polyglycol with 50 ml of both solvents (the solvents had previously been shaken to equilibrium in separatory funnels). If nothing to the contrary is stated, the amount of polyglycol in each experiment was 500 mg. A 25 ml portion of each phase was evaporated to dryness and weighed after drying in vacuum at 50°C.

The countercurrent experiments. An all-glass countercurrent apparatus according to Craig could not be used because the transfer of liquid could not be performed without preventing parts of the lower phase following the upper phase and parts of the upper phase remaining with the lower stationary phase. For this reason, separatory funnels of the type described above were used as contacting vessels. The transfer of liquid was of course more laborious and time-consuming but the phases could be separated almost exactly from each other.

The shaking device consisted of a horizontal metal rod along which 20 clamps for the separatory funnels were attached on opposite sides. The funnels were situated at right angles to the rod at a distance of about 20 cm from the rod. Equilibrium was obtained by automatically turning the rod back and forth around its axis through an angle of $\pm$ 40° for 30 min, the mean position of the funnels being horizontal. The number of periods per min was 33. After allowing the two liquid layers to separate, the lower phases were transferred according to the general fundamental distribution pattern.

The substance to be distributed was added in the solid state to tube No. 0 which contained 50 ml of both phases. Equal volumes were used in all steps. At the end of the distribution experiment, the contents of the twenty tubes together with the necessary rinsing liquid were evaporated to dryness in warm air. The fractions were finally dried in vacuum at 50°C and weighed.

In order to show that the phases separate well and that no appreciable loss of solvents by evaporation or other reasons occurs, the volumes of both phases in each tube were determined at the end of a distribution experiment. The losses were small and were partly due to the liquid adhering to the walls of the funnels.

In order to demonstrate that the apparatus can be used as an analytical tool, a pure compound, N,N-dimethylaniline, twice distilled in vacuum, was distributed in the apparatus. The solvents used were *cyclo*hexane and aqueous phosphate-citrate buffer according

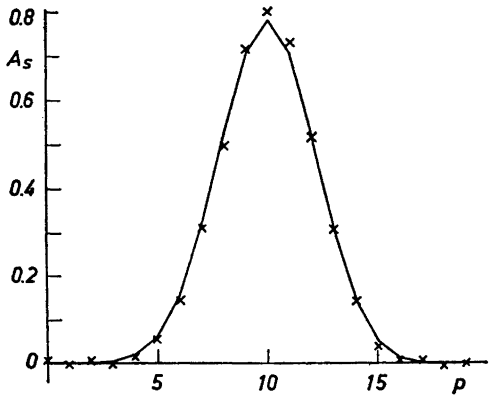


Fig. 6. Comparison at 251 mμ between experimental (points) and theoretical (curve) absorbancies of the upper phase for countercurrent distribution of N,N-dimethylaniline. Solvent system: *Cyclo*hexane aqueous buffer (pH = 2.75). Equal volumes of both phases.

to McIlvaine with pH = 2.75, the two liquids being shaken to equilibrium before use. A solution containing about 3×10^{-5} g N,N-dimethylaniline in 50 ml of the equilibrated *cyclo*hexane was prepared and used as the upper phase in tube No. 0. After completion of the distribution, the upper phases were collected and their absorbancies measured at 250 mμ in a Beckman spectrophotometer, the equilibrated *cyclo*hexane being used as the reference substance. The results are given in Fig. 6 where a comparison can be made with the theoretical values for a compound with a partition coefficient = 1.10_4. This value is in fairly good agreement with the value 1.16 which can be calculated from the paper of Columbic and Goldbach [8]. The agreement between experimental and calculated absorbancies must be regarded as good.

Acknowledgement. The author is indebted to Dr. Börje Steenberg for his encouraging interest in this work and to Mo och Domsjö AB for their kindness in providing the polyglycol preparations.

REFERENCES

1. Almin, K. E. and Steenberg, B. *Acta Chem. Scand.* **11** (1957) 936.
2. Almin, K. E. *Acta Chem. Scand.* **11** (1957) 1541.
3. Schulz, C. V. and Nordt, E. *J. prakt. Chem.* **155** (1940) 115.
4. Schulz, C. V. *Z. physik. Chem.* **A 179** (1937) 321.
5. Brønsted, J. N. *Z. physik. Chem., Bodenstein-Festband* **1931** 257.
6. Craig, L. C. *Anal. Chem.* **22** (1950) 1346.
7. Craig, L. C. *J. Biol Chem.* **155** (1944) 519.
8. Columbic, C. and Goldbach, G. *J. Am. Chem. Soc.* **73** (1951) 3966.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1274–1277

Fractionation of Polymers by Liquid-Liquid Distribution

III. The Interaction Problem

KARL ERIK ALMIN and RUNE LUNDBERG

Swedish Forest Products Research Laboratory, Paper Technology Department, and Royal Institute of Technology, Department of Paper Technology, Stockholm, Sweden

Fractionated polyglycols have been countercurrent distributed both separately and in mixtures. From the experimental data the conclusion has been drawn that no interferring interaction occurs between molecules of different sizes.

In polymer countercurrent distribution experiments, association phenomena between molecules of different sizes can be expected to influence the results [1]. It was suggested that such phenomena should result in effects closely related to the coprecipitation occurring during the precipitation fractionation of polymers.

This paper consists of a report on a theoretical and experimental investigation performed by the authors on this association effect. Because polymer specimens are always inhomogeneous with respect to molecular weight, the effect must be studied on samples containing molecules of different sizes.

We consider two polymer samples of the same monomer with frequency functions, $f_1(k)$ and $f_2(k)$, with respect to the partition coefficient and, in addition, a sample made up by mixing equal parts of these samples. These three preparations are then fractionated in a Craig apparatus. If ideal conditions prevail, the relative amount of polymer in each fraction of the three experimental series can be written [2].

$$W(p)^{(1)} = \int_0^\infty F(k,p) \cdot f_1(k)\ \mathrm{d}k \tag{1}$$

$$W(p)^{(2)} = \int_0^\infty F(k,p) \cdot f_2(k)\ \mathrm{d}k \tag{2}$$

$$W(p)^{(3)} = \int_0^\infty F(k,p) \cdot \frac{f_1(k) + f_2(k)}{2}\ \mathrm{d}k \tag{3}$$

where $F(k,p) = \binom{n}{p} \frac{k^p}{(1+k)^n}$.

It is easily deduced that $W(p)^{(3)}$ is equal to the arithmetical mean of $W(p)^{(1)}$ and $W(p)^{(2)}$. If interaction occurs, then the eqns. 1—3 become more complicated. The function $F(k,p)$ cannot then be identical for the three cases. This means that $W(p)^{(3)}$ must differ from the arithmetical mean of the two other $W(p)$ values.

The same type of discussion can be applied to the mean molecular weight of the fractions, using eqn. 13 of Ref.[2]:

$$\overline{M(p)}^{a} = \frac{1}{W(p)} \cdot \binom{n}{p} \cdot \int_0^\infty \frac{k^p}{(1+k)^n} \cdot f(k) \cdot M^a \cdot \mathrm{d}k \qquad (4)$$

as a basis for the discussion. Such a discussion leads to essentially the same result, *viz.* that the product of the measured property, which is proportional to $\overline{M(p)}^{a}$, and $W(p)$ in the third series should be equal to the arithmetical mean of the corresponding quantities in the other two series provided that no interaction occurs, but should differ from the mean if interaction occurs.

It is important that not only the relative weights of the different fractions in the three experimental series are compared but also the quantities closely related to the mean molecular weight of the fractions. From eqns. 1 and 4, it can be seen that the expression within the integral contains the molecular weight in the second case but not in the first. This indicates that, if the arithmetical mean law is valid by mere accident in one case, then it must be practically impossible for this law to be valid in the other case if interaction occurs.

In order to make any possible interaction effect easy to observe, polymer specimens with greatly different mean molecular weights and rather narrow molecular frequency functions should be used. The polymer used in this investigation was polyglycol. The solvent system was prepared by mixing trichloroethylene, alcohol (95 %) and water in the volume ratio 2 : 2 : 1. The viscosity numbers were used as a measure of the molecular weight.

Two preparations with relatively sharp-peaked frequency curves were obtained as fractions in a large-scale countercurrent fractionation of two polyglycol preparations with number-average molecular weights of about 1 500 and 6 000 (called A 1 500 and A 6 000). These two fractions were then countercurrent fractionated according to the fundamental Craig procedure ($n = 19$) both separately and using a mixture of equal parts. The weights of the fractions obtained were then determined, *cf.* Figs. 1 and 2. The curve in Fig. 2 was obtained as the arithmetical mean of the two curves in Fig. 1. The viscosity numbers of those fractions in which sufficient polymer was present were also measured. The results are given in Figs. 3 and 4. In Fig. 4, there is also shown a curve which was calculated from the curves (not the points) in Fig. 3 assuming that the viscosity numbers are additive with respect to weight. The experimental $W(p)$ values were used for the calculations.

The experimental points in Figs. 2 and 4 agree, within the experimental accuracy, with the corresponding calculated curves. The conclusion must be drawn that no interfering interaction occurs between molecules of different sizes apart from the concentration-dependent interaction discussed in Ref.[1] This means that intermolecular association, which results in irreversible

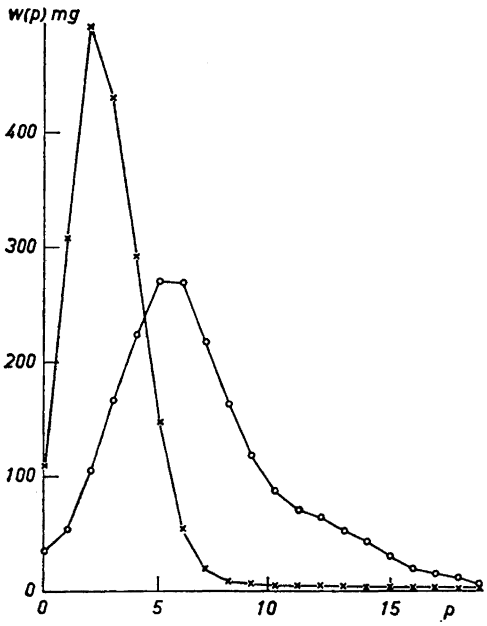


Fig. 1. Countercurrent distribution of Preps. I and II. Solvent system: trichloroethylene-alcohol-water = 2:2:1.

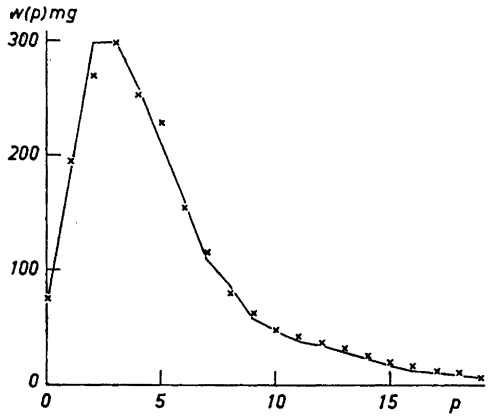


Fig. 2. Countercurrent distribution of Prep. III. Solvent system: trichloroethylene-alcohol-water = 2:2:1. Points: experimental values. Curve: calculated values.

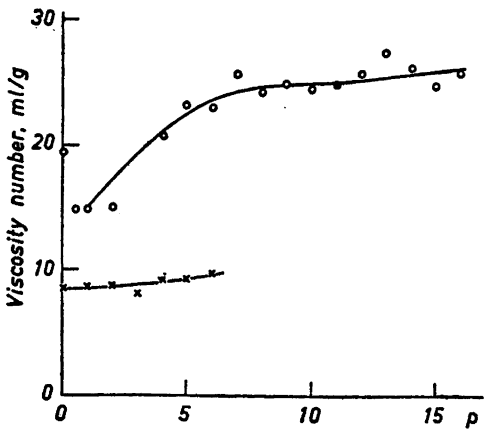


Fig. 3. Viscosity numbers for the fractions obtained from Preps. I and II.

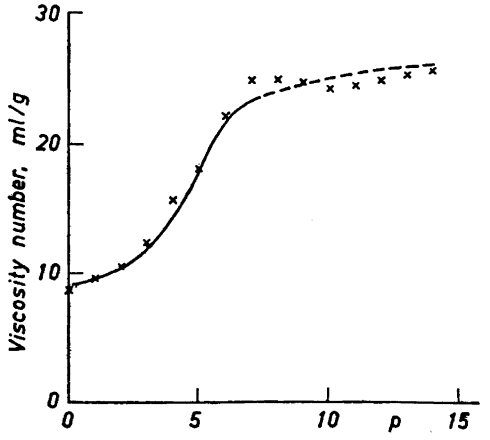


Fig. 4. Viscosity numbers for the fractions obtained from Prep. III. Points: experimental values. Curve: calculated values.

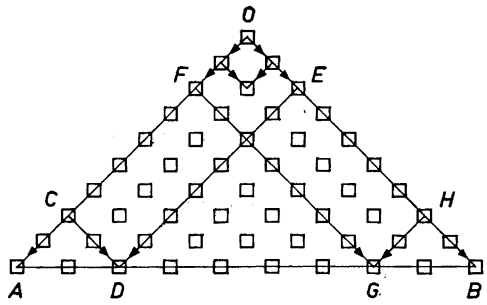


Fig. 5. Schematic diagram for large scale fractionation of polyglycol samples A 1 500 and A 6 000.

equlibrium conditions for the distribution of the polymer between the two solvents does not occur in this case. In the subsequent work, it will be assumed that this is valid at all compositions of the solvent system.

EXPERIMENTAL

Concerning temperature, solvents and purification of the polyglycols, see Experimental of Ref.[1] The solvent system used in this investigation was prepared by mixing trichloroethylene, alcohol (95 %) and water in the volume ratio 2:2:1. This volume ratio corresponds closely to $v = 0.39$ (*cf.* Ref.[1]).

Large scale fractionation. The purpose of this experiment was to prepare two polyglycol specimens, with relatively narrow molecular frequency curves and with greatly different mean molecular weights, by means of a 10 tube ($n = 9$) countercurrent fractionation according to the fundamental Craig procedure. The low molecular weight specimen was prepared from polyglycol A 1 500 and the high molecular weight specimen from polyglycol A 6 000. The procedure could be appreciably shortened since not more than one fraction was of interest in each fractionation series. The complete fractionation scheme for a 10 tube fractionation is shown by the triangle OAB in Fig. 5. The low molecular preparation from polyglycol A 1 500 was obtained by performing the operations within the rectangle OCDE and the high molecular weight preparation from polyglycol A 6 000 by performing the operations within the rectangle OFGH. The experiments were carried out using large separatory funnels.

The weight of sample was 200 g for A 1 500 and 600 g for A 6 000. The yields were 57.7 g and 11.8 g respectively. These two fractions (called Prep. I and Prep. II) were used for the three series of countercurrent experiments. (A mixture of equal parts of these two fractions was designated Prep. III.)

The countercurrent experiments were carried out as described in Ref.[1] The weight of sample was in all three cases 2.0000 g. The difference between the sample weight and the sum of the weights of the fractions was for Prep. I 5.6 mg, for Prep. II 19.0 mg and for Prep. III 31.6 mg.

Viscosity measurements were performed on solutions obtained by dissolving about 50 mg of the different fractions in 10.00 ml chloroform. Cannon-Fenske pipettes recommended for the range between 0.3 and 5.0 centipoises were used at 25°C ± 0.02.

REFERENCES

1. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1263.
2. Almin, K. E. *Acta Chem. Scand.* **11** (1957) 1541.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1278–1286

Fractionation of Polymers by Liquid-Liquid Distribution

IV. Countercurrent Fractionation of Polyglycols

KARL ERIK ALMIN

Swedish Forest Products Research Laboratory, Paper Technology Department, Stockholm, Sweden

Three polyglycol preparations have been countercurrent distributed in the solvent system trichloroethylene-alcohol-water. Weights and limiting viscosity numbers of the different fractions have been determined. Curves of weight frequency *vs.* limiting viscosity number have been calculated.

In this paper, experiments will be reported in which polyglycols have been fractionated by means of liquid-liquid countercurrent distribution. The method has been described in earlier papers of this series from both theoretical and practical points of view [1,2]. Three different preparations, A 6 000, A 4 000 and A 1 500, have been investigated and equal volumes of the two phases obtained by mixing trichloroethylene, alcohol and water (TAW) in the volume ratio 0.414 : 0.43 : 0.156 have been used as solvent system.

In Tables 1—3, the experimental results from experiments with different weights of samples can be seen in columns 2—5. The notations are in agreement with those of Ref.[1]. Col. 6 of these tables contains $W(p)$ values at infinite dilution calculated by means of eqn. 4 in Ref.[2]. The method of least squares was applied. The slopes of the lines obtained in this way are given in Fig. 1 (points). In this figure, the $\Delta W(p)$ values calculated from eqn. 5 of Ref.[2]

$$\Delta W(p) = W(p) \cdot \left(\frac{p}{k_o} - \frac{n}{1+k_o} \right) \cdot \Delta k_o \qquad (1)$$

are also given (curves), suitable values for k_o and Δk_o being assumed. The statement in Ref.[2] that the concentration dependence of the partition coefficient results in an effect which can be compared with an overall change in partition coefficient has thus been experimentally verified. It can further be noted in the case of the preparation A 6 000 that the peak of the $W(p)$ *vs.* p curve moves to higher p values as the sample weight is increased while

Table 1. Countercurrent distribution of polyglycol A 6 000. Solvent system: TAW (v = 0.43). $w(p)$, $W(p)$ = amount and relative amount, respectively, of polyglycol in fraction no. p. w_o = initial weight of sample.

p	$W(p) \times 10^3$ (exptl.)				$W(p) \times 10^3$ (calc.) (w_o = 0)	$\Delta W(p) \times 10^3$
	w_o = 5 g	w_o = 2 g	w_o = 1 g	w_o = 0.5 g		
0	0.5	1.1	1.5	3.0	2.5	−0.4
1	0.9	1.6	1.9	3.2	2.8	−0.4
2	2.5	2.7	3.4	4.1	3.8	0.0
3	7.2	6.9	7.4	8.5	7.9	0.7
4	17.0	16.4	15.3	16.8	16.0	0.4
5	34.8	32.2	31.8	32.3	31.4	0.2
6	61.0	54.3	53.6	56.3	53.4	−0.6
7	89.9	85.7	82.7	83.0	81.9	0.4
8	116.5	108.5	106.9	106.9	104.9	−2.1
9	128.4	129.6	128.0	124.9	126.7	3.5
10	133.4	128.4	126.8	126.4	125.4	−1.0
11	121.9	119.1	118.1	115.9	116.2	−0.9
12	94.3	101.6	100.9	99.3	101.9	1.9
13	74.1	78.2	79.7	76.8	79.2	−0.6
14	50.5	56.7	57.2	57.3	58.8	−0.5
15	32.6	36.9	39.1	38.4	39.8	−0.3
16	18.9	21.7	23.8	24.1	24.6	0.1
17	9.6	11.7	12.8	13.4	13.6	0.2
18	4.6	4.7	6.9	7.3	7.1	0.0
19	1.5	2.2	2.1	1.8	2.1	−0.5
$\sum w(p) - w_o$ mg	−76.2	−1.2	−2.4	6.4		$\sum$ = 0.1

Table 2. Countercurrent distribution of polyglycol A 4 000. Solvent system: TAW (v = 0.43). For symbols see Table 1.

p	$W(p) \times 10^3$ (exptl.)				$W(p) \times 10^3$ (calc.) (w_o = 0)	$\Delta W(p) \times 10^3$
	w_o = 5 g	w_o = 2 g	w_o = 1 g	w_o = 0.5 g		
0	0.7	1.2	1.6	2.3	2.1	1.1
1	4.0	6.0	7.2	8.3	8.3	0.6
2	18.1	22.5	24.2	27.9	27.2	−1.1
3	43.7	57.4	58.6	66.5	66.2	−2.2
4	88.0	107.0	112.3	117.1	119.5	−0.9
5	128.7	152.8	159.5	161.4	166.4	2.3
6	168.8	174.0	176.0	179.5	179.1	−0.1
7	169.3	172.0	165.6	167.8	167.6	7.4
8	151.1	134.1	123.8	122.0	118.8	0.0
9	111.0	87.9	76.6	72.7	68.9	−4.9
10	64.5	46.9	39.8	37.7	34.4	−4.6
11	30.6	21.7	22.0	17.4	17.3	−0.6
12	12.6	8.8	13.5	7.6	9.3	1.4
13	4.9	3.8	8.5	3.6	5.5	1.1
14	2.1	1.7	4.7	2.5	3.4	0.2
15	0.9	0.8	2.6	1.7	2.1	−0.4
16	0.4	0.4	1.3	1.5	1.4	−0.3
17	0.2	0.7	0.9	1.1	1.1	0.2
18	0.2	0.2	0.9	1.3	1.1	0.8
19	0.1	0.1	0.3	0.3	0.3	0.2
$\sum w(p) - w_o$ mg	−88.0	−36.4	6.1	2.0		$\sum$ = 0.2

Table 3. Countercurrent distribution of polyglycol A 1 500. Solvent system: TAW (v = 0.43). For symbols see Table 1.

p	$W(p) \times 10^3$ (exptl.)				$W(p) \times 10^3$ (calc.) (w_o = 0)	$\Delta W(p) \times 10^3$
	w_o = 5 g	w_o = 2 g	w_o = 1 g	w_o = 0.5 g		
0	3.6	5.2	7.9	9.4	9.1	0.6
1	34.3	35.8	43.8	48.4	46.5	0.3
2	83.6	100.1	104.1	120.2	116.7	−1.8
3	155.2	164.0	185.9	184.3	186.8	−4.5
4	201.4	208.4	210.3	209.6	211.6	−6.5
5	199.9	196.3	188.7	179.7	183.1	−3.4
6	157.2	143.5	125.7	121.3	120.3	−3.7
7	93.3	82.3	72.0	64.6	65.5	−0.1
8	42.4	37.7	32.6	30.0	30.1	2.0
9	17.2	15.4	13.4	12.3	12.4	2.6
10	6.3	5.8	4.3	5.7	5.0	2.2
11	2.4	2.3	2.7	2.8	2.7	2.0
12	1.1	1.3	2.1	1.8	2.0	1.9
13	0.6	0.5	1.0	1.7	1.3	1.3
14	0.3	0.3	0.8	1.6	1.2	1.2
15	0.3	0.3	0.9	1.4	1.2	1.2
16	0.2	0.2	0.9	1.5	1.2	1.2
17	0.2	0.2	0.9	1.3	1.1	1.1
18	0.2	0.3	1.0	1.6	1.3	1.3
19	0.2	0.1	0.8	0.6	0.7	0.7
$\sum w(p) - w_o$ mg	5.8	−16.8	7.8	5.3		$\sum = -0.4$

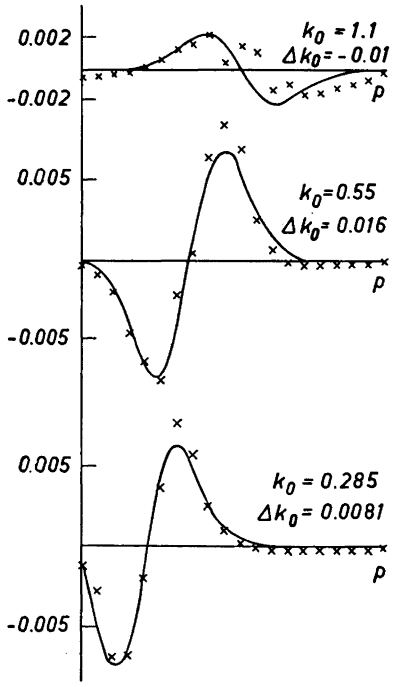


Fig. 1. Changes in the $W(p)$ values due to the concentration dependence of the partition coefficient (*cf.* text).

Table 4. Parameters of the formulas giving the mathematical expressions for the frequency functions of polyglycols A 6 000 and A 4 000. (*Cf.* eqn. 2).

Sample	i	A_i	r_i	a_i	s_i
A 6 000	1	4.08	1	38	1
	2	7.684×10^{-4}	1	5.9	2
	3	9.53×10^{-5}	1	7	1
	4	1.9×10^{-15}	1	2	1
A 4 000	1	9.84×10^{-1}	12	40	2
	2	1.6×10^{-2}	1	6	1

the opposite is the case for the two other preparations. This is in accordance with the signs of the slopes of the lines in Fig. 4 of Ref.[2]

The calculations of the frequency functions of the partition coefficient have been based on the calculated $W(p)$ values at infinite dilution. In accordance with Ref.[1], it is assumed that the frequency functions of the preparations A 6 000 and A 4 000 can be expressed as a sum of functions of the type

$$A_i \cdot k^{r_i} \cdot (1+k)^{19} \cdot \exp(-a_i \cdot k^{s_i}) \qquad (2)$$

The results of these calculations are given in Table 4. The differences between the $W(p)$ values in col. 6 of Tables 1 and 2 and the $W(p)$ values calculated from the calculated frequency functions can be seen in col. 7 of Tables 1 and 2. These differences are small considering the experimental errors and hence the calculated frequency functions must be regarded as satisfactory. The frequency curve of preparation A 6 000 shows a small peak at low k values.

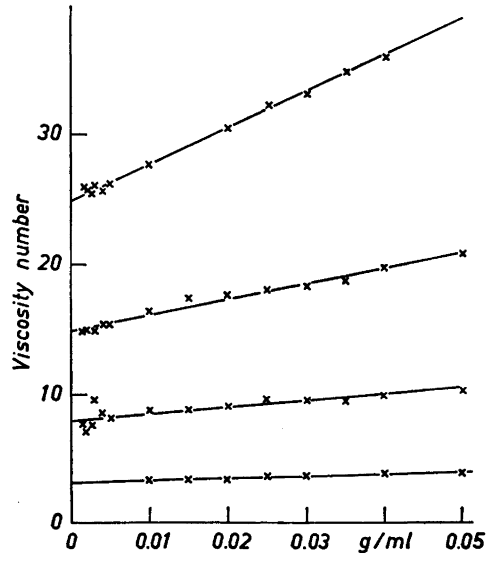


Fig. 2. Viscosity number of the polyglycol samples as a function of concentration. Solvent: chloroform.

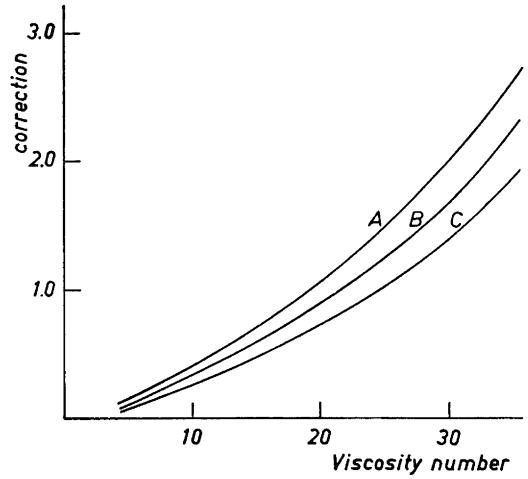


Fig. 3. Curves giving the correction to be subtracted from an experimentally determined viscosity number for polyglycol in chloroform in order to obtain the limiting viscosity number. A = 0.006 g/ml; B = 0.005 g/ml; C = 0.004 g/ml.

This peak must be a consequence of slight errors in the experimental and numerical methods.

No attempts were made to calculate a frequency curve for preparation A 1500. The reason for this was that the $W(p)$ *vs.* p curve for this preparation agreed so closely with the corresponding curve for a preparation containing molecules of only one size. The differences between the $W(p)$ values in col. 6 of Table 3 and the $W(p)$ values expected for a substance with $k = 0.285$ can be seen in col. 7 of Table 3. These differences are only slightly greater than the experimental errors. They show, however, a pronounced trend, thus indicating that molecules of different sizes are present. The frequency curve must therefore have been very narrow. The resolving power was thus too low in these experiments to permit a determination of the shape of the frequency curve. One of the reasons for this is the disadvantageous volume ratio of the phases in the experiment. It can be calculated that a volume ratio of about 1 : 0.285 for the two phases would increase the resolving power by about 45 %. Such an increase cannot, however, be regarded as sufficient. Thus, some other solvent system must be used. The results of the distribution experiment with polyglycol A 1 500 can therefore only serve as an aid in determining the relation between partition coefficient and molecular weight.

In order to determine the frequency functions of the limiting viscosity number, the latter quantity was calculated from the viscosities of chloroform solutions, for each of the fractions containing more than about 25 mg polyglycol. The calculations of the limiting viscosity numbers were carried out under the assumption that the slope of the line giving the relation between viscosity number and concentration is a function solely of the limiting viscosity number of the preparation. These slopes were determined from the relation

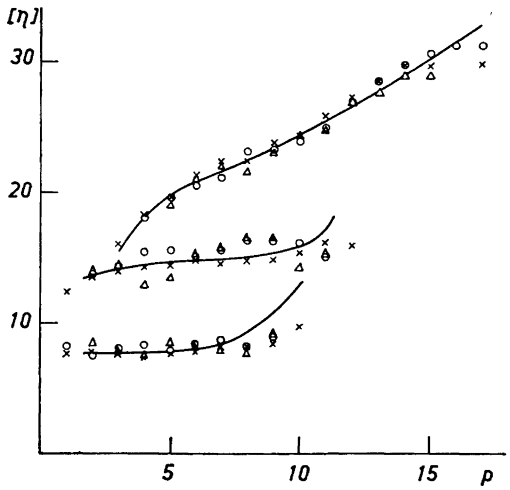


Fig. 4. Comparison between experimentally determined (points) and calculated (curves) limiting viscosity numbers for the polyglycol fractions obtained from polyglycols A 6 000, A 4 000 and A 1 500. × 5 g, O 2 g and △ 1 g experiments.

giving the viscosity number as a function of the concentration for the preparations A 6 000, A 4 000, A 1 500 and A 300. These relations can be seen in Fig. 2. The difference between the viscosity number and the limiting viscosity number was then plotted as a function of the viscosity number at different concentrations (Fig. 3). Using this figure, it is possible to find the correction which must be subtracted from a certain vicsosity number determined at a known concentration in order to obtain the limiting viscosity number.

In Fig. 4, the limiting viscosity numbers of the different fractions obtained from the 5 g, 2 g and 1 g distribution experiments are plotted as a function of the tube number. No systematic differences between results obtained from countercurrent experiments with different sample weights can be detected. The author has therefore regarded all the experimental points as being of

Table 5. Comparison between limiting viscosity numbers calculated from the fractions in the countercurrent experiments and the limiting viscosity numbers obtained from Fig. 2.

w_0	A 6 000	A 4 000	A 1 500
5 g	25.0	14.8	7.8
2 g	24.9	15.7	8.3
1 g	24.7	15.6	8.1
[η] from Fig. 2	24.8_8	14.8_8	7.9_0

equal statistical weight and equal to the values which would have been obtained in a fictitious countercurrent experiment carried out at infinite dilution.

In Table 5, limiting viscosity numbers for the three fractionated samples, calculated from the limiting viscosity numbers and the corresponding fraction weights according to Philippoff [3], are compared with the directly determined limiting viscosity numbers of the original polyglycol preparations. Considering the accuracy of the viscosity determinations, the agreement must be regarded as satisfactory.

The relation between the limiting viscosity number and the partition coefficient was determined according to the method described in Ref.[1] The experimentally determined values for the limiting viscosity numbers of the fractions, $\overline{[\eta]}_p$, and the corresponding $S(p)$ values calculated from the $W(p)$ values at infinite dilution were used. The values of $\frac{\mathrm{d} \ln S(p)}{\mathrm{d}p}$ were obtained by an interpolation method. The i values chosen were -2, -1, 0, 1 and 2. In this way, a set of equations was obtained from which the constants of the relation

$$[\eta] = B \cdot \ln k + \sum_i C_i \cdot k^i \qquad (i = -2, -1, 0, 1, 2) \tag{3}$$

were obtained using the method of least squares. Because of the large number of equations and variables, an electronic computor (BESK) was used for the calculations. The results of these calculations can be seen in Table 6. The

Table 6. Numerical values of the constants of eqn. 3.

B	5.38	($\pm$ 6.05)
C_{-2}	0.2821	($\pm$ 0.1501)
C_{-1}	-4.519	($\pm$ 2.420)
C_0	26.52	($\pm$ 2.45)
C_1	1.608	($\pm$ 4.377)
C_2	-0.2649	($\pm$ 0.3456)
Standard deviation (eqn. 4)	1.02	

errors in the constants B and C_i are considerable. The reason for these large errors is that ln k can be expressed as a series of the type $\Sigma C_i k^i$ in a limited range of k and this makes it difficult for the numerical method to »decide» which term in the author's formula shall predominate. This difficulty can be overcome by decreasing the number of terms in eqn. 1, but then the solution would not be free from assumptions that may be biased. The reliability of the final relation between the limiting viscosity number and the partition coefficient should be judged from the standard deviation, σ, calculated from the formula

$$\sigma^2 = \frac{1}{m-n} \cdot \sum(\overline{[\eta]}_{p,\,\mathrm{obs}} - \overline{[\eta]}_{p,\,\mathrm{calc}})^2 \tag{4}$$

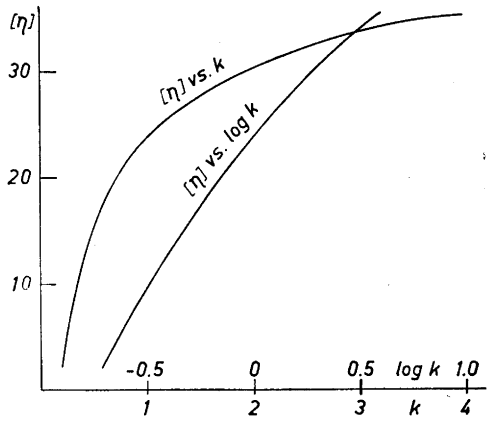


Fig. 5. Relation between the limiting viscosity number of polyglycol in chloroform and the partition coefficient of polyglycol in the solvent system TAW (v = 0.43).

where m = number of equations and n = number of coefficients. The value of σ (Table 6) must be considered consistent with the spread of the experimental $\overline{[\eta]}_p$ values. It should be pointed out that the final relation and its derivative are monotonic. The corresponding curve is shown in Fig. 5. The $[\eta]$ *vs.* log k curve is almost a straight line but it does not pass through the origin. This indicates that Brønsteds exponential formula for the partition coeffcient of polymers [4] has to be multiplied by a constant if it is to represent approximately the experimental results of this investigation. This circumstance must also be responsible for some of the discrepancies given by the partition coefficient formula of Schulz [5].

The $\overline{[\eta]}_p$ values calculated using the constants in Table 6 and the $W(p)$ values at infinite dilution are plotted in Fig. 4 (curves). The agreement between

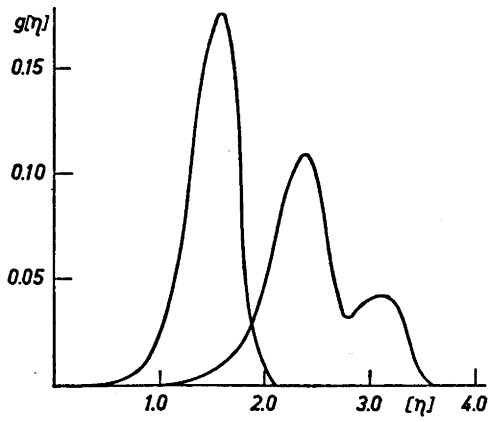


Fig. 6. Curves of weight frequency *vs.* limiting viscosity number in chloroform solutions for the polyglycol samples A 4 000 and A 6 000.

the experimental points and the calculated curves must be regarded as good, especially considering the appreciable molecular weight range of the substances investigated.

The derivative $\frac{d[\eta]}{dk}$ can now be calculated from the constants of eqn. 3. The curves of frequency *vs.* the limiting viscosity number in chloroform can thus be calculated using the method given in Ref.[1] The results of these calculations are given in Fig. 6 for the samples A 4 000 and A 6 000.

The accuracy of the frequency curves obtained will not be discussed completely in this paper because, as earlier reported [1], the results can only be checked by comparing them with results obtained under different experimental conditions. However, a few remarks can be made. The double peak in the frequency curve for polyglycol A 6 000 must be considered to be rather well established. This double peak can also be seen in the curve of frequency *vs.* partition coefficient and it has not been possible for the author to make the $W(p)$ values obtained consistent with an unimodal frequency curve. According to Flory [6], polymers prepared using a type of reaction which involves the successive addition of the monomer to a functional group and which can be characterized by a single rate constant have unimodal frequency curves. Polyglycol belongs to such a group of polymers. From this point of view, the double peak in the frequency curve for sample A 6 000 is suspect. However, the manufacturer of the samples used has informed the author that A 6 000 was produced by adding ethylene oxide to a mixture of two different polyglycol preparations of lower molecular weights and thus a double peak should appear. For the preparations A 4 000 and A 1 500, on the other hand, unimodal frequency curves should, according to the manufacturer, be expected.

In the case of sample A 4 000 the frequency curve is, as can be expected from the theory of Flory [6], rather narrow. It should, however, be pointed out that if the relation between limiting viscosity number and partition coeffient is calculated from only the experimental data obtained for preparation A 4 000, then a $[\eta]$ *versus* k relation with a smaller slope and thus a somewhat more sharply peaked curve is obtained. Such a treatment of the problem cannot be considered justified even if, as will be shown in a later paper, a sharper peaked curve is probably more correct.

EXPERIMENTAL

For experimental details: see Experimental of Refs.[2,7].

REFERENCES

1. Almin, K. E. *Acta Chem. Scand.* **11** (1957) 1541.
2. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1263.
3. Philippoff, W. *Ber.* **70** (1937) 827.
4. Brønsted, J. N. *Z. physik. Chem., Bodenstein-Festband* **1931** 257.
5. Schulz, G. V. *Z. physik. Chem.* **A 179** (1937) 321.
6. Flory, P. J. *J. Am. Chem. Soc.* **62** (1940) 1561.
7. Almin, K. E. and Lundberg, R. *Acta Chem. Scand.* **13** (1959) 1274.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1287–1292

Fractionation of Polymers by Liquid-Liquid Distribution

V. Countercurrent Fractionation of Polyglycols in the Solvent System Trichloroethylene-Chloroform-Water

KARL ERIK ALMIN

Swedish Forest Products Research Laboratory, Paper Technology Department, Stockholm, Sweden

Three polyglycol preparations have been countercurrent distributed in the solvent system trichloroethylene-chloroform-water. Weights and limiting viscosity numbers of the different fractions have been determined. Curves of weight frequency *vs.* limiting viscosity number have been calculated. The influence of formation of emulsions in the distribution experiments is discussed.

In the preceding paper of this series[1], data from the liquid-liquid countercurrent distribution analysis of three polyglycol preparations in the solvent system trichloroethylene-alcohol-water (TAW) were reported. The present paper will deal with analogous experiments in another solvent system, trichloroethylene-chloroform-water (TCW).

Preliminary liquid-liquid distribution experiments with the solvent system TCW have shown that the partition coefficients of polyglycols are more dependent on the temperature than is the case with the solvent system trichloroethylene-alcohol-water (TAW) [2]. For this reason, the countercurrent experiments with the solvent system TCW were carried out in a thermostat set at 25°C. This temperature is about five degrees higher than that used in the preliminary experiments reported in Ref.[2]

Difficulties have, however, arisen because of the ease of formation of emulsions in the solvent system TCW. The emulsions were especially prone to appear when the concentration was high, when the molecular weight of the polyglycol was large and when the partition coefficient of the polyglycols differed from unity. In these countercurrent experiments, the emulsions were allowed to remain with the stationary upper phases since the lower surfaces of the emulsions were smoother than the upper ones. The amount of emulsion was, however, found to decrease during the course of the distribution experiment. This was probably due to the decrease in the concentration of polyglycol. The volumes of especially the first ($p = 0$) and the last ($p = 19$) fractions

were thus appreciably less than the others at the end of the distribution experiments, these volume losses being more pronounced when the amount of emulsion formed was larger.

The influence of the emulsion formation can also be seen in the $W(p)$ *vs.* p curves and in the $\overline{[\eta]}_p$ values. For symbol notation see Ref.[3] The $W(p)$ *vs.* p curves became shifted towards lower p values and the relation between $\overline{[\eta]}_p$ and p became steeper when the sample weight and thus the amount of emulsion were increased. These changes can be explained in the following way. The emulsion layers contain varying amounts of the high molecular weight parts of the preparation which should belong to the lower phases. When the phases are separated, these high molecular weight parts remain with the upper phases. The possibility of accumulation of polymer and/or either of the solvents in the emulsion layer on account of surface phenomena will not be discussed.

The experimental data from experiments in which the mean partition coefficient differed appreciably from unity had to be discarded because of the very large emulsion formation. It has therefore not been possible to extend the range of validity of the relation between partition coefficient and limiting viscosity number in the same way as was possible in the case of the solvent system TAW [1].

In the cases where the formation of emulsion was less pronounced, the data from each experimental series were treated individually, exactly as if no emulsion formation had occurred. The curves of frequency *vs.* partition coefficient, $f(k)$, thus obtained are consequently more compressed than in the cases where no emulsions were obtained. At the same time, the curves of $[\eta]$ *vs.* partition coefficient, k, are steeper. The final frequency functions in terms of the limiting viscosity number can, however, be expected to be correct or nearly correct since the two above-mentioned effects counteract each other.

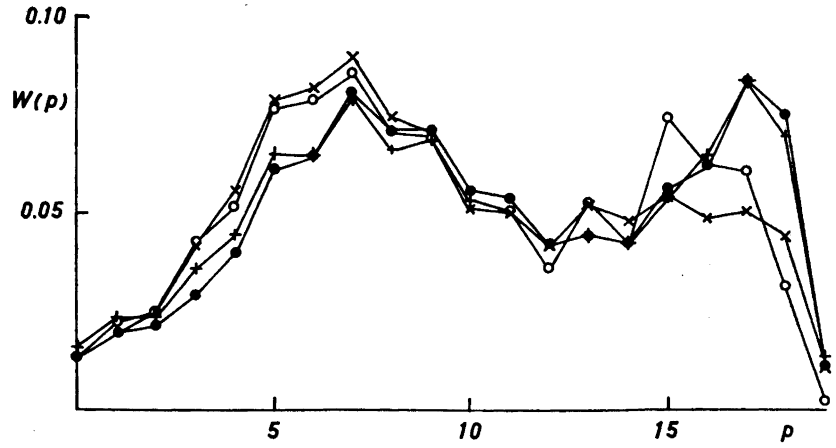


Fig. 1. Experimental $W(p)$ values obtained in the countercurrent experiments in TCW ($v = 0.165$) with polyglycol A 6 000. × 5 g, O 2 g, ● 1 g and + 0.5 g sample weight.

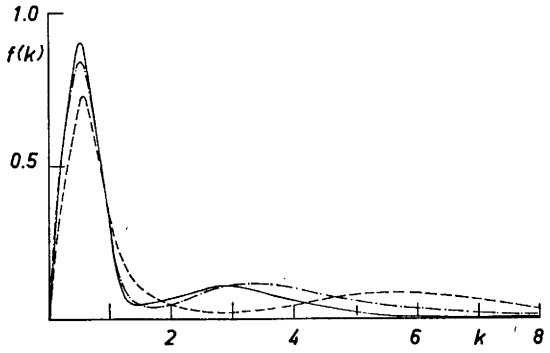


Fig. 2. Curves of weight frequency *vs.* partition coefficient, calculated from the experimental $W(p)$ values obtained in the experiments with polyglycol A 6 000. ——— 5 g, —.—.—.— 2 g and — — — — — 1.0 and 0.5 g sample weight.

The calculation of the relation between $[\eta]$ and k was found to be much simpler with the present solvent system than with the solvent system TAW [1] inasmuch as $[\eta]_p$ proved to be, in all cases, a linear function of the derivative $\frac{\mathrm{d} \ln S(p)}{\mathrm{d}\, p}$ (*cf.* Ref.[3]). The relation between limiting viscosity number and partition coefficient can thus be written,

$$[\eta] = C_0 + B \ln k \qquad (1)$$

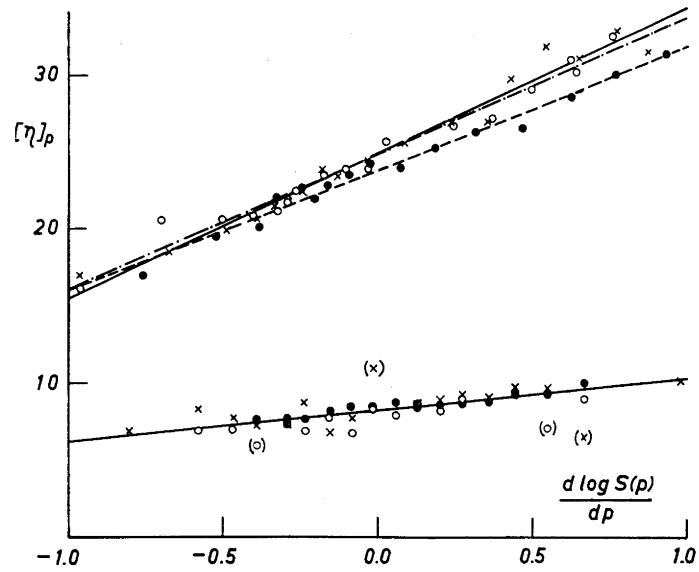


Fig. 3. Relation between limiting viscosity number and the derivative d log $S(p)$/d p for polyglycol A 6 000 and A 1 500 (Sample weights: *cf.* Fig. 1).

Table 1. The constants B and C_0 of eqn. 2 calculated from viscosity data.

Sample	Weight of sample, g	C_0	B
A 6 000	5	24.90	4.03
	2	24.87	3.86
	1 and 0.5	23.78	3.47
A 4 000	5	15.41	2.03
	2	14.88	2.23
	1 and 0.5	15.10	1.96
A 1 500	5, 2 and 1	8.19	0.93

The $W(p)$ values obtained from the experiments with polyglycol A 6 000 are given in Fig. 1. The composition of the solvents corresponds to $v = 0.165$ (*cf.* Ref.[2]). The influence of emulsion formation is obvious — especially in the 5 g and 2 g experiments. From the curves, it can be seen that the $W(p)$ values of every second fraction seem too low and those of every other one too high. The cause of these irregularities is unknown to the author. It may be pointed out that they seem to be more pronounced when the emulsion formation is large. The $f(k)$ curves calculated from the experimental data are given in Fig. 2. The experimental $\overline{[\eta]}_p$ values are plotted in Fig. 3 as a function of the derivative $\frac{\mathrm{d} \ln S(p)}{\mathrm{d} p}$. The numerical values of the constants C_0 and B, calculated from the lines in Fig. 3, are given in Table 1. In Fig 4, the frequency *vs.* limiting viscosity number curves are reproduced. The agreement is good in spite of the differences in the $f(k)$ curves. The differences in the frequency curves in the range $[\eta] > 32$ must be ascribed to slight errors in the experimental and numerical methods.

The results of the experiments with polyglycyol A 4 000 in the solvent system TCW ($v = 0.192$) are quite analogous to those reported for polyglycol

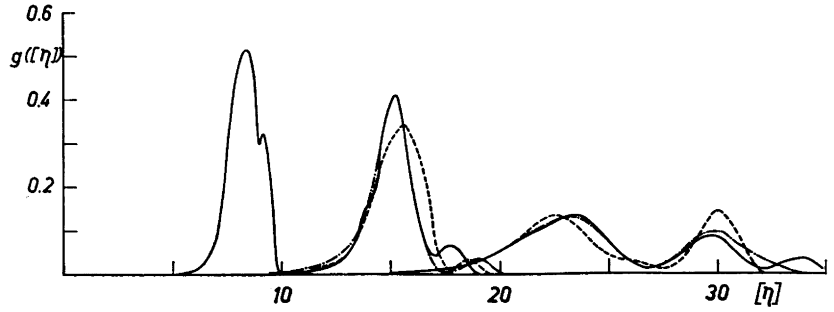


Fig. 4. Curves of weight frequency *vs.* limiting viscosity number for polyglycols A 6 000, A 4 000 and A 1 500 calculated from the corresponding curves in terms of partition coefficient. (Sample weights: *cf.* Fig. 2.)

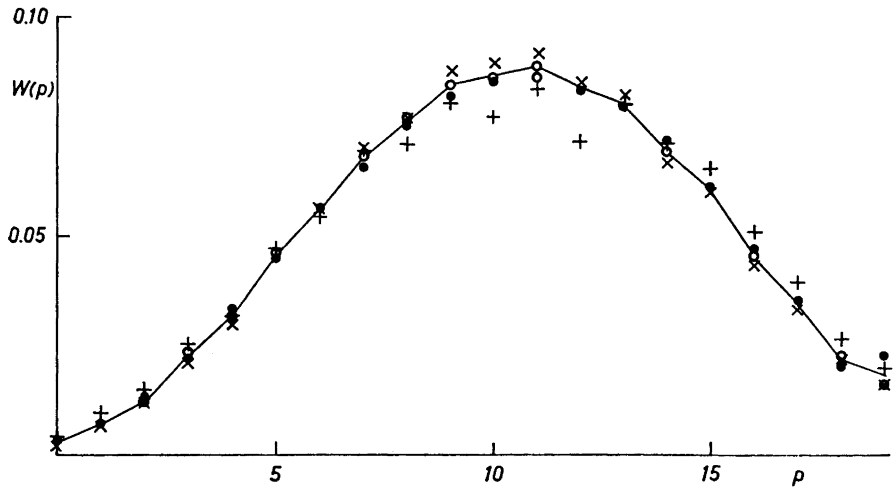


Fig. 5. $W(p)$ values obtained in the countercurrent experiments in TCW ($v = 0.83$) with polyglycol A 1 500. (Sample weights: *cf.* Fig. 1.) The curve represents the arithmetical mean of the $W(p)$ values from the 5 g, 2 g and 1 g experiments.

A 6 000. The irregularities in the experimental $W(p)$ *vs.* p curves were, however, less pronounced. The final results of the calculations can be seen in Table 1 and Fig. 4. The small peaks in the frequency curves at about $[\eta] = 18$ must, once again, be ascribed to slight errors in the experimental and numerical methods.

No emulsions appeared in the experiments in the solvent system TCW ($v = 0.283$) with polyglycol A 1 500. The experimental $W(p)$ values are given in Fig. 5. It can be seen that the points form curves with somewhat higher maxima at high concentrations. Craig [4] ascribes such a phenomenon to a nonmonotonic partition isotherm. The phenomenon is thus not equivalent to the concentration effect found in the experiments with the solvent system TAW [2]. The differences between the $W(p)$ values obtained at different sample weights are, however, small and the limiting viscosity numbers of the fractions proved to be independent of the sample weights. The calculations have therefore been made using the arithmetical mean of the experimental $W(p)$ values. The final results can be seen in Table 1 and Figs. 3 and 4. The small peak at $[\eta] = 9$ in Fig. 3 must be regarded as insignificant.

The final discussion concerning the accuracy of the frequency curves obtained will be given in the next paper of this series. It shall only be pointed out here that the double peak of the frequency curve of polyglycol A 6 000, as found in earlier experiments [1], has been verified once more.

From the experimental point of view, the work with the solvent system TCW did not differ from that with TAW, apart from the ease of emulsion formation and the dependence on temperature. As a compensation, however, the solvent system TCW has a higher resolving power. Furthermore, it has

been shown that small deviations from ideality do not appear in the final results provided that the experimental data are treated in the proper way.

EXPERIMENTAL

For experimental details, see earlier papers of this series [1,2]. The constant temperature (25°C) in the countercurrent experiments was obtained by placing the shaking device in an air thermostat.

The frequency *vs.* limiting viscosity number curves were calculated from the corresponding curves with respect to partition coefficient by means of a Litton digital differential analyzer equipped with graph follower and graph plotter.

REFERENCES

1. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1278.
2. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1263.
3. Almin, K. E. *Acta Chem. Scand.* **11** (1957) 1541.
4. Haussmann, W. and Craig, L. C. *J. Am. Chem. Soc.* **80** (1958) 2703.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1293–1298

Fractionation of Polymers by Liquid-Liquid Distribution

VI. Molecular Weight Frequency Curves for Polyglycols

KARL ERIK ALMIN

Swedish Forest Products Research Laboratory, Paper Technology Department, Stockholm, Sweden

The relation between the molecular weight and limiting viscosity number has been determined on unfractionated polyglycol preparations. By means of this relation earlier determined frequency functions of limiting viscosity number have been transformed into molecular weight frequency curves. The accuracy of the curves obtained is discussed.

Molecular weight frequency curves for polymers can be obtained from liquid-liquid countercurrent experiments in which the weights and the molecular weights of the fractions obtained have been determined. The mathematical treatment of the experimental data has been described in Ref.[1] The method for the molecular weight determination must be chosen so that absolute values are obtained for the molecular weights. Such determinations either require relatively large quantities of the samples or are time-consuming and require special techniques. In the experiments reported in the previous papers of this series [2,3], it was found that often only small quantities of the samples were obtained in the fractions. For this reason, the present author has chosen to determine the relation between the molecular weight and the limiting viscosity number on unfractionated samples and to then apply this relation to the previously determined curves of frequency *vs.* limiting viscosity number [2,3].

We assume that Mark's [4] modification of the Staudinger relation

$$[\eta] = K \cdot M^{x} \tag{1}$$

is valid *.

* In connection with work on polyglycols, the relation

$$[\eta] = K \cdot M + l$$

has been used by some other authors. Because the constants in eqn. 1 are sometimes of greater theoretical interest, this mathematical form has been preferred by the present author.

The average molecular weight of the sample can be written

$$\overline{M}^{a} = \int_{0}^{\infty} M^{a}\, g\, ([\eta])\, \mathrm{d}[\eta] \qquad (2)$$

where a is fixed by the conditions of the experimental method [4] and $g([\eta])$ is the weight frequency function in terms of the limiting viscosity number.

From eqns. 1 and 2, we obtain

$$\frac{1}{\overline{M}^{a}} \cdot \int_{0}^{\infty} [\eta]^{a/x} \cdot g([\eta]) \cdot \mathrm{d}[\eta] = K^{a/x} \qquad (3)$$

If $\overline{M}$ is measured for a number of different samples of the polymer with known $g([\eta])$ functions, the left hand side of eqn. 3 can be calculated for different x values. The correct value for x is the one which makes the value of the left hand side independent of the choice of sample.

Such a treatment of the problem has — as far as the author knows — not been performed earlier. The method generally used nowadays is to determine the molecular weight and the limiting viscosity number for fractionated polymer samples and to then regard the fractions as samples containing molecules of only one size. Eqn. 1 is then directly applicable to the experimental $\overline{[\eta]}$ values. Considering that the polymer samples prepared up to the present are far from homogeneous with respect to molecular weight, this method cannot be regarded as free from objections.

In this investigation, the end group molecular weight of the polyglycol preparations examined has been determined (Table 1). Thus $a = -1$ in the formulae above. In Table 2, the numerical values of the left hand side of eqn. 3 are given for different values of x for the polyglycol preparations reported in Parts IV and V [2,3] of this series. As can be seen, it is difficult to estimate the correct x value directly from the table. Because of this, the coefficient of variation ($= v$) has been calculated for the apparent constant at each x value. The magnitude v^2 has been plotted as a function of x. The values of x at which the minimum of this curve occurs is regarded as the correct value. The minimum value was determined under the assumption that the law concerning the rectilinear diameter is valid (Fig. 1). The value found is

Table 1. Number average molecular weights of the polyglycol preparations determined according to the end group method.

Sample	Number of dets.	Number average mol. weight
A 6 000	8	6 600 ± 40
A 4 000	9	3 620 ± 40
A 1 500	10	1 590 ± 30

Table 2. Left member of eqn. 3 calculated for the polyglycol preparations for different values of x.

	Left member of eqn. 3 ($a = -1$)				
Sample	A 1 500	A 4 000	A 4 000	A 6 000	A 6 000
Solvent system	TCW	TAW	TCW	TAW	TCW
$x = 0.5$	21.7	17.3	16.4	11.6	11.6
0.6	45.1	42.1	36.8	33.1	33.6
0.7	74.2	78.9	76.4	69.9	71.1
0.75	95.3	104.1	98.1	89.9	96.7
0.8	113.4	129.9	122.0	125.8	125.7
0.9	150.7	188.3	179.0	189.0	196.1
1.0	195.4	251.3	240.5	278.1	277.4
(∞)	(1 591)	(3 619)	(3 619)	(6 593)	(6 594)

$x = 0.74_5$. The arithmetical mean of the values of $K^{-1/x}$ interpolated from Table 2 was found to be 94.5. The Staudinger relation can thus be written

$$[\eta] = 3.38 \times 10^{-2} M^{0.75} \quad (4)$$

for polyglycol. The range of validity of this relation is difficult to estimate but a lower limit of about $M = 1\ 200$ and an upper limit of about $M = 9\ 000$ seem reasonable.

It is of interest to compare the above constants with those obtained when the calculations were carried out without regard to the width of the frequency curves. The $\overline{[\eta]}$ values used in these latter calculations were the arithmetical means of the values calculated from the experimental $[\eta]_p$ values for the fractions in the countercurrent experiments. The values of x and K were calculated according to the same principles as above. The v^2 *vs.* x curve obtained is given in Fig. 1 (dotted curve). The values found were $x = 0.77_4$ and $K = 2.68 \times 10^{-2}$. (The same result is obtained if the method of least squares is applied to the Staudinger relation after taking the logarithm of both sides). The $[\eta]$ *versus* M curve constructed using these latter x and K values differs only slightly from

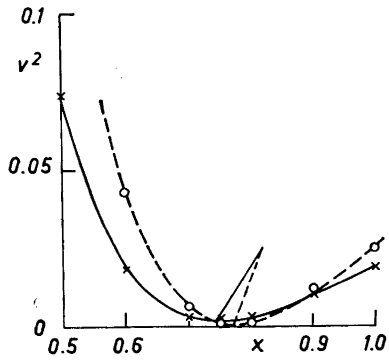


Fig. 1. Graphic determination of the exponent x in eqn. 1 for polyglycols.

the curve obtained from eqn. 4. In the range of validity, the differences amount to 0—3 %. However, the change in K is about 25 %. The experimental data on which the above calculations have been based are too few to permit a penetrating discussion, but the tendency is nevertheless clear.

Eqn. 4 has been used to transform the frequency curves. The results can be seen in Fig. 2. The full-drawn curves are the experimental curves. In the case of polyglycol A 4 000 and A 6 000, the curves from the experiments in the solvent system TCW are the arithmetical means of the curves obtained from the corresponding curves of frequency *vs.* limiting viscosity number. The dotted curves have been calculated using the formula derived by Flory [5].

The curves obtained with the solvent system TCW are more sharply peaked than those obtained with the solvent system TAW. This effect is more pronounced for polyglycol A 4 000 than for polyglycol A 6 000. The ratio of the resolving powers [6] of the systems TAW and TCW was calculated to be 1 : 7.4 in the case of polyglycol A 4 000, the molecules being assumed to have a molecular weight identical to the number average molecular weight of the sample. The curve obtained with the solvent system TCW must therefore be regarded to be the more reliable one. In the case of polyglycol A 6 000, the corresponding ratio was 1 : 3.0. The differences in the curves obtained are less pronounced than in the case of polyglycol A 4 000. The chief differences appear in the high molecular weight parts of the curves. The double peak of polyglycol A 6 000 could not be detected directly in the experimental $W(p)$ curves obtained in the TAW experiments [2]. This implies that the numerical treatment of the data from the TAW system, as well as the lower resolving power, can influence the results. In the case of polyglycol A 6 000, the results from the solvent system TCW must therefore be regarded as somewhat more reliable.

For polyglycol A 1 500, the resolving power is about 25 times higher in the solvent system TCW than that in the solvent system TAW. In view of the narrowness of the frequency curve, this ratio explains why it was impossible to perform any calculations of the frequency function using data from the TAW experiments.

The ratio of the resolving powers for the experiments with polyglycols A 6 000, A 4 000 and A 1 500 in the solvent system TCW was found to be 1 : 1.5 : 2.6. The final frequency curves obtained from data from the solvent system TCW must thus be regarded as nearly equivalent with regard to accuracy although, especially in the experiments with A 6 000, the formation of emulsions interfered considerably.

The agreement between the experimental frequency curves from the TCW experiments and the theoretically calculated frequency curves in Fig. 2 must be regarded as very good. The differences still remaining may be due to errors either in the author's experimental and theoretical treatment of the problem or in the theories of Flory [5]. The latter worker assumes that all steps in the polyreaction in which polyglycol is formed from glycol and ethylene oxide can be characterized by a single rate constant. If one assumes that the first steps of the reaction proceed with a higher velocity than the subsequent steps, then a sharper peaked frequency curve would be obtained. This assumption is only one of the possibilities for explaining the differences. Another

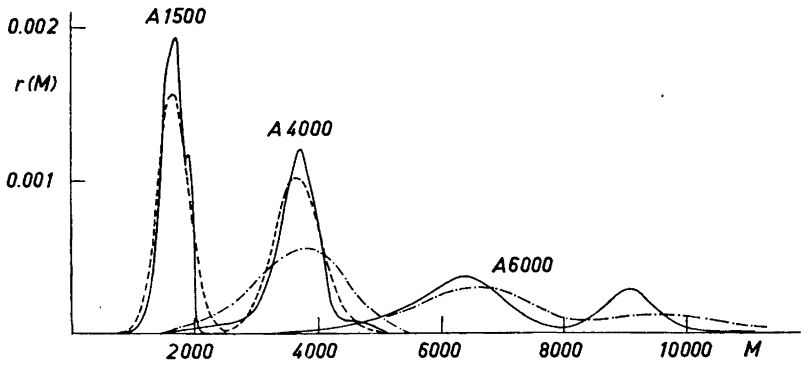


Fig. 2. Molecular weight frequency curves for the three polyglycol preparations investigated. ———— Curves obtained from the solvent system TCW; —·—·—·— Curves obtained from the solvent system TAW; — — — — — — Curves according to the theory of Flory.

possible reason for the differences between the calculated and experimental curves in Fig. 2 might be that the frequency curve of the sample is changed by the purification [2].

In any case, the frequency curves calculated using the theory of Flory are not consistent with the experimental $W(p)$ values if it is assumed that the experimentally obtained relation between limiting viscosity number and partition coefficient is correct. Approximate calculations show that the $W(p)$ values for preparation A 1 500 obtained from the theoretical frequency curve according to Flory [5], are about 0.010 lower than the experimental values for $8 < p < 11$. This difference must be considered to be far outside the experimental accuracy.

DISCUSSION

The countercurrent distribution of polymers between two immiscible solvents seems to lend itself admirably to the determination of molecular frequency functions. The chief difficulty is to find the two immiscible solvents. This problem will be treated in a forthcoming paper.

From the practical point of view, the method has the advantage over conventional methods that it is completely reproducible and this means that it is not necessary to make any time consuming calculations to decide whether two preparations of the same polymer are equal or not from the molecular frequency point of view. The equilibrium between the two phases is rapidly established, thus making it possible to obtain a large number of fractions in a relatively short time. The sensitivity to temperature is not so great in the investigated cases that an extremely accurate temperature control is required. In a given solvent system, the relation between molecular weight and partition coefficient is fixed. This means that, once this relation is known, only the relative amounts of polymer in each fraction have

to be determined. From these data, the frequency function can be calculated in terms of the partition coefficient and then converted, if required, into the molecular frequency curve. However, for a comparative study, such a recalculation may not be necessary. Sometimes the $W(p)$ *vs.* p curve gives all the information required.

The experimental results of this investigation confirm Flory's theoretical interpretation [5] of the reaction mechanism for the synthesis of polymers which are formed exclusively by the addition of monomers to a fixed number of polymer molecules.

Except for the highest molecular weight preparation examined in this investigation, the solvent system trichloroethylene-alcohol-water was shown to have a resolving power which was too low compared with the solvent system trichloroethylene-chloroform-water. The former solvent system, however, must not be regarded as useless. If the frequency curve of the polyglycol is broader than that for the preparations used in this investigation, then the system TCW might be unsuitable. Such polyglycol preparations can be obtained, for example, either from mixtures of preparations prepared in the common way or as a condensation polymer synthesized according to the method proposed by Flory [5]. It is then possible that all of the preparation may find its way into the first and last tubes of the countercurrent apparatus. An investigation in the solvent system TAW would, however, in such a case give the desired information.

For polyglycol preparations with molecular weights higher than the ones investigated in this series of articles it seems probable that the solvent system TAW or a solvent system composed of a mixture of trichloroethylene, chloroform, alcohol and water would be most suitable.

EXPERIMENTAL

The number average molecular weights of the polyglycol preparations investigated here were determined by titrimetric end group determinations. A weighed quantity of the preparation was heated under reflux for 1 h with 20 ml 6 % acetic anhydride solution in pyridine on a water bath. After cooling, 15 ml water was added and the refluxing continued for a further 10 min. The remaining liquid was then titrated with 0.5 N KOH in alcohol. A blank titration was carried out on the acetic anhydride-pyridine mixture under the same conditions as those for the sample. Phenolphthalein was used as indicator.

The apparent constants in Table 2 and the molecular weight frequency curves were calculated by means of a Litton digital differential analyzer equipped with graph follower and graph plotter.

REFERENCES

1. Almin, K. E. *Acta Chem. Scand.* **11** (1957) 1541.
2. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1278.
3. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1287.
4. Mark, H. *Die festen Körper*, Hirzel, Leipzig 1938.
5. Flory, P. J. *J. Am. Chem. Soc.* **62** (1940) 1561.
6. Almin, K. E. *Acta Chem. Scand.* **13** (1959) 1263.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1299–1304

Structure of a Glucomannan from *Pinus silvestris* L.

INGEMAR CROON, BENGT LINDBERG and HANS MEIER

Träkemiska avdelningen, Svenska Träforskningsinstitutet, Stockholm Ö, Sweden

Investigation of a glucomannan from *Pinus silvestris* has shown that it is built up very largely of β-1,4-linked sugar residues with about three branch points per molecule, most of which are attached to glucose residues at $C_{(3)}$.

A fractionation of the hemicellulose constituents of *Pinus silvestris* L. (Scots Pine) was reported in a previous communication from this Department [1]. A relatively pure glucomannan has now been prepared by similar methods from wood meal delignified by the chlorite method [2]. The polysaccharide fraction contained galactose, glucose, mannose, arabinose and xylose in the relative proportions 3.9:20.6:72.5:1.5:1.5 and had a $\overline{DP}_n$, determined osmometrically on the nitrate ester, of 97. It showed a typical lignin spectrum in the UV, with a lignin content estimated from the extinction at 280 mμ as 3—4 %. This lignin did not affect the DP-determination as it was completely lost during preparation of the nitrate ester. The lignin may be linked to the polysaccharide, and it seemed desirable to remove it before undertaking the structural investigation. Treatment with chlorine and ethanolic ethanolamine in a homogeneous medium repeated three times reduced the lignin content to about 0.2 %. The $\overline{DP}_n$ was reduced only to 93 by this treatment, which is an indication of the merit of this delignification method. Timell and Jahn [3] found that it causes only slight depolymerisation of polysaccharides, and this has been further confirmed by Glaudemans and Timell [4] in a recent investigation of birch wood hemicelluloses. — The relative proportions of galactose, glucose, mannose, arabinose and xylose in our delignified glucomannan sample were 3.0:21.1:74.0:traces:1.9, and were thus practically unchanged. The specific rotation was −31° in 2 N sodium hydroxide.

The glucomannan was methylated, first with methyl sulphate in sodium hydroxide and then with methyl iodide and silver oxide in dimethyl formamide. The product had a methoxyl content of 43.9 % and a $\overline{DP}_n$ value of 44. The methylation thus involved considerable degradation, which might also account for the rather low yield (45 %) of the methylated product. In a previous study [5] of the glucomannans from spruce (*Picea abies*), the $\overline{DP}_n$-values of the methylated products were not determined, but it must be assumed that the methylation, done in the same way as above, involved a similar depolymerisation.

The methylated polysaccharide was hydrolysed and the monomers obtained were fractionated by chromatography on a carbon-Celite column. The di-*O*-methyl ethers were only partially resolved and were further fractionated by paper chromatography and paper electrophoresis. This gave 2,6-di-*O*-methyl-D--glucose, which was the main component, smaller amounts of unidentified di-*O*-methyl-D-mannoses and of 2,3-di-*O*-methyl-D-xylose. 2,3,6-Tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-glucose were obtained in a pure state, followed by a small fraction that on demethylation gave galactose only. This latter fraction appeared homogeneous on chromatography in ordinary solvent systems but was resolved into two components by chromatography with *iso*propyl ether on dimethylsulphoxide-impregnated paper [6]. These had the same R_F-values as 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose, the substance corresponding to the first of these giving a much stronger spot than that corresponding to the second. 2,3,4,6-Tetra-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-methyl-D-glucose were obtained in separate fractions. In a previous investigation [5] these components were obtained as a mixture, the successful fraction in the present case is probably due to eluation with aqueous acetone, which gives more distinct fractions of highly methylated ethers than the aqueous ethanol previously used.

2,3-Di-*O*-methyl-D-xylose is the predominant xylose ether expected from a methylated xylan. The tri-*O*-methyl-D-galactose ethers are also normal products from the arabogalactans isolated from various coniferous woods. Polysaccharides of this type have recently been isolated from two pine species [7,8]. As these polysaccharides are highly branched, one should expect to find some di- and tetra-*O*-methyl-D-galactose ethers too, but as the percentage of galactose residues in the original material was quite low, these ethers might easily have been overlooked. We believe that the xylose and galactose ethers arose from contaminating polysaccharides and they are therefore not taken into account in Table 1, which gives the molar percentages of the ethers in the hydrolysates.

Table 1. Molar percentages of monomers in the hydrolysate of the methylated glucomannan.

Ether	%
Di-*O*-methyl-D-mannoses	2.6
2,6-Di-*O*-methyl-D-glucose	5.3
2,3,6-Tri-*O*-methyl-D-mannose	67.6
2,3,6-Tri-*O*-methyl-D-glucose	19.5
2,3,4,6-Tetra-*O*-methyl-D-mannose	4.2
2,3,4,6-Tetra-*O*-methyl-D-glucose	0.8

These percentages are quite similar to those found in studies on the glucomannans from *Picea abies* [5], and it can be concluded that the polysaccharides have a similar structure with chains of 1,4-β-linked mannose and glucose residues. The high percentage of tetra-*O*-methyl-hexoses indicates that the molecules are branched, containing about 3 branches per 100 hexose residues. The percentage of di-*O*-methyl-hexoses found is certainly too high, due to incomplete methylation and possibly also to some demethylation during the

hydrolysis. As 2,6-di-*O*-methyl-D-glucose predominates among the di-*O*-methyl-hexoses, it is reasonable to assume that most of the branches start from 3-positions in glucose residues. Studies on partially methylated celluloses [9] have shown, however, that the hydroxyl in the 3-position is methylated at a slower rate than those in the 2- and 6-positions, so that 2,6-di-*O*-methyl-D-glucose is the only di-*O*-methyl-D-glucose which should occur in significant amounts in the hydrolysate of a slightly undermethylated product. It can also be seen from Table 1 that most of the non-reducing end groups are mannose residues, as would be expected if a statistical distribution of mannose and glucose residues in the polysaccharide is assumed.

On periodate oxidation the glucomannan consumed 0.90 moles of periodate per hexose residue, with the formation of 0.12 moles of acids. This result indicates a higher branching than was found by the methylation study, and most probably is due to over-oxidation. The hydrolysate of the oxidised polysaccharide contained more glucose than mannose, their relative proportions being about 2:1. This provides further support for the assumption that the polysaccharide is branched and that most branches start from glucose residues.

The chain of 1,4-β-linked glucose and mannose residues seems to be a common feature of glucomannans isolated from coniferous woods [5,10–14]. These show glucose to mannose ratios of between 1:4 and 1:2. The proportion of glucose is increased in the partially hydrolysed polysaccharides [5]. A high glucose content in a glucomannan might indicate that its isolation from the wood has involved degradation or that the material is contaminated with cellulose. It seems fairly certain that the glucomannans from pine and spruce [5,12] have a slightly branched structure. The partially degraded polysaccharide from a hemlock sulphite pulp [13] showed no such branching. The undegraded glucomannan from spruce was amorphous but the partially hydrolysed product obtained from it was highly crystalline [15]. This difference is easily understood if the crystalline material is made up of linear fragments of the branched polysaccharide.

Indications have been recorded that coniferous woods contain galactoglucomannans as well as glucomannans [14,16–18]. Studies here on hemicellulose fractions isolated from holocellulose from pine and spruce wood have not given any support for this hypothesis. It is true that glucomannan fractions often contain considerable amounts of galactose and xylose residues which cannot be removed by further fractionation. We found, however, that these samples always contained a small percentage of lignin. After further delignification the contaminating polysaccharides could usually be separated from the glucomannans. A typical example of such a fractionation is given in the experimental part. A glucomannan that had been extracted with alkali from a pine holocellulose, after fractionation with barium hydroxide, contained 11 % galactose- and 5.5 % xylose residues. After delignification of the sample the galactose residues could be reduced to about 1 to 2 % and the xylose residues to traces by precipitation with barium hydroxide followed by precipitation twice with Fehling's solution. At the same time an arabogalactan or galactan was obtained which was free from glucose and mannose residues and had not been precipitated on the addition of Fehling's solution. We therefore believe that different polysaccharides, glucomannans and xylans as well as galactans

or arabogalactans may be linked to the same lignin molecule, which of course would make fractionation prior to further delignification impossible. The existence of linkage between lignin and hemicelluloses in woods has been strongly indicated by recent investigations by Björkman [19] and Lindgren [20]. The small amounts of galactose and xylose residues in our glucomannan used for methylation could be explained in the same way. The isolation, after partial hydrolysis of the polysaccharide, of oligosaccharides containing galactose and either glucose or mannose residues would of course prove the presence of galactoglucomannans but such proof has not yet been obtained*.

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 and on 3 MM filter papers. Chromatographic solvents: (*a*) ethyl acetate-acetic acid-water (3:1:3)
(*b*) butan-2-one, saturated with water
(*c*) 4 % dimethyl sulphoxide in benzene

Paper electrophoresis was carried out on Whatman No. 3 MM filter paper in borate buffer at pH 10.

Solutions were always concentrated under reduced pressure at a bath temperature of about 40°C.

Isolation of the glucomannan. Wood meal (18—35 mesh) from *Pinus silvestris* was extracted with acetone in a Soxhlet-type extractor and the residue was delignified by the chlorite method [8]. The product remaining after delignification amounted to 67.2 % of the acetone-extracted wood and contained less than 1 % Klason lignin. The holocellulose (297 g) was extracted successively under nitrogen with dimethyl sulphoxide, boiling water, 14 % potassium hydroxide and finally with 24 % potassium hydroxide and 3 % boric acid. The hemicelluloses from the last extract after acidification of the solution were precipitated with ethanol, dissolved in water and a glucomannan was precipitated by the addition of barium hydroxide [21]. The glucomannan was further purified by redissolving in water and precipitating once more with barium hydroxide. A sample (5.3 g) on hydrolysis gave 3.9 % galactose, 20.6 % glucose, 72.5 % mannose, 1.5 % arabinose and 1.5 % xylose. It contained between 3 and 4 % lignin (determined by UV-absorption [1]) and was therefore dissolved in water, and delignified by three successive chlorine-ethanolamine treatments as described earlier [1]. This reduced the lignin content to about 0.2 %. The delignified product used in the following methylation study had $[\alpha]_D^{20}$ −31° (c = 0.8, in 2.5 N sodium hydroxide) and on hydrolysis gave 3.0 % galactose, 21.1 % glucose, 74.0 % mannose, a trace of arabinose and 1.9 % xylose.

Determination of the $\overline{DP}_n$-values. The $\overline{DP}_n$-values were determined osmometrically on the nitrate-esters before and after the final delignification of the glucomannan and also on the methylated product. These gave values of 97.6, 93.0 and 43.7, respectively. The determinations were carried out in a Zim and Myerson osmometer and butyl acetate was used as solvent. The procedure has been described in an earlier paper [15].

Fractionation of a galactose-rich glucomannan. A glucomannan fraction which had been extracted with alkali from a pine holocellulose was purified by two precipitations with barium hydroxide [21] and then contained galactose, glucose, mannose, arabinose and xylose in the proportions 11.0:21.3:59.7:2.5:5.5. The lignin content was 5.3 %. After delignification by the chlorine-ethanolamine method, the sample was precipitated once with barium hydroxide and then contained galactose, glucose, mannose, arabinose and xylose in the proportions 10.1:20.6:64:2.6:2.7. The lignin had been reduced to 0.3 %. Part of the fraction (800 mg) was dissolved in water (100 ml), Fehling's solution (30 ml)

* *Added in proof.* From a partial hydrolysate of an easily soluble spruce hemicellulose fraction, rich in galactose residues, we have now isolated a galactosyl-mannose and a galactosyl-mannobiose. From these results, the presence of either a galactoglucomannan or a galactomannan, in addition to the previously known glucomannan is indicated.

was added and the precipitate was centrifuged. After washing once with water it was dissolved in 1 N hydrochloric acid, the hemicelluloses were precipitated with ethanol and the whole procedure was repeated. This sample (420 mg) was then hydrolysed and was found to be an almost pure glucomannan containing 1—2 % galactose and only traces of arabinose and xylose. After centrifuging the first precipitate with Fehling's solution, the solution was acidified with hydrochloric acid and dialysed, acidified again and then precipitated with ethanol, yielding a polysaccharide fraction (140 mg) which, on hydrolysis, gave about 50 % galactose, 10 % glucose, 30 % mannose, 5 % arabinose and 5 % xylose. This hemicellulose in aqueous solution was again treated with Fehling's solution. The precipitate formed was found to be a rather pure glucomannan but the centrifugate was free from mannose- and glucose residues and contained a galactan or arabogalactan contaminated with an araboxylan. A synthetic mixture of a pure glucomannan and a galactan from spruce wood [22] was easily separated into pure components by the same method.

Methylation of the glucomannan. The glucomannan (2 g) was dissolved in 22.5 % sodium hydroxide (50 ml), under nitrogen, and methyl sulphate (10 ml) was added over 1 h with vigorous stirring while the temperature was kept at 20° by external cooling. After 6 h the solution was neutralised with 6 N sulphuric acid and the polysaccharide was recovered by dialysis. Yield 1.98 g (OCH_3, 26.1 %).

The partially methylated product was dissolved in dimethyl formamide (30 ml) and methyl iodide (6 ml) was added in one portion. Silver oxide (6 g) was added over 1 h with vigorous stirring. After 20 h the insoluble material was centrifuged off and washed with dimethyl formamide (10 ml) and chloroform (10 ml). The combined centrifugates were concentrated. The resulting syrup was treated with benzene-chloroform (1:1, 20 ml) and the undissolved salts were centrifuged off. The solid residue was washed with benzene (20 ml). The combined centrifugates were evaporated to dryness and then methylated again three times using the procedure described above. The product finally obtained (0.89 g) had a OCH_3-content of 43.9.

Hydrolysis of the methylated glucomannan. The methylated glucomannan (0.89 g) was dissolved in anhydrous formic acid and kept at 100°C for 6 h. The solution was concentrated and the resulting syrup was dissolved in 0.5 N sulphuric acid and kept at 100°C for 15 h. This solution was then neutralised with barium carbonate, giving finally a syrup (0.75 g). Chromatographic examination of the hydrolysate (Solvent *a*) revealed the presence of di-, tri- and tetra-*O*-methyl hexoses but no glucose, mannose or mono-*O*-methyl hexoses.

Examination of the hydrolysis products from methylated glucomannan. The hydrolysate from the methylated glucomannan (753 mg) was added to the top of a carbon-Celite column (43 × 3.5 cm) which was then developed by gradient elution with the following solvents:

10 % → 30 % aqueous ethanol	5 000 ml
30 % aqueous ethanol → 30 % aqueous acetone	3 000 »

The eluate was divided into fractions (30 ml) which were examined on paper chromatograms (Solvent *a*). Those fractions which were indistinguishable were combined and taken to dryness.

Fraction 1. (54 mg.) Five components were isolated by paper electrophoresis. The principal component on demethylation gave glucose only and was chromatographically and electrophoretically indistinguishable from authentic 2,6-di-*O*-methyl-D-glucose. The components were fractionated, in two separate experiments, on paper chromatograms (Solvent *b*) and on paper electrophoretograms, and quantitative determinations of the principal components were made by hypoiodite oxidation [23]. Three of the components isolated gave mannose only on demethylation but were not further investigated. The fifth component was shown to be 2,3-di-*O*-methyl-D-xylose (17 mg).

Fraction 2. (395 mg.) This fraction on demethylation yielded mannose only. An aniline derivative was prepared, m. p. 125—126°, alone or in admixture with authentic 2,3,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine. This fraction was chromatographically homogeneous.

Fraction 3. (99 mg.) The material crystallised when the solvent was evaporated. One crystallisation from ethyl acetate yielded the pure substance, m. p. 117—119°, undepressed on admixture with authentic 2,3,6-tri-*O*-methyl-D-glucose. Chromatograms of the mother liquor indicated traces of an unknown substance with a slightly higher

R_F-value (Solvent *a*) than 2,3,6-tri-*D*-methyl-D-glucose. This was probably the same substance as was previously isolated [5] and that had been shown to be a tri-*O*-methyl-D-glucose, distinguishable from 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose.

Fraction 4. (17 mg) on demethylation yielded galactose only. Chromatography on dimethyl sulphoxide treated paper with solvent *c* separated the fraction into two components with the same R_F-values as 2,3,4-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose. The spot corresponding to the former had the lowest R_F-value and was the principal substance in this fraction.

Fraction 5. (26 mg) gave mannose only on demethylation. The aniline derivative, m. p. 142—144°C, was not depressed on admixture with the aniline derivative of 2,3,4,6-tetra-*O*-methyl-D-mannose.

Fraction 6. (5 mg) on demethylation yielded glucose only. It was chromatographed on dimethyl sulphoxide treated paper using *iso*propyl ether as solvent, and gave an elongated spot with the same R_F-values as authentic 2,3,4,6-tetra-*O*-methyl-D-glucose (0.56) but higher than 2,3,4,6-tetra-*O*-methyl-D-mannose (0.47).

Periodate oxidation studies. The glucomannan (373 mg) was kept in water (50 ml) overnight in the refrigerator (2—4°). Sodium metaperiodate solution (0.32 M, 25 ml) was then added and the volume was adjusted to 125 ml. The solution was kept in the refrigerator and samples (5 ml) were withdrawn at intervals and analysed for periodate consumption and for the formation of acid. The results are summarised in Table 2.

Table 2. Periodate oxidation of the glucomannan.

Time, h	Moles of periodate consumed per $C_6H_{10}O_5$	Acid equivalents per $C_6H_{10}O_5$
60	0.71	0.061
72	0.86	0.076
100	0.89	0.108
140	0.92	0.136
200	0.95	0.144
350	0.97	0.148

REFERENCES

1. Meier, H. *Acta Chem. Scand.* **12** (1958) 1911.
2. Wise, L. E., Murphy, M. and D'Addieco, A. A. *Paper Trade J.* **122** (1946) No. 2, 35.
3. Timell, T. E. and Jahn, E. C. *Svensk Papperstidn.* **54** (1951) 831.
4. Glaudemans, C. P. J. and Timell, T. E. *Svensk Papperstidn.* **60** (1957) 869.
5. Croon, I. and Lindberg, B. *Acta Chem. Scand.* **12** (1958) 453.
6. Wickberg, B. *Acta Chem. Scand.* **12** (1958) 615.
7. Wadman, W. H., Anderson, A. B. and Hassid, W. Z. *J. Am. Chem. Soc.* **76** (1954) 4097.
8. Bishop. C. T. *Can. J. Chem.* **35** (1957) 1010.
9. Croon, I. *Svensk Papperstidn.* **61** (1958) 919.
10. Hamilton, J. K., Kirchner, H. N. and Thompson, N. S. *J. Am. Chem. Soc.* **78** (1956) 2508.
11. Aspinall, G. O., Laidlaw, R. A. and Rashbrook, R. B. *J. Chem. Soc.* **1957** 4444.
12. Merler, E. and Wise, L. E. *Tappi* **41** (1958) 80.
13. Hamilton, J. K. and Kirchner, H. N. *J. Am. Chem. Soc.* **80** (1958) 4703.
14. Hamilton, J. K. and Partlow, E. V. *J. Am. Chem. Soc.* **80** (1958) 4880.
15. Lindberg, B. and Meier, H. *Svensk Papperstidn.* **60** (1957) 785.
16. Ball, D. J., Jones, J. K. N., Nicholson, W. H. and Painter, T. J. *Tappi* **39** (1956) 438.
17. Hamilton, J. K. and Thompson, N. S. *Pulp and Paper Mag. Canada* **59** (1958) 233.
18. Adams, G. A. *Can. J. Chem.* **36** (1957) 721.
19. Björkman, A. *Svensk Papperstidn.* **60** (1957) 243.
20. Lindgren, B. O. *Acta Chem. Scand.* **12** (1958) 447.
21. Meier, H. *Acta Chem. Scand.* **12** (1958) 144.
22. Bouveng, H.O. and Meier, H. *Acta Chem. Scand.* **13** (1959). *In press.*
23. Hirst, E. L., Hough, L. and Jones, J. K. N. *J. Chem. Soc.* **1949** 59.

Received December 23, 1958.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1305–1308

Fungus Pigments

XI.* 2-Amino-3-hydroxymethylphenol from Cinnabarin

JARL GRIPENBERG

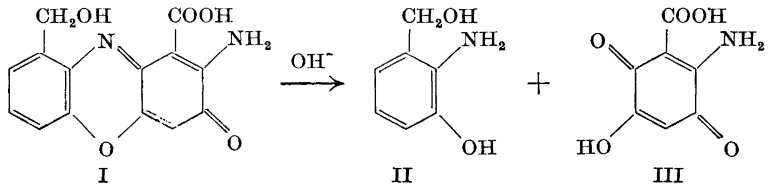
Department of Chemistry, Finland Institute of Technology, Helsingfors, Finland

Alkaline hydrolysis of cinnabarin has yielded 2-amino-3-hydroxymethylphenol, whose structure was established by synthesis. This definitely proved the presence of a hydroxymethyl group in cinnabarin.

In earlier papers of this series [1–3] evidence has been advanced supporting structure I for cinnabarin. The same structure was independently proposed by Cavill *et al.* [4,5].

Direct proof for the presence of the hydroxymethyl group was, however, lacking. That cinnabarin has a side chain in position 9 was shown by the formation of benzoxalone-4-carboxylic acid upon oxidation [1], but its nature was only indirectly deduced [2,6].

As has already been described,[2,3] cinnabarin treated with alkali yields cinnaquinone, which is most probably 3-amino-6-hydroxybenzoquinone-2-carboxylic acid (III). Cinnaquinone originates evidently from the quinonoid part of the phenoxazinone ring system. Assuming that its formation is due to a simple hydrolytic cleavage of the central ring, 2-amino-3-hydroxymethylphenol (II), should be formed from the benzenoid ring.



I II III

The mother liquor remaining after the sodium salt of cinnaquinone had been removed [2], upon saturation with carbon dioxide and extraction with ether did indeed give a compound with the properties to be expected of II.

Part X, *Acta Chem. Scand.* **12** (1958) 1762.

It had the composition $C_7H_9NO_2$ and was readily soluble in water but difficultly soluble in hydrocarbons. Acetylation gave a tri-acetate. The U.V.-spectrum had a maximum at 290 mμ (log ε 3.78) and an inflexion at $\sim$ 230 mμ. It showed great similarity to the spectrum of *o*-aminophenol [7]. In alkaline solution a bathochromic shift of the maximum to 302 mμ (log ε 3.63) occurred. In acidic solution the maximum was shifted to 275 mμ (log ε 3.36). The spectrum was then practically identical with that of *m*-cresol [8].

The spectrum of the acetate was somewhat anomalous. One would have expected it to be similar to the spectrum of *o*-aminophenol diacetate, which has a maximum at 236 mμ (log ε 4.06) with a very weak inflexion at 280 mμ. (Le Rosen and Smith [9] report 238 mμ (log ε 3.93) for this maximum). Instead, it had a weak maximum at 260 mμ (log ε 2.74) and only end absorption down to 220 mμ.

The maximum of *o*-aminophenol diacetate is certainly due to the acetanilide group, as shown by the very similar absorption of acetanilide, with maxima at 242 mμ (log ε 4.16) and 280 mμ (log ε 2.70) [10]. Apparently the acetoxymethyl group in 2-amino-3-hydroxymethylphenol triacetate sterically hinders the conjugation between the aromatic ring and the acetamide group and the absorption is probably essentially that of the benzene ring. Alkyl groups in the *ortho*-position interfere sterically with this conjugation as has been noted earlier [10,11].

The fact that the compound obtained from cinnabarin was indeed 2-amino-3-hydroxymethylphenol (II) was finally proven through its synthesis by the lithium aluminium hydride reduction of methyl 3-hydroxyanthranilate. This was prepared according to Angyal *et al.*[12], with minor modifications, as indicated in the experimental part.

The reduction of methyl 3-hydroxyanthranilate did not proceed very well. The reduction was first carried out in boiling ether using a large excess of lithium aluminium hydride. Although this method gave some of the desired compound, considerable hydrogenolysis to 2-amino-*m*-cresol occurred. This supports the results of Conover and Tarbell [13], who report considerable hydrogenolysis in the reduction of methyl anthranilate, particularily if the reaction time is extended to several hours. In order to minimise the hydrogenolysis, attempts were made to carry out the reduction by reverse addition of the theoretical amount of lithium aluminium hydride. A heavy precipitate was formed at once, however, and even after boiling the mixture for several hours, considerable unreacted lithium aluminium hydride remained in the solution. Only a very minute amount of 2-amino-3-hydroxymethylphenol could be isolated using this particular method. The use of methyl 3-acetoxy anthranilate was similarly unsatisfactory.

The best procedure was finally found to be by slowly adding methyl 3-hydroxy anthranilate at room temperature to a moderate excess of lithium aluminium hydride, and allowing the mixture to stand for some hours at room temperature. In this way, a yield of approximately 50 % 2-amino-3-hydroxymethylphenol was obtained. Its m.p. was slightly lower than that found for the substance obtained from cinnabarin but no m.p. depression was observed in a mixture test. Its rather high carbon content indicated the presence of some 2-amino-*m*-cresol. It was best characterised as its triacetate,

which was readily obtained in a pure state. There was no m.p. depression when this was mixed with the corresponding derivative obtained from cinnabarin, and their I.R.-spectra were identical.

EXPERIMENTAL

3-Methoxy-2-nitrobenzoic acid was prepared according to Nyc and Mitchell [14]. For the separation of 3-methoxy-2-nitrobenzoic acid from the other isomers, the reaction mixture was completely dissolved in the minimum quantity of hot ethyl alcohol. Upon cooling, large glass-like crystals first appeared. When small clusters of white needles began to separate on the surface of the big ones, the crystals were filtered off and recrystallised once from ethyl alcohol. In this way a very consistent yield of 1.15 g of 3-methoxy-2-nitrobenzoic acid (m. p. 260—265°; lit.[14] 260—263°) was obtained from 10 g *m*-methoxybenzoic acid. When attempts were made to carry out the reaction on a larger scale, difficulties were encountered in regulating the reaction temperature.

3-Hydroxy-2-nitrobenzoic acid was prepared according to Angyal *et al.*[12] The pyridine hydrochloride melt was dissolved in water and the solution thoroughly extracted with ethyl acetate. After drying this was evaporated under vacuum to a small volume. Light petroleum was then added. 3-Hydroxy-2-nitrobenzoic acid crystallised as yellow crystals, m. p. 178—180° (Hegedüs [15] gives m. p. 176—178° for a crude product). Yield 1.6 g from 2 g 3-methoxy-2-nitrobenzoic acid.

Methyl 3-hydroxy-2-nitrobenzoate was prepared from the foregoing by esterification with methyl alcohol and sulphuric acid. Recrystallised from water, it formed colourless needles, of m. p. 114—115° (lit.[16] 115°).

Methyl 3-hydroxy anthranilate. Methyl 3-hydroxy-2-nitrobenzoate (2 g) dissolved in alcohol was hydrogenated with a palladium-on-charcoal catalyst at room temperature and ordinary pressure. In four hours 680 ml of hydrogen were taken up. The solution was evaporated to dryness under vacuum. The residue was recrystallised from an ethyl-acetate-light petroleum mixture, giving almost colourless crystals (0.8 g), m. p. 97—98° (lit.[17] 94°).

The mother liquor contained more of the same substance, but this was contaminated with considerable red-coloured material, from which it was difficult to free the crystals.

Methyl 3-acetoxy-2-nitrobenzoate. Methyl 3-hydroxy-2-nitrobenzoate was acetylated with acetic anhydride and a drop of pyridine. After standing over night the mixture was poured into water. Methyl 3-acetoxy-2-nitrobenzoate separated as an oil which solidified on scratching the container. Recrystallisation from light petroleum gave colourless crystals m. p. 54—54.5°. (Found: C 50.58; H 3.89. $C_{10}H_9NO_6$ requires C 50.21; H 3.79).

Methyl 3-acetoxyanthranilate. Methyl 3-acetoxy-2-nitrobenzoate (2.25 g) dissolved in ethyl alcohol was hydrogenated with a palladium-on-charcoal catalyst at room temperature and ordinary pressure. The hydrogen consumption amounted to 600 ml (Theor. 635 ml). The solution was evaporated under vacuum and turned to a dark red colour. Benzene was added to the residue and a red precipitate was filtered off. Light petroleum was carefully added to the benzene solution precipitating dark impurities. The almost colourless solution was evaporated to dryness under vacuum. Sublimation (90—100°/0.02 mm) and further crystallisation from ligroin gave colourless crystals (0.66 g), m. p. 94—95°. (Found: C 57.05; H 5.36. $C_{10}H_{11}NO_4$ requires C 57.41; H 5.30). It gave a large m. p. depression when mixed with methyl 3-hydroxyanthranilate, m. p. 97—98°.

2-Amino-3-hydroxymethylphenol (II). a) Methyl-3-hydroxyanthranilate (500 mg) dissolved in ether was added dropwise to a stirred suspension of lithium aluminium hydride (500 mg) in ether. The mixture was allowed to stand 2 h. Water was then added carefully to destroy the excess of lithium aluminium hydride. A little sodium hydroxide was added to facilitate the dissolving of the precipitate. The aqueous layer was separated and saturated with carbon dioxide. It was then extracted thoroughly with ether until a sample of the extract left no crystalline residue. The precipitate which formed during the introduction of carbon dioxide did not interfere with the extraction. It was found advisable to introduce more carbon dioxide from time to time during the extraction because the pH showed a tendency to rise.

The ether extract was evaporated under vacuum and the residue dissolved in a small amount of water, filtered and evaporated under vacuum. The red oily residue was treated with benzene whereupon it crystallised. The crystals were filtered off and addition of light petroleum to the mother liquor gave more of the same crystals. Altogether 190 mg were obtained. Recrystallisation from ethyl acetate-benzene and subsequent sublimation (120—130°/0.01 mm) yielded colourless needles, m. p. 124—125°. (Found: C 61.37; H 6.49; N 10.33. $C_7H_9NO_2$ requires C 60.42; H 6.52; N 10.07). Main I.R.-maxima (KBr-disc): 3 510, 3 430, 3 330, 3 100, 2 880, 1 630, 1 583, 1 500, 1 472, 1 373, 1 305, 1 280, 1 265, 1 168, 1 080, 1 000, 980, 938, 925, 865, 773 and 721 cm^{-1}.

The acetate of 2-amino-3-hydroxymethylphenol was prepared by acetylation with acetic anhydride and a drop of pyridine. After standing over night the mixture was evaporated under vacuum and the crystalline residue recrystallised from benzene-light petroleum, m. p. 117°. (Found: C 58.64; H 5.66; N 5.69. $C_{13}H_{15}NO_5$ requires C 58.86; H 5.70; N 5.28.) Main I.R.-maxima (KBr-disc): 3 280, 3 040, 2 800, 1 778, 1 748, 1 656, 1 590, 1 517, 1 469, 1 451, 1 432, 1 371, 1 265, 1 218, 1 192, 1 088, 1 062, 1 018, 965, 860, 783, 753 and 733 cm^{-1}.

b) Cinnabarin (400 mg) was treated with 2 N sodium hydroxide as previously described [3]. After filtering off the sodium salt of cinnaquinone, the alkaline solution remaining was saturated with carbon dioxide, and thoroughly extracted with ether. The residue from the ether extract was dissolved in a small amount of water, filtered and evaporated under vacuum. The crystalline residue was sublimed at 100—110°/0.01 mm. The sublimate (40 mg) was recrystallised from ethyl acetate-benzene and found to have a m. p. of 125—126°. (Found: C 60.70; H 6.35; N 9.91. $C_7H_9NO_2$ requires C 60.42; H 6.52; N 10.07.) No m. p. depression was observed when the sublimate was mixed with material prepared according to a).

The acetate was prepared as described above and had m. p. 117°. It gave no m. p. depression when mixed with the corresponding derivative described under a), and their I.R.-spectra were identical.

The analyses have been carried out by Dr. A. Bernhardt, Mülheim, Germany. The I.R.-spectra have been taken by Mr. J. Miettinen of this department.

REFERENCES

1. Gripenberg, J., Honkanen, E. and Patoharju, O. *Acta Chem. Scand.* **11** (1957) 1485.
2. Gripenberg, J. *Proc. Chem. Soc.* **1957** 233.
3. Gripenberg, J. *Acta Chem. Scand.* **12** (1958) 603.
4. Cavill, G. W. K., Clezy, P. S. and Tetaz, J. R. *Proc. Chem. Soc.* **1957** 346.
5. Cavill, G. W. K., Clezy, P. S., Tetaz, J. R. and Werner, R. L. *Tetrahedron* **5** (1959) 275.
6. Gripenberg, J. and Kivalo, P. *Suomen Kemistilehti* **B 30** (1957) 134.
7. Morton, R. A. and McGookin, A. *J. Chem. Soc.* **1934** 901.
8. Hodgson, H. H. *J. Chem. Soc.* **1943** 380.
9. Le Rosen, A. L. and Smith, E. D. *J. Am. Chem. Soc.* **70** (1948) 2705.
10. Ungnade, H. E. *J. Am. Chem. Soc.* **76** (1954) 5133.
11. Grammatikacis, P. *Bull. soc. chim. France* **1949** 134.
12. Angyal, S. J., Bullock, E., Hanger, W. G., Howell, W. C. and Johnson, A. W. *J. Chem. Soc.* **1957** 1592.
13. Conover, L. H. and Tarbell, D. S. *J. Am. Chem. Soc.* **70** (1948) 3586.
14. Nyc, J. F. and Mitchell, H. K. *J. Am. Chem. Soc.* **70** (1948) 1847.
15. Hegedüs, B. *Helv. Chim. Acta* **34** (1951) 611.
16. Senoh, S., Seki, F. and Kikkawa, H. *J. Inst. Polytech., Osaka City Univ., Ser. C* **3** (1952) 59.
17. Boyland, E. and Sims, P. *J. Chem. Soc.* **1954** 980.

Received May 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1309–1331

Benzaurinstudien

II. Gleichgewichte und Reaktionskinetik des 3,3′,5,5′-Tetrabrom-benzaurins und des 3,3′-Dibrom-5,5′-dimethyl-benzaurins in wässrigen Lösungen

HANS GUSTAF HANSSON

Abteilung für anorganische und physikalische Chemie des Chemischen Instituts der Universität Lund, Schweden

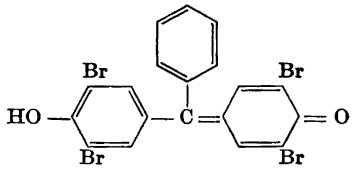
The equilibrium conditions of 3,3',5,5'-tetrabromo-benzaurin and 3,3'-dibromo-5,5'-dimethyl-benzaurin in aqueous solutions have been quantitatively investigated. They can be expressed in the scheme stated. The scheme is made up as follows. Three coloured forms are in instantaneous pH-equilibrium with each other. Further, there are three colourless forms likewise in instantaneous pH-equilibrium with each other. A coloured form is converted into the corresponding colourless form by the reaction with water. The taking up of water is, according to circumstances, a time reaction which appears as a fading of the colour of the solution. All the reactions are reversible.

By measurements of the light absorption at equilibrium in a series of solutions with different pH values and by determination of the reaction rate of the fading process at different pH values the author has succeeded in determining the equilibrium and velocity constants of the scheme.

The constants determined are referred to the ionic strength 0.50 M and a solvent with the composition 98 % water + 2 % acetone. The light absorption measurements were performed with a Beckman Quartz Spectrophotometer Model DU. The temperature was 20°C.

Während der letzten 'Jahre sind mehrere wichtige quantitative Untersuchungen über das Gleichgewichtsschema von Triphenylmethanfarbstoffen in wässrigen Lösungen ausgeführt worden. Bisher sind vor allem basische Triphenylmethanfarbstoffe vom Malachitgrüntypus untersucht worden [1–4]. Eine frühere Arbeit [5] über das Aurin, 4,4'-Dihydroxy-fuchson, (vgl. auch die Beobachtungen bei Phenolphtalein [6] und frühere einzelne Ergebnisse bei Benzaurin [7–14]) machen es wahrscheinlich, dass bei den sauren Triphenylmenthanfarbstoffen vom Benzaurintypus das Gleichgewichtsschema dem der basischen Triphenylmethanfarbstoffe vom Malachitgrüntypus ähnelt.

Die vorliegende Untersuchung über Tetrabrom-benzaurin und Dibrom-dimethyl-benzaurin hat diese Annahme bestätigt.



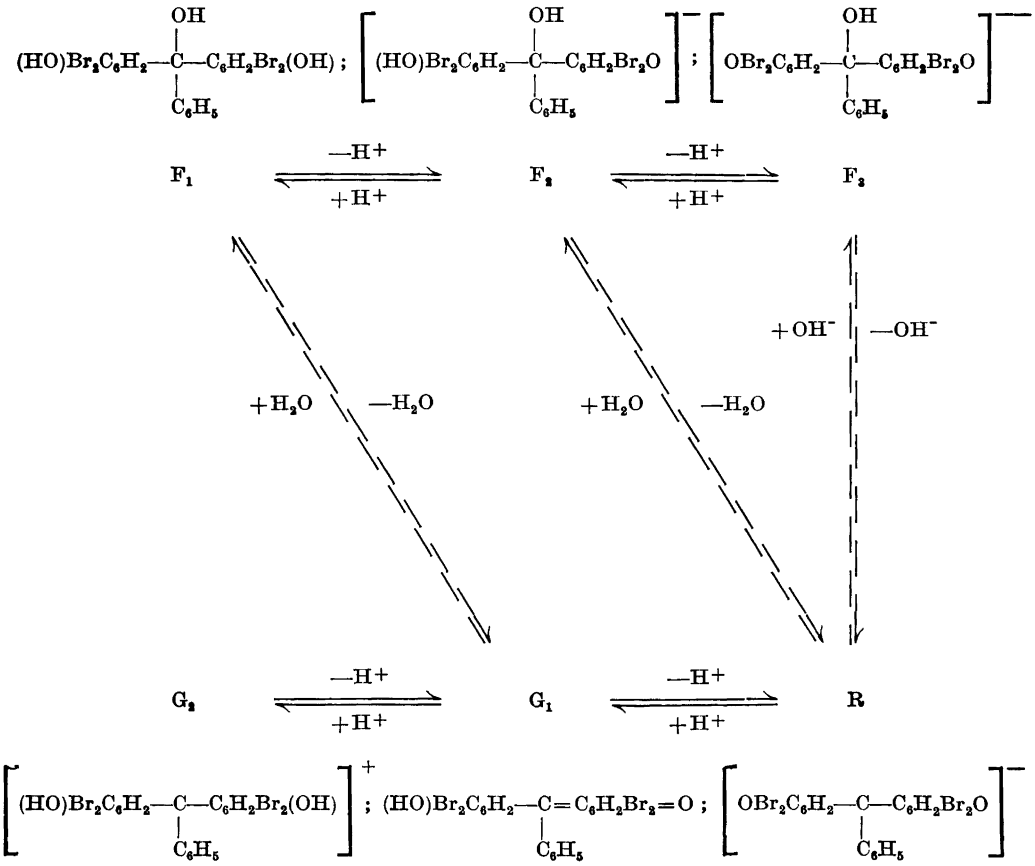
Tetrabrom-benzaurin (Anhydrid)

Das System kann also in wässrigen Lösungen durch das folgende Reaktionsschema beschrieben werden. Das Schema ist das möglichst Einfache und sagt über den Reaktionsverlauf in den einzelnen Stufen nichts aus.

In der Folge werden schematisch die Symbole R, G_1, G_2, F_1, F_2 und F_3 statt der vollständigen Formeln benutzt. Die Gleichgewichtskonstanten des Schemas werden wie folgt definiert:

Die Protolysenkonstanten

$$K_1 = \frac{[G_1]}{[H^+]\cdot[R]}\,;\; K_7 = \frac{[H^+]\cdot[G_1]}{[G_2]}\,;\; K_3 = \frac{[H^+]\cdot[F_2]}{[F_1]}\,;\; K_4 = \frac{[H^+]\cdot[F_3]}{[F_2]}$$



Die Hydratationskonstanten bei konstanter Aktivität des Wassers

$$K_2 = \frac{[F_1]}{[G_1]}\,;\, K_6 = \frac{[R]}{[F_2]}$$

Aus den Definitionen ergibt sich $K_1 \cdot K_2 \cdot K_3 \cdot K_6 = 1$. Ferner ist $K_5 = K_6/K_4$.

Die Geschwindigkeitskonstanten

k_1 für die Reaktion $G_1 + H_2O \rightarrow F_1$
k_2 » » » $F_1 \rightarrow G_1 + H_2O$
k_3 » » » $R + H_2O \rightarrow F_2$ einschliesslich $R + OH^- \rightarrow F_3$
k_4 » » » $F_2 \rightarrow R + H_2O$ » $F_3 \rightarrow R + OH^-$

k_1 und k_3 enthalten die Konzentration des Wassers. Es gilt:
$k_2 = k_1 \cdot 1/K_2$ und $k_4 = k_3 \cdot K_6$.

Folgende generelle Annahmen, die durch halbquantitative Vorversuche bestätigt worden sind, liegen der Bestimmung der zu dem Schema gehörigen Konstanten zu Grunde.

1. Die Reaktionen $R \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} G_1 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} G_2$ sowie $F_3 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} F_2 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} F_1$ sind momentan. Die Reaktionen $G_1 + H_2O \rightleftharpoons F_1$ und $R + H_2O \rightleftharpoons F_2$ (einschliesslich $R + OH^- \rightleftharpoons F_3$) sind aber Zeitreaktionen, die wenigstens in gewissen pH-Gebieten * mit messbarer Geschwindigkeit verlaufen. Der Unterschied ist im vorangehenden Schema durch ausgezogene bzw. gestrichelte Reaktionspfeile angedeutet.

2. Die Anhydridformen R, G_1 und G_2 sind farbig, die Karbinolformen F_1, F_2 und F_3 dagegen farblos.

3. Eine Lösung der Farbstoffe in wasserfreiem Aceton enthält nicht die farblosen Karbinolformen F_1, F_2 und F_3.

Es wird auf diese Annahmen hier nicht näher eingegangen; es soll aber kurz darauf hingewiesen werden, dass die gute optische Beständigkeit und Reproduzierbarkeit von Lösungen des Farbstoffes in Aceton die Annahme 3 fast völlig begründet.

Von diesen Annahmen her kann man das Gleichgewichtssystem durch Messungen von zusammengehörigen Werten der Lichtabsorption und Wasserstoffionenkonzentration aufklären, da der molekulare Extinktionskoeffizient einer Lösung des Farbstoffes beim Gleichgewicht — bzw. bei der Zeit $t = 0$ (Moment des Mischens) — immer eine eindeutige Funktion der Wasserstoffionenkonzentration ist. In einigen Fällen ist es dabei möglich, die von Cigén [3] abgeleiteten Formeln bei den Malachitgrünfarbstoffen zur Berechnung zu verwenden.

Zur Ermittelung der Geschwindigkeitskonstanten, die im Gegensatz zum Verhalten der Malachitgrünfarbstoffe [3] von der Wasserstoffionen-(bzw. Hydroxylionen-)konzentration abhängig sind, misst man bei verschiedenen Wasserstoffionenkonzentrationen die Geschwindigkeit mit welcher sich ein frisch bereitetes Gemisch von 1—2 ml einer Acetonstammlösung des Farbstoffes mit einem geeigneten wässrigen Puffer (99—98 ml) durch teilweise Umlagerung in die farblosen Formen aufhellt.

* pH bedeutet in dieser Arbeit immer $-\log[H^+]$.

ABLEITUNG VON FORMELN

I. Berechnungen der Anfangsextinktionen. Bestimmung von K_1

Beim Mischen einer Pufferlösung mit einer Stammlösung des Farbstoffes in Aceton stellt sich das Gleichgewicht $R + H^+ \rightleftharpoons G_1$ momentan ein. Die Mischung wird allmählich durch die Reaktionen

$$G_1 + H_2O \rightarrow F_1 \text{ und } R + H_2O \rightarrow F_2$$

aufgehellt. Die Aufhellung, die ohne Änderung des Farbtons stattfindet, wird durch Messung der Lichtextinktion E der Lösung verfolgt. Log $(E - E_\infty)$, als Funktion der Zeit (t) aufgetragen, ergibt eine Gerade. Extrapolation auf $t = 0$ gibt E_0, welches die Lichtextinktion im Augenblick des Mischens ist. Bei $t = 0$ liegt der Farbstoff nur als G_1 und R vor *. E wird bei einer Wellenlänge gemessen, wo nur R absorbiert. Also gilt

$$K_1 = \frac{[G_1]}{[H^+] \cdot [R]} \; ; \; E_0 = E_R \cdot \frac{[R]}{C}$$

wo die Gesamtkonzentration des Farbstoffes $C = [R] + [G_1]$ ist und $E_R =$ die Extinktion für eine Lösung, in der $[R] = C$ ist (bei unveränderter Schichtdicke). Man bekommt

$$\frac{1}{E_0} = \frac{1}{E_R} + \frac{[K_1] \cdot [H^+]}{E_R} \qquad (1)$$

Für eine Reihe von Lösungen mit verschiedenen, geeignet gewählten $[H^+]$-Werten und mit gleicher Gesamtkonzentration des Farbstoffes werden bei gleicher Schichtdicke teils $[H^+]$, teils E_0 (durch Extrapolation wie oben erwähnt) bestimmt. $1/E_0$ wird als Funktion von $[H^+]$ aufgetragen und gibt nach (1) eine Gerade; aus deren Schnittpunkt a_1 mit der Ordinate und dem Richtungskoeffizienten b_1 erhält man

$$E_R = 1/a_1 \; ; \; K_1 = b_1/a_1$$

II. Berechnungen aus den Gleichgewichtsextinktionen

Beim Mischen von einer Lösung des Farbstoffes in Aceton mit einer Pufferlösung erfolgt, wie oben erwähnt, eine Aufhellung. Nachdem Gleichgewicht eingetreten ist, liegen in der Lösung die Formen G_1, R, F_1, F_2 und F_3 vor *.

A. Wenn $[H^+] \ll 1/K_1$ ist, kann $[G_1]$ vernachlässigt werden und man hat mit den Formen R, F_1, F_2 und F_3 im Gleichgewicht zu rechnen. Wenn C die Gesamtkonzentration des Farbstoffes, E_∞ die Lichtextinktion für eine Lösung beim Gleichgewicht und E_R wie oben (I) definiert ist, gilt:

* G_2 tritt erst in sehr stark sauren Lösungen auf.

$$E_\infty = E_R \cdot \frac{[R]}{C} \; ; \; C = [R] + [F_1] + [F_2] + [F_3]$$

$$K_3 = \frac{[H^+] \cdot [F_2]}{[F_1]} \; ; \; K_4 = \frac{[H^+] \cdot [F_3]}{[F_2]} \; ; \; K_6 = \frac{[R]}{[F_2]}$$

Aus diesen Relationen erhält man

$$E_\infty = E_R \cdot \frac{K_6}{1 + K_6 + [H^+]/K_3 + K_4/[H^+]} \qquad (2)$$

1. *Bestimmung von K_3K_6 und K_6.* Im allgemeinen kann man ein pH-Gebiet finden, wo (2) weiter vereinfacht werden kann, dadurch, dass $[H^+] \gg K_4$ wird. Dann wird $[F_3] \ll [F_1] + [F_2] + [R]$ und $K_4/[H^+]$ ist in Formel (2) zu vernachlässigen. Man bekommt

$$E_\infty = E_R \cdot \frac{K_6}{1 + K_6 + [H^+]/K_3}$$

welches umgeformt wird zu

$$\frac{1}{E_\infty} = \frac{1 + 1/K_6}{E_R} + \frac{1}{E_R \cdot K_3K_6} \cdot [H^+] \qquad (3)$$

Für eine Reihe von Lösungen werden $[H^+]$ und E_∞ bei gleicher Schichtdicke und Gesamtkonzentration des Farbstoffes bestimmt. $1/E_\infty$ wird als Funktion von $[H^+]$ aufgetragen. Man bekommt nach (3) eine Gerade. Aus deren Schnittpunkt a_2 mit der Ordinate und dem Richtungskoeffizienten b_2 erhält man

$$K_6 = 1/(a_2 \cdot E_R - 1) \; ; \; K_3K_6 = 1/b_2 \cdot E_R$$

Der E_R-Wert wurde bei Bestimmung von K_1 (siehe oben) erhalten. Er kann aber auch durch Messung der Aufhellungsgeschwindigkeit bei genügend kleiner $[H^+]$ ($[H^+] \ll 1/K_1$) erhalten werden. Extrapolation auf $t = 0$ gibt dann (bei evtl. grösserer $[H^+]$ nach Korrektion für vorhandenes G_1) E_R.

Anm. Aus praktischen Gründen mussten die Messungen sich auf ein Gebiet erstrecken, wo $[G_1]$ nicht ganz zu vernachlässigen ist. Da die Formeln (2) und (3) voraussetzen, dass $[G_1]$ vernachlässigt werden kann, soll man in diesen Fällen E_∞ durch einen korrigierten Wert E'_∞ ersetzen, wo $E'_\infty - E_\infty \approx E_\infty \cdot K_1 \cdot [H^+] \cdot E_\infty/E_R$ ist. Evtl. muss weiter korrigiert werden (siehe Anm. unten!).

2. *Bestimmung von $K_6/K_4 = K_5$ und K_6.* Wenn

$$[H^+] \ll K_3, \text{ ist } [F_1] \ll [F_2] + [F_3] + [R] \text{ und } [H^+]/K_3$$

in Formel (2) zu vernachlässigen. Man bekommt

$$E_\infty = E_R \cdot \frac{K_6}{1 + K_6 + K_4/[H^+]}$$

welches umgeformt wird zu

$$\frac{[H^+]}{E_\infty} = \frac{1}{E_R \cdot K_5} + \frac{1 + 1/K_6}{E_R} \cdot [H^+] \qquad (4)$$

Man bestimmt wie in 1. (siehe oben) die zusammengehörigen Werte von $[H^+]$ und E_∞. $[H^+]/E_\infty$ als Funktion von $[H^+]$ aufgetragen, gibt nach (4) eine Gerade; aus deren Schnittpunkt a_3 mit der Ordinate und dem Richtungskoeffizienten b_3 erhält man (mit bekanntem E_R)

$$K_5 = 1/a_3 \cdot E_R \; ; \; K_6 = 1/(b_3 \cdot E_R - 1)$$

Nachdem K_6 nach 1. und 2. bestimmt worden ist, werden K_3 und K_4 aus den Relationen $K_3 K_6 = 1/b_2 \cdot E_R$ bzw. $K_6/K_4 = K_5 = 1/a_3 \cdot E_R$ berechnet.

Anm. Nachdem K_3, K_4 und K_6 bestimmt worden sind, wird untersucht, ob die ausgeführten Näherungen (Vernachlässigung von $[F_3]$ in 1. und $[F_1]$ in 2.) erlaubt waren. Im allgemeinen ist dies bei den kleinsten (bzw. grössten) Werten von $[H^+]$ in den Mess-serien nicht ganz der Fall. Man wiederholt deshalb die Berechnungen, nachdem die Werte von E'_∞ bzw. E_∞ durch die korrigierten Werte E''_∞ bzw. E'''_∞ ersetzt worden sind. Diese sind mit Hilfe von den in der ersten Stufe berechneten Werten von K_3, K_4 und K_6 in entsprechender Weise wie oben (1. Anm.) erhalten. Man bekommt:

$$E''_\infty - E'_\infty \approx (E_\infty \cdot K_4/K_6 \cdot [H^+]) \cdot (E_\infty/E_R) \text{ und } E'''_\infty - E_\infty \approx (E_\infty \cdot [H^+]/K_3 \cdot K_6) \cdot (E_\infty/E_R).$$

Die Korrektionen unter 1. und 2. betragen in den vorliegenden Fällen nur einige Prozent in E_∞.

3. *Bestimmung des Maximums für* E_∞. Die Formel (2) definiert E_∞ als Funktion von $[H^+]$. Die Ableitung dieser Funktion nach $[H^+]$ hat die Nullstelle $[H^+] = \sqrt{K_3 \cdot K_4}$. Die Nullstelle entspricht dem Maximum bei E_∞. Man bekommt

$$\frac{(E_\infty)_{max}}{E_R} = \frac{K_6}{1 + K_6 + 2 \cdot \sqrt{K_4/K_3}} \tag{5}$$

Dadurch, dass man für die gleiche Gesamtkonzentration, Schichtdicke und Wellenlänge wie früher E_∞ als Funktion vom pH in der Nähe des Maximums aufträgt, kann $(E_\infty)_{max}$ graphisch ermittelt werden. Da E_R bekannt ist, kann das linke Glied in (5) getrennt berechnet werden, wobei der erhaltene Wert mit dem Wert übereinstimmen soll, welchen man vom rechten Glied in (5) nach Einsetzen der berechneten Werte von K_3, K_4 und K_6 erhält. Das rechte Glied in (5) ist jedoch in den vorliegenden Fällen verhältnismässig unempfindlich gegen Veränderungen in den Konstanten K_3 und K_4, weshalb oben erwähntes Verfahren als Kontrolle von begrenztem Wert ist.

B. Bestimmung von K_2. In einem Gebiet, in dem $[H^+]$ grösser ist als in A und in dem es als sicher gilt, dass $[H^+] \gg K_4$ ist, kann $[F_3]$ vernachlässigt werden, und man hat beim Gleichgewicht die Formen R, G_1, F_1 und F_2. Es gilt:

$$K_2 = \frac{[F_1]}{[G_1]} \; ; \; K_6 = \frac{[R]}{[F_2]} \; ; \; K_1 = \frac{[G_1]}{[H^+] \cdot [R]}$$

$$E_\infty = E_R \cdot \frac{[R]^*}{C} \; ; \; C = [R] + [G_1] + [F_1] + [F_2]$$

* Die Messungen finden bei einer Wellenlänge statt, wo nur R absorbiert.

Vereinfachung ergibt

$$K_2 = \frac{E_R - E_\infty(1 + K_1 \cdot [H^+] + 1/K_6)}{E_\infty \cdot K_1 \cdot [H^+]} \quad (6)$$

Nach Messung der zusammengehörigen Werte von $[H^+]$ und E_∞ wird K_2 aus (6) mit dem entsprechenden Wert von E_R und den oben bestimmten Werten von K_1 und K_6 eingesetzt, berechnet.

Schliesslich wird kontrolliert, dass, den Definitionen gemäss, $K_1 \cdot K_2 \cdot K_3 \cdot K_6 = 1$ ist.

C. Die Form G_2 bildet sich in stark sauren Lösungen, wobei man beim Gleichgewicht, das sich momentan einstellt, die Formen F_1, G_1 und G_2 hat. Da indessen G_2 erst dann nachzuweisen ist, wenn $[H^+] > 5$ bzw. 2 M ist, sind auf diesem Gebiet nur qualitative Versuche im Reagenzglas ausgeführt worden. K_7 ist also nicht bestimmt worden.

III. Berechnungen der Geschwindigkeitskonstanten

Beim Mischen von einer Stammlösung des Farbstoffes in Aceton mit einer Pufferlösung hellt sich die resultierende Lösung wegen der zwei Zeitreaktionen $G_1 + H_2O \rightarrow F_1$ und $R + H_2O \rightarrow F_2$ auf. Durch Messen der Lichtextinktion E als Funktion der Zeit t kann die Konstante k für die Reaktions-(Aufhellungs-) geschwindigkeit aus der Formel wie folgt [3,4] bestimmt werden.

$$k = \frac{1}{t} \cdot \ln \frac{E_0 - E_\infty}{E - E_\infty} \quad (7)$$

Ferner gilt:

$$k = \frac{k_2 \cdot [H^+]^2 + k_4 \cdot K_3 \cdot [H^+]}{[H^+]^2 + K_3 \cdot [H^+] + K_3 \cdot K_4} + \frac{k_1 \cdot K_1 \cdot [H^+] + k_3}{1 + K_1 \cdot [H^+]} \quad (8)$$

Bei der Ableitung von (7) und (8) geht man von der Differentialgleichung für die Aufhellung, nämlich

$$-\frac{d}{dt}([R] + [G_1]) = k_1 \cdot [G_1] - k_2 \cdot [F_1] + k_3 \cdot [R] - k_4 \cdot [F_2],$$

ferner von den im System geltenden Gleichgewichtsgleichungen und dem generellen Ausdruck $E = E_R \cdot [R]/C + E_{G_1} \cdot [G_1]/C$ aus, indem E_{G_1} auf entsprechende Weise wie E_R (I. oben) definiert wird.

k ist über einem pH-Gebiet von insgesamt 8—9 Einheiten experimentell bestimmt worden *. In dem Gebiet, wo $[R] \geq [G_1]$ ist, wird E im Absorptionsmaximum von R, bei geringerem pH im Absorptionsmaximum von G_1 gemessen.

A. Bestimmung von k_1 und k_2. In Lösungen, die so sauer sind, dass die Reaktion $G_1 + H_2O \rightleftharpoons F_1$ dominierend wird und folglich keine Rücksicht auf die Reaktion $R + H_2O \rightleftharpoons F_2$ genommen werden muss, geht Formel (8) in

$$k = k_1 \cdot \frac{K_1 \cdot [H^+]}{1 + K_1 \cdot [H^+]} + k_2 \quad (9)$$

über.

* Log $(E - E_\infty)$ wird als Funktion von t aufgetragen.

Da $k_2 = k_1 \cdot 1/K_2$ ist, kann man aus (9) k_1 berechnen, durch Einsetzen dieser Relation und durch Einsetzen von zusammengehörigen Werten von k und $[H^+]$, die experimentell bestimmt werden. Man findet dann dass k_1 linear mit $[H^+]$ gemäss der Formel

$$k_1 = k_1^0 + k_1' \cdot [H^+] \tag{10}$$

ansteigt.

Die Reaktion $G_1 + H_2O \rightleftharpoons F_1$ wird folglich durch die H^+-Ionen katalysiert. Ferner findet man, dass k_1^0 sicher von Null verschieden ist. k_2 erhält man aus der Relation $k_2 = k_1 \cdot 1/K_2$.

B. Bestimmung von k_3 und k_4. Bei höherem pH-Wert, wo die Reaktion $G_1 + H_2O \rightleftharpoons F_1$ nicht länger in erwähnenswertem Grad auf k einwirkt, geht Formel (8) in

$$k = k_4 \cdot \frac{K_3 \cdot [H^+]}{[H^+]^2 + K_3[H^+] + K_3 \cdot K_4} + k_3 \tag{11}$$

über.

Wenn weiter $[H^+] \ll K_3$ ist ($[F_1]$ kann vernachlässigt werden), bekommt man

$$k = k_4 \cdot \frac{[H^+]}{[H^+] + K_4} + k_3 \tag{12}$$

Aus Formel (12) kann k_3 durch Einsetzen von zusammengehörigen (experimentell bestimmten) Werten von k und $[H^+]$ und der Relation $k_4 = K_6 \cdot k_3$ berechnet werden. Unter Umständen wird diese Berechnung dadurch erleichtert, dass der erste Ausdruck des rechten Gliedes von (12) zu vernachlässigen und folglich $k = k_3$ ist.

Man findet, dass k_3, das wächst, wenn $[H^+]$ abnimmt, mit der folgenden Formel wiedergegeben werden kann:

$$k_3 = k_3^0 + k_3' \cdot [OH^-] \tag{13}*$$

Die Reaktion $R + H_2O \rightleftharpoons F_2$ wird also von OH^--Ionen katalysiert. Die Katalyse entspricht wahrscheinlich der Reaktion $R + OH^- \rightleftharpoons F_3$. Es soll doch bemerkt werden, dass k_3^0 nicht gleich Null ist. Man erhält k_4 aus der Relation $k_4 = K_6 \cdot k_3$.

C. k-Maximum. Wenn man auf dem Gebiet, wo Formel (11) gilt, annehmen kann, dass die OH^--Ionenkatalyse zu vernachlässigen ist (d.h., dass $k_3 = k_3^0$ und $k_4 = k_4^0$ ist), kann man zeigen [3], dass k das Maximum für $[H^+] = \sqrt{K_3 \cdot K_4}$ hat. Das Vorhandensein eines Maximums in k bei $[H^+] \approx \sqrt{K_3 K_4}$ ist in diesem Falle experimentell verifiziert worden.

D. Kontrolle. Nachdem sämtliche im Reaktionsschema eingehenden Protolysen-, Hydrations- und Geschwindigkeitskonstanten bestimmt worden sind, kann man mit Hilfe von Formel (8) die Aufhellungsgeschwindigkeit bei einem beliebigen $[H^+]$-Wert errechnen. Der errechnete Wert muss dann mit dem experimentell gefundenen übereinstimmen.

---

* $[OH^-]$ erhält man aus $[H^+] \cdot [OH^-] = K_w \cdot K_w$ wird separat bestimmt.

ALLGEMEINES ÜBER DEN EXPERIMENTELLEN TEIL

Es wurden Stammlösungen des Farbstoffes in wasserfreiem Aceton mit einer Farbstoffkonzentration von $(1/3-1) \times 10^{-4}$ M bereitet. Bei der Bereitung der Messlösungen wurden 1,050 ml Farbstoffstammlösung mit 49,0 ml Pufferlösung gemischt, oder — wenn die halbe Farbstoffkonzentration erwünscht war — 98,0 ml Pufferlösung und 1,050 ml Aceton. Die Mischung wurde in einem Kolben aus Pyrex- oder Jenaer Glas vorgenommen. Das Lösungsmittel in den Messlösungen hatte durchgehend die Zusammensetzung 98 % Wasser + 2 % Aceton. Die Gesamtionenstärke der Pufferlösungen wurde durch Zusatz von Natriumchlorid so gewählt, dass sie in den Messlösungen stets 0,50 M betrug. Der Beitrag von Natriumchlorid zur Gesamtionenstärke war fast immer mindestens 80 %. Man kann deshalb die Aktivitätskoeffizienten als konstant betrachten. Die bestimmten Gleichgewichtskonstanten sind also die sog. Konzentrationskonstanten bei der Ionenstärke 0,50 M.

Um reproduzierbare und definierte Mengen der Farbstoffstammlösung zu erhalten, wurde bei Bereitung der Messlösungen die Stammlösung stets mit einer 1 ml-Injektionsspritze aus Pyrex-Glas hinzugefügt. Beim Leeren der Spritze wurde ihre Kapillare etwa in die Mitte der Pufferlösung gebracht. Das Leeren dauerte 5—10 Sek. Auf diese Weise erhielt man schon von Anfang an eine gute Durchmischung, ohne den Kolben stark schütteln zu müssen. Die Spritze war mit besonderen, aussen angebrachten Vorrichtungen aus Metall versehen, die eine Volumengenauigkeit von 0,1 % gaben, was durch Auswägen mit Wasser kontrolliert wurde.

Für die Messungen der Lichtextinktion wurde ein Beckman-Quarz-Spektrophotometer, Modell DU, benutzt. Als Küvetten dienten verschiedene Paare Quarz- und Glasküvetten mit Schliffstopfen. Von jedem Paar diente eine, mit dest. Wasser gefüllt, stets als Nullreferenz. Im Gebiet $pH > 2,7$ wurde die Wasserstoffionkonzentration potentiometrisch bestimmt. Als Vergleichslösung wurde eine Lösung von der Zusammensetzung 0,480 M NaCl + 0,020 M HCl und als Trennlösung gesättigte KCl-Lösung verwendet. Die Elektroden waren blanke Platinbleche und Chinhydron für $pH \leqq 8$ und Platinwasserstoffgaselektroden für $pH > 8$. Meistens wurde die Wasserstoffionenkonzentration in den Endmesslösungen gemessen. Im Gebiet $pH < 2,7$ wurde die Wasserstoffionenkonzentration durch Zusatz von bestimmten Mengen HCl definiert. $K_w = [H^+] \cdot [OH^-]$ wurde für sich bei der Ionenstärke 0,50 M durch Messung von $[H^+]$ in einer Lösung der Zusammensetzung 0,480 M NaCl + 0,020 M NaOH bestimmt. $K_w = 1,22 \times 10^{-14}$.

Bei Messungen über die kinetischen Verläufe wurde die Zeit, t, gleich Null in dem Moment gesetzt, wo die Injektionsspritze zur Hälfte geleert war. Sobald die Messlösung gemischt war, wurde dann eine Küvette gefüllt und in die Beckmann-Apparatur gesetzt. Darauf wurde die Aufhellung durch Messung der Extinktion E (bei einer angemessen gewählten Wellenlänge) als Funktion von t verfolgt.

Die ausserordentlich kleine Konzentration des Farbstoffes ($3,5 \times 10^{-7}$ M) in gewissen Messreihen ist dadurch notwendigerweise bedingt, dass die Form G_1 sehr schwer löslich ist. Schon bei der doppelten Konzentration ($7,0 \times 10^{-7}$ M) erhielt man in sauren Lösungen ($pH < 3,0$) bei Messungen über die Geschwindigkeit der Reaktion $G_1 + H_2O \rightleftharpoons F_1$ Abweichungen vom linearen Verlauf der t-Funktion $\log(E-E_\infty)$. Die Abweichungen, die kleineren Geschwindigkeiten entsprachen, waren regelmässig nur im Anfang des kinetischen Verlaufes vorhanden. Nach ein paar Minuten nahm die Funktion einen geradlinigen Verlauf an. Die Neigung der Linie war jedoch um ein Geringes zu klein. Diese Beobachtungen lassen sich völlig dadurch erklären, dass der Farbstoff, wenn er aus der Injektionsspritze in die Lösung gelangt, wegen lokaler Überschüsse teilweise kristallisiert, um dann wieder bei vollständiger Mischung allmählich aufgelöst zu werden.

Obwohl die ersten Ablesungen der Totalextinktion wegen der kleinen Farbstoffkonzentration nur von einer ungefähren Grösse von 0,050 waren, war eine Reproduzierbarkeit der Geschwindigkeitskonstanten innerhalb einer Fehlergrenze von etwa 5 % möglich. Die Totalextinktionen in diesem Gebiet liegen keinen anderen Berechnungen zugrunde.

Die Gültigkeit des Beer'schen Gesetzes ist durch separate Messungen bei konstanter Extinktion und veränderlicher Konzentration und optischer Länge bestätigt.

Sämtliche untersuchten Reaktionen sind im vorliegenden pH-Gebiet völlig reversibel. Bei längerer Aufbewahrung in Lösungen mit $pH > 10$ scheinen doch irreversible Reaktio-

nen einzutreten. Die Messungen wurden bei 20°C ausgeführt. Besondere Anordnungen zur Gewinnung einer guten Temperaturkonstanz sind von Cigén [3] beschrieben worden. Vor Einstellung des Gleichgewichtes wurden die Lösungen im Thermostaten bei 20°C aufbewahrt. Die Wartezeit beim Messen von E_∞ war in der Regel 9—10 Halbwertzeiten.

Den Versuchsserien, die den Berechnungen der verschiedenen Konstanten zugrunde lagen, waren systematische, halbquantitative Versuche im Reagenzglas vorangegangen, wodurch die geeignetsten Bedingungen für die endgültigen Versuchsserien ermittelt wurden.

Die Geschwindigkeitskonstanten sind durchgehend mit Brigg'schen Logarithmen berechnet worden und enthalten also die Grösse $^{10}\log e$.

Die verwendeten Chemikalien waren pro-analysi-Produkte von Baker oder Merck.

Messfehler. Durch Untersuchung der Reproduzierbarkeit an verschiedenen Stellen in den Messungen und durch Schätzung der Messfehler, zufälliger sowohl als auch eventuell systematischer, ist versucht worden, ein Urteil über die Genauigkeit der Resultate zu gewinnen. Die bestimmten Konstanten sind demzufolge so angegeben worden, dass die Unsicherheit normalerweise 2—5 Einheiten in der letzten Ziffer beträgt. Bei evtl. grösserer Unsicherheit wurde die Ziffer in Klammern gesetzt.

MESSUNGEN UND ERGEBNISSE

I. 3, 3', 5, 5'-Tetrabrom-benzaurin

Präparat: Tetrabrom-benzaurin wurde durch Bromierung von Benzaurin* hergestellt [12], und durch mehrmaliges Umkristallisieren aus Nitrobenzol bis zu extinktiometrischer Konstanz gereinigt. (Gefunden: Br 54,35; 54,11. Ber. für $C_{19}H_{10}O_2Br_4$: Br 54,20).

Als *Puffersubstanzen* wurden Dinatriumhydrogenphosphat-Natriumhydroxyd, Borsäure-Natriumhydroxyd, Natriumdihydrogenphosphat-Dinatriumhydrogenphosphat, Essigsäure-Natriumacetat, Citronensäure-Natriumhydroxyd in folgenden ungefähren pH-Gebieten verwendet: 9,9—10,9; 8,1—9,9; 6,2—7,9; 3,6—6,2; 2,7—3,3. Für $pH < 2{,}7$ wurde $[H^+]$ durch Zusatz einer bestimmten Menge Salzsäure definiert.

Lichtabsorptionskurven. Fig. 1 stellt die Absorptionskurven vom blauen Ion R und vom gelben Molekül G_1 (den molekularen Lichtextinktionskoeffizienten ε als Funktion der Wellenlänge λ in mμ) dar. Die Kurve für R wurde durch Messung der Gleichgewichtsextinktion (E_∞) bei 10 cm Schichtdicke (d) in einer Lösung, in der der pH-Wert 6,32 betrug und die Gesamtkonzentration C des Farbstoffes $2{,}10 \times 10^{-6}$ M war, aufgenommen. ε wurde aus $E_R = \varepsilon \times 10 \times 2{,}1 \times 10^{-6}$ berechnet und E_R aus E_∞ aus Formel (2). Weiter fand man $(\varepsilon_R)_{max} = 7{,}7 \times 10^4$ für $\lambda \approx 580$ mμ.

Die Kurve für G_1 wurde in Lösungen (Acetatpuffer) mit pH = 3,085 aufgenommen, $C = 1{,}07 \times 10^{-6}$ M und $d = 10$ cm. Da G_1 bei Gleichgewicht nur in sehr kleiner Konzentration vorhanden ist (K_2 ist gross), wurden sämtliche (sieben) Punkte auf der Kurve durch Messung der Aufhellungsgeschwindigkeit und durch Extrapolation auf die Zeit Null erhalten. Aus dem so erhaltenen E_0 wurde ε_0 aus $E_0 = \varepsilon_0 \times 10 \times 1{,}07 \times 10^{-6}$ berechnet und ε_{G_1} aus

$$\varepsilon_0 = \frac{[H^+]}{[H^+] + 1/K_1} \cdot \varepsilon_{G_1} + \frac{1/K_1}{[H^+] + 1/K_1} \cdot \varepsilon_R$$

Es ging hervor, dass $(\varepsilon_{G_1})_{max} = 2{,}7 \times 10^4$ für $\lambda \approx 450$ mμ war.

* Benzaurin wurde nach Döbner [10] hergestellt.

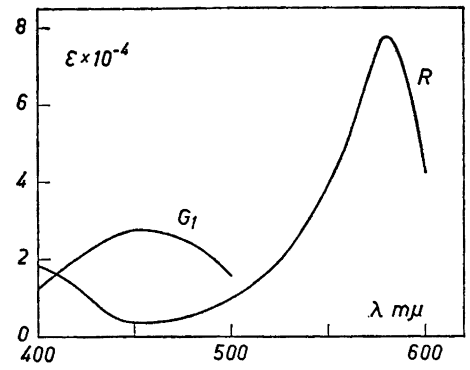


Fig. 1. Tetrabrom-benzaurin. Lichtabsorptionskurven. R ist das blaue Ion $[(OBr_2C_6H_2)_2C(C_6H_5)]^-$; G_1 ist das gelbe Molekül $(HOBr_2C_6H_2)C(C_6H_5)(C_6H_2Br_2O)$.

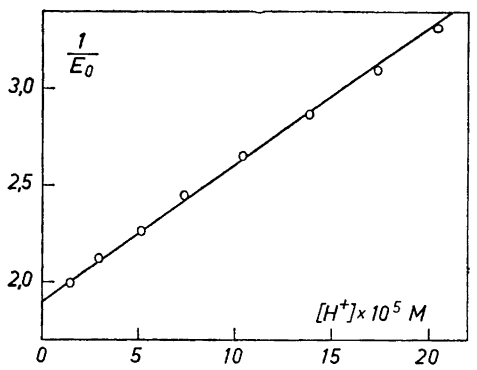


Fig. 2. Tetrabrom-benzaurin. $1/E_0$ als Funktion von $[H^+]$ für die Bestimmung von K_1 (und E_R). λ = 580 mμ, d = 10 cm und C = 7,0 × 10^{-7} M

Bestimmung von K_1 aus Formel (1). Aus einer Serie von Acetatpuffern (3,6<pH<4,8) wurden durch Mischung von 98 ml Puffer mit 1 ml Aceton und 1 ml einer Stammlösung des Farbstoffes in Aceton Messlösungen bereitet. Die Aufhellung wurde bei der Wellenlänge 580 mμ extinktiometrisch verfolgt und E_0 durch Extrapolation berechnet. Die Messungen fanden bei 10 cm Schichtdicke (d) statt. Die Gesamtkonzentration C des Farbstoffes war 7,0 × 10^{-7} M. Fig. 2 gibt $1/E_0$ als Funktion von $[H^+]$ wieder.

Resultat: E_R = 0,526 ; K_1 = 3,7(3) × 10^3.

In einer Serie, wo C 50,0 % grösser war (1,05 × 10^{-6} M), erhielt man: E_R = 0,792 ; K_1 = 3,9(7) × 10^3.

Mittelwerte: K_1 = 3,8(5) × 10^3, $1/K_1$ = 2,6(0) × 10^{-4}.

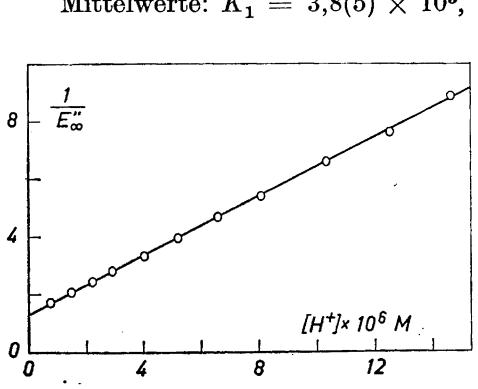


Fig. 3. Tetrabrom-benzaurin. $1/E''_\infty$ als Funktion von $[H^+]$ für die Bestimmung von K_3K_6 und K_6. λ = 580 mμ, d = 10 cm und C = 2,12 × 10^{-6} M

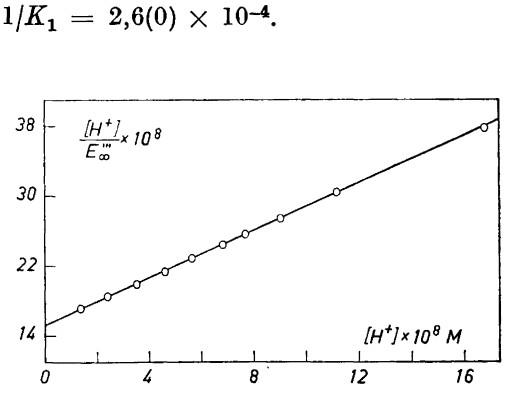


Fig. 4. Tetrabrom-benzaurin. $[H^+]/E'''_\infty$ als Funktion von $[H^+]$ für die Bestimmung von K_5 und K_6. λ = 580 mμ, d = 10 cm und C = 2,12 × 10^{-6} M

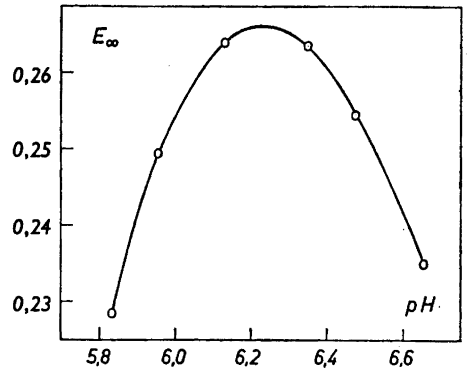


Fig. 5. Tetrabrom-benzaurin. E_∞ als Funktion von pH für die graphische Bestimmung von $(E_\infty)_{max}$. λ = 580 mμ, d = 10 cm und C = 1,06 × 10^{-6} M $(E_\infty)_{max}$ = 0,266.

Bestimmung von K_3K_6 *und* K_6 aus Formel (3). Im Gebiet 4,8<pH<6,1 wurden Messlösungen durch Zusatz von 2 ml Acetonstammlösung * des Farbstoffes zu 98 ml eines geeigneten Acetatpuffers bereitet; in den Messlösungen war $C = 2{,}12 \times 10^{-6}$ M. Nachdem Gleichgewicht eingetreten war, wurde die Extinktion (E_∞) bei 10 cm Schichtdicke und der Wellenlänge 580 mμ gemessen. Fig. 3 beschreibt $1/E''_\infty$ als Funktion von $[H^+]$. Das entsprechende E_R wurde bei 5,6<pH<6,2 durch Messung der Aufhellungsgeschwindigkeit und durch Extrapolation auf die Zeit Null bestimmt. Nach Korrektion für G_1 erhielt man aus sechs Versuchen bei d = 10 cm und mit $C = 1{,}06 \times 10^{-6}$ M (50,0 % des Obenerwähnten) im Durchschnitt $1/2 \cdot E_R = 0{,}810$ ($\pm$ 0,002).

Resultat: $K_6 = 0{,}88$; $K_3K_6 = 1{,}21 \times 10^{-6}$.

Bei Reproduzierung wurde

$$K_6 = 0{,}89 \; ; \; K_3K_6 = 1{,}22 \times 10^{-6}$$

erhalten.

Bestimmung von $K_5 = K_6/K_4$ *und* K_6 aus Formel (4) im Gebiet 6,8<pH<7,9. Die Versuche wurden mit derselben Stammlösung bei derselben Wellenlänge und mit gleichem C, d (und E_R) wie bei der bereits erwähnten Bestimmung von K_3K_6 und K_6 ausgeführt. Als Puffer dienten NaH_2PO_4—Na_2HPO_4-Lösungen. Fig. 4 gibt $[H^+]/E'''_\infty$ als Funktion von $[H^+]$ wieder.

Resultat: $K_5 = 4{,}06 \times 10^6$; $K_6 = 0{,}84(5)$.

Die Reproduzierung der Serie führte zu denselben Ergebnissen.

Aus den obenstehenden Resultaten wurden
$K_6 = 0{,}86(5)$; $K_3 = 1{,}40 \times 10^{-6}$; $K_4 = 2{,}1(3) \times 10^{-7}$ erhalten.

Bestimmung von $(E_\infty)_{max}$. Im Intervall 5,8<pH<6,7, wo E_∞ Formel (2) folgt, hatte E_∞ sein Maximum. Fig. 5 gibt E_∞ bei 580 mμ als Funktion vom pH-Wert in diesem Gebiet wieder. E_∞ wurde in sechs verschiedenen Lösungen gemessen, die für pH<6,2 aus Acetatpuffern und für pH>6,2 aus Phosphatpuffern bereitet waren, bei d = 10 cm und $C = 1{,}06 \times 10^{-6}$ M. Unter diesen

* nicht identisch mit der Stammlösung bei der Bestimmung von K_1.

Tabelle 1. Tetrabrom-benzaurin. Messungen bei 580 mμ zur Bestimmung von K_2; $d = 10$ cm; $C = 2{,}12 \times 10^{-6}$ M

$[H^+] \times 10^5$ M	2,96	1,46(0)	1,25(5)	1,03(5)	0,80(8)
E_∞	0,062	0,115	0,130	0,151	0,184
K_2	211	211	211	213	212

Bedingungen wurde oben E_R zu 0,810 bestimmt. Aus der Kurve in Fig. 5 wurde $(E_\infty)_{max} = 0{,}266$ bei pH≈6,24 (≈ —log $\sqrt{K_3 K_4}$) erhalten. Die beiden Glieder der Formel (2) wurden zu

$$\frac{(E_\infty)_{max}}{E_R} = 0{,}328 \; ; \qquad \frac{K_6}{1 + K_6 + 2\sqrt{K_4/K_3}} = 0{,}327$$

berechnet.

Bestimmung von K_2. Im Gebiet 4,5<pH<5,1 konnte K_2 aus Formel (6) berechnet werden. Die Messlösungen wurden wie früher aus Acetatpuffern und Farbstoffstammlösung bereitet und die Extinktionen (E_∞) beim Gleichgewicht bei der Wellenlänge 580 mμ gemessen. Ferner war $d = 10$ cm, $C = 2{,}12 \times 10^{-6}$ M und $E_R = 1{,}620$. Für K_1 und K_6 wurden die oben erhaltenen Werte verwendet. Die Ergebnisse sind in Tabelle 1 aufgeführt. Als Mittelwert wurde $K_2 = 212$ erhalten.

Die oben erhaltenen Konstanten ergeben das Produkt $K_1 \cdot K_2 \cdot K_3 \cdot K_6 = 0{,}99$.

Die Form G_2: Zu etwa 20 ml von teils einer Serie HCl-Lösungen, teils einer Serie $HClO_4$-Lösungen, mit der Säurestärke variierend zwischen 6 und 12 M, wurde 1/2 ml Farbstoffstammlösung zugesetzt. Die Lösungen der beiden Serien nahmen eine beständige rotgelbe Farbe der Form G_2 an. Die Farbe war stärker bei grösserer Säurestärke und, was den Farbton anbetrifft, deutlich unterschiedlich von der Farbe bei G_1.

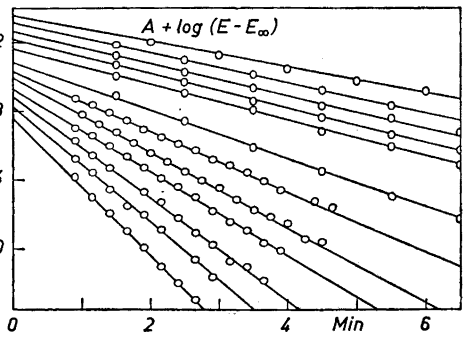


Fig. 6. Tetrabrom-benzaurin. Reaktionskinetik bei $\lambda = 450$ mμ, $d = 10$ cm und $C = 3{,}50 \times 10^{-7}$ M. Für die Linien gelten in der Ordnung von oben nach unten die folgenden Daten:

A	3,55	3,48	3,43	3,35	3,30	3,20
	3,10	3,05	3,00	2,95	2,85	2,80
pH	3,235	3,100	3,030	2,960	2,884	2,610
	2,485	2,388	2,274	2,184	2,087	2,008

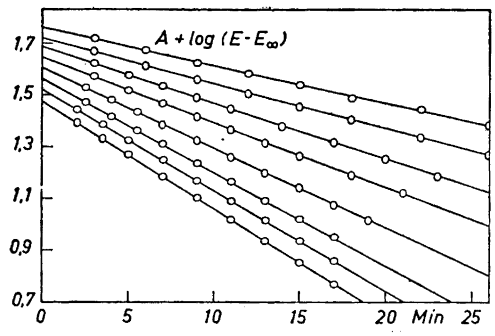


Fig. 7. Tetrabrom-benzaurin. Reaktionskinetik bei $\lambda = 580$ mμ, $d = 10$ cm und $C = 7{,}0 \times 10^{-7}$ M. Von oben nach unten gelten:

A	2,10	2,07	2,06	2,05	2,04	2,03
	2,02	2,00				
pH	4,832	4,531	4,289	4,132	3,983	3,859
	3,761	3,691				

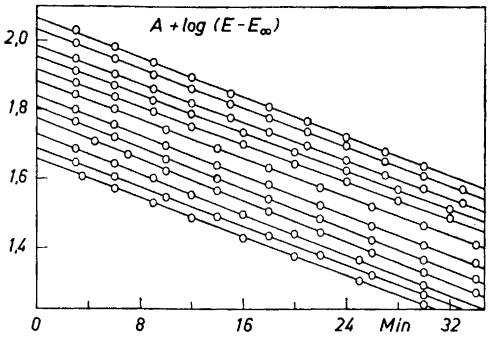


Fig. 8. Tetrabrom-benzaurin. Reaktionskinetik bei $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}06 \times 10^{-6}$ M. Von oben nach unten gelten:

A	2,23	2,20	2,16	2,14	2,12	2,10
	2,08	2,06	2,04	2,00	1,95	1,90
pH	4,909	5,099	5,181	5,393	5,531	5,653
	5,834	5,955	6,131	6,325	6,476	6,627

Messungen der Reaktions-(Aufhellungs-)Geschwindigkeiten. Die Geschwindigkeitskonstante k wurde über das ganze in Frage kommende pH-Gebiet (2,0—10,9) für mehr als fünfzig verschiedene Lösungen bestimmt, welche aus 98 ml Puffer + 1 ml Aceton + 1 ml Acetonstammlösung des Farbstoffes bereitet waren. Die Messungen der Extinktionen (E) wurden durchgehend bei einer Schichtdicke von 10 cm gemacht. Die Wellenlänge war 580 mμ (R's

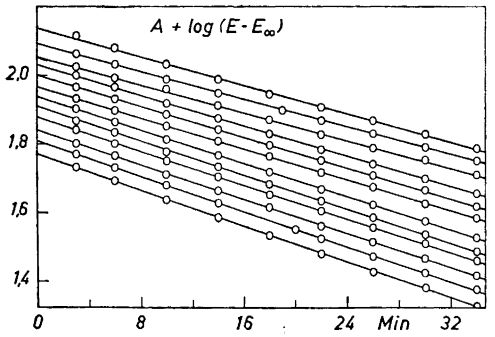


Fig. 9. Tetrabrom-benzaurin. Reaktionskinetik bei $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}06 \times 10^{-6}$ M. Von oben nach unten gelten:

A	2,25	2,20	2,17	2,15	2,13	2,11
	2,10	2,08	2,06	2,03	2,01	2,00
pH	9,01	8,58	8,175	7,860	7,618	7,454
	7,257	7,168	7,115	7,045	6,952	6,772

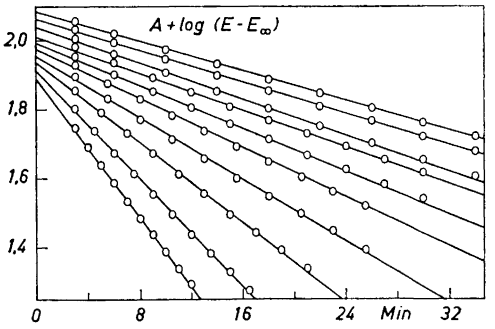


Fig. 10. Tetrabrom-benzaurin. Reaktionskinetik bei $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}06 \times 10^{-6}$ M. Von oben nach unten gelten:

A	2,19	2,17	2,15	2,13	2,11	2,09
	2,07	2,05	2,03	2,00		
pH	9,31	9,58(5)	9,83	9,88	10,03	10,21
	10,39	10,58	10,77(5)	10,89		

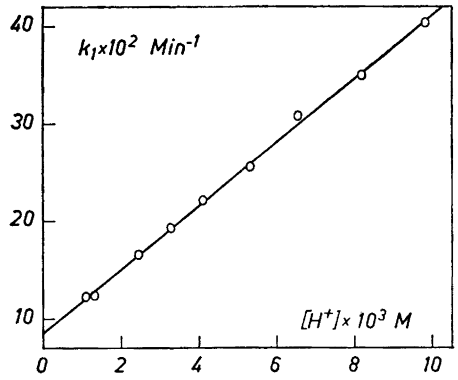


Fig. 11. Tetrabrom-benzaurin. Die Geschwindigkeitskonstante k_1 als Funktion von $[H^+]$.

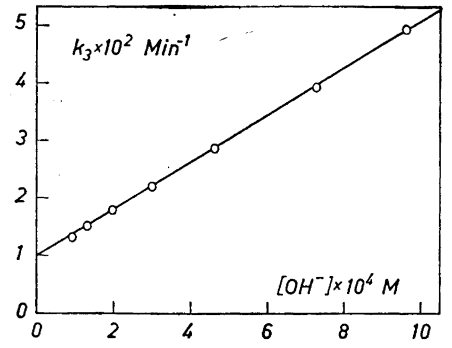


Fig. 12. Tetrabrom-benzaurin. Die Geschwindigkeitskonstante k_3 als Funktion von $[OH^-]$.

Absorptionsmaximum) für pH>3,6, 450 mμ (G_1's Absorptionsmaximum) für pH<3,6. C war $3{,}50 \times 10^{-7}$ M für pH<3,6, $7{,}0 \times 10^{-7}$ M für 3,6<pH<4,9 und $1{,}06 \times 10^{-6}$ M für pH>4,9. Die Resultate sind graphisch in den Figuren 6—10 aufgeführt; Fig. 13 zeigt k's Abhängigkeit vom pH-Wert.

Bestimmung von k_1 und k_2. Im Gebiet 2,0<pH<3,0 ist k_1 mit Hilfe der Formel (9) durch Einsetzen der Relation $k_2 = k_1 \cdot 1/K_2$ und zusammengehörigen Werten von $[H^+]$ und k berechnet worden. Fig. 11 zeigt, dass k_1 als Funktion von $[H^+]$ eine gerade Linie ist. Graphische Bestimmung gab als Resultat: $k_1^0 = 8{,}6 \times 10^{-2}$; $k_1' = 32{,}(5)$. Man erhält also $k_1 = 8{,}6 \times 10^{-2} + 32 \cdot [H^+]$; $k_2 = 4{,}1 \times 10^{-4} + 0{,}15 \cdot [H^+]$ (Min^{-1}); $[H^+]$ in Mol/l.

Bestimmung von k_3 und k_4. Im Gebiet pH>9,8 geht Formel (12) in $k = k_3$ über. Es geht aus Fig. 10 hervor, dass k_3 mit dem pH-Wert wächst. Fig. 12 zeigt, dass k_3 als Funktion von $[OH^-]$ eine Gerade ist. Durch graphische Bestimmung wurde das Resultat $k_3^0 = 1{,}00 \times 10^{-2}$, $k_3' = 40{,}8$ erhalten. Man bekommt also: $k_3 = 1{,}00 \times 10^{-2} + 41 \cdot [OH^-]$, $k_4 = 0{,}86 \times 10^{-2} + 35 \cdot [OH^-]$ (Min^{-1}); $[OH^-]$ in Mol/l.

k als Funktion von pH. In Fig. 13 sind die experimentell bestimmten Werte von k im ganzen untersuchten pH-Gebiet eingetragen. Aus Formel (8) ist ferner k bei verschiedenen pH-Werten, durch Einsetzen der experimentell bestimmten Werte der in die Formel eingehenden Konstanten, berechnet worden. Die Berechnungen sind nur bei den pH-Werten ausgeführt worden, wo das Verfahren wirkliche Kontrolle bedeutet. Die extrem sauren bzw. alkalischen Gebiete, die bei den früheren experimentellen Bestimmungen von k_1 und k_2 bzw. k_3 und k_4 verwendet wurden, sind demnach nicht berücksichtigt worden. Der ausgezogenen Kurve liegen diese Berechnungen zu Grunde. Man findet gute Übereinstimmung, bei pH<5 jedoch einige Diskrepanz zwischen berechneten und experimentell bestimmten Werten. Die nach (8) berechneten Werte von k sind bei pH<5 von K_1 stark abhängig, daher ist es möglich, dass die Diskrepanz nur auf einen geringeren Fehler in K_1 zurückzuführen ist.

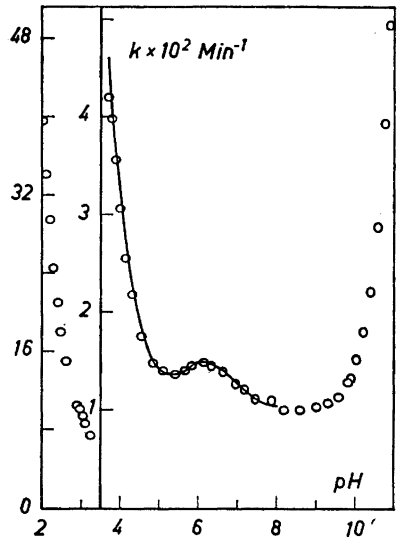


Fig. 13. Tetrabrom-benzaurin. Die Geschwindigkeitskonstante k als Funktion von pH. Die experimentellen Werte sind O. Die ausgezogene Kurve ist aus Formel (8) berechnet.

Aus der Kurve und den experimentellen Werten geht hervor, dass k sein Maximum bei pH$\approx$6,2 ($\approx -\log \sqrt{K_3 K_4}$) hat; im grossen und ganzen mit der Lage für $(E_\infty)_{max}$ übereinstimmend.

II. 3, 3'-Dibrom-5, 5'-dimethyl-benzaurin

Präparat: Dibrom-dimethyl-benzaurin wurde durch Bromierung von Dimethyl-benzaurin [15,*] hergestellt und aus wasserhaltigem Eisessig zu extinktiometrischer Konstanz umkristallisiert. Das Endprodukt bestand aus kompakten Kristallen mit blauem metallischem Glanz. (Gefunden: C 54,67; H 3,41; O 7,23; Br 35,61. Berechnet für $C_{21}H_{16}O_2Br_2$: C 54,80; H 3,50; O 6,95; Br 34,73.) Um die evtl. Empfindlichkeit der Acetonstammlösungen gegen Spuren von Wasser herabzusetzen, wurden sie mit Triäthylamin versetzt, wobei die molekulare Konzentration von Triäthylamin der des Farbstoffes etwa entsprach. Die Hydratation ist nämlich bedeutend kleiner für das Gleichgewicht R + $H_2O \rightleftharpoons F_2$ als für das Gleichgewicht $G_1 + H_2O \rightleftharpoons F_1$.

Puffersubstanzen: Dinatriumhydrogenphosphat-Natriumhydroxyd, Borsäure-Natriumhydroxyd, Natriumdihydrogenphosphat-Dinatriumhydrogenphosphat, Essigsäure-Natriumacetat in den pH-Gebieten 9,9—11,1; 7,9—9,8; 5,5—7,9; 3,4—4,6.

Lichtabsorptionskurven: Die Lichtabsorptionskurven der Formen G_1 (gelb) und R (blau) finden sich in Fig. 14 wieder, die den molekularen Lichtextinktionskoeffizienten ε als Funktion der Wellenlänge λ in mμ darstellt. Die Aufnahme der R-Kurve wurde in einer Lösung mit pH = 8,44 und $C = 1{,}09 \times 10^{-6}$ M vorgenommen. Die G_1-Kurve, die durch Extrapolationsverfahren er-

* Dimethyl-benzaurin wurde nach Schroeter [16] hergestellt. S. auch Hansson und Nilsson [17].

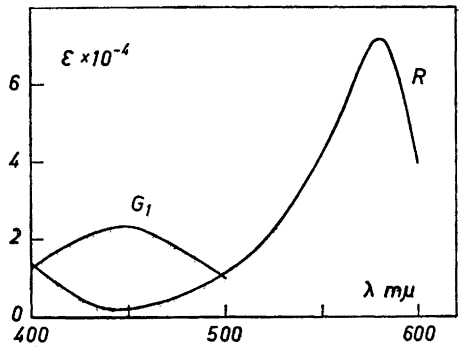


Fig. 14. Dibrom-dimethyl-benzaurin. Lichtabsoptionskurven. R ist das blaue Ion $[(OBrCH_3C_6H_3)_2C(C_6H_5)]^+$; G_1 ist das gelbe Molekül $(HOBrCH_3C_6H_3)C(C_6H_5)(C_6H_3CH_3BrO)$

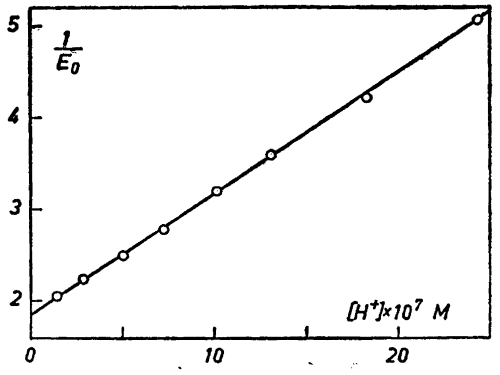


Fig. 15. Dibrom-dimethyl-benzaurin. $1/E_0$ als Funktion von $[H^+]$ für die Bestimmung von K_1 (und E_R). $\lambda = 580$ mμ, $d = 10$ cm und $C = 7{,}3 \times 10^{-7}$ M.

halten wurde, wurde bei pH = 3,87 und $C = 3{,}88 \times 10^{-7}$ M aufgenommen. Die Schichtdicke betrug in beiden Fällen 10 cm. Die Berechnungen wurden auf dieselbe Weise wie für Tetrabrom-benzaurin ausgeführt. Die Daten für die Maxima betrugen

für R: $(\varepsilon_R)_{max} = 7{,}2 \times 10^4$ bei $\lambda \approx 580$ mμ
für G_1: $(\varepsilon_{G_1})_{max} = 2{,}3(4) \times 10^4$ bei $\lambda \approx 450$ mμ.

Bestimmung von K_1 aus Formel (1) im Gebiet $5{,}6 < pH < 6{,}9$. Die Messlösungen wurden aus Phosphatpuffern (49 ml) und einer Stammlösung (1 ml) des Farbstoffes in Aceton bereitet. Die Extinktionsmessungen wurden wie bei Tetrabrom-benzaurin bei 580 mμ und 10 cm Schichtdicke ausgeführt. Fig. 15 gibt $1/E_0$ als Funktion von $[H^+]$ in einer Serie wieder, in der C $7{,}3 \times 10^{-7}$ M betrug.

Resultat: $E_R = 0{,}541$; $K_1 = 7{,}14 \times 10^5$.
In einer Serie, wo C 50,0 % grösser war ($1{,}09 \times 10^{-6}$ M), erhielt man: $E_R = 0{,}800$; $K_1 = 7{,}57 \times 10^5$.

Mittelwerte: $K_1 = 7{,}3(5) \times 10^5$; $1/K_1 = 1{,}36 \times 10^{-6}$.

Bestimmung von K_3K_6 *und* K_6 aus Formel (3) im Gebiet $6{,}8 < pH < 7{,}9$. Die Messlösungen wurden von 49 ml Phosphatpuffern + 1 ml Acetonstammlösung erhalten. Die Messungen wurden wie bei Tetrabrom-benzaurin ausgeführt; $\lambda = 580$ mμ; $C = 1{,}09 \times 10^{-6}$ M, $d = 10$ cm. Das entsprechende E_R wurde aus fünf Versuchen bei gleichem C und d im Gebiet $7{,}3 < pH < 7{,}9$ im Mittel zu 0,786 (± 0,002) erhalten. Fig. 16 zeigt $1/E''_\infty$ als Funktion von $[H^+]$. Resultat: $K_6 = 5{,}(7)$; $K_3K_6 = 5{,}4(2) \times 10^{-8}$.

Bei Reproduzierung erhielt man: $K_6 = 5{,}(6)$; $K_3K_6 = 5{,}3(6) \times 10^{-8}$.

Bestimmung von $K_5 = K_6/K_4$ *und* K_6 aus Formel (4) im Gebiet $8{,}7 < pH < 9{,}8$. Die Messlösungen wurden aus 49 ml Boratpuffer + 1 ml Acetonstamm-

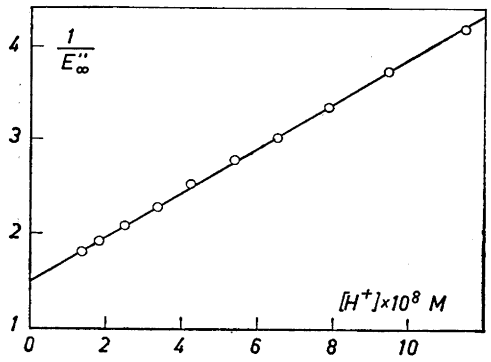


Fig. 16. Dibrom-dimethyl-benzaurin. $1/E''_\infty$ als Funktion von $[H^+]$ für die Bestimmung von K_3K_6 und K_6. $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}09 \times 10^{-6}$ M.

lösung bereitet. E_∞ wurde bei $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}09 \times 10^{-6}$ M gemessen. Da die Stammlösung nicht diegleiche war wie früher, wurde E_R aufs Neue bestimmt. Es wurde aus fünf Versuchen im Gebiet $8{,}5 < \text{pH} < 9{,}8$ mit obenstehendem C und d im Mittel $E_R = 0{,}781$ erhalten. Fig. 17 zeigt $[H^+]/E'''_\infty$ als Funktion von $[H^+]$.

Resultat: $K_5 = 2{,}7(8) \times 10^9$; $K_6 = 5{,}(1)$.

Aus obenstehenden Resultaten * wurde $K_6 = 5{,}(4)$; $K_3 = 1{,}0(0) \times 10^{-8}$; $K_4 = 1{,}9(4) \times 10^{-9}$ erhalten.

Bestimmung von $(E_\infty)_{max}$. Fig. 18 zeigt E_∞ als Funktion vom pH-Wert im Gebiet $7{,}8 < \text{pH} < 8{,}9$, in dem Formel (2) als geltend angesehen werden kann. E_∞ wurde in Boratpuffern bei $\lambda = 580$ mμ, $C = 1{,}09 \times 10^{-6}$ M und $d = 10$ cm gemessen. Aus der Kurve in Fig. 18 wurde $(E_\infty)_{max} = 0{,}574$ bei $\text{pH} \approx 8{,}36$ $(= -\log\sqrt{K_3K_4})$ erhalten. Mit $E_R = 0{,}781$ wurden die zwei Glieder der Formel (2) zu

$$\frac{(E_\infty)_{max}}{E_R} = 0{,}734 \; ; \quad \frac{K_6}{1 + K_6 + 2\sqrt{K_4/K_3}} = 0{,}742$$

berechnet.

Bestimmung von K_2 aus Formel (6) bei $5{,}7 < \text{pH} < 6{,}6$. Die Messlösungen wurden aus 49 ml Phosphatpuffer + 1 ml Acetonstammlösung bereitet. E_∞ wurde bei $\lambda = 580$ mμ gemessen. Ferner war $d = 10$ cm, $C = 1{,}09 \times 10^{-6}$ M und $E_R = 0{,}786$. Für K_1 und K_6 wurden die oben bestimmten Werte verwendet. Die Resultate sind in Tabelle 2 aufgeführt. Als Mittelwert wurde $K_2 = 26{,}(2)$ erhalten.

Aus den obenstehenden Konstanten wurde das Produkt $K_1 \cdot K_2 \cdot K_3 \cdot K_6 = 1{,}04$ *erhalten.*

* Bei beiden Bestimmungen wird K_6 aus dem Ausdruck $1 + 1/K_6$ erhalten und ist daher mit relativ grosser Unsicherheit behaftet.

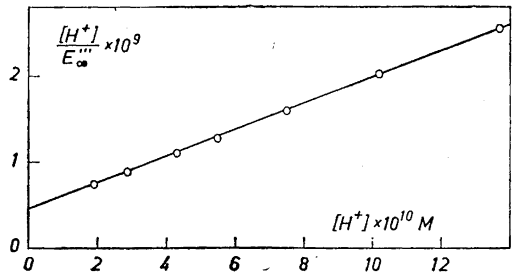


Fig. 17. Dibrom-dimethyl-benzaurin. $[H^+]/E'''_\infty$ als Funktion von $[H^+]$ für die Bestimmung von K_5 und K_6. $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}09 \times 10^{-6}$ M.

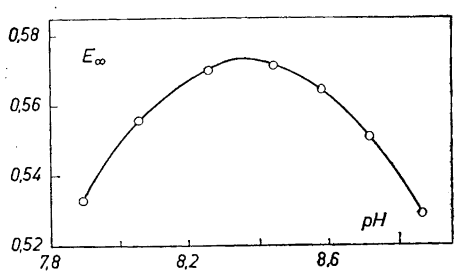


Fig. 18. Dibrom-dimethyl-benzaurin. E_∞ als Funktion von pH für die graphische Bestimmung von $(E_\infty)_{max}$. $\lambda = 580$ mμ, $d = 10$ cm und $C = 1{,}09 \times 10^{-6}$ M. $(E_\infty)_{max} = 0{,}574$.

Die Form G_2 wurde beim Zusatz von Farbstoffstammlösung zu HCl-Lösungen mit der Säurestärke ≥ 2 M qualitativ nachgewiesen. Dabei konnten dieselben Beobachtungen wie bei Tetrabrom-benzaurin gemacht werden.

Messungen der Aufhellungsgeschwindigkeit. Die Geschwindigkeitskonstante k wurde im Gebiet $3{,}4 < \text{pH} < 11{,}1$ bestimmt. Die Messlösungen wurden aus 49 ml Puffer + 1 ml Acetonstammlösung des Farbstoffes bereitet. Die Extinktionsmessungen wurden bei 10 cm Schichtdicke ausgeführt. Die Wellenlänge war 580 mμ für pH > 5, 450 mμ für pH < 5; C betrug $3{,}8(8) \times 10^{-7}$ M für pH < 5,0; $7{,}3 \times 10^{-7}$ M für $5{,}0 < \text{pH} < 7{,}0$ und $1{,}09 \times 10^{-6}$ M für pH > 7,0. Die reaktionskinetischen Verläufe wichen von denen des Tetrabrom-benzaurins nicht prinzipiell ab. Sie werden daher nur für diejenigen sauren bzw. alkalischen Lösungen dargestellt (Fig. 19—20), in denen die Messungen der Berechnung von k_1 und k_2 bzw. k_3 und k_4 zu Grunde lagen. Fig. 23 zeigt k's Ahbängigkeit vom pH-Wert.

Bestimmung von k_1 *und* k_2. Im Gebiet $3{,}4 < \text{pH} < 4{,}6$ ist k_1 aus Formel (9) durch Einsetzen der Relation $k_2 = k_1 \cdot 1/K_2$ und zusammengehörigen Werten

Tabelle 2. Dibrom-dimethyl-benzaurin. Messungen bei 580 mμ zur Bestimmung von K_2; $d = 10$ cm, $C = 1{,}09 \times 10^{-6}$ M

$[H^+] \times 10^7$ M	13,2(5)	10,2(0)	7,2(5)	5,00	2,86(5)	1,42(5)
E_∞	0,030(5)	0,034(5)	0,051(5)	0,068(5)	0,112(5)	0,194(5)
K_2	24,3	27,8	25,4	27,0	26,5	26,3

Tabelle 3. Zusammenfassung der

	$1/K_1$	K_3	K_4	K_5	K_6
Tetrabrom-benzaurin	$2{,}6(0)\times10^{-4}$	$1{,}40\times10^{-6}$	$2{,}1(3)\times10^{-7}$	212	0,86
Dibrom-dimethyl-benzaurin	$1{,}36\times10^{-6}$	$1{,}0\times10^{-8}$	$1{,}9\times10^{-9}$	26	5,(4)

von k und $[H^+]$ berechnet worden. Fig. 21 zeigt, dass k_1 annähernd linear mit $[H^+]$ wächst. Die graphische Bestimmung ergab das Resultat *:

$$k_1^0 = 0{,}3(1) \times 10^{-2} \; ; \; k_1' = 3{,}4 \times 10^2$$

Man erhält also

$k_1 = 3{,}(1)\times10^{-3} + 340 \cdot [H^+]$; $k_2 = 0{,}12\times10^{-3} + 13 \cdot [H^+]$ (Min^{-1}) ; $[H^+]$ in Mol/l.

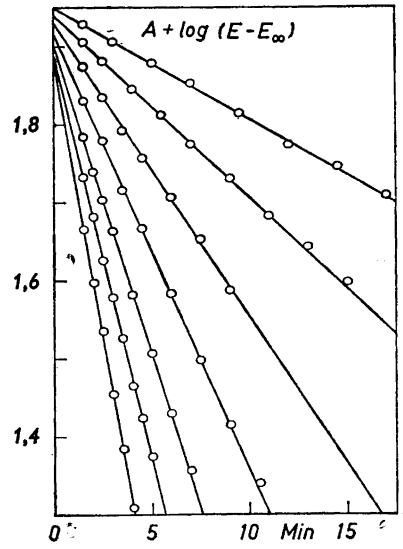


Fig. 19. Dibrom-dimethyl-benzaurin. Reaktionskinetik bei λ = 450 mμ, d = 10 cm und $C = 3{,}88 \times 10^{-7}$ M. Von oben nach unten gelten:

A 3,04 3,015 3,00 2,96 2,95 2,94, 2,91
pH 4,525, 4,292 4,045 3,866 3,690 3,535 3,408

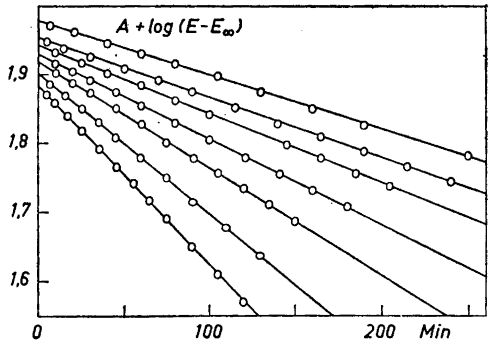


Fig. 20. Dibrom-dimethyl-benzaurin. Reaktionskinetik bei λ = 580 mμ, d = 10 cm und $C = 1{,}09 \times 10^{-6}$ M. Von oben nach unten gelten:

A 2,19 2,13 2,10 2,07 2,05 2,02 2,00
pH 9,99 10,20 10,38 10,57 10,72 10,90 11,04

* Bei der Bestimmung von k_1^0 wurden nur die vier ersten Messpunkte mit einbezogen, wobei das Bild vergrössert wurde. Da k_1^0 klein ist, wird die Bestimmung relativ unsicher.

Ergebnisse. [H+] und [OH⁻] in Mol/l.

k_1 Min^{-1}	k_2 Min^{-1}	k_3 Min^{-1}	k_4 Min^{-1}
$8{,}6\times10^{-2}+32\times[H^+]$	$4{,}1\times10^{-4}+0{,}15\times[H^+]$	$1{,}00\times10^{-2}+41\times[OH^-]$	$0{,}86\times10^{-2}+35\times[OH^-]$
$3{,}(1)\times10^{-3}+340\times[H^+]$	$0{,}12\times10^{-3}+13\times[H^+]$	$0{,}37\times10^{-3}+1{,}58\times[OH^-]$	$2{,}0\times10^{-3}+8{,}5\times[OH^-]$

Bestimmung von k_3 und k_4. Durch Einsetzen der Relation $k_4 = k_3 \cdot K_6$ und zusammengehöriger Werte von k und $[H^+]$ ist k_3 aus Formel (12) im Gebiet $9{,}9<pH<11{,}1$ berechnet worden. Fig. 22 gibt k_3 als Funktion von $[OH^-]$ wieder. Durch graphische Bestimmung wurde $k_3^0 = 0{,}44 \times 10^{-3}$; $k_3' = 1{,}58$ erhalten. Man bekommt also

$$k_3 = 0{,}44\times10^{-3} + 1{,}5(8)\times[OH^-]\,;\; k_4 = 2{,}4\times10^{-3} + 8{,}5\cdot[OH^-]\ (\text{Min}^{-1})\,;$$
$$[OH^-] \text{ in Mol/l.}$$

k als Funktion von pH. In Fig. 23 wird k als Funktion von pH wiedergegeben, wobei die experimentell bestimmten Werte im ganzen untersuchten Gebiet eingetragen sind. Wenn man in derselben Weise wie bei Tetrabrombenzaurin k aus Formel (8) berechnet, erhält man eine Kurve, die sich bei $pH>6{,}5$ von den experimentell bestimmten Werten von k erheblich unterscheidet. Wenn aber bei der Berechnung die Konstanten k_3^0 und k_4^0 korrigiert werden, schliesst sich die berechnete Kurve den experimentell bestimmten Werten an. Die ausgezogene Kurve in Fig. 23 ist mit $k_3 = 0{,}37 \times 10^{-3} + 1{,}58 \cdot [OH^-]$ und $k_4 = 2{,}0 \times 10^{-3} + 8{,}5 \cdot [OH^-]$ (Min^{-1}) berechnet; für die übrigen Konstanten sind die früher berechneten Werte verwendet worden. Bis auf einige Ausnahmen schliessen sich die experimentellen Punkte dieser Kurve gut an. Der graphisch bestimmte Wert von k_3^0 ist also fast 20 % zu

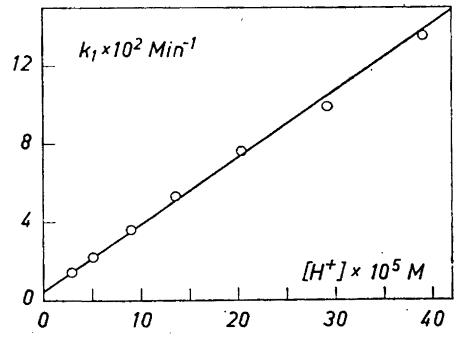


Fig. 21. Dibrom-dimethyl-benzaurin. Die Geschwindigkeitskonstante k_1 als Funktion von [H+].

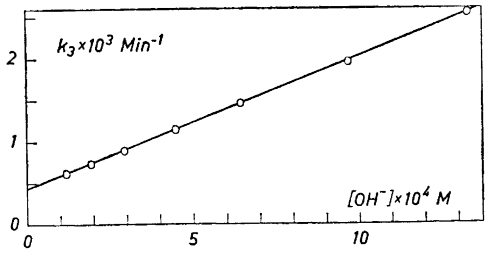


Fig. 22. Dibrom-dimethyl-benzaurin. Die Geschwindigkeitskonstante k_3 als Funktion von [OH⁻].

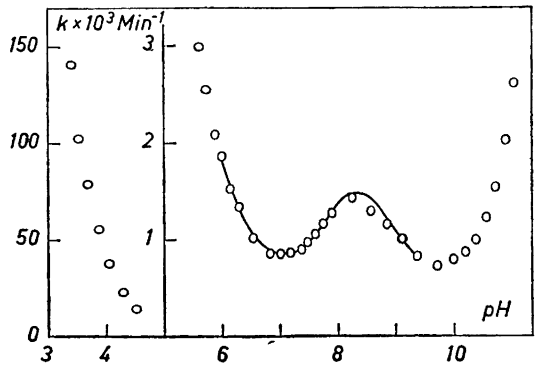


Fig. 23. Dibrom-dimethyl-benzaurin. Die Geschwindigkeitskonstante k als Funktion von pH. Die experimentellen Werte sind O. Die ausgezogene Kurve ist aus Formel (8) berechnet.

gross geworden! ($0{,}44 \times 10^{-3}$ statt $0{,}37 \times 10^{-3}$), als dass die berechnete k-Kurve mit den gefundenen Werten von k übereinstimmt. Warum das so ist, kann nicht mit Bestimmtheit ausgesagt werden. Eine Möglichkeit ist, dass k_3 nicht, wie angenommen wurde, eine linear ansteigende Funktion von $[OH^-]$ auch für *kleine Werte* von $[OH^-]$ ist, sondern dann stärker wächst. k_{max} findet sich bei pH≈8,35 wieder.

DISKUSSION

Tabelle 3 enthält eine Zusammenfassung der erhaltenen Konstanten. Aus dem Vergleich zwischen den Ergebnissen für Tetrabrom-benzaurin und Dibrom-dimethyl-benzaurin ist folgendes zu entnehmen: Eine Substitution von 2 Brom gegen 2 Methyl verringert im vorliegenden Falle die Protolysenkonstanten $1/K_1$, K_3 und K_4 um mehr als 2 Zehnerpotenzen. Gleichzeitig werden beide Hydratationsgleichgewichte stark gegen geringere Hydratation verschoben; K_2 nimmt ab, bzw. K_6 wächst mit dem Faktor 8,1 bzw. 6,2. Gleich auffallend ist die Wirkung auf die Hydratations-(Aufhellungs-)Geschwindigkeit, die sich um eine gute Zehnerpotenz verringert; k_1^0 und k_3^0 nehmen beide mit dem Faktor 27 ab. Dagegen wirkt die Substitution nur unbedeutend auf die Lichtabsorption sowohl bei R als auch bei G_1 ein.

Herrn Professor Sven Bodforss möchte ich meinen besonderen Dank dafür sagen, dass er mir die Anregung zu dieser Untersuchung gegeben und ein stetes Interesse für ihren Fortgang gezeigt hat. Ebenso danke ich Herrn Professor Sture Fronæus und Herrn Dozent Rune Cigén für wertvolle Ratschläge.

Fräulein Lena Hansson war als Assistentin bei Messungen und Berechnungen behilflich.

Die Synthese von Dibrom-dimethyl-benzaurin wurde von Herrn Olle Nilsson, die Mikroelementaranalysen wurden von Herrn Dr. Alfred Bernhardt, Max-Planck-Institut für Kohlenforschung, Mühlheim/Ruhr, ausgeführt.

Die Untersuchung wurde mit finanzieller Hilfe von *Statens Naturvetenskapliga Forskningsråd* und *Kungliga Fysiografiska Sällskapet i Lund* ausgeführt.

Die Übersetzung ins Deutsche ist von fil. mag. Ingrid Wartenberg ausgeführt.

LITERATUR

1. Bodforss, S., Ahrland, S. und Cigén, R. *Z.physik. Chem.* (Leipzig) **203** (1954) 73.
2. Bodforss, S. und Cigén, R. *Kgl. Fysiograf. Sällskap. Lund, Förh.* **26** (1956) No. 6.
3. Cigén, R. *Protolysengleichgewichte und Reaktionsgeschwindigkeiten einiger basischer Triphenylmethanfarbstoffe* (Diss.), Universität Lund 1956.
4. Cigén, R. *Acta Chem. Scand.* **12** (1958) 1456.
5. Bodforss, S. und Hansson, H. G. *Kgl. Fysiograf. Sällskap. Lund, Förh.* **24** (1954) No. 10.
6. Lund, H. *J. Chem. Soc.* **1930** 1844.
7. Meyer, R. und Fischer, O. *Ber.* **46** (1913) 70.
8. Orndorff, W. R., Gibbs, R. C. und Mc Nulty, S. A. *J. Am. Chem. Soc.* **47** (1925) 2767.
9. Orndorff, W. R., Gibbs, R. C., Mc Nulty, S. A. und Shapiro, C. V. *J. Am. Chem. Soc.* **49** (1927) 1541.
10. Döbner, O. *Ann.* **217** (1883) 223.
11. Meyer, R. und Gerloff, W. *Ber.* **56** (1923) 98.
12. Meyer, R. und Gerloff, W. *Ber.* **57** (1924) 591.
13. Lifschitz, J. *Ber.* **58** (1925) 2434.
14. Ramart-Lucas, P. *Bull. soc. chim. France* **8** (1941) 865.
15. Meyer, R. und Funke, E. *Ber.* **57** (1924) 1360.
16. Schroeter, G. *Ann.* **257** (1890) 68.
17. Hansson, H. G. und Nilsson, O. *Kgl. Fysiograf. Sällskap. Lund, Förh.* **29** (1959) No. 9. S. 93 (= Benzaurinstudien I.)

Eingegangen am 3. April 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1332–1346

The Reaction between Streptokinase and Human Plasma

MOGENS LASSEN

Biological Institute of the Carlsberg Foundation, and University Institute of Physical Chemistry, Copenhagen, Denmark

1. The amount of activator formed from a given amount of streptokinase mixed with an excess of proactivator depends on the preparation of proactivator used. A probable explanation is that streptokinase is consumed by two competing reactions, the relative rates of which depend on the preparation of proactivator. The first reaction leads to the formation of the activator; the other is mentioned below (2).

2. Streptokinase is destroyed by small amounts of plasma. The reaction is probably catalyzed by the plasmin formed.

3. The reaction between streptokinase and proactivator initially leads to the formation of activator which is relatively stable at extreme pH values. The rapid transformation of the stable into the well known labile modification is probably catalyzed by the contaminating plasmin, the reaction resambling that mentioned above. Both reactions can be considered side reactions in so far as they are not involved in the formation of the activator.

The mixing of human plasma with the extracellular streptococcal product termed streptokinase results in the formation of a proteolytic enzyme termed plasmin. The following schemes summarize our present knowledge of the mechanism of this reaction:

$$\text{streptokinase} + \text{proactivator} \rightleftharpoons \text{activator}$$

$$\text{plasminogen} \xrightarrow{\text{activator}} \text{plasmin}$$

References and detailed informations are given by Lassen [1]. Yet, it must be emphasized that there is some uncertainty on the nature of the proactivator and the plasminogen, since it is not known wether we are concerned with two different compounds, or with a single precursor which can participate in both reactions. From the results of this paper it is apparent that large systematical errors may interfere with the estimations of proactivator and of streptokinase. Therefore, the author does not feel absolutely sure that the results of the stability experiments previously reported (Lassen [2]) substantiate the two component-theory; but to facilitate the discussion he wants to keep the double terminology.

The present paper deals with the reaction possibilities of the streptokinase after mixing with human plasma, including the first of the above reaction schemes, but excluding the last. The problem mentioned above is of minor importance for the conclusions drawn as the main assumptions are:

1. Activator and plasmin are chemically different. This assumption is based upon the fact that the activator can be destroyed without destroying the plasmin (Müllertz [3]).

2. When streptokinase and human plasma are mixed at neutral pH, activator as well as plasmin are always formed.

3. The activator is formed through a stoicheiometric reaction between streptokinase and a plasma factor (termed proactivator). The most striking evidence for this mode of reaction was given by Troll and Sherry [4], and in essentials their experiments were reproduced by Lassen (Ref.[5], and present paper).

MATERIALS AND METHODS

Veronal-gelatin buffer. 0.05 M sodium diethyl barbiturate buffer pH 7.75 to which was added NaCl to give a final ionic strength of 0.15. Furthermore gelatin to a concentration of 0.2 % was added in order to prevent adsorption of the fibrinolytic factors on glass surfaces (Lassen [8]).

Streptokinase (SK). "Varidase" was kindly placed at my disposal by the Lederle Laboratories Division, American Cyanamid Company. The units are those stated on the packages as determined according to Christensen [6].

Proactivator (PA). Human plasma or serum or acetone powders of these prepared as described by Lassen [1].

L-*Lysine ethyl ester (LEe).* The dihydrochloride of this compound was prepared according to the method of Werbin and Palm [7].

Witte peptone (WP). A commercial product (made by pepsin hydrolysis of bovine fibrin). Before use the powder was dissolved in veronal buffer, heated at 100° for 15 min, and cleared by centrifuging.

Measuring methods. The activator was determined by the lysis time method as described by Lassen [8]. The reported methods for measuring the precursors were modified by adding an equal volume of a 10 % solution of WP to the incubation mixtures of SK and PA. The advantage of this addition will be obvious from the experimental results presented in section 1.

RESULTS

1. Amount of activator formed in mixtures of its precursors.

The rate of formation. These experiments are preliminary investigations made in order to find an appropriate time of incubation usable in "equilibrium" studies. The rate of formation of the activator (A) was found to depend largely on the preparation of proactivator (PA) used. When different plasma samples were mixed with streptokinase (SK) in concentrated solutions the time necessary for obtaining maximum activity varied from 0 to 7 min at room temperature. With serum, the time was prolonged to 10 to 25 min. The rate of deterioration of A was found to depend mainly on the proportion of SK and PA, most commenly being at a maximum with equivalent amounts. Fig. 1 shows the results obtained by estimating A at different times in concentrated and diluted mixtures of SK with plasma or serum. The curves begin

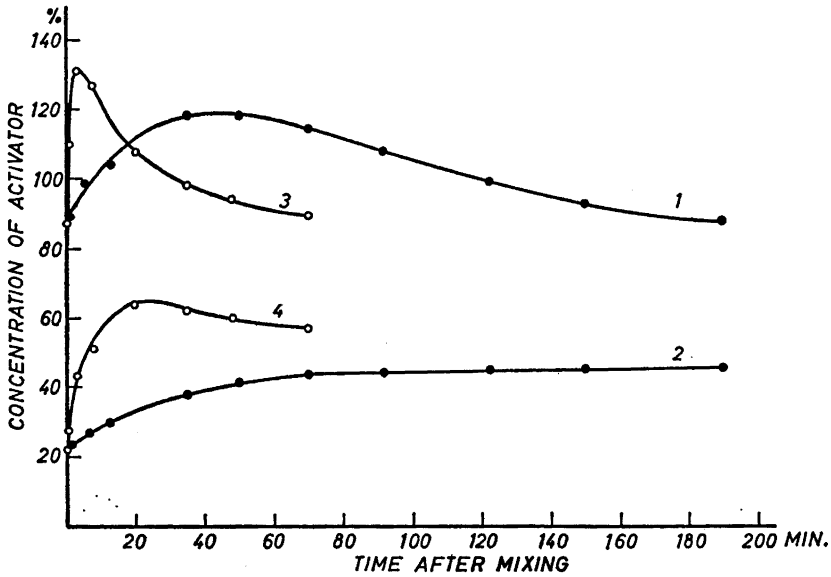


Fig. 1. Formation and deterioration of activator at room temperature. *Diluted incubation mixtures:* 20 ml veronal-gelatin buffer + 500 units of SK + 100 µl plasma (Curve 1) or serum (Curve 2). A was estimated without further dilution. *Concentrated incubation mixtures*: 500 µl plasma (Curve 3) or serum (Curve 4) + 500 µl buffer containing 2 500 units of SK. 50 µl aliquots were diluted with 5 ml buffer before the estimation of A.

above zero concentration, indicating that the formation of A continues during the lysis time determinations. Accordingly, the experimental results do not represent the true variation of the concentration of A in the incubation mixtures.

However, an impression of the "time of formation" is obtained, and since concentrated mixtures of SK and PA generally form maximum amount of A within 20 min at room temperature, this incubation time was used in the experiments described below.

Relationship between the concentration of activator formed and the concentrations of streptokinase and proactivator used. Müllertz [3] estimated the concentration of A formed in different mixtures of SK and human globulin and found it independent of the concentration of the precursor in excess and proportional to the concentration of the other precursor. The results obtained by investigating the relationship between the concentration of lysine esterase and of SK (Lassen [1]), and between the concentration of A and of SK (Lassen [5]) are in accordance with the results of Müllertz. However, later experiments have shown that the results depend largely on the precursor preparations used. Thus, the results presented below differ considerably from those obtained by Müllertz, and show the reaction to be much more complex than assumed earlier. In Fig. 2 A the concentration of SK was constant and the concentration of PA (plasma, curve 1 and 2; serum, curve 3 and 4) was varied, and in Fig. 2B PA

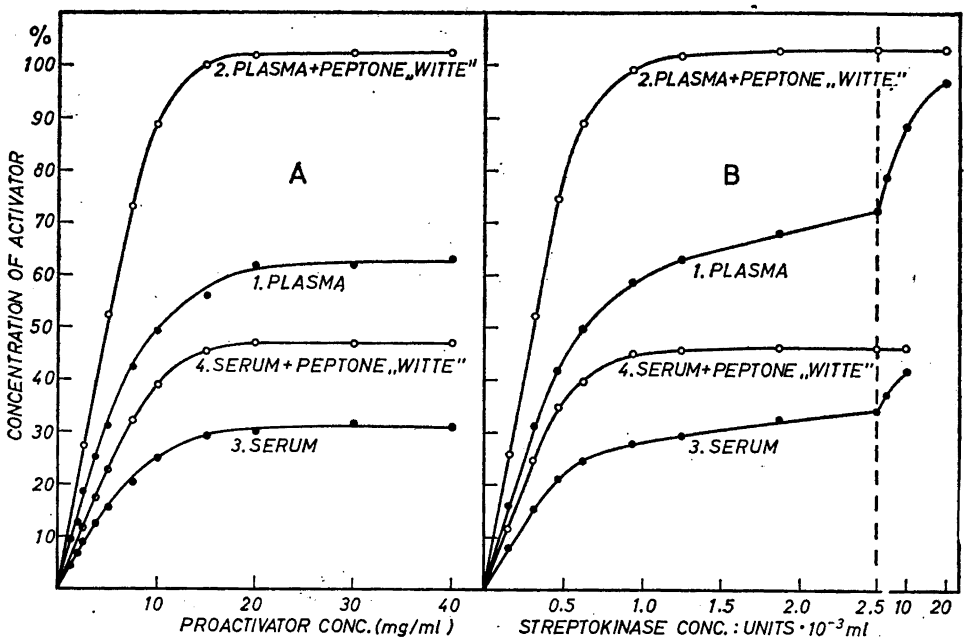


Fig. 2. Relationship between the concentration of activator and of PA (A) or SK (B) in solutions containing a constant concentration of the other precursor. All mixtures were incubated for 20 min at room temperature before estimating A. The mixtures contained: *A*: 625 units of SK per ml + the concentration of plasma acetone powder (Curves 1 and 2) or serum acetone powder (Curves 3 and 4) denoted on the abscissa. The mixtures used for the curves 2 and 4 contained additionally 25 mg of WP per ml. *B*: The concentration of SK denoted on the abscissa + 10 mg of plasma acetone powder (Curves 1 and 2) or 10 mg of serum acetone powder (Curves 3 and 4). The mixtures used for the curves 2 and 4 contained additionally 25 mg of WP per ml.

(plasma, curves 1 and 2; serum, curves 3 and 4) was constant and SK varied. For the curves designated 2 and 4, 2.5 % Witte peptone (WP) was present in all incubation mixtures. These curves resemble the curves obtained by Müllertz, but those without WP differ. The main differences in the shape of the curves are the obviously smaller equilibrium constants and the much more asymmetric behaviour of PA and SK in the experiments without addition of WP. The principal difficulties in understanding these curves become obvious by comparing the "saturation values" in the curves in Fig. 2 A. These values are the maximum amounts of A obtained in mixtures containing an identical amount of SK and excess of PA. If the large differences found are real (that is; really different amounts of A are present in the solutions) it means that the assumption that we are concerned with a simple equilibrium system must be wrong, since this assumption would require identical "saturation values" with different preparations of PA. That the difference between curves 1 and 2 is real is revealed by the experiment represented by Fig. 3. The figure shows

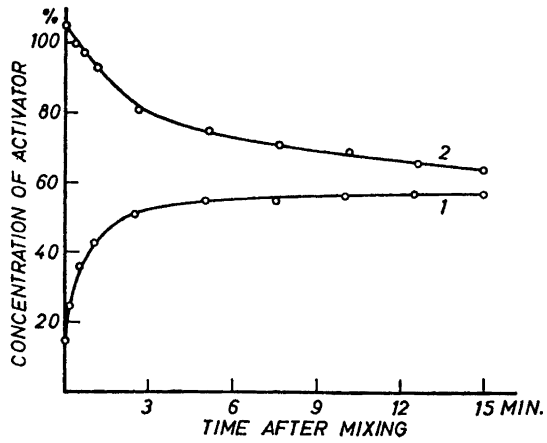


Fig. 3. At room temperature 75 mg of plasma acetone powder dissolved in 1 ml buffer were mixed with 1 000 units of SK dissolved in 1 ml buffer. At different intervals of time 100 µl aliquots were withdrawn and added to either 10 ml buffer for immediate estimation of A (Curve 1) or to 50 µl 10 % WP. After 20 min at room temperature the last samples were diluted with 10 ml buffer, and A was estimated (Curve 2).

that the effect of WP approaches zero, coincident with the completion of the reaction leading to the formation of A, and that WP is without effect on the activity of A already formed.

The stabilizing effect of WP. In addition to its activity-increasing effect WP was also found to have a stabilizing effect on A made from plasma but not on that made from serum. Table 1 shows that A in the case of serum with and without addition of WP, and in the case of plasma without addition of WP is very labile at 37° and nearly disappears within 24 h. By contrast A is nearly stable for 24 h at 37° in the case of plasma plus WP.

Calculation of the equivalent amounts of proactivator and streptokinase. This expression is defined on the basis of the following: From "saturation values" obtained with excess of one precursor in the presence of WP (curves 4 in Fig. 2) the maximum amounts of A which could be obtained from 1 ml of plasma (or from 1 mg of plasma acetone powder) and from 1 unit of SK were calculated. The proportion between these maximum amounts gives the equivalent amounts in units of SK per ml of plasma (or per mg of plasma acetone powder). Due to the uncertainty about the meaning of this value it has been used only for qualitative evaluations of the proportion between SK and PA in given mixtures. With six different plasma samples the equivalent amounts were found to be within the range of 3 500 to 6 000 units of SK per ml plasma.

Discussion. The results presented in this section indicate that the reactions which are initiated by the addition of SK to plasma or serum are much more complex than hitherto assumed. Especially, it must be emphasized that the results in Fig. 2 disagree with the simple introductory scheme. The assumption that A is formed through a stoicheiometric reversible reaction between PA and SK or modifications of these compounds is probably still valid, since

Table 1. The stabilizing effect of Witte peptone.

The following solutions were incubated at 37°.
1. 100 µl plasma + 100 µl SK solution containing 10 000 units per ml + 200 µl veronal-gelatin buffer.
2. 100 µl plasma + 100 µl SK solution as above + 200 µl 10 % WP.
3. 100 µl serum + 100 µl SK solution as above + 200 µl veronal-gelatin buffer.
4. 100 µl serum + 100 µl SK solution as above + 200 µl 10 % WP.

Incubation mixture	Incubation time	Relative amount of activator
1	2 min	45
2	»	98
3	»	28
4	»	32
1	50 »	19
2	»	102
3	»	13
4	»	16
1	24 h	0.2
2	»	97
3	»	0.5
4	»	0.5

this assumption is strongly supported by experiments presented by Troll and Sherry [4]. However, it is evident that this reaction and the subsequent conversion of plasminogen into plasmin are only two of more reactions which are initiated by the mixing of SK and plasma. As an example the different "saturation values" of curves 1 and 2 in Fig. 2, A can most easily be explained by assuming that, while perhaps all SK is used for the formation of A when mixed with excess of plasma + WP, a considerable part of the SK must be

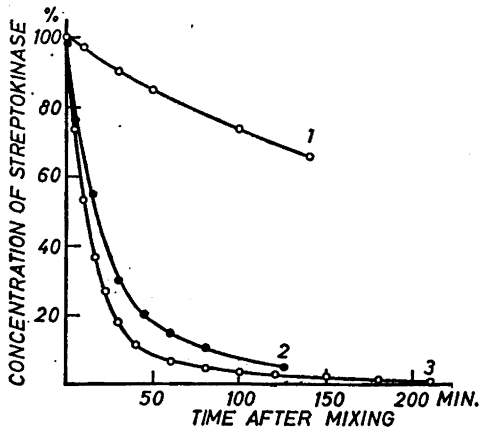


Fig. 4. Destruction of SK with small amounts of plasma. *Curve 1:* 1 ml of a solution containing 10 000 units of SK + 1 ml 20 % plasma were incubated at 25°. *Curve 2:* The same mixture as used for curve 1, but incubated at 37°. *Curve 3:* As for curve 2, but another plasma sample had been used.

consumed by another reaction when WP is omitted. More detailed investigations of this problem will be presented in the subsequent section.

2. Destruction of streptokinase.

This section deals with the possible side reaction mentioned in the above discussion. Below is shown that SK after mixing with plasma is consumed by two competing reactions; the first being the known reaction leading to the formation of A while the second leads to the destruction of SK. SK dissolved in veronal-gelatin buffer was found to be stable for hours at all temperatures below 50°. However, when small amounts of plasma or serum were added SK was found to be labile (Fig. 4). Comparing curves 1 and 2 of Fig. 4 it is apparent that for a rise of 10° the rate of reaction is increased tenfold.

In the next experiment (Fig. 5) solutions of SK were mixed with increasing small amounts of plasma acetone powder and incubated for 60 min at 37°, and the remaining concentrations of SK were then estimated. Although the reaction rates cannot be evaluated from these 60 min values it is evident however, that with small amounts of plasma acetone powder, the reaction rate is more than doubled when the concentration of plasma is doubled. With higher concentrations of plasma acetone powder this phenomenon is less significant, but a limited reaction rate was not obtained within the concentration range used.

Determination of SK destroying factor. The SK destroying factor present in plasma can be measured by determining the destroying effect in defined manner, provided that the relationship between concentration and effect is known. The following method was found convenient: The experimental sample was diluted to give a concentration of PA equivalent to less than 500 units of SK per ml. By reason of accuracy the concentration of destroying factor

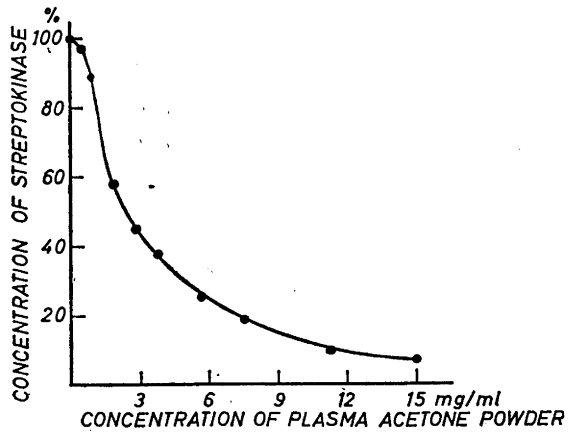


Fig. 5. Destruction of SK at 37° with small amounts of plasma. Mixtures containing 5 000 units of SK per ml and the concentrations of plasma acetone powder denoted on the abscissa were incubated for 60 min at 37°, and the residual concentrations of SK were then estimated.

must be within the range of 2 to 10 % of that found in plasma. 50 μl of the diluted sample was incubated for 60 min at 37° with 50 μl of a solution containing 5 000 units of SK per ml, which is a large excess of SK. The SK not destroyed was then converted into activator by adding 100 μl plasma, which now provides an excess of proactivator, and incubating for 20 min at room temperature. Finally, 10 ml veronal-gelatin buffer was added, and lysis time determined as described. Lysis times were converted into concentrations of destroying factor by interpolation on a standard curve obtained by testing serial dilutions of plasma as described for the experimental sample. Good results could be obtained within the range of 2 to 10 % of undiluted plasma (before mixing with SK).

Attempts to identify the streptokinase destroying component. 1 a. Plasmin (Müllertz [9]) and A (Müllertz [9], Lassen [5]) are adsorbed on fibrin. To find out whether the SK destroying factor is also adsorbed the following experiment was performed: 1 ml of plasma was mixed with 1 ml of a solution containing 5 000 units of SK (nearly equivalent amounts) plus 50 μl thrombin solution. After incubation for 20 min the fibrin clot was removed. Estimations revealed that plasmin, A, PA, and SK destroying component were completely absent from the serum (plasmin was estimated by the manometric method described by Lassen [1]).

1 b. In a control experiment in which no SK was added only slight differences between plasma and serum were found. The activities in serum expressed as percentages of the corresponding activities in plasma were: PA 55 %, plasminogen 90 %, and SK destroying component 70 %. Plasminogen was measured by adding an excess of SK and estimating the plasmin activities as above, ignoring the effect of plasmin inhibitors. These two experiments show that the SK destroying component resembles plasminogen and PA by being present in plasma as a precursor which is not adsorbed to fibrin, and which is transformed into another component by addition of SK; this component is strongly adsorbed to fibrin.

2. SK was found to be rapidly destroyed by preparations of human as well as bovine plasmin. The human plasmin was prepared according to Müllertz [3], but since the preparation contained considerable amounts of PA the observations obtained with this did not give further information as to the nature of the destroying component. The bovine plasmin was prepared according to Lassen [1] with the modification that a smaller amount of activator was employed (100 μl human plasma + 1 000 units of SK (excess) to 10 ml of 1.3 % of bovine fibrinogen). The plasmin activity of the final preparation was compared to that of SK activated human plasma. Assuming no inactivation of the original amount of A present in the preparation the proportion A/plasmin was about 1 /50 of that in SK activated human plasma. Solutions of bovine plasmin containing the same plasmin activity as did SK activated human plasma were found to destroy SK somewhat faster than did human plasma.

3. Inhibition studies: The destruction of SK by human plasma was found to be inhibited by L-lysine ethyl ester (Fig. 6) by WP (Fig. 7), by benzoyl-L-arginine ethyl ester and, to a smaller extent, by casein. The synthetic esters are known to inhibit plasmin and A (Troll and Sherry [4], Lassen [1]), and in the

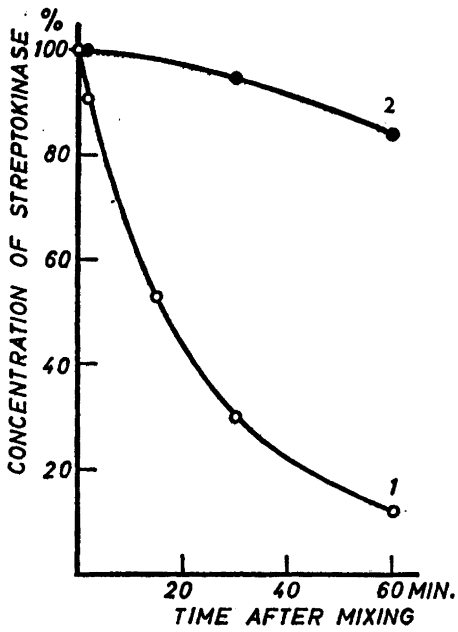


Fig. 6. The effect of LEe on the destruction of SK with small amounts of plasma at 37°. The following mixtures were employed. *Curve 1:* 1 000 units of SK dissolved in 100 µl buffer + 100 µl 10 % plasma + 100 µl 0.3 M NaCl. *Curve 2:* As for curve 1, except that 100 µl 0.1 M LEe were substituted for the NaCl solution.

lysis time method WP and casein were found to inhibit the fibrinolytic effect of bovine as well as human plasmin. Contrary to the inhibition of plasma neither WP nor casein seem to inhibit A as judged from preliminary experiments on the activation rate of bovine plasminogen in the presence or absence of these compounds. The formation of plasmin was followed by the semi-quantitative method described by Lassen [1].

Conclusion. SK is destroyed by small amounts of plasma. Studies on the adsorption on fibrin indicate that the destroying factor is identical with plasmin and/or activator. The isolated effects of human plasmin and activator on SK could not be studied as the necessary preparations were not available. However, the fact that the SK destroying activity of bovine plasmin is comparable to that of human plasma gives some evidence for the assumption that the destruction of SK caused by human plasma, is due to the proteolytic effect of the plasmin formed. This assumption was further supported by inhibition studies. Accordingly, the reaction may be described by:

$$\text{SK} \xrightarrow{\text{plasmin}} \text{destroyed SK}$$

As emphasized in the discussion of the preceding section the existence of a reaction like this is a necessary condition for explaining the fact that the maximum amount of A which can be formed from a given amount of SK depends on the source of PA (Fig. 2 A). The amount of A formed in a given mixture of PA and SK depends on the original concentrations of the precursors

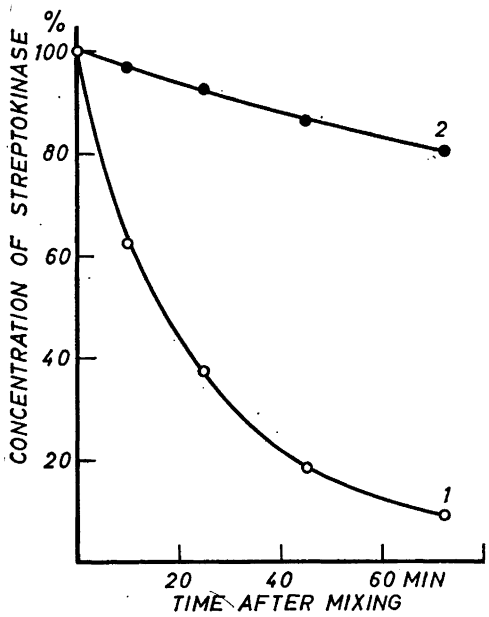


Fig. 7. The effect of WP on the destruction of SK with small amounts of plasma at 37°. The following mixtures were employed. *Curve 1:* 200 units of SK dissolved in 200 µl buffer + 200 µl 10 % plasma + 200 µl veronal buffer. *Curve 2:* As for curve 1, except that 20 mg of WP were dissolved in the buffer solution.

as well as on the relative rates of the two competing reactions; namely the stoicheiometric reaction leading to the formation of A and the above mentioned destruction of SK which is probably caused by the plasmin formed. The formation of A proceeds at a slower rate in serum than in plasma and supposing nearly identical rates of destruction the different "saturation values" of curve 1 and 2 in Fig. 2 A becomes understandable. The effect of WP on the "saturation values" can at least partly be explained by its inhibitory effect on the destruction of SK.

3. The transformation of stable into labile activator.

Troll and Sherry [4] found that a transformation of A into PA could be achieved by heating at 100° for 15 min at pH 2. Since SK is labile under these conditions they assumed the reaction to be caused by the isolated destruction of SK, leaving the more stable PA unchanged in the solution. Lassen [5] found the same transformation to proceed at milder conditions (37° and pH 3.5). The inactivation did not run to completion as a certain part of A was found to be stable under the experimental conditions used. It was later found that the same reaction can proceed at alkaline pH. Fig. 8, curve 1, shows the inactiva-

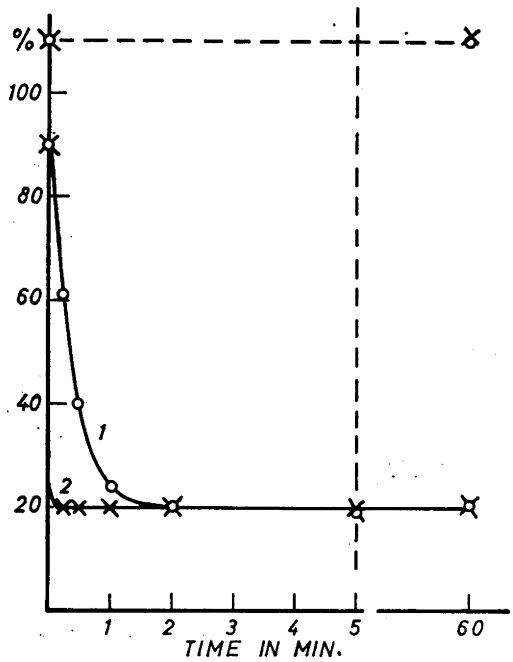


Fig. 8. Inactivation of activator in acid and alkaline solutions. Ordinate: Concentration of A (full lines) or PA plus A (dotted line) in per cent of the untreated solutions of A. The activator solution was prepared by adding 1 ml plasma to 1 ml of a solution containing 5 000 units of SK and incubating for 20 min at room temperature. The inactivation at acid reaction (Curve 1, (O)) was performed by adding 1 ml A solution to 1 ml 0.1 M HCl in water bath at 37°, yielding a pH of about 3. The inactivation at alkaline reaction (Curve 2, (×)) was performed by adding 1 ml A to 1 ml 0.1 M NaOH in an ice bath, yielding a pH of about 13. A was estimated in 100 μl aliquots diluted with 10 ml veronal gelatin buffer. For the estimations of PA + A 100 μl samples were diluted with10 ml-veronal-gelatin buffer containing 500 units of SK. These solutions were incubated for 60 min at room temperature before the lysis time determinations.

tion of A at 37° and pH about 3.0, while curve 2 shows the inactivation of the same preparation of A at 0° and at a pH between 12 and 13. The amount of stable A is seen to be independent of the mode of inactivation. This probably excludes the interpretation that the stable residue represents an equilibrium concentration in a reversible denaturation, and supports the assumption that labile and stable A are chemically different components. SK itself was found to be stable for at least 1 h under the conditions used for the inactivation of A. (At the acid pH this is true only when proactivator or denaturated activator is present in the solution, at the alkaline pH also uncontaminated SK was found to be stable). This makes the situation a little more complicated than assumed by Troll and Sherry [4]. The assumption that the denaturation of the activator at extreme pH values is caused by the isolated destruction of the SK part of the activator molecule may still be valid; but this SK part

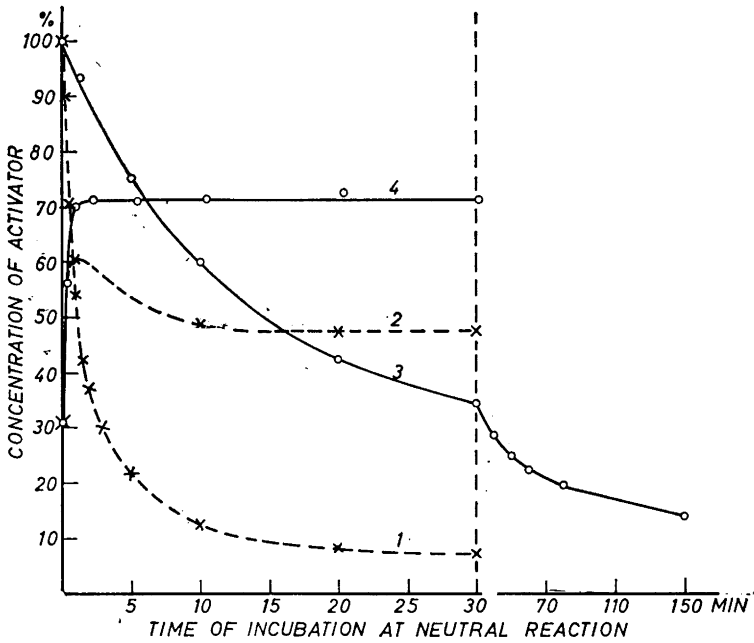


Fig. 9. The formation (and deterioration) of A (Curves 2 and 4), and the degree of inactivation at alkaline reaction (Curves 1 and 3) followed with time in mixtures of SK and PA without addition of WP (dotted lines), and with addition of WP (full lines). The experiments were performed at room temperature (22°). For the dotted curves a mixture of 1 ml plasma with 0.5 ml of a solution containing 4 000 units of SK was used. The mixture used for the full drawn curves contained in addition a final concentration of WP equal to 2.5 %. The activator was estimated in 100 μl aliquots diluted to 10 ml with veronal-gelatin buffer. The inactivations were performed by adding 100 μl samples to 50 μl 0.2 M NaOH at 0°. After standing for 10 min at 0° the residual amount of A plus SK was estimated by adding 50 μl 0.2 M HCl + 200 μl buffer containing 25 % plasma (v/v) and 10 % WP (w/v), incubating for about 20 min at room temperature, diluting with 10 ml veronal-gelatin buffer, and finally determining the lysis times as described. All results were converted into concentrations of A by interpolation on the same standard curve.

is not identical with the original SK-molecule, as some transformation into a more labile modification must have occurred during the previous incubation at neutral reactions. The nature of the stable residue cannot be evaluated on the basis of the above experiments. We may be concerned either with a stable activator molecule or with a residue of unreacted SK which during the assay reacts with PA to form A.

Since total A and labile A can be measured independently it might be possible to obtain further information of the whole problem by comparing the relative rates of the formation of these quantities, especially if it could be possible to find conditions under wich these formation rates differ significantly. Nearly equivalent amounts of plasma and SK were mixed at room temperature and at a neutral pH. At different intervals of time samples of this mixture

were treated at alkaline reaction, and the residual amounts of A plus SK were estimated by neutralizing, adding an excess of proactivator and measuring the total amount of activator formed (Fig. 9, curve 1). On the condition that SK and the activator formed is stable in the neutral mixture of plasma and SK the difference between the ordinate at zero time and that at the time t represents the amount of labile A present in the neutral solution at the time t. Since this condition is not fulfilled the curve obtained does not permit an exact calculation of the amounts of labile A formed, but a rough picture of the rate of formation can be obtained. Simultaneously with the performance of the estimations used for curve 1 the formation and deterioration of A in the original mixture were followed with time (Curve 2). Unfortunately, there is a high initial lability of A, and therefore the curve does give any information of the time of formation. Accordingly, no conclusions can be drawn from the results of the whole experiment.

More valuable information was obtained by adding Witte peptone (WP) to the neutral incubation mixture of SK and plasma. The rate of formation of labile A appears from curve 3 of Fig. 9 which correspond to curve 1 in the above experiment. Obviously the formation of labile A was strongly inhibited by WP, the rate of formation being reduced to about 1/10 of before. The formation of A in the mixture of plasma SK and WP is represented by curve 4. The activator formed is seen to be stable throughout the whole experiment and evidently the formation ceased 2 to 3 min after mixing. At this time nearly all activator was present as a stable modification (stable at alkaline pH), but during the next few hours this modification was transformed into the known labile modification. These results strongly suggest that PA and SK initially react to form an activator which is stable at extreme pH values. In mixtures of SK and plasma, this primarily formed activator is rapidly transformed into a labile modification having the same activating properties. (The transformation of the stable into the labile modification does not alter the activities as judged from curve 4). The rate of the formation of stable A is not significantly influenced by WP, while the secondary transformation is inhibited by this agent.

The effect of L-lysine-ethylester (LEe) was found to be comparable to that of WP. The experiment was performed as described in the legend to Fig. 9, except that 0.05 M LEe was substituted for WP. After incubation for 30 min only 5 % of the total amount of A plus SK were present as labile A. However, after 1 h the rate of reaction increased, and after 3 h only 10 % remained in the form of stable A. This increased rate was accomplished by a drift downwards in pH due to hydrolysis of the ester, and therefore quantitative details other than those given above are probably without significance.

Conclusion. The activator formed by the reaction between SK and PA is extremely labile at acid and alkaline reactions. The experimental results presented above strongly indicate that the reaction sequence leading to the formation of this labile A involves at least two consequtive steps, the first being the stoicheiometric reaction between the precursors, during which a more stable modification of A is formed, while the last step is a transformation of the stable into the well known labile modification. The temporary existance of the stable A was demonstrable in mixtures of plasma and SK to which

Witte peptone or L-lysine-ethylester was added. This was possible since these agents proved to reduce strongly the rate of the secondary transformation without significantly affecting the rate of the primary formation of the stable A.

DISCUSSION

The present work is an experimental investigation on the reaction possibilities of streptokinase (SK) after mixing with the fibrinolytic precursor(s) contained in human plasma. It is considered an experimental fact that SK reacts stoicheiometrically with a precursor in human plasma termed proactivator (PA) giving rise to the formation of an activator of plasminogen (A) (Müllertz and Lassen [10], Troll and Sherry [4] and Müllertz [3]). It seems probable that this reaction can be described by one of the following two schemes:

$$PA + SK \rightleftharpoons A \quad (1)$$

$$PA + SK \rightleftharpoons A + \text{inactive component} \quad (2)$$

This reaction is followed by the catalyzed transformation of plasminogen into plasmin:

$$\text{plasminogen} \xrightarrow{A} \text{plasmin}$$

In all probability the last reaction involves the splitting of a peptide bond (Troll and Sherry [4]).

The "equilibrium" studies presented in the first section of this paper indicate that these schemes do not represent all reaction possibilities of the precursors concerned, and therefore suggested a search for competing reactions. Evidence for the existence of such competing reactions were obtained in the case of SK as well as in the case of PA (not published) but owing to the irritating situation that the nature of the PA is still unknown a discussion of the last case is omitted. In the case of SK the competing reaction probably consists in a proteolytic splitting of one or more peptide bonds in the SK molecule caused by the plasmin formed, which is not surprising since SK is a protein and plasmin is a proteolytic enzyme. The quantitative importance of this reaction appears from Fig. 2 A. With excess of PA supplied as serum at least 70 % of the SK is consumed by the side reaction and only 30 % by the main reaction leading to the formation of A. Substitution of plasma for serum results in a higher rate of the main reaction and accordingly a smaller fraction of the SK is destroyed by the side reaction.

The last section deals with a reaction which seems to have a certain resemblance to this side reaction. The results show that the reaction between SK and plasma results in the formation of two chemically different modifications of A. One of these is relatively stable at extreme pH values, and may be considered the primary product of the reaction between SK and PA. This modification does have a temporary existence only, since it is rapidly transformed into the labile A ordinarily dealt with. If the above mentioned reaction (1) is valid the formation of labile A may be described by:

$$PA + SK \rightleftharpoons \text{stable A} \rightleftharpoons \text{labile A}$$

During the inactivation of labile A at acid and alkaline pH the sum of A and PA is constant, which means that the reaction consists in a transformation of labile A into PA plus some sort of inactivated SK.

$$\text{labile A} \rightarrow \text{PA} + \text{inactivated SK}$$

Since stable A as well as the original SK is stable under the conditions used for the inactivation of labile A the difference between stable and labile A must be due to a difference in the SK part of the activator molecule. Still supposing that the reaction (1) is valid the whole series of reactions may be described by:

at neutral pH: $\underset{\text{stable A}}{\text{PA} + \text{SK}} \rightleftharpoons \underset{\text{labile A}}{\text{PA, SK}'}$

at acid pH: $\underset{\text{labile A}}{\text{PA,SK}'} \rightarrow \underset{\text{inactivated SK}}{\text{PA} + \text{SK}''}$

If reaction (2) is valid the SK molecule must be present — partly or as a whole — in either the A molecule or in the inactive component. In this case more detailed conclusions must be omitted, but the general conclusion that it is this "modified SK molecule" on the right side of the equilibrium sign which is changed during the formation of labile A is still probable.

Since we have already seen that plasmin may attack SK there is some probability that the formation of labile A is due to a similar effect of plasmin:

$$\text{stable A} \xrightarrow{\text{plasmin}} \text{labile A}$$

This probability is further supported by the fact that both reactions are inhibited by L-lysine-ethylester and by Witte peptone.

Acknowledgements. The author is indebted to Dr. Tage Astrup of the Biological Institute and to Dr. Daniel L. Kline, Yale University, U.S.A. for valuable discussions and helpful advice. This investigation was made possible by a grant from *Statens almindelige Videnskabsfond*, Denmark. It forms part of the investigations on fibrinolysis for which Dr. Astrup receives support from the *Josiah Macy, Jr. Foundation*, New York, and the *National Danish Association against Rheumatic Diseases.*

REFERENCES

1. Lassen, M. *Biochem. J.* **69** (1958) 360.
2. Lassen, M. *Acta Chem. Scand.* **13** (1959) 1064.
3. Müllertz, S. *Biochem. J.* **61** (1955) 424.
4. Troll, W. and Sherry, S. *J. Biol. Chem.* **213** (1955) 881.
5. Lassen, M. *Acta Chem. Scand.* **12** (1958) 1825.
6. Christensen, L. R. *J. Clin. Invest.* **28** (1949) 163.
7. Werbin, H. and Palm, A. *J. Am. Chem. Soc.* **73** (1951) 1382.
8. Lassen, M. *Scand. J. Clin. Lab. Invest.* **10** (1958) 384.
9. Müllertz, S. *Mechanism of activation and effect of plasmin in blood* (Diss); *Acta Physiol. Scand.* **38** (1956) Suppl. No. 130.
10. Müllertz, S. and Lassen, M. *Proc. Soc. Exptl. Biol. Med.* **82** (1953) 64.

Received April 29, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1347–1357

Solvent Extraction of Metal Chelates

I. Application of a Titration Procedure to the Study of the Extraction of Metal Chelates

BROR SKYTTE JENSEN

Danish Atomic Energy Commission, Risø, Denmark

A two-phase titration procedure for the study of metalchelate equilibria is presented. Equations expressing the relationship between the apparent mean complexity constant in the two-phase system, the mean complexity constant in a water phase and the distribution coefficients of the uncharged species are derived. The pH-value of 50 % extraction of a metal ion is determined with good approximation by this method. The error introduced by the simplified theoretical treatment is discussed and the predictions are confirmed by independent distribution experiments done, employing lanthanum-140 as tracer.

Extraction is among the few separation procedures where in principle the same laws are valid both for macro and tracer concentrations. Its application thus ranges from large scale technical separations to radiochemical work with practically weightless amounts. In recent years extraction procedures have especially been applied for the separation of metals, thus in the reprocessing of spent reactor fuel and in numerous analytical and radiochemical separations.[1–4]

In the reprocessing of spent reactor fuel by extraction, methods based on the extractability of certain mixed complexes of UO_2^{+2} or Pu^{+4} with nitrate ion and organic solvents containing oxygen are presently the most favoured. The advantage of this method is an extraordinary selectivity of the metals which can be extracted.

The extraction and separation, on a tehnical scale, of actinides from fission products by chelating agents have been performed with thenoyltrifluoroacetone, an extractive agent evaluated by Reid and Calvin [5,6]. An advantage of this process is that there is no need for a salting-out agent, thus reducing waste disposal problems. A significant disadvantage, however, is that the rate of chelation is slow, which has serious consequences for a large scale countercurrent extraction.

Other chelating agents of possible use for reprocessing have been investigated mostly by Swedish chemists [7–9].

Apart from its possible use in the reprocessing of spent reactor fuels, an extractive chelating agent might possess other valuable properties, *e.g.* as an analytical reagent. It seems well worth to continue the investigations in a systematic manner to gain more insight into the influence of the properties of the molecule on its usefulness as an extractive agent.

The chelating agents, previously investigated, constitute a rather inhomogeneous assembly of chemically very different compounds, and only uncertain predictions can be made as to the improvement of an existing chelating agent in one or other respects by modifying its molecular structure. Therefore it seems interesting to perform an analysis on series of closely related compounds in the expectation that secondary effects of any nature will remain constant or nearly so throughout the series. The comparison of chelates is then expected to give a more clear picture of the trends in extractive behaviour as a function of the constants of the chelating agent, and as far as their magnitudes are influenced by substituents or steric arrangements of the molecules, as a function of those.

In a systematic investigation of a large assembly there is always the need for a rapid and reliable method to estimate the values to be compared. In the study of metal ion extraction by chelating agents rather laborious methods have been used to determine the distribution coefficients. The most favoured ones seem to be photometric and radiometric methods. This paper presents an easy titration procedure for the study of the extraction of metal chelates, and the signification of the measured constants is discussed and related to the extractive behaviour of the chelating agent. The method has been used on some 1-phenyl-3-methyl-4-acyl pyrazolones [10,11] and on some O-oxy-azocompounds.

THEORY

The formation of a complex of type MA_q from a metal ion M^{N+} and the anion of a monobasic chelating agent HA is now generally believed to occur stepwise through intermediates of composition

$$MA_i \; ; \; (i = 0,1,2,\text{---},q) \tag{1}$$

where q is the maximum coordination number of the metalion for the anion A^-. The notation of charge and of coordinated water has been omitted for simplicity.

Assuming the formation of polynuclear complexes, hydrolysis products and competing complexes negligible — an assumption which can be regarded valid in dilute acid solutions of a metalnitrate or metal-perchlorate and a chelating agent — the total concentration C_M of metal in a homogeneous solution can be expressed as

$$C_M = \sum_{i=0}^{i=q} [MA_i] = [M] \sum_{i=0}^{i=q} \beta_i [A]^i \tag{2}$$

where we have introduced the formation constants, K_i, and the complexity products, β_i [12,13], defined as

$$K_i = \frac{[MA_i]}{[A][MA_{i-1}]} \qquad (3) \qquad\qquad \beta_i = K_1 K_2 - - - K_i \qquad (4)$$

$$\beta_0 = 1 \qquad (5)$$

Similarily we may express the concentration $[A_b]$ of ligands A bound to metal ions as

$$[A_b] = \sum_{i=0}^{i=q} i\,[MA_i] = [M] \sum_{i=0}^{i=q} i\ \beta_i [A]^i \qquad (6)$$

The formation function $\bar{n}$ for a system of metal ions and ligands defined as the average number of ligands bound per metal ion present [12,13] can be expressed as

$$\bar{n} = \frac{[A_b]}{C_M} = \frac{\sum_{i=0}^{i=q} i\,[MA_i]}{\sum_{i=0}^{i=q} [MA_i]} = \frac{\sum_{i=0}^{i=q} i\,\beta_i [A]^i}{\sum_{i=0}^{i=q} \beta_i [A]^i}$$

$$= \frac{K_1[A] + 2K_1K_2[A]^2 + ..+ NK_1 - - K_N[A]^N + ..+ qK_1 - - K_q\,[A]^q}{1 + K_1\,[A] + K_1K_2[A]^2 + ..+ K_1 - - K_N[A]^N + ..+ K_1 - - K_q[A]^q} \qquad (7)$$

In a two phase system of equal columes of the phases, where only the uncharged species MA_N is assumed to be distributed between the organic phase and water with a distribution coefficient λ defined as

$$\lambda = \frac{[MA_N]^{org}}{[MA_N]} \qquad (8)$$

a new formation function $\bar{n}_E$ may be defined

$$\bar{n}_E = \frac{[A_{b,E}]}{C_M} = \frac{K_1[A] + 2K_1K_2[A]^2 + (1+\lambda)NK_1K_2 - - K_N[A]^N + - - - - - -}{1 + K_1[A] + K_1K_2[A]^2 + ..+ (1+\lambda)K_1K_2 - - K_N[A]^N + - - -} \qquad (9)$$

valid for the heterogeneous system, but expressed in variables occurring only in the water phase. The system will now behave as if the formation constant K_N has been multiplied by the factor $(1 + \lambda)$. If, as is often the case for chelates,

$$N = q \qquad (10)$$

then the mean complexity constant K_{av} for the two phase system determined by standard procedures [12–16] will be

$$K_{av} = \sqrt[N]{\beta_N(1 + \lambda)} \qquad (11$$

The spreading factor [12,13], x, which is a measure of chemical effects on the successive formation constants, is defined as

$$\frac{(i+1)(N-i+1)}{i(N-i)}\,x^2 = \frac{K_i}{K_{i+1}} \tag{12}$$

In a two phase system for $1 + i = N$ we get

$$\frac{2N}{N-1}\,x^2 = \frac{K_{N-1}}{K_N(1+\lambda)} \tag{13}$$

As $(1 + \lambda) \gg 1$ for systems of practical interest the spreading factor will be small. This means, according to Bjerrum [12,13], that a plot of $\bar{n}_E$ against log [A] will be steep at the inflection point, corresponding to almost simultaneous dissociation of the N ligands from the complex; especially so because the formation constants $K_1, K_2, \cdots K_N$ usually do not differ very much [18].

To determine $K_{av} = \sqrt[N]{\beta_N(1+\lambda)}$, $\bar{n}_E$ must be known as a function of [A].

In a two phase system containing HA and metal salt MX_N it is possible to write the material balance regarding HA in a way that relates the concentration of some species in the organic phase to the actual concentrations of the water phase, simply by dividing by the volume ratio of the phases, thus

$$C_A = [A] + [HA]^{org} + [A_{b,E}] + [HA] \tag{14}$$

In this expression [A] and [HA] are solely contained in the water phase, $[HA]^{org}$ represents the amount HA extracted into the organic phase and $[A_{b,E}]$ represents the sum of intermediary complexes in both phases.

An expression describing the behaviour of the chelating agent in the above system can be obtained from the following equations combined with (14) and (9)

$$K_d = \frac{[HA]^{org}}{[HA]} \tag{15}$$

$$K_a = \frac{[H][A]}{[HA]} \tag{16}$$

defining the acid dissociation constant K_a and the distribution coefficient K_d of the chelating agent.

We get

$$K_b = \frac{K_a}{1+K_d} = \frac{[H][A]}{C_A - \bar{n}_E \cdot C_M - [A]} = \frac{[H](C_A - [HA] - [HA]^{org} - [A_{b,E}])}{[HA] + [HA]^{org}} \tag{17}$$

This is an equation from which to determine [A], when $[A_{b,E}]$ or $\bar{n}_E$ is known.

For $[A_{b,E}] = 0$ this equation shows that an acid in a two phase system will behave as if it has an acid dissociation constant K_b, where

$$pK_b = pK_a + \log\,(1 + K_d) \tag{18}$$

This equation is derived under the assumption that no association or dissociation of HA occur in the organic phase (eqn. 15).

To determine $[A_{b,E}]$ a method proposed by Calvin and Melchior [14] has been further evaluated.

Assuming only uncharged species to be extracted it is possible to express the electroneutrality equation for the water phase during a titration with NaOH by

$$[H] + [Na] + \sum_{i=0}^{i=N} (N-i)\,[MA_i] = [X] + [A] + [OH] \tag{19}$$

Inserting

$$[X] = N \cdot C_M \tag{20}$$

and eqns (2) and (6) this reduces to

$$[H] + [Na] - [A_{b,E}] - [OH] = [A] \tag{21}$$

From (14) and (21) we obtain

$$C_A = [H] + [Na] + [HA] + [HA]^{org} - [OH] \tag{22}$$

Now assuming C_A constant, [H] constant and therefore [OH] constant we get, by differentiation

$$dC_A = 0 = d[Na] + d([HA] + [HA]^{org}) \tag{23}$$

This represents a relation between the change in $([HA] + [HA]^{org})$ as a function of a change in [Na] during a simultaneous addition of NaOH and MX_N to the system in such a way that pH is kept constant. Integrating between the case where no metal salt has been added and the case where C_M has been added we obtain

$$\int_a^b d[Na] = [Na_2] - [Na_1] = -\int_a^b d([HA] + [HA]^{org})$$

$$= -\int_0^{A_b} \frac{d([HA] + [HA]^{org})}{d[A_{b,E}]}\, d[A_{b,E}] \tag{24}$$

where the integration limit a corresponds to a point on the titration curve of the chelating agent without metal salt added and b to a point at the same pH on the titration curve obtained with MX_N added in total concentration C_M. It is assumed that no Na-complexes have been formed.

From eqn (17) upon differentiation with respect to $[A_{b,E}]$ we obtain

$$\frac{d([HA] + [HA]^{org})}{d[A_{b,E}]} = -\frac{[H]}{[H] + K_b} \tag{25}$$

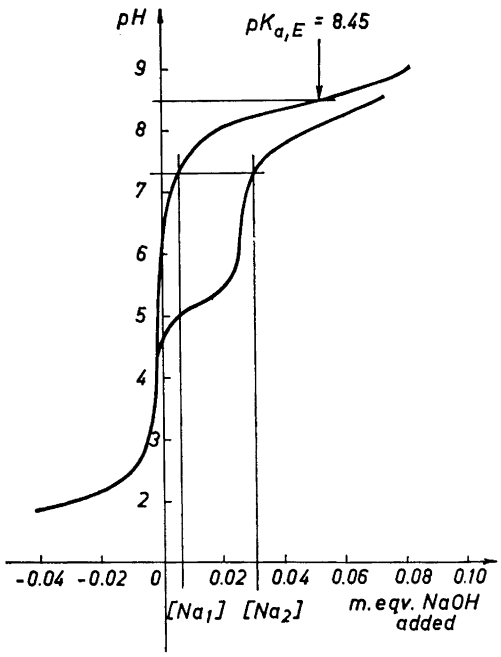


Fig. 1. Titration curve and complex-extraction titration curve for the system TTA, lanthanum ion, chloroform-water.

It is thus possible to integrate eqn (24) for [H] = constant leading to

$$([Na_2] - [Na_1]) \frac{[H]+K_b}{[H]} = [A_{b,E}] \tag{26}$$

As corresponding values are $[A_{b,E}] = 0$, and [Na] = $[Na_1]$, and [H] and [OH] may be neglected at not extreme pH values, we get

$$[A_{b,E}] = ([Na_2] - [Na_1]) \frac{C_A}{C_A - [Na_1]} \tag{27}$$

$([Na_2]-[Na_1])$ is the difference between the titration curves with and without added metal salt on a line of constant pH. An actual example of a two phase titration is shown in Fig. 1. The values necessary to calculate $[A_{b,E}]$ are indicated.

As a log [A] *versus* pH diagram for an acid is linear except at pH values near the pK_a value of the acid, a plot of $\bar{n}_E$ or $[A_{b,E}]$ against pH will have the same form as the formation function except at pH = $pK_a + \log(1 + K_d)$.

At the inflection point $\bar{n}_E = N/2$. Inserting this value into eqn (17) it is possible to calculate the log [A] value corresponding to the inflection point. This determines log K_{av} by the relation

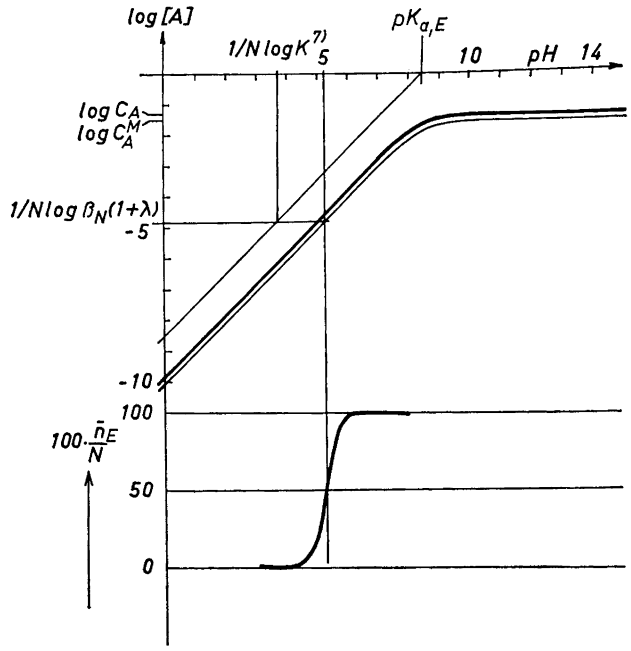


Fig. 2. The percent extraction curve for lanthanum ion in the TTA, chloroform-water system.

$$\log\,[\mathrm{A}'] = -\log K_{\mathrm{av}} = -\frac{1}{N}\log\,\beta_N\,(1+\lambda) \tag{28}$$

A graphical solution is most convenient and is shown in Fig. 2.
Assuming $\lambda \gg 1$ and [A] not too small, eqn (9) may be approximated to

$$\bar{n}_{\mathrm{E}} = \frac{[\mathrm{MA}] + 2[\mathrm{MA}_2] + \cdots + N[\mathrm{MA}_N] + \lambda N[\mathrm{MA}_N]}{C_{\mathrm{M}}} \cong \frac{\lambda N[\mathrm{MA}_N]}{C_{\mathrm{M}}} \tag{29}$$

Introducing an expression for the per cent metal extracted, % E, as

$$\%\ \mathrm{E} = \frac{[\mathrm{MA}_N]^{\mathrm{org}}}{C_{\mathrm{M}}}\ 100\ \% \tag{30}$$

and combining it with eqns (8) and (29) we obtain

$$\%\ \mathrm{E} = \frac{\bar{n}_{\mathrm{E}}}{N}\ 100\ \% \tag{31}$$

Eqn (31) shows that a plot of $100\ \bar{n}_{\mathrm{E}}/N$ against pH will conform with the per cent extraction curve for $\lambda \gg 1$.

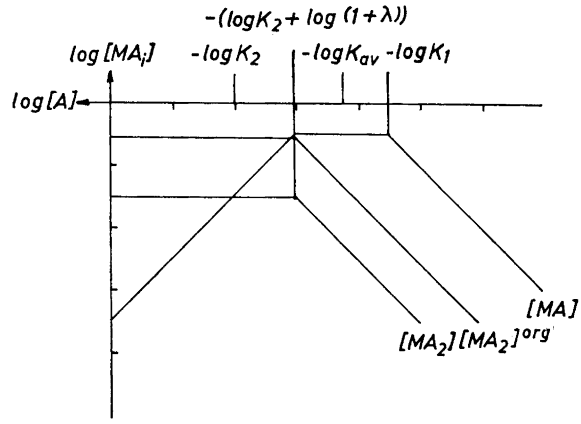


Fig. 3. A logarithmic diagram of metal chelate formation in a two phase system.

The log [A] *versus* pH diagram [17] in Fig. 2 is drawn using the experimental pK_b value determined graphically as shown in Fig. 1. $[A_b]$ is calculated by use eqn (27), and % E determined from eqn (31) is then plotted against pH as shown in Fig. 2.

A new log [A] *versus* pH diagram is then constructed according to a total concentration $C_A - \bar{n}_E \cdot C_M$, where $\bar{n}_E = N/2$, and the log [A'] value corresponding to % E = 50 % is found graphically. This represents a determination of the mean complexity constant K_{av} by relation (28). This constant is independent of the total concentration of the chelating agent. Assuming $C_A = 1$ and $[A_b]$ negligible, the pH of 50 % extraction of tracer concentrations of metal by a 1 M solution of extractive agent can be found graphically. This constant is equal to $\frac{1}{N} \log K$ defined by Dyrssen [7] and used by him to compare the chelating agents.

The general case where λ can have any value is better surveyed on a $\log[A] - \log[MA_i]$ ($i = 0, 1, 2, --, N$) diagram. For simplicity only the case $N = 2$ will be treated.

Noting the formal analogy between acid dissociation and complex dissociation, Fig. 3 can be constructed. The diagram resembles that of a dibasic acid [17]. The species MA_2, which alone is assumed to be extractable, have to be shared between the two phases with the distribution coefficient λ defined by eqn (8).

If λ is small the inflection point on the actual % E curve will correspond more to the second formation constant K_2 than to the mean complexity constant K_{av}, and accordingly, the inflection point on the actual extraction curve is expected to occur [12,13] for $\bar{n}_E = 3/2$.

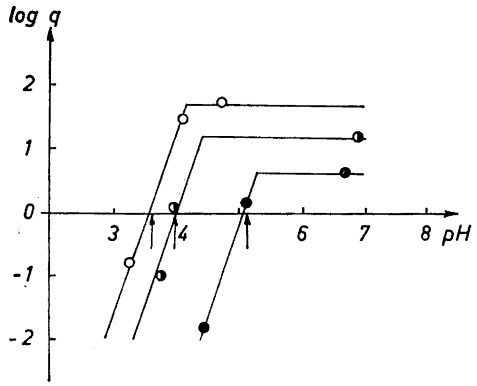


Fig. 4. Distribution data for the lanthanum ion with different chelating agents in chloroform. ○ 1-Phenyl-3-methyl-4-chloroacetyl-pyrazolone-5. ◑ Ethyl ester of 1-phenyl-3-methyl-4-carboxy-pyrazolone-5. ● Thenoyltrifluoroacetone.

If the formation curve ($\bar{n}_E$ — log[A]) has a rather large slope at the inflection point (larger than 0.5) indicating a small spreading factor x (less than 1) [12,13], then from eqn (13)

$$\frac{K_1}{K_2(1+\lambda)} < 4 \qquad (32) \qquad \log K_1 - \log K_2(1+\lambda) < 0.6 \qquad (33)$$

$$\log K_{av} = \frac{1}{2}[\log K_1 + \log K_2(1+\lambda)] < 0.3 + \log K_2(1+\lambda) \qquad (34)$$

$$\log K_{av} - \log K_2(1+\lambda) < 0.3 \qquad (35)$$

Eqn (35) indicates a systematic error in this case less than 0.3 pH units in the determination of the 50 % extraction value by this simple procedure. A correction may be made by the use of the relation between the average spreading factor and the midpoint slope of the formation curve [12,13], but if the slope is well above 0.5 a correction should not be nessessary, as in this case other errors are expected to dominate.

In the theoretical considerations given above certain approximations have been introduced. Generally they can be assumed to be justified under the specified conditions, but as actual systems deviate more or less from this simplified system, the ultimate precision of the equations obtained is hard to judge from theoretical considerations alone. Therefore as a check of the reliability of the method, some distribution experiments have been performed with ^{140}La as a tracer and with some chelating agents, to determine the actual pH's of 50 % extraction of La^{+3} to see how they correspond to the values obtained by the titration procedure.

METHODS

Titration procedures. The two phase titrations were made by equilibrating 2 ml of a 0.05 M solution of the chelate in chloroform with 2 ml of a water phase containing varying amounts of 0.1 N NaOH (or 0.1 N $HClO_4$) and 0.1 M $NaClO_4$ solution corresponding to different points on the titration curve. A set of 8—12 points were chosen corresponding to increasing amounts of base added, and the whole titration curve was obtained by simultaneous equilibration of the phases. Containers were 6 ml weighing bottles. After shaking to equilibrium — in most cases 10 min were sufficient — the pH of the water phase was measured when in contact with the organic layer.

The complex-extraction titrations were performed in the same manner except that 250 μl of the 0.1 M $NaClO_4$ solution was omitted and instead 250 μl of a *ca.* 0.05 M metal nitrate or metal perchlorate solution added. In the case of Th^{+4} only 100 μl was added. To be sure that equilibrium had been obtained some points were remeasured after a certain interval of time.

All measurements were performed at room temperature. (22 $\pm$ 1°C).

A Radiometer PHM-22 with a Radiometer glass-electrode type G 202 B and a Radiometer calomel electrode type K 100 with capillary drawn tip was used for the measurements.

Fig. 4 shows an actual set of curves obtained with thenoyltrifluoroacetone and $La(NO_3)_3$. Fig. 2 shows the plot of % E for the same combination.

Distribution experiments. 15 ml of a 0.05 M solution of the chelating agent in chloroform were shaken to equilibrium in a separatory funnel with 15 ml of a water phase containing varying amounts of $NaClO_4$ and NaOH at ionic strength 0.1 and tracer amounts of ^{140}La. (^{140}La was obtained by 2 h irradiation of 50 mg of $La(NO_3)_3$, 6 H_2O in D.R.1, Risø, at a flux of *ca.* 10^{10} n/sec.cm^2.) The sample was dissolved in 5 ml of water and 50 μl of the solution added to the separatory funnel.

The distribution coefficient q of La^{+3} was determined by measuring the γ-activity of 10 ml samples of the centrifuged water and chloroform phases with a conventional γ-counting unit equipped with a well-crystal. The pH of the water phase was measured for each distribution determination. The pH of 50 % extraction of La^{+3} was determined as the point where a curve representing a plot of log q against pH intersected the line log $q = 0$ shown in Fig. 4.

CONCLUSION

In Fig. 4 the pH's of 50 % extraction of La^{+3}, determined by the titration method is indicated by an arrow. The agreement between the two procedures is thus very good, as is to be expected, as often as the formation function $\bar{n}_E$ shows a large slope at the inflection point.

The present titration procedure is expected to offer an easy method by use of which the possibility of a given separation of metal ions by extraction with a given chelating agent can be checked, and a method by use of which the search for a useful extractive agent for a given separation of metal ions has become less tedious.

REFERENCES

1. Kuznetsov, V. I. *A.E.R.E. Lib/Trans 532.*
2. Irving, H. *Quart. Revs. London* **5** (1951) 206.
3. Morrison, G. H. and Greiser, H. *Solvent Extraction in Analytical Chemistry.* G. Wiley and Sons, N.Y. 1957, where an exhaustive series of references may be found.
4. Glasstone, S. *Principles Nucl. Reactor Eng.* McMillan and Co, London 1956.
5. Seaborg, G. *The Transuranium Elements,* Yale Univ. Press. 1958.
6. Reid, J. C. and Calvin, M. *J. Am. Chem. Soc.* **72** (1950) 2948.
7. Dyrssen, D. *Svensk Kem. Tidskr.* **68** (1956) 212.

8. Haeffner, E., Nilsson, G. and Hultgren, Å. *Intern. Conf. Peaceful Uses Atomic Energy, Geneve 1955*, P/785.
9. Rydberg, J. *Svensk. Kem. Tidskr.* **67** (1955) 499.
10. Skytte Jensen B. *To be published.*
11. Skytte Jensen B. *To be published.*
12. Bjerrum, J. *Metal Ammine Formation in Aqueous Solution*, Haase og Søn, Copenhagen 1941. Reprinted 1957.
13. Martell, A. E. and Calvin, M. *The Chemistry of the Metal Chelate Compounds*, Prentice Hall, N.Y. 1953, where an exhaustive series of references may be found.
14. Calvin, M. and Melchior, N. C. *J. Am. Chem. Soc.* **70** (1948) 3270.
15. Calvin, M. and Melchior, N. C. *J. Am. Chem. Soc.* **67** (1945) 2003.
16. Schwarzenbach, G. and Ackermann, H. *Helv. Chim. Acta* **30** (1947) 1798.
17. Hägg, G. *Kemisk Reaktionslära.* Hugo Gebers Förlag, Stockholm 1943.
18. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants I.* The Chemical Society, London 1957.

Received April 29, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1358–1365

Synthesis of Thiamine Phosphates in Baker's Yeast

KARL-HEINZ KIESSLING

Institute of Zoophysiology, University of Uppsala, Sweden

The synthesis of thiamine di- and triphosphate has been investigated for Swedish and English baker's yeast. Four different types of buffers were used. The most suitable medium for both compounds was a succinate buffer, pH 3.7.

The biosynthesis of thiamine triphosphate was found to start with the formation of thiamine diphosphate. Then, by addition of another orthophosphate, this compound was transformed into thiamine triphosphate.

As to the biosynthesis of thiamine diphosphate the results agree with the earlier opinion that the compound is formed mainly by a transfer of a pyrophosphate group to thiamine.

The results give no definite information about the biosynthesis of thiamine monophosphate. Different alternatives are discussed.

The synthesis of thiamine diphosphate * by yeast enzymes has been thoroughly investigated in intact and plasmolyzed yeast cells [1–4] as well as in purified enzyme systems [5–9]. The major amount of TDP is synthesized in yeast by a transfer of a pyrophosphate group from ATP to thiamine [1,5,6,8]. Only a smaller amount of TDP can be synthesized by transfer of an orthophosphate group to TMP [4].

About the biosynthesis of TTP not very much has been published. It was shown that baker's yeast in addition to TDP also accumulates TTP, when incubated with an excess of thiamine [10,11]. Further Greiling [9] recently showed that a partly purified enzyme system from brewer's yeast could synthesize TTP from thiamine and ATP.

In the present paper the synthesis of TMP, TDP, and TTP in intact baker's yeast has been investigated.

MATERIALS AND METHODS

Yeast. The yeasts used are the English baker's yeast from the Distillers Company Ltd, London **, and the Swedish baker's yeast from The Yeast Company at Rotebro.

Incubation. 20 g of baker's yeast were suspended in a 0.1 M buffer, 0.2 g thiamine added, and the suspension incubated at 27°C with oxygen as the gas phase.

* Abbreviations: T thiamine, TMP thiamine monophosphate, TDP thiamine diphosphate, TTP thiamine triphosphate, AMP adenosine monophosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, TCA trichloroacetic acid, $^{32}P_i$ radioactive inorganic phosphate.

** Kindly supplied by Dr. E. R. Dawson, The Distillers Company Ltd, London.

When searching for the best medium for the biosynthesis of TTP different buffers were used: sodium succinate buffer 0.1 M, pH 3.7 or 6.2, pyrophosphate buffer 0.1 M, pH 6.2, prepared from sodium pyrophosphate and HCl, and monophosphate buffer 0.1 M, pH 6.2, compounded from Na_2HPO_4 and NaH_2PO_4.

Samples taken from the yeast incubations were immediately centrifuged in the cold, the supernatant discarded, and the yeast cells suspended in cold 40 % TCA to a final concentration of 8 %. After 15 min at −10°C the yeast suspension was stirred strongly, centrifuged, and TCA removed from the yellow extract with ether.

Isolation of thiamine phosphates. From 20 g of yeast a final extract of about 15 ml was obtained. 0.25 ml were spread as a narrow line on a Whatman 52 filter paper washed with versene according to Eggleston and Hems [12]. The chromatograms were developed according to Kiessling [13], and the compounds localized by cutting off a strip from each chromatogram and spraying it with an alkaline potassium ferricyanide solution. The areas, containing TTP, TDP, and TMP were cut out, the compounds removed with water, and then re-chromatographed in the same solvent. The chromatography was repeated a third time in the solvent used by Greiling [14] or according to Siliprandi and Siliprandi [15] (*n*-butanol — 1 M HAc pH 5 — H_2O, 70 : 10 : 20). Finally TTP was purified by paper electrophoresis on Whatman 52 washed as described above. The solvent was an acetate buffer pH 5,4 (*cf.* Ref.[15]), the current 3 mA and the time 9 h.

Isolation of ATP and ADP. ATP and ADP were isolated by ion exchange chromatography according to Herbert and Potter [16].

Phosphate determination. Orthophosphate was determined according to Ernster *et al.*[17] whose method is a modification of that of Martin and Doty [18]. Hydrolyzable phosphate of TDP was determined after 15 min at 100°C in 0.5 M H_2SO_4, non-hydrolyzable phosphate of TTP, TDP, and TMP after wet ashing in 0.3 ml conc. $HClO_4$. γ and βP of TTP were removed by hydrolysis by means of potato apyrase according to Kiessling [11], or by hydrolysis for 3 min at 100°C in 0.1 M HCl for γP and for 15 min in 0.5 M H_2SO_4 for $\gamma + \beta$P.

The radioactivity of the phosphate was measured with an M-6 liquid counter tube on the samples used for ^{32}P determinations after dilution to 10 ml with ethanol.

Thiochrome method. In order to determine the amounts of TTP, TDP, and TMP synthesized in yeast after different time intervals the compounds were isolated by paper chromatography, hydrolyzed to thiamine by a yeast phosphatase according to Westenbrink *et al.*[19], transformed into the thiochrome form according to Hjarde, [20] and measured in a Beckman spectrophotometer with a special fluorescence attachment.

RESULTS AND DISCUSSION

Westenbrink has shown that, in the presence of an excess of thiamine, Dutch baker's yeast could go on synthesizing TDP for several hours [21]. Also for the Swedish baker's yeast I found this to be the case for at least 14 h (Fig. 1).

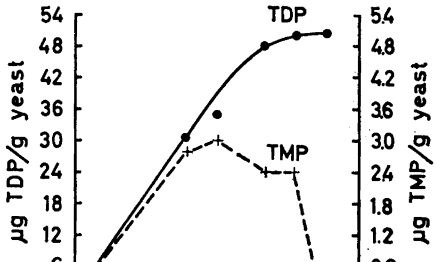


Fig. 1. Synthesis of TDP and TMP in baker's yeast. The yeast is the Swedish baker's yeast, incubated with an excess of thiamine in a 0.1 M succinate buffer, pH 3.7, at 27°C with oxygen as the gas phase. The compounds have been separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

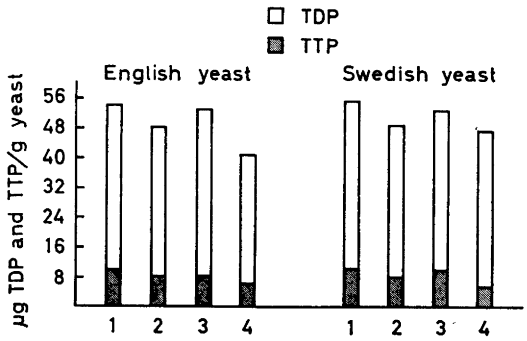


Fig. 2. Synthesis of TTP and TDP in baker's yeast. The yeasts used are an English and a Swedish baker's yeast. The yeasts have been incubated with an excess of thiamine in different buffers (1—4) during 16 h at 27°C with oxygen as the gas phase. The buffers are:

1. 0.1 M succinate buffer, pH 3.7
2. 0.1 M succinate buffer, pH 6.2
3. 0.1 M monophosphate buffer, pH 6.2
4. 0.1 M pyrophosphate buffer, pH 6.2

The compounds have been separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

At the same time the Swedish yeast as well as the English baker's yeast has been shown to synthesize TTP [10,11]. Without addition of special substrates the best medium for synthesis of TTP in the Swedish baker's yeast was found to be either succinate buffer, pH 3.7, or a monophosphate buffer, pH 6.2, and in the English yeast succinate buffer, pH 3.7 (Fig. 2). The same media were also the most favourable for the biosynthesis of TDP (Fig. 2). Buffers tested were succinate buffers, pH 3.7 and 6.2, monophosphate buffer pH 6.2 and pyrophosphate buffer, 6.2, all 0.1 M. In the following experiments with Swedish baker's yeast 0.1 M succinate buffer, pH 3.7, has been used.

Addition of thiamine phosphates to yeast cells immediately results in a hydrolysis of the phosphate by phosphatases, very likely situated at the cell

Table 1. Radioactivity of the different phosphate groups in thiamine phosphates isolated from baker's yeast. Incubation at 27°C with oxygen as the gas phase in a 0.1 M succinate buffer, pH 3.7 and with an excess of thiamine. $^{32}P_i$ was added after 4.5 h incubation, and samples for analysis were taken after another 2 and 4 h, respectively.

Thiamine compound	4.5 + 2 h (counts/5 min/µg P)			4.5 + 4 h (counts/5 min/µg P)		
	αP	βP	γP	αP	βP	γP
TTP	485	819	2 265	1 188	1 690	6 100
TDP	1 315	1 765	—	3 260	3 360	—
TMP	140	—	—	1 440	—	—

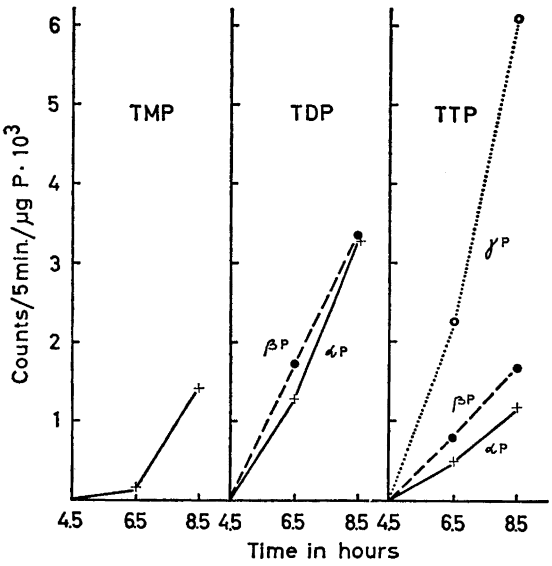


Fig. 3. Radioactivity of α, β and γP from TMP, TDP and TTP. Swedish baker's yeast was incubated with an excess of thiamine in 0.1 M succinate buffer, pH 3.7, with oxygen as the gas phase at 27°C. The first part of the incubation (up to 4.5 h) was performed in the absence, and the second part in the presence of radioactive phosphate. The compounds have been separated by means of paper chromatography.

surface. Besides it is doubtful, whether or not the phosphorylated compounds could penetrate the cell membrane. The yeast cells, however, transfer added thiamine, which easily penetrates the cell membranes, into thiamine phosphates which give origin to starting-points for further synthesis. Thus yeast was allowed to accumulate thiamine phosphates for a certain interval. Then radio-

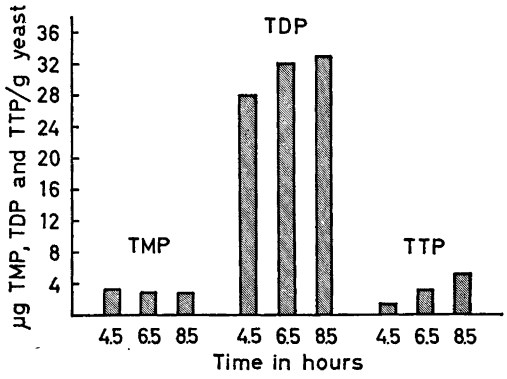


Fig. 4. Amount of TMP, TDP and TTP in baker's yeast after different time intervals. Incubation as in Fig. 3. The compounds were separated by means of paper chromatography, and measured as thiochrome after hydrolysis with yeast phosphatase.

active inorganic phosphate was added, and the incubation continued. Samples were taken after different time intervals, the thiamine phosphates isolated, and the radioactivity of the different phosphate groups determined. This permits to see how TTP and possibly also TDP are synthesized from already formed thiamine phosphates.

Results from an experiment are shown in Table 1 and Fig. 3. After 4.5 h incubation in the absence of $^{32}P_i$, radioactive phosphate was added, and samples taken after another 2 and 4 h incubation. Fig. 4 shows the amounts of TMP, TDP, and TTP at the end of the different intervals.

Two ways are open for the biosynthesis of TTP: either by a transfer of a pyrophosphate group to TMP or a monophosphate group to TDP. The biosynthesis of TDP is thought to be brought about by a transfer of a pyrophosphate group to thiamine. About the way of formation of TMP nothing is known, but it is generally assumed to be generated by hydrolysis of TDP.

From Table 1 and Fig. 3 it is seen that the radioactivity of γP of TTP increases much more rapidly than that of β and αP. As ATP has been shown to be the phosphate donor at the synthesis of TDP from thiamine as well as at the synthesis of TTP in isolated systems, the synthesis of TTP in intact yeast cells, according to Table 1 and Fig. 3 very probably may be written:

$$TDP + ATP \longrightarrow TTP + ADP$$

that is, TDP, already synthesized from thiamine and ATP, receives a third phosphate from ATP.

Another possibility for the synthesis of TTP is:

$$TMP + ATP \longrightarrow TTP + AMP$$

This possibility is, however, excluded by the circumstances that βP in TTP is much less labelled than γP. Very probably the βP of ATP becomes labelled somewhat slower than the γP, but the differences can't suffice for the explanation of the different labelling of β and γP of TTP. This becomes obvious from the synthesis of TDP. From experiments with partly isolated thiamino kinases [5,6,8,9], and with autholyzed yeast cells [4] the synthesis of TDP was found mainly to proceed according to the reaction:

$$T + ATP \longrightarrow TDP + AMP$$

The results in Table 1 and Fig. 3 show that αP of TDP is only slightly less radioactive than βP. The differences can be explained by one of two assumptions: either that βP of ATP does not exchange its phosphate as rapidly as γP, or that TDP to a small extent is also synthesized from TMP. Whatever the explanation may be the fact remains that the differences in radioactivity between βP and γP of ATP are too small to account for the differences between β and γP of TTP. It therefore seems most satisfactory to explain the results by ascribing the synthesis of TTP to a transfer of a phosphate group to previously formed TDP.

It is difficult to give a satisfactory explanation for the figures in Table 1 on the radioactivity of TMP. The general opinion is that TMP is more likely formed by hydrolysis of TDP than by a direct phosphorylation of thiamine. On the contrary Greiling reported that he had found an enzyme in brewer's

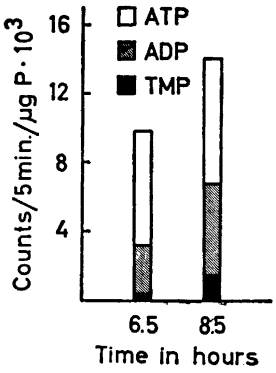


Fig. 5. Radioactivity of TMP and of hydrolyzable phosphate of ATP and ADP after 2 and 4 h incubation respectively. The samples are from the experiment described in Fig. 3. ATP and ADP were isolated on a Dowex 1 column according to Herbert and Potter [16], and the radioactivity measured on the phosphate hydrolyzed during 10 min at 100°C in 1 M H_2SO_4. TMP was isolated by paper chromatography (see Methods), and the radioactivity determined after wet ashing in $HClO_4$.

yeast which, at most to a small extent, is able to synthesize TMP from thiamine and ATP [9].

TMP accumulates only during the first hours, when yeast is incubated with thiamine (Figs. 1 and 4). Further incubation causes a decrease of the amounts of TMP. The decrease may either depend on the fact that TMP is hydrolyzed to thiamine or that it is transformed into another thiamine phosphate (TDP or TTP). However, it does not necessarily mean that TMP is no longer formed. The increasing radioactivity of TMP, isolated from the yeast during periods when the total amount of TMP is decreasing, indicates that a formation of the compound still takes place.

If TMP, as is generally believed, is formed from TDP, the quantity and also the radioactivity, as with αP of TDP, should increase altogether continuously. This is, however, not the case (Figs. 1 and 3). If TMP is synthesized by a transfer of a phosphate from ATP to thiamine, the radioactivity should likewise increase nearly straight-lined as in βP of TDP (the decrease in the total amount of TMP should cause the curve to bend only slightly upwards). The results in Fig. 3 makes, however, ATP a less probable phosphate donor at a possibly occurring synthesis of TMP.

Table 2. The radioactivity of TTP and TDP from yeast after further incubation with an excess of non-radioactive phosphate. First part of the incubation was performed as described in Table 1. Incubation time 16 h. $^{32}P_i$ was added after 8 h incubation and a sample taken after another 8 h. The remaining yeast was then washed twice with a succinate buffer, and the incubation continued in the presence of an excess of non-radioactive phosphate. Samples were taken after 1 and 2 h, respectively.

Thiamine compound	Counts of hydrolyzable P/5 min/µg P		
	16 h	17 h	18 h
TTP	743	762	808
TDP	440	503	495

From Fig. 5 it is seen that ADP becomes labelled at a slower rate than ATP. The increase along a straight line of the radioactivity of ADP eliminates also ADP as a possible phosphate donor.

Thus the results give no information about the way in which TMP is formed in baker's yeast. The comparison of the curve for TMP and αP of TDP (Fig. 3) allows only the conclusion that TMP is not at all, or only to a small extent, an intermediate in the biosynthesis of TDP from thiamine in intact baker's yeast when present in biological concentrations (*cf.* Ref.[4]).

Previously it has been shown that γP in yeast TTP rapidly becomes radioactive, when glucose is added together with radioactive phosphate to a yeast suspension.[22] This is not the case in the absence of glucose. The increase of radioactivity in the γP of TTP in the presence of glucose was therefore looked upon as depending on a turnover of the terminal phosphate in already existing TTP rather than on a further synthesis of TTP.

In order to make sure that in the present experiments (Table 1), performed in the absence of glucose, the high radioactivity of γP in TTP compared with that in β and αP did not depend on a turnover of the type described above, the following control was made: yeast was incubated 16 h as described in Table 1. After 8 h radioactive inorganic phosphate was added. At 16 h the yeast was washed twice with succinate buffer to remove excess of thiamine and $^{32}P_i$. It was then suspended in a new buffer consisting of three parts 0.1 M succinate buffer and one part 0.1 M phosphate buffer, pH 3.7. Samples were taken after 1 and 2 h incubation. The results are seen in Table 2. No decrease of the radioactivity in the hydrolyzable phosphate of TTP and TDP was found. Neither does any remarkable synthesis of TTP take place any longer, and the relatively constant radioactivity in its phosphate indicates that no exchange of the terminal phosphate is going on.

Greiling recently reported that he had been able to synthesize TTP from thiamine with a purified enzyme system from brewer's yeast [9]. He used ATP labelled in the terminal phosphate, and found the ratio between the activities of γ, β and αP in TTP to be 3:3:1. In TDP the ratio between β and αP was 3:1. The conclusions drawn from his results agree with those for the synthesis of TTP in intact yeast cells reported in the present paper.

Acknowledgements. I am indebted to Prof. P. E. Lindahl for the interest he has shown in my work and to Mr. Bo Molin for valuable technical assistance.

This work was made possible by a grant from the *Swedish Natural Science Research Council.*

REFERENCES

1. Lipton, M. A. and Elvehjem, C. A. *Nature* **145** (1940) 226.
2. Sperber, E. and Renvall, S. *Biochem. Z.* **310** (1941) 160.
3. Westenbrink, H. G. K., Steyn-Parvé, E. P. and Veldman, H. *Biochim. et Biophys. Acta* **1** (1947) 154.
4. Kiessling, K.-H. *Arkiv Kemi* **10** (1956) 279.
5. Weil-Malherbe, H. *Biochem. J.* **33** (1939) 1997; *J. Soc. Chem. Ind. (London)* **58** (1939) 1021.
6. Steyn-Parvé, E. P. *Biochim. et Biophys. Acta* **8** (1952) 310.
7. Van Thoai, N. and Chevillard, L. *Compt rend. soc. biol.* **232** (1951) 1444.
8. Forsander, O. *Soc Sci. Fennica, Commentationes Phys. Math.* 19. 2. (1956).

9. Greiling, H. *Reported at the 4th Intern. Congr. Biochem.*, Vienna 1958.
10. Kiessling, K.-H. *Nature* **172** (1953) 1187.
11. Kiessling, K.-H. *Biochim. et Biophys. Acta* **20** (1956) 293.
12. Eggleston, L. V. and Hems, R. *Biochem. J.* **52** (1952) 156.
13. Kiessling, K.-H. *Acta Chem. Scand.* **10** (1956) 1356.
14. Greiling, H. *Z. Naturforsch.* **12 b** (1957) 605.
15. Siliprandi, D. and Siliprandi, N. *Biochim. et Biophys. Acta* **14** (1954) 52.
16. Herbert, E. and Potter, V. R. *J. Biol. Chem.* **222** (1956) 453.
17. Ernster, L., Zetterström, R. and Lindberg, O. *Acta Chem. Scand.* **4** (1950) 942.
18. Martin, J. M. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.
19. Westenbrink, H. G. K. and Steyn-Parvé, E. P. *Intern. Rev. Vitamin Research* **21** (1950) 461.
20. Hjarde, W. *Nogle undersøgelser over B_1-vitaminet in vitro og in vivo.* Diss. Copenhagen 1950.
21. Westenbrink, H. G. K., Steyn-Parvé, E. P. and Veldman, H. *Biochim. et Biophys. Acta* **1** (1947) 154.
22. Kiessling, K.-H. *Acta Chem. Scand.* **10** (1956) 831.

Received May 8, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1366–1374

Action of Phenylalanine Metabolites on Glutamic Acid Decarboxylase and γ-Aminobutyric Acid—α-Ketoglutaric Acid Transaminase in Brain

ARNE HANSON

Department of Clinical Chemistry, General Hospital, Malmö, Sweden

Several intermediary metabolites of phenylalanine, namely phenylpyruvic, *p*-hydroxyphenylpyruvic, phenylacetic, *p*-hydroxyphenylacetic, and *o*-hydroxyphenylacetic acids have been found to inhibit the glutamic acid decarboxylase of brain *in vitro*. The inhibition is of the competitive type and appears to be localized in the apoenzyme.

No inhibitory effect could be demonstrated when the same metabolites were tested on the γ-aminobutyric acid—α-ketoglutaric acid transaminase system.

The significance of these findings in relation to phenylketonuria is briefly discussed.

In phenylketonuria hydroxylation of phenylalanine to tyrosine is markedly reduced. This results in an excess of phenylalanine in the tissues and an overproduction of intermediary metabolites of phenylalanine catabolism. The major metabolites of phenylalanine found in abnormal amounts in phenylketonuria are phenylpyruvic, phenyllactic and phenylacetic acids [1]. It has been suggested [1] that the mental deficiency is caused by an overproduction of one of these metabolites or by an excess of phenylalanine.

These aromatic metabolites of phenylalanine have recently been found to inhibit dihydroxyphenylalanine decarboxylase of beef adrenal medulla [2] and 5-hydroxytryptophan decarboxylase of guinea-pig kidney [3] *in vitro*. This inhibitory effect has been offered as an explanation of the low plasma levels of adrenaline [4] and 5-hydroxytryptamine [5] in phenylketonuria. Since much argues that both adrenaline and 5-hydroxytryptamine play a rôle in normal brain function [6], it appears that inhibition of these enzymes might reasonably be a contributing cause of mental deficiency.

The reported inhibition of dihydroxyphenylalanine decarboxylase and 5-hydroxytryptophan decarboxylase by aromatic phenylalanine metabolites

prompted us to examine the effects on GAD * and GABA-T of brain of these metabolites as well as of some others also occurring in phenylketonuria, namely *p*-hydroxyphenylpyruvic, *p*-hydroxyphenylacetic, and *o*-hydroxyphenylacetic acids and *o*-tyrosine.

The presence, in brain, of very large amounts of GAD and of ABA — the product formed by its action on GA — suggests that decarboxylation of GA may be a reaction of major importance in the metabolism of the central nervous system [7]. No other tissue has been found to show as high GAD activity as brain. This enzyme requires the presence of a coenzyme, namely pyridoxal-5-phosphate.

In studies on the metabolic fate of GABA evidence has also been obtained for its transamination with KGA in brain to form SSA and GA [7,8]. The transaminase catalyzing this reaction also requires pyridoxal-5-phosphate as coenzyme which, however, appears to be more firmly bound to the apoenzyme than is the case in the decarboxylase [9,10]. The SSA can be oxidized further to succinic acid and so enter the tricarboxylic acid cycle. This latter reaction appears to be catalyzed by a diphosphopyridine nucleotide dependent dehydrogenase [11].

In view of these findings it has been suggested that in brain a shunt operates around the KGA oxidase system of the tricarboxylic acid cycle by which KGA could be withdrawn through formation of GA by transamination with GABA and the carbon chain of GABA could enter the cycle at the succinic acid level. It is of special interest that this step in the tricarboxylic acid cycle has been considered to be the rate limiting reaction of the cycle; thus the whole cycle could be secondarily regulated by the activity of "the GABA cycle".

Recent findings also suggest that GABA may act in the cortex as an inhibitory transmitter substance [12,13]. Interference with its formation and/or metabolism may therefore be an example of a biochemical lesion of the central nervous system.

MATERIALS AND METHODS

Materials. The phenylalanine metabolites used were obtained from commercial sources. Phenylacetic acid from Eastman-Kodak Co.; phenylpyruvic acid, sodium salt, from Hoffman-La Roche & Co. Ltd.; *p*-hydroxyphenylacetic acid from K and K Laboratories, Inc.; *p*-hydroxyphenylpyruvic acid, sodium salt, from Sigma Chemical Co.; *o*-hydroxyphenylacetic acid and DL-a-phenyllactic acid from Light & Co. Ltd.; DL-*o*-tyrosine from California Foundation for Biochemical Research; DL-phenylalanine and L-GA from Merck & Co. Ltd.; GABA from Hoffman-La Roche & Co. Ltd.; KGA from Sigma Chemical Co. Pyridoxal-5-phosphate was generously supplied by Dr. W. W. Umbreit, Merck Institute for Therapeutic Research, Rahway, N. J.

The metabolites obtained as free acids were transformed into their sodium salts before use.

SSA was synthesized from glutamic acid according to Mosbach *et al.*[14] and isolated as 2,4-dinitrophenylhydrazone. After recrystallization from ethanol the product had a melting point of 200°.

Other chemicals used in the experiments were of reagent grade.

---

* The following abbreviations have been used: GA, glutamic acid; GABA, γ-aminobutyric acid; KGA, α-ketoglutaric acid; GABA-T, γ-aminobutyric acid — α-ketoglutaric acid transaminase; GAD, glutamic acid decarboxylase; SSA, succinic semialdehyde.

Enzyme preparations. GAD was obtained from either rat or guinea-pig brain. The animals were killed by decapitation and the brains immediately placed in small beakers containing cracked ice. Homogenates containing 500 mg of fresh weight of brain per ml were prepared in ice-cold 0.5 M phosphate buffer pH 5.9.

GABA-T was obtained from mitochondria isolated from either rat or guinea pig brains. For the isolation of mitochondria the brains were disrupted in 9 volumes of 0.25 M sucrose in a Potter-Elvehjelm homogenizer. The homogenate was centrifuged twice at 1 500 × *g* for 5 min to remove nuclear and cellular debris. The final supernatant was then centrifuged at 12 000 × *g* for 20 min to sediment the mitochondria. After being washed once in 0.25 M sucrose the mitochondria were suspended in 0.25 M sucrose and the suspension diluted so that 1 ml corresponded to 5 g of brain. The centrifugations were carried out in an International Refrigerated Centrifuge with multispeed attachment.

All steps in the preparation of the enzymes were carried out at 0 – 4°.

Assay of enzyme activity. The rate of GA decarboxylation was determined with a Warburg apparatus in an N_2 atmosphere at 37°. The main compartment of the flasks contained 1 ml of brain homogenate, 200 μg of pyridoxal-5-phosphate and inhibitor dissolved in phosphate buffer to make a final volume of 2.65 ml. One side arm contained GA solution (pH 7.0 to 7.4) to give a final concentration of 0.03 M. The substrate was added after an equilibration time of 10 min. The other side arm contained 0.3 ml of 3 N H_2SO_4. This was added to stop the reaction and to liberate bound CO_2, for which suitable corrections were made. Carbon dioxide output was determined for 60 mins.

To ascertain whether the formation of CO_2 and GABA took place in stoichiometric amounts under the conditions employed, the quantity of GABA in the incubation fluids was determined in some of the experiments. The GABA was separated by means of high voltage electrophoresis and determined quantitatively by treating the ninhydrin-developed spots with a copper nitrate solution, eluting the red colour of the copperninhydrin-complex with methanol and measuring the colour intensity spectrophotometrically [15].

The formation of SSA and GA was taken as a measure of the GABA-T activity. The assay method was as follows: Incubations were carried out, in air, with shaking in small conical flasks for 2 h at 37°. The incubation mixture contained 1.5 ml of 0.1 M phosphate buffer pH 7.4, 0.5 ml of mitochondria suspension, 0.2 ml of 0.1 M KGA (20 μM), 0.4 ml of 0.2 M GABA (80 μM), and inhibitor (usually in a volume of 0.6 ml). The final volume of the incubation mixture was usually 3.2 ml. At the end of the incubation period 1 ml of 15 % trichloroacetic acid was added to deproteinize the contents which were then centrifuged for 10 min.

For estimation of the SSA formation an aliquot of the supernatant was pipetted into a glass-stoppered test-tube placed in an ice-bath and 3 ml of a 0.5 % solution of 2,4-dinitrophenylhydrazine in 5 N HCl added. After 10 min 5 ml of xylene were added to the tube for extraction of the hydrazone formed. This extraction was repeated once. The pooled extracts were then extracted with 5 ml of a 0.1 M borate buffer pH 10. An aliquot of the borate extract was diluted with borate buffer to a volume of 6 ml and this was made alkaline with 0.5 ml of a 5 % alcoholic sodium hydroxide solution.

Exactly 2 min later the colour density was measured in a Beckman DU spectrophotometer at 420 mμ against a blank consisting of borate buffer and alcoholic sodium hydroxide. The concentration of SSA in the extract was assessed from a calibration curve made up from known amounts of a synthetic sample of SSA-2,4-dinitrophenylhydrazone. Only small amounts of the KGA present in the incubation mixture was found to react with the 2,4-dinitrophenylhydrazine to give a hydrazone under the conditions described, and xylene extracted but little of this hydrazone.

In some of the experiments the presence of SSA-2,4-dinitrophenylhydrazone in the borate extract was confirmed by paper chromatography. For this the extract was acidified to pH 2 with HCl and extracted with a mixture of ethanol and chloroform (20:80). This extract was evaporated to dryness and the residue taken up in a small volume of ethyl acetate and applied to the paper. Ascending chromatography with a mixture of *n*-butanol, ethanol and 0.5 N ammonia (70:10:20) as developing system was used. In this system SSA-2,4-dinitrophenylhydrazone gave two spots having the R_F values 0.09 and 0.43, respectively.

When the inhibitor tested was a carbonyl compound, such as phenylpyruvic and *p*-hydroxyphenylpyruvic acids, the transamination assay could not be based on the for-

mation of SSA since the inhibitors formed hydrazones. Here only the formation of GA was used for the assay.

The GA formed in the transamination was measured in the deproteinized incubation mixture by the high voltage electrophoresis method mentioned above [15].

RESULTS AND DISCUSSION

Several of the phenylalanine metabolites were found to inhibit the GAD. Some of the results of these experiments are given in Table 1. Phenylacetic

Table 1. Inhibition of glutamic acid decarboxylase of guinea-pig brain by phenylalanine metabolites.

Additions	Concentration of inhibitor μmole/l	CO_2 output (μl/60 min)	Inhibition %
Control		174	
Phenylpyruvic acid	25	82	47
	50	76	56
	100	67	61
Control		172	
p-OH-Phenylpyruvic acid	25	150	13
	50	137	20
	100	107	38
Control		119 *	
Phenylacetic acid	25	106	11
	50	60	50
	100	14	96
Control		119 *	
p-OH-Phenylacetic acid	25	55	54
	50	29	76
	100	15	92
Control		173	
Phenyllactic acid	25	169	2
	50	167	3
	100	157	9
Control		130 *	
o-OH-Phenylacetic acid	125	126	3
	50	99	24
	100	64	50
Control		183	
o-Tyrosine	100	178	3
Control		164	
Phenylalanine	100	151	8

* These experiments were performed with rat brain.

Table 2. Equivalence of formation of CO_2 and γ-aminobutyric acid by guinea-pig brain homogenate.

Expt. No.	Incubation time, min.	CO_2 formed μmole	γ-Aminobutyric acid formed μmole
1	60	8	8.3
2	60	6.9	7.1
3	30	3.9	4
4	30	3.6	3.6
5 *	60	2.2	2.4
6 *	30	1.6	1.7

* In experiments 5 and 6 the incubation mixture contained *p*-OH-phenylacetic acid (50 μmole/l).

and phenylpyruvic acids were found to be the most effective inhibitors of the unsubstituted compounds. Phenyllactic acid and phenylalanine had very little activity. Of the *para*-hydroxylated compounds studied, *p*-hydroxyphenylacetic acid proved a more active inhibitor than *p*-hydroxyphenylpyruvic acid. Two *ortho* compounds were also tested. Of these, *o*-hydroxyphenylacetic acid was rather active, while its precursor, *o*-tyrosine, was inactive.

Table 2 gives the results of experiments performed to determine whether the formation of CO_2 and GABA takes place in stoichiometric amounts under the conditions employed. The experiments were performed both with and without added inhibitor. Good equivalence was found and it may be said that, within experimental error, there was a mole for mole formation of CO_2 and GABA.

It may be of interest to compare some of these results with those obtained from similar studies on other decarboxylases. Davison and Sandler [3] in their study of 5-hydroxytryptophan decarboxylase from guinea-pig kidney as well as Hartman *et al.*[16] in their study of dihydroxyphenylalanine decarboxylase from hog kidney obtained comparable inhibitions of their enzyme preparations with phenylacetic and phenylpyruvic acids and less or no inhibition with phenyllactic acid. In Fellman's study of the dihydroxyphenylalanine decarboxylase from beef adrenals phenylpyruvic and phenyllactic acids were the most effective inhibitors while phenylacetic acid was a poor inhibitor [2]. An inhibitory effect of *p*-hydroxyphenylpyruvic acid on dihydroxyphenylalanine decarboxylase has been reported by Hartman *et al.*[16]. Its effect was equal to that of phenylpyruvic acid.

The equations of Hunter and Downs [17] were used for characterizing the type of inhibition observed in the present experiments. If α is defined as the fractional activity, *i. e.* v_1/v, where v_1 is the activity in the presence of inhibitor and v the activity in its absence, with the same substrate concentration, these equations are, for a competitive inhibition,

$$[I]\ \frac{\alpha}{1-\alpha} = K_i + \frac{K_i}{K_m}\ [S]$$

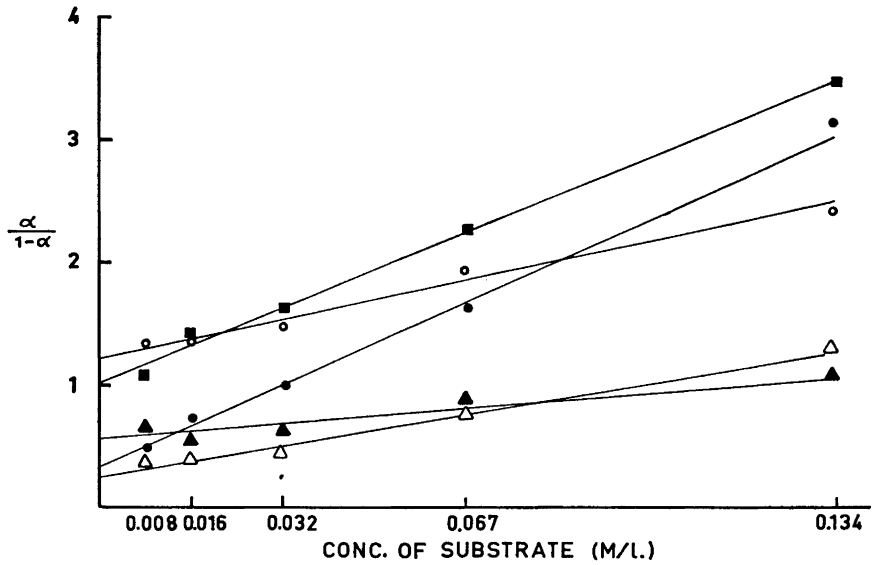


Fig. 1. Inhibition of glutamic acid decarboxylase of brain by phenylalanine metabolites. Experimental conditions as described in text. The results are plotted according to the equation derived by Hunter and Downs [17]: $a/(1-a)$ against substrate concentration. Values of a are determined as the ratio of inhibited velocity to uninhibited velocity with the same concentration of the substrate. Inhibitor concentration 100 μmole/l. Inhibitors: ● Phenylpyruvic acid; ○ *p*-OH-Phenylpyruvic acid; ▲ Phenylacetic acid; △ *p*-OH-Phenylacetic acid; ■ *o*-OH-Phenylacetic acid.

and, for a non-competitive inhibition,

$$[I]\ \frac{a}{1-a} = K_i$$

where [I] is the inhibitor concentration, K_i the dissociation constant of the enzyme-inhibitor complex, [S] the substrate concentration, and K_m the Michaelis constant. By plotting $a/(1-a)$ against [S] one obtains for competitive inhibition a straight line with slope K_i/K_m and intercept K_i; and, for non-competitive inhibition, a straight line of zero slope and intercept K_i. The value of K_i represents the amount of inhibitor required for 50 % inhibition.

When $a/(1-a)$ was plotted against [S] with the different inhibitors the graphs obtained were found to be straight lines with different slopes (Fig. 1). This indicates that the inhibition is dependent of the substrate concentration, [S], *i. e.* it is of the competitive type. The value of K_i can be calculated from each of the lines by multiplying the value of [I] by the value of the intersection point obtained by extrapolating the line to the vertical axis (Table 3).

To ascertain whether the inhibition noted was due to any interference with the coenzyme of GAD, *i. e.* pyridoxal-5-phosphate, determinations were made of the influence of increasing amounts of the latter on systems inhibited by the

Table 3. K_i values of different competitive inhibitors of glutamic acid decarboxylase. (Values of K_i were calculated form $a/(1-a)$ values obtained by the graphical method shown in Fig. 1).

Inhibitor	Inhibitor conc. (μmole/l)	Calculated K_i (μmole/l)
Phenylpyruvic acid	100	30
p-OH-Phenylpyruvic acid	100	120
Phenylacetic acid	100	55
p-OH-Phenylacetic acid	100	25
o-OH-Phenylacetic acid	100	100

various inhibitors. It is clear from Table 4 that increasing concentration of the pyridoxal-5-phosphate produced no demonstrable effect. The inhibition thus seems to be localized in the apoenzyme and not to be of the type caused by hydrazides or certain pyrocatechol derivatives. The former compounds have been shown by Killam and Bain [18] to inhibit the brain GAD, the inhibition being competitively reversed by pyridoxal-5-phosphate, however. Of the pyrocatechol derivatives, primary phenylalkylamines containing a phenolic hydroxy group in *meta* position such as dopamine, noradrenaline, and *m*-hydroxyphenylserine have been found by Holtz and Westermann [19] to inhibit the same enzyme. In this case, however, the inhibition is not of the competitive type but has been interpreted as a reaction of the inhibitor with the coenzyme with formation — *via* Schiff base formation — of tetrahydro*iso*-quinoline derivatives resulting in strong inhibition of the enzyme. If added in excess, pyridoxal-5-phosphate can counteract the inhibition.

When tested on the GABA-T system none of the inhibitors had any effect in the concentrations found to inhibit the GAD. Some of the results of these experiments are shown in Table 5. It appears from the data that there is a formation of about 0.5 μM of SSA for each micromole of GA. This difference in equivalence between the compounds formed in the transamination reaction is probably due to the SSA being utilized by the brain mitochondria and thus

Table 4. Influence of increasing amounts of pyridoxal-5-phosphate on the inhibition of glutamic acid decarboxylase. (The reaction mixture was as described above. Concentration of inhibitor 100 μmole/l. The figures refer to CO_2 output in μl).

	Without inhibitor	Concentration of pyridoxal-5-phosphate (μmole/3.05 ml)				
		1	2	4	6	8
Phenylpyruvic acid	165	62	64	65	66	66
p-OH-phenylpyruvic acid	161	96	92	95	93	94
Phenylacetic acid	176	21	23	25	28	25
p-OH-Phenylacetic acid	177	66	70	74	78	71
o-OH-Phenylacetic acid	156	76	73	78	81	80

Table 5. Influence of different phenylalanine metabolites on the α-aminobutyric acid-α-ketoglutaric acid transaminase. (Reaction mixture as described above. Concentration of inhibitor 100 μmole/l).

Additions	Products formed μmole	
	Succinic semialdehyde *	Glutamic acid
Control	3.4	6.3
Phenylpyruvic acid	— **	6.1
p-OH-Phenylpyruvic acid	— **	5.9
Phenylacetic acid	3.1	6.4
p-OH-Phenylacetic acid	2.8	6.0
o-OH-Phenylacetic acid	3.0	5.9

* Calculated from its hydrazone.
** Addition interfered with method of estimation.

partially disappearing spontaneously. Oxygen uptake of guinea-pig brain slices [20] as well as esterification of phosphate by cat cortex mitochondria [21] have been shown to be supported by SSA and brain homogenates have also been found to catalyze a diphosphopyridine nucleotide dependent oxidation of SSA [11].

It is difficult to say whether these findings are of any significance in the investigation of the cause of the mental defect in phenylketonuria because no figures are available on the concentration of the different phenylalanine metabolites in the central nervous system. It is therefore impossible to decide whether the *in vitro* findings reported here have any counterpart *in vivo*.

In a search for the cause of the mental defect in phenylketonuria, however, it seems reasonable to consider the rôle played by the phenylalanine metabolites as inhibitors of enzymes believed to be important for normal function of the central nervous system. The fact that these metabolites are able to inhibit three different decarboxylases, all producers of possible transmitter substances, *i. e.* adrenaline, 5-hydroxytryptamine and GABA, is not without interest.

This work was supported by a grant from the *Magnus Bergvall Foundation*, Stockholm.

REFERENCES

1. Knox, W. E. and Hsia, D.Y.Y. *Am. J. Med.* **22** (1957) 687.
2. Fellman, J. H. *Proc. Soc. Exptl. Biol. Med.* **93** (1956) 413.
3. Davison, A. N. and Sandler, M. *Nature* **181** (1958) 186.
4. Weil-Malherbe, H. and Bone, A. D. *J. Mental Sci.* **101** (1955) 733.
5. Pare, C. M. B., Sandler, M. and Stacey, R. S. *Lancet* **1** (1957) 551.
6. Brodie, B. B. and Shore, P. A. in Hoagland, H. *Hormones, Brain Function, and Behavior*, New York 1957, p. 161.
7. Roberts, E. in Korey, S. R. and Nurnberger, J. I. *Neurochemistry*, New York 1956, p. 11.

8. Bessman, S. P., Rossen, J. and Layne, E. C. *J. Biol. Chem.* **201** (1953) 385.
9. Roberts, E., Rothstein, M. and Baxter, C. F. *Proc. Soc. Exptl. Biol. Med.* **97** (1958) 796.
10. Baxter, C. F. and Roberts, E. *J. Biol. Chem.* **233** (1958) 1135.
11. Albers, R. W. and Salvador, R. A. *Science* **128** (1958) 359.
12. Killam, K. E. *Federation Proc.* **17** (1958) 1018.
13. Elliott, K. A. C. *Rev. can. biol.* **17** (1958) 367.
14. Mosbach, E. H., Phares, E. F. and Carson, S. F. *Arch. Biochem. Biophys.* **33** (1951) 179.
15. Hanson, A. and von Studnitz, W. *Acta Chem. Scand.* **12** (1958) 1332.
16. Hartman, W. J., Akawie, R. I. and Clark, W. G. *J. Biol. Chem.* **216** (1955) 507.
17. Hunter, A. and Downs, C. E. *J. Biol. Chem.* **157** (1945) 427.
18. Killam, K. F. and Bain, J. A. *J. Pharmacol. Exptl. Therap.* **119** (1957) 255.
19. Holtz, P. and Westermann, E. *Arch. exptl. Pathol. Pharmakol. Naunyn-Schmiedberg's* **231** (1957) 311.
20. Tsukada, Y., Nagata, Y. and Takagaki, G. *Proc. Japan. Acad.* **33** (1957) 510.
21. McKhann, G. M. and Tower, D. B. *Am. J. Physiol.* **196** (1959) 36.

Received May 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1375–1382

Determination of L-5-Vinyl-2-thiooxazolidone from Plant Material and Milk

MATTI KREULA and MARTTI KIESVAARA

Laboratory of Valio and Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

A quantitative micromethod for the determination of L-5-vinyl-2-thiooxazolidone in different parts of plants, in silage, and in milk, is presented. The method is based on the separation of this substance by paper chromatography and its spectrophotometric determination. The absorption maximum is at 240 mμ.

Astwood *et al.*[1] have presented a quantitative method for the determination of the L-5-vinyl-2-thiooxazolidone (VTO) contents in the seeds and the roots of cruciferous plants. No method for the determination of the small amounts of VTO formed enzymatically in the green parts of cruciferous plants has, however, been described earlier. The formation of this substance in the green parts of many cruciferous plants was in fact found in this laboratory only two years ago [2]. Later Long *et al.*[3] also isolated VTO from cabbage. The development of a method for the determination of VTO in milk, in which it can be present only in extremely low concentrations [4], has met with considerable difficulties.

EXPERIMENTAL

I. VTO in green parts of plants.

Equipment (*cf.* Fig. 1). 1. A Mojonnier flask (12 ml); the volume of the lower part 2 ml and that of the upper part 10 ml; glass plug.
2. A conical flask for vacuum evaporation (100 ml) and a glass suction adapter.
3. Chromatography equipment for ascending development; a. A glass cylinder, ø 11 cm, height 26 cm, b. Paper, Whatman No. 1.

Reagents. 1 Water saturated ethyl acetate.
2. Phosphate buffer, pH 6.5, M/15 KH_2PO_4 + M/15 Na_2HPO_4.
3. Ethyl ether-methanol, 1:1. Ether shaken peroxide-free with $FeSO_4$ and distilled.

Determination. 10 g of minced plant material is allowed to stand for 7 h at room temperature in a beaker covered with a polytene membrane. The beaker with its contents is heated on a water bath for 5 min, the plant material is transferred to a piece of cloth (gauze) in which it is tightly pressed. 1.5 ml of the filtered press juice is measured into a Mojonnier flask as well as 0.5 ml of phosphate buffer. The mixture, the pH of which is 6.3–6.5, is extracted with 5 ml of water saturated ethyl acetate by thorough

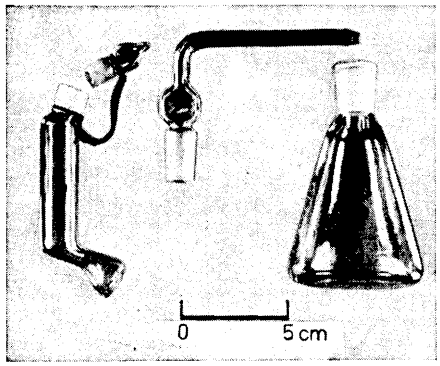


Fig. 1. Equipment for the determination of VTO. A Mojonnier flask (1), a glass adapter (2), and a 100 ml vacuum evaporation flask.

shaking for 20 sec. The clarified extract is poured into a 100 ml conical flask. A second extraction is performed with another 5 ml amount of ethyl acetate. The combined extracts are evaporated to dryness *in vacuo* (water pump). To prevent overheating, the liquid in the conical flask has to be kept in rotatory motion. Using water bath heating as well, evaporation can be effectively speeded up. The residue is now dissolved in 0.5 ml of hot water and 250 μl of the extract obtained is used in two-dimensional paper chromatography, first in methanol-water and then in methanol-ether. Ascending development is used.

On the papers dried at room temperature the boundaries of the spots are drawn using UV-light. The spot of VTO is first cut off and then cut into small slips which are extracted twice with 2.5 ml of hot water in a test tube on a boiling water bath. The combined extracts are estimated photometrically with a Beckmann Spectrophotometer at 220, 240, and 260 mμ.

In calculating the results the method reported by Astwood *et al.*[1] is used. The average of the extinction values at 220 and 260 mμ are subtracted from the extinction value at 240 mμ and the obtained difference is multiplied with the factor 1.16. The correction is justified since the VTO separated by paper chromatography is not yet quite pure. A calibration curve is constructed with pure VTO. The μg-values of the solutions to be investigated are read from this curve, and the results are multiplied with the dilution factor. The results are given in μg/ml of press juice.

In calculating the results *Lamber-Beer*'s law can also be used, in which case the *E*-value obtained by the correction made according to Astwood is multiplied with the factor 7.5. The concentration of the solution to be measured is obtained in μg/ml. The coefficient is equivalent to log *E* 4.23 of the VTO isolated from the seeds of pure big-leafed turnip, rutabaga (Bangholm), and winter turnip rape (Lembke).

II. VTO in silage.

From silage the analysis is made in much the same way as is reported above in connection with the investigation of green parts of plants. Since silage is acid (pH in good silage 3—4) the superfluous acid has to be neutralized first. Such an amount of 1 N NaOH is then added to the juice, obtained from the silage by pressing, that the pH reaches the value 5.5—6. 1.5 ml of the press juice thus treated is pipetted into a Mojonnier flask as well as 0.5 ml of M/15 phosphate buffer, whereafter the analysis is continued in the normal way.

III. VTO in seeds.

1.0 g of finely ground (Willey laboratory mill) seeds are moistened with 5.0 ml of water in a 30 ml beaker, covered with a polytene membrane, and allowed to stand for 9 h at room temperature. The beaker with its contents is heated on a boiling water

bath for 5 min and filtered. The residue on the filter is washed twice with in all 3 ml of hot water. 1.0 ml of the combined filtrates is pipetted into a Mojonnier flask, and the analysis is then continued in the same way as is reported for the analysis of green parts of plants. This time a smaller amount, *e.g.* 100 μl, will, however, suffice in the paper chromatography run.

IV. VTO in milk.

A. Whole milk.

Equipment. Equipments 2 and 3 from section I, and in addition:

4. 3 l and 60 ml separatory funnels.
5. An all-glass vacuum evaporating apparatus with a 1 l flask.

Reagents. 1. Water saturated ethyl acetate.
2. 1 % ammonia.
3. 18 % and 0.5 % acetic acid.
4. Methanol-ether, 1:1.
5. Water-saturated chloroform.

Determination. 300 ml of milk, heated to 85°C and rapidly cooled, is shaken twice with 500 ml of ethyl acetate in a separatory funnel. The combined extracts are evaporated almost to dryness *in vacuo* by slight warming. The residue is treated with 5 ml of 1 % ammonia by gentle shaking and warming in a water bath simultaneously. The ammonia treatment is repeated four times in all. The ammonia extracts obtained are combined and neutralized with 18 % acetic acid to pH 6.5—7.5. Milk fat is removed by treating the extract twice in a separatory funnel with 5 ml of petroleum ether. To avoid emulsion this extraction has to be performed very carefully. The water phase, the volume of which is about 20 ml, is extracted in a small separatory funnel twice with 30 ml of chloroform. The chloroform extract is evaporated to dryness *in vacuo* by slight warming in a 100 ml conical flask. The residue is dissolved in 0.5 ml of hot water and 0.3 ml of it is used in the chromatography run. In the ascending two-dimensional run 1) 0.5 % acetic acid, and 2) methanol-ether, are used as solvents.

A control solution for spectrophotometric measurement is prepared thus that beside the spot of VTO an equally large piece of filter paper is cut out, which piece is then treated in the same way as the spot. The spectrum is measured with a Beckman Spectrophotometer at 220—290 mμ. In calculating the results the extinction value between the maximum point at 240 mμ and the straight line connecting the points at 225 and 260 mμ is used. (E_0, *cf.* Fig. 2).

This extinction value is multiplied with the correction factor 1.16 and the product further with the constant 7.5. The content of VTO in the measuring solution is thus given in μg/ml. The content of the substance to be determined in the milk is obtained when the product is multiplied with the dilution factor.

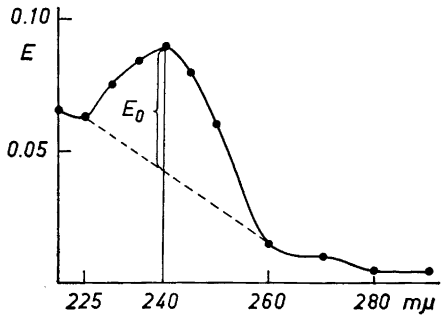


Fig. 2. Extraction curve of the VTO excreted from milk. E_0 used in the calculations, *cf.* text.

B. Skim milk.

This method has been used by Virtanen *et al.*[4]

Equipment and reagents. Equipments 1, 2, and 3 from section I and a 3 l separatory funnel as well as an all-glass vacuum evaporating apparatus with a 1 l flask. Reagents 1, 2, and 4 from section IV and 0.5 % acetic acid.

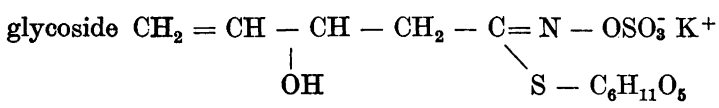
Determination. 500 ml of skim milk is extracted twice in a separatory funnel with 500 ml of ethyl acetate. The acetate is evaporated to dryness *in vacuo* by slight warming, and the residue is treated with 5 ml of 1 % ammonia, and after that also twice with 5 ml of hot water. The extracts are evaporated to dryness in a 100 ml conical flask (vacuum). The residue is washed quantitatively with 2 ml of 0.5 % acetic acid in small amounts into a Mojonnier flask and are shaken twice with 5 ml of ethyl acetate. The ethyl acetate extracts are evaporated to dryness in a 100 ml conical flask (vacuum) and dissolved in 0.5 ml of water. In paper chromatography a two-dimensional ascending run is made from as large a part of the extract as possible in 0.5 % acetic acid and methanol-water. The place for the VTO spot is examined in UV-light whereafter it is cut out from the paper and dissolved in 5 ml of hot water (2 × 2.5 ml). The UV-spectrum is measured at 220—290 mμ. Distilled water is used as control solution. In calculating the results the method described in the section for green parts of plants is used.

RESULTS

Green parts of plants.

Paper chromatograms. In the paper chromatographic run a chromatogram with several spots is obtained from green parts of plants. Fig. 3 shows such a chromatogram. The spot of VTO is marked with S. In the seeds the share of interfering substances is very small although the concentration of VTO is very high.

Enzymatic formation of VTO. VTO is rapidly formed [5] from the progoitrin glycoside



The progress of the reaction can be seen in Fig. 4 which shows experiments performed with (1) rape seeds, and (2) green parts of rape plants.

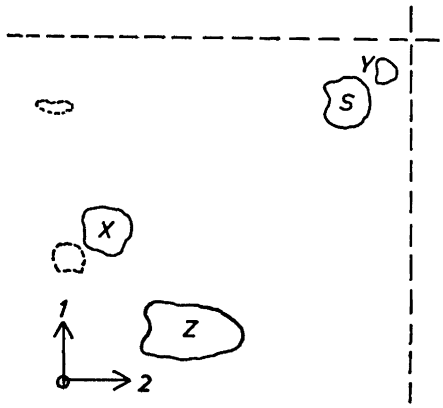


Fig. 3. A two-dimensional paper chromatogram of green parts of winter turnip rape. Solvents used in direction 1: methanol-water, and in direction 2: methanol-ether. Spot S is VTO

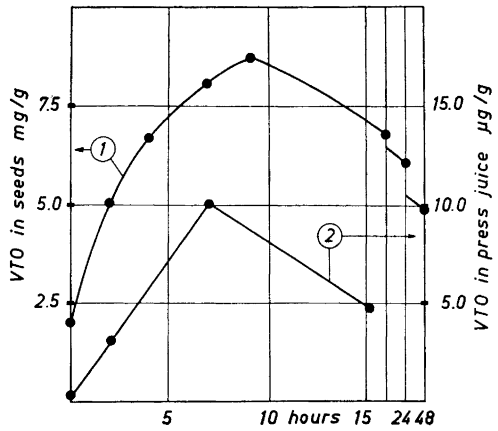


Fig. 4. Enzymatic liberation of VTO at room temperature (1) in crushed seeds and (2) in crushed green parts of Liho rape.

A very rapid enzyme action is obtained when higher temperature is used. In the experiments the maximum value has been obtained after an incubation time of 2 h at 42°C.

The accuracy of the determination of VTO in green parts of plants and seeds. To test the accuracy of the method known amounts of pure VTO was added to the water extract of crushed seeds and to the press juice of fresh as well as preserved green parts of plants. The results of the tests can be seen in Table 1.

The table shows that the developed analytical method worked quantitatively. In the column for µg-amounts in the table pure VTO could also be recovered quantitatively in a determination performed with reagents only.

VTO in the plant material. Using the above methods of determination, the seeds of various cruciferous plants, their green parts, and silage prepared

Table 1. L-5-Vinyl-2-thiooxazolidone contents in water extracts of green parts of rape, silage, and rape seeds as well as the values found after the addition of VTO.

Material	VTO in the extract µg/ml	VTO addition µg/ml	VTO total 1 + 2 µg/ml	VTO found µg/ml	VTO found %
	1	2	3	4	5
Green parts of rape	3.9	1.2	5.1	5.2	102
	3.9	2.7	6.6	6.5	99
Silage *	23.5	15.0	38.5	38.6	100
Rape seeds	15.8	2.3	18.1	17.9	99
	25.6	6.8	32.4	32.4	100
	25.6	22.5	48.1	46.9	98

* Prepared by the AIV method using NH_4HSO_4 as a preservative.

Table 2. VTO values found in crushed seeds of cruciferous plants.

Species	Samples	VTO	
		Ranges of variation mg/g	Average mg/g
Spring rape	8	2.3 — 4.2	3.16
Winter rape	11	7.7 —12.3	8.96
Spring turnip rape	3	0.2 — 0.8	0.46
Winter turnip rape	16	0.04— 0.7	0.27
Big-leafed turnip	9	0.2 — 3.5	1.16
Turnip	2	0.5 — 0.9	0.70
Swede	9	3.2 —14.5	6.70
Marrow kale	1	—	1.07
Radish	1	—	0.04

Table 3. VTO values of green parts of cruciferous plants. Samples from field cultivations and greenhouse. Contents given in μg/ml press juice.

Species	Samples	VTO	
		Ranges of variation μg/ml	Average μg/ml
Spring rape V *	1	—	4
» » K **	8	2— 40	14
Winter rape V	9	7— 73	33
» » K	4	23—135	69
Spring turnip rape K	2	3— 60	32
Winter » » K	1	—	24
Turnip K	1	—	72
Big-leafed turnip V	4	11— 55	25
Marrow kale V	3	0— 8	3
» » K	1	—	9

* V = sample from field cultivation.
** K = sample from greenhouse.

Table 4. VTO values of silage prepared from cruciferous plants by the AIV-method. Contents given in μg/ml press juice from silage.

Raw material	Samples	VTO	
		Ranges of variation μg/ml	Average μg/ml
Spring rape	4	0— 8	3
Fodder rape	22	0—37	5
Winter rape	15	0—56	21
Big-leafed turnip	5	0—33	11
Swede (tops)	3	9—80	28

from cruciferous plants by the AIV method, were investigated. Part of the plants were grown in a greenhouse, but the bulk of the samples investigated came from field cultivations, however. The results of the tests are collected in Tables 2, 3, and 4.

VTO in milk.

Whole milk. Preliminary experiments showed that VTO very rapidly disappears in the milk. To eliminate this factor which hampered the determination, milk into which a known amount of the substance was added under different test conditions was investigated. Heating of the milk appeared to be of most decisive importance for the stability of VTO. Also by deep freezing the milk the stability could be improved. An artificial lowering of the pH again had a harmful effect on the yield. The effect of heating and the test temperature is shown in Tables 5 and 6. From the results shown in the tables

Table 5. The effect of heating on the yield of VTO in whole milk. 300 ml of milk heated immediately after the addition of the substance and cooled rapidly.

Treatment °C	VTO		
	Added μg	Yield	
		μg	%
Unheated	48	22	46
60°	48	29	60
85°	48	37	77
100°	48	37	77
Unheated	30	15	50
85°	30	19	63
100°	30	23	77

Table 6. The stability of VTO at + 20°C and −15°C in unheated whole milk and in whole milk heated to 85°C. The milk hot treated before the addition. Milk amounts 300 ml.

Treatment	Time of keeping h	Addition μg	Found			
			+20°C		−15°C	
			μg	%	μg	%
Unheated	0	43	28	65	—	—
	24	43	3 *	7	13	30
	48	43	2.5 *	6	10	23
	144	43	—	—	8	19
Heated to 85°C	0	65	48	74	—	—
	24	65	—	—	49	75
	48	65	40	62	—	—
	96	65	26	40	48	74
	192	65	—	—	46	71

* sour milk.

it appears that about 75 % of the VTO added to milk is recovered, and that hence about 25 % remains as a loss of analysis. A considerable part of this loss is due to the chloroform extraction used in the method, which causes a loss of about 10 %. When the milk is heated to 85°C and is kept at −15°C, the substance to be determined is very stable and even when kept at room temperature it disappears comparatively slowly.

In the experiment with reagents only, 154 μg of VTO were recovered from the 170 μg eluted into 300 ml of distilled water, corresponding to 90.5 % of the amount added.

Skim milk. In order to test the accuracy of the method 110 μg of VTO was added to 500 ml of skim milk the fat content of which was 0.06 %. Of the amount added 84 % were recovered in the analysis. The result corresponds to the yield in the method for whole milk when the methodical loss of 10 % in this method is taken into consideration. When, in addition to fat, casein is also removed from the milk recovery is not improved.

The determinations performed on skim milk or whey do not yet show with certainty the real content of VTO in the milk used in the experiment. Because the substance is fat soluble it can, for instance, be retained in the milk fat to a large extent. This is also indicated by an experiment in which melted milk fat was mixed into a water solution of VTO. When analyzing the water phase after shaking, it did not contain more than 80 % of the original amount of VTO.

A fellowship from the *Finnish Government Scientific Commission (Valtion luonnontieteellinen toimikunta)* to the senior author is gratefully acknowledged.

REFERENCES

1. Astwood, E. B., Greer, M. A. and Ettlinger, M. G. *J. Biol. Chem.* **181** (1949) 121.
2. Virtanen, A. I. *Suomen Kemistilehti A* **31** (1958) 96 (*lecture given at the meeting of Societas Biochemica, Biophysica et Microbiologica Fenniae*, March 19, 1958); *Schweiz. Z. allgem. Pathol. u. Bakteriol.* **21** (1958) 970.
3. Altamura, M. R., Long, Jr. L. and Hasselström, T. *Paper presented at the 134th National Meeting of the American Chemical Society Division of Agricultural and Food Chemistry*, Chicago, Illinois, 7−12 September 1958.
4. Virtanen, A. I., Kreula, M. and Kiesvaara, M. *Suomen Kemistilehti A* **32** (1959) 45 (*communication at the meeting of Societas Biochemica, Biophysica et Microbiologica Fenniae*, October 27, 1958); *Acta Chem. Scand.* **12** (1958) 580.
5. Greer, M. A., Ettlinger, M. G. and Astwood, E. B. *J. Clin. Endocrinol.* **9** (1949) 1069; Greer, M. A. *J. Am. Chem. Soc.* **78** (1956) 1260; Kjær, A., Gmelin, R. and Jensen, R. B. *Acta Chem. Scand.* **10** (1956) 432; Schultz, O.-E. and Wagner, W. *Arch. Pharm.* **289/61** (1956) 597; Ettlinger, M. G. and Lundeen, A. J. *J. Am. Chem. Soc.* **78** (1956) 4172; ibid. **79** (1957) 1764.

Received May 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1383–1394

Proton Magnetic Resonance Studies of Enolised β-Triketones

STURE FORSÉN and MARTIN NILSSON

Research Group for Nuclear Magnetic Resonance, Division of Physical Chemistry and Division of Organic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Triacetylmethane together with some 2-acyl*cyclo*hexane-1,3-diones and the fern pigment ceroptene and its transformation products have been investigated by proton magnetic resonance (PMR) and infrared spectroscopy.

In the PMR spectra, very large shifts ($-\delta_{aq}$ = 12.6–14.1) were observed for the enolic protons, the largest shifts being shown by the conjugated β-triketones. The shifts were correlated with the infrared chelate carbonyl absorptions.

The results indicate that, in solution, ceroptene is present as a mixture of two enol forms of 5-methoxy-2-cinnamoyl-6,6-dimethyl-*cyclo*hex-4-ene-1,3-dione (IV, R = Ph–CH=CH–).

Interest in β-triketones (α-acyl-β-diketones) has been mainly devoted to the 2-acyl*cyclo*hexane-1,3-diones and -1,3,5-triones. These types of β-triketones form an important group of natural products; *cf.* the recent review by Hassall [1].

Our interest in this type of compound arose from work on the natural colouring matter of the fern *Pityrogramma triangularis*. This pigment, ceroptene, was recently investigated by one of us and was shown to be a methyl enol ether of 2-cinnamoyl-6,6-dimethyl*cyclo*hexane-1,3,5,-trione (III, R=Ph–CH=CH–), either 5-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-4-ene-1,3-dione (IV,R = Ph–CH = CH–) or 3-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-3-ene-1,5-dione (V, R = Ph–CH = CH–) [2]. Of these, structure IV was considered more likely since the infrared data indicated a pronounced conjugated chelation (*cf.* discussion below) similar to that suggested by Chan and Hassall [3] for 2-acyl*cyclo*hexane-1,3-diones. However the chelate carbonyl absorption (1 515 cm^{-1}) occurred at a lower frequency than any previously reported and it was considered desirable to check this assignment by some independent method.

An opportunity arose of studying this problem by nuclear magnetic resonance as part of the general hydrogen bond research programme of the NMR group at this institute.

To provide background information a survey was made of the hydrogen bonding of some simple β-triketones as well as of ceroptene and its transformation products. The basic compound of the series, triacetylmethane (I), was prepared in a state of purity. 2-Acetyl-5,5-dimethyl*cyclo*hexane-1,3-dione ("acetyldimedone", II, R = CH_3) which was readily accessible by synthesis was taken as a convenient model compound for ceroptene, especially since condensation with aromatic aldehydes afforded conjugated analogues.

METHODS

Proton magnetic resonance measurements. The measurements were made with a Varian V—4 300 NMR spectrometer operating at a fixed frequency of 40 Mc/s and equipped with a Varian V-K 3 506 Super Stabilizer.

The sample cells used in all low concentration measurements were thin-walled glass tubes with a good filling factor [4]. Measurements against an external water standard were made in coaxial glass tubes containing water in the central capillary and the solution to be measured in the annular space. Using the notation of Reilly, McConell and Meisenheimer [5], the concentric tube had the following dimensions: $2\,a = 1.3$ mm, $2\,b = 1.8$ mm and $2\,r = 2.45$ mm.

The resonance shifts were determined in c/s by the audiofrequency sideband method employing a Hewlett-Packard 200 CD Audio Oscillator. The sign of the shift was taken as positive when the resonance occurred at a higher field than that of the water reference. The shift measurements were generally reproducible to within $\pm$ 1 c/s.

Resonance shifts are sometimes discussed in terms of the dimensionless unit δ defined by the expression

$$\delta = 10^6 \frac{H_{\text{sample}} - H_{\text{ref}}}{H_{\text{ref}}}$$

To avoid confusion, the reference substance is specified here by a lower index, *e.g.* δ_{aq}.

Where not otherwise stated the sample temperature was 22 $\pm$ 1°. For the measurement at —30°, an apparatus permitting simultaneous cooling and spinning of the sample was employed.

As has been pointed out by Allred and Rochow [6], diamagnetic anisotropy effects in the interaction between solvent and solute may introduce errors and make the direct comparison of different PMR spectra less satisfactory. Thus benzene is known to cause solvent shifts that vary with the nature of the solute. This is probably due to the magnetic anisotropy effects of the benzene ring [7]. The PMR shifts of all compounds were therefore determined using dilute carbon tetrachloride solutions against an external water standard in order to keep the bulk diamagnetic susceptibility practically constant in all measurements and, also, to eliminate to some extent the anisotropy effects of the solvent.

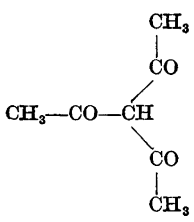
Infrared measurements. The infrared spectra were recorded on a Perkin-Elmer No 21 instrument using a sodium chloride prism, carbon tetrachloride solutions (*ca.* 0.1 M) and rock salt cells of 0.1 and 1 mm thickness. The solvent absorption was compensated with a Hilger-Watts variable cell. The calibration of the wavelength scale was checked against atmospheric moisture. The frequencies should be accurate to within $\pm$ 5 cm^{-1}. The sample temperature was *ca.* 25°.

MATERIALS

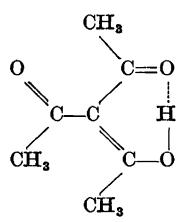
Solvents. For the PMR measurements, carbon tetrachloride (*Merck pro analysi*) was dried over phosphorus(V)oxide and distilled. The benzene used was a redistilled product (*Merck pro analysi*). For the IR measurements, commercial carbon tetrachloride as above was used without further treatment.

Triketones. Melting points were determined on a Kofler block.

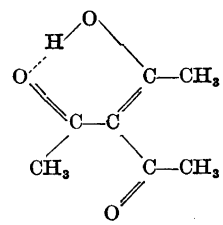
3-Acetylpentane-2,4-dione (»triacetylmethane», I). Since there is some discussion in the literature about this compound, the detailed experimental procedure will be given.



a

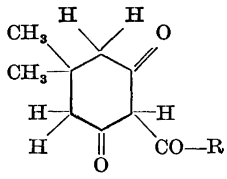


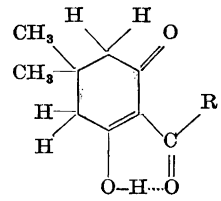
b



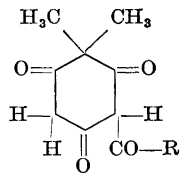
c

I

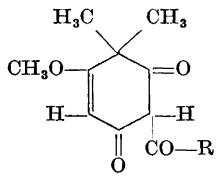




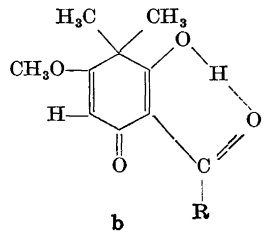
II



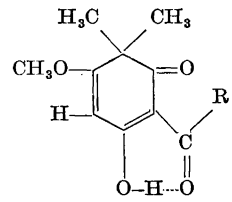
III



a

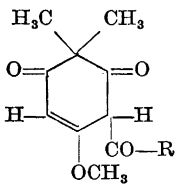


b

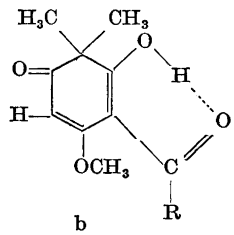


c

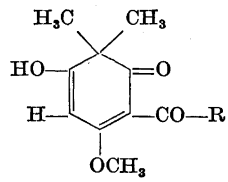
IV



a



b



c

V

A suspension of sodium hydride (1 mole, 50 % in paraffin oil) in dry ether (1 000 ml) was cooled to 0° and acetylacetone (1 mole) in ether (100 ml) was added during 30 min. When hydrogen evolution had ceased, acetyl chloride (1 mole) in ether (100 ml) was added during 30 min. The reaction mixture was left overnight. Water (500 ml) was then added to dissolve the sodium chloride, and the ethereal solution obtained was washed with water and exhaustively extracted with sodium hydrogen carbonate (sat.). The hydrogen carbonate extract was washed thoroughly with ether to remove all traces of neutral material, acidified with dilute sulphuric acid and the triacetylmethane was extracted with ether. The ether extract was washed with several portions of water, dried and evaporated giving crude triacetylmethane (*ca.* 20—25 %) in which no enol acetate could be detected (IR). Distillation gave pure triacetylmethane in 15—20 % yield. For the NMR and IR measurements a fraction was taken of b.p. 85—86°/7 mm, n_D^{25} 1.4893, d_4^{20} 1.096, equiv. wt. 142 (by titration with standard alkali against phenolphtalein; calc. 142). The b.p. has been given as 104°/19 mm [8,9] and the density as d_4^{20} 1.0658 and d_4^{22} 1.0742 [9]. The measurements were made within 24 h of the preparation because of the instability of the compound.

The ether phase remaining after the hydrogen carbonate extraction contained neutral material (*ca.* 60 g, excluding paraffin), which according to its IR spectrum (compare Rasmussen *et al.*[10]), consisted mainly of the enol acetate of acetylacetone. This decomposed both on distillation and on extraction with 2 M sodium hydroxide. Repeated extraction with sodium carbonate (5 %) gave additional quantities of triacetylmethane, thus indicating that the enol acetate is slowly converted to the triketone.

Essentially the same results were obtained using powdered sodium. This shows the correctness of the original work by Nef [8] and also indicates the cause of the aberrant results of Birkenbach *et al.*[9] (see also Schwarzenbach and Lutz [11]).

*2-Acetyl-5,5-dimethyl*cyclo*hexane-1,3-dione* ("acetyldimedone", II, R = CH_3) was prepared according to Dieckmann and Stein [12]. It was purified *via* the copper salt and then distilled, b.p. 132—133°/20 mm, m.p. 36—38°. The authors cited give m.p. 36°.

*2-Cinnamoyl-5,5-dimethyl*cyclo*hexane-1,3-dione* ("cinnamoyldimedone", II, R = Ph—CH=CH—) was prepared by condensation of acetyldimedone with benzaldehyde on the water bath for 1 h using piperidine as catalyst. The product was distilled, b.p. *ca.* 190°/1 mm, and recrystallised from methanol, m.p. 98—100°. Ukita *et al.*[13] report m.p. 96.5°. Yield 50 %.

*2-Hydrocinnamoyl-5,5-dimethyl*cyclo*hexane-1,3-dione* (II, R = Ph—CH_2—CH_2—) was obtained in 70 % yield from cinnamoyldimedone by hydrogenation over palladised charcoal in ethanol. It was recrystallised from *cyclo*hexane and sublimed, m.p. 57—58.5°. (Found: C 74.9; H 7.4. Calc. for $C_{17}H_{20}O_3$: C 75.0; H 7.4.)

*2-(4-Methoxycinnamoyl)-5,5-dimethyl*cyclo*hexane-1,3-dione* was obtained from acetyldimedone and anisaldehyde using piperidine as catalyst. It was recrystallised from methanol and sublimed, m.p. 136—138°. Yield 60 %. (Found: C 71.8; H 6.6. Calc. for $C_{18}H_{20}O_4$: C 72.0; H 6.7.)

*2-(4-Methoxyhydrocinnamoyl)-5,5-dimethyl*cyclo*hexane-1,3-dione* was prepared, in 60 % yield, by hydrogenation of the above compound. It was recrystallised from *cyclo*hexane and sublimed, m.p. 71—72.5°. (Found: C 70.8; H 7.4. Calc. for $C_{18}H_{22}O_4$: C 71.5; H 7.3.)

For details of the ceroptene derivatives, *cf.* Ref.[3]

O-Methyl-3-acetylfilicinic acid. A synthetic product was recrystallised from *cyclo*hexane and sublimed, m.p. 107—109°.

Dihydroceroptene was obtained by hydrogenation of the natural product. It was recrystallised from methanol, m.p. 98—100°.

Ceroptene. Samples of the natural product were recrystallised from methanol, m.p. 137—140°.

RESULTS

Proton magnetic resonance measurements

Triacetylmethane (I). Investigations by Schwarzenbach and Lutz [11] indicate that this compound is completely enolised in aqueous solution. As indicated

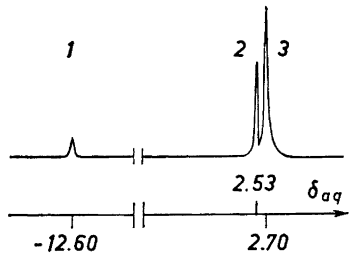


Fig. 1. PMR spectrum of triacetylmethane in carbon tetrachloride (mole fraction $x = 0.1$). Chemical shifts given as δ_{aq}.

above, the "triketo form" discussed by Birkenbach *et al.* [9] is apparently the enol acetate of acetylacetone. The PMR spectrum of the pure liquid showed a minor peak ($\delta_{aq} = 0.3$) which may be due to the central hydrogen atom of the triketo form and corresponds to the presence of *ca.* 5 % of non-enolised material. This signal could not be detected in the spectra of the solutions and it may be concluded that triacetylmethane is almost completely enolised in solution. In the IR spectrum of the pure liquid, there was a shoulder at 1 700 cm^{-1}, possibly also due to the triketo form.

The PMR spectrum of the carbon tetrachloride solution was simple (Fig. 1). Signal 1 was assigned to the enolic proton. The chemical shift was large ($\delta_{aq} = -12.6$) compared with that for acetylacetone ($\delta_{aq} = -10$, *cf.* Reeves [14]). In acetylacetone, the methyl groups are identical in the PMR spectrum and Reeves has attributed this to the rapid movement of the enolic proton along the hydrogen bond. In the PMR spectrum of triacetylmethane, there were two methyl signals (2 and 3) and the intensity ratio was 1:2. Signal 2 was attributed to the methyl group not directly attached to the hydrogen bond system and signal 3 to the two methyl groups adjacent to the intramolecular hydrogen bond. The existence of two separate methyl signals indicated that the interconversion of identical forms (for instance I b and c) was slow.

In benzene solution the methyl signals coincided. This may be due to a solvent anisotropy effect or to the rapid interconversion of identical forms. It was interesting to note that the enol proton signal occurred at a still lower field ($\delta_{aq} = -13.1$).

Acetyldimedone (II, R = CH_3). This compound is known to be completely enolised in both polar and non-polar solvents [3].

The PMR spectra of solutions in carbon tetrachloride, benzene and a mixture of these solvents are shown in Fig. 2. Signal 1 in the first spectrum was considered to be due to the enolic proton. The chemical shift was larger than that for triacetylmethane ($\delta_{aq} = -13.3$). Signal 4 was evidently caused by the gem-dimethyl group since the chemical shift was close to the expected value and the intensity corresponded to six of the fourteen protons. Signal 3 ($\delta_{aq} = 2.69$) judging by its intensity, should correspond to two protons and should therefore be due to a ring methylene group. Signal 2 was at first difficult to explain. The intensity indicated, however, that it was due to five protons. In benzene solution, the magnitudes of peaks 2 and 3 were reversed and, in mixtures of these solvents, they were resolved into three peaks with relative intensities 3:2:2. This clearly indicated that peak 2 in the first spectrum was

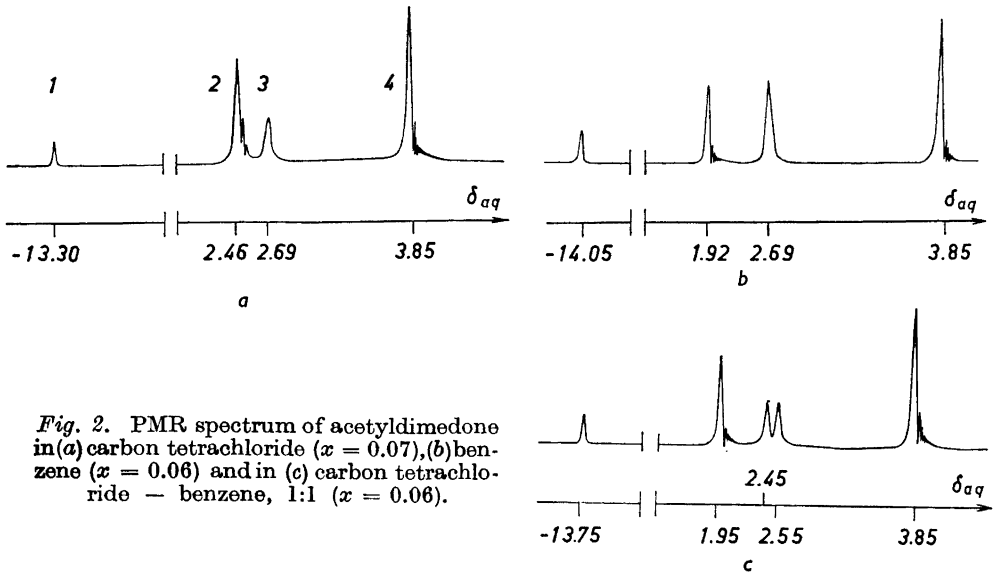


Fig. 2. PMR spectrum of acetyldimedone in (*a*) carbon tetrachloride ($x = 0.07$), (*b*) benzene ($x = 0.06$) and in (*c*) carbon tetrachloride — benzene, 1:1 ($x = 0.06$).

the sum of signals from the acetyl group and one methylene group. In the PMR measurements on the benzene solution, the methylene groups were equivalent; this is analogous to the results obtained with triacetylmethane. The separation of signals 2 and 3 in the carbon tetrachloride solution was not affected by lowering the sample temperature to −30°.

Hydrocinnamoyldimedone (II, R = Ph—CH_2—CH_2—). The PMR spectrum (Fig. 3) was clearly reminiscent of that of acetyldimedone. The protons of the benzene ring and the methylene groups between the rings gave signals at the expected field (signals 2 and 3). The enol proton signal (1) occurred at the same field as in acetyldimedone. Approximately the same splitting and shifts for the methylene groups in the ring (4) were recognised.

Cinnamoyldimedone (II, R = Ph—CH = CH—). In the spectrum for this compound (Fig. 3), the enolic proton appeared as a signal at a very low field ($\delta_{aq} = -13.8$). The gem-dimethyl group gave a signal at the same field as in acetyldimedone (5) and the methylene signals (4) were quite separate. Signal group 2, consisting of two signals of high intensity ($\delta_{aq} = -3.23$ and -3.10,) and two signals of lower intensity ($\delta_{aq} = -3.80$ and -2.70 hidden in signal group 3), possessed the characteristic appearance of two spin-coupled non-identical protons of a —CH = CH— group [15]. The spin-coupling constant, J, was 15 c/s, indicating *trans*-configuration [16]. The PMR spectrum of ethyl cinnamate ($x = 0.1$ in carbon tetrachloride) was recorded for comparison. It showed a spin-coupling constant for the *trans*-ethylenic double bond protons of 16.0 c/s.

The benzenoid protons in cinnamoyldimedone gave a complex signal (3) of the type often obtained from conjugated aromatic nuclei.

4-Methoxycinnamoyldimedone and its dihydroderivative showed no relevant differences and the enol proton resonances occurred at the same fields as those for cinnamoyldimedone and hydrocinnamoyldimedone respectively.

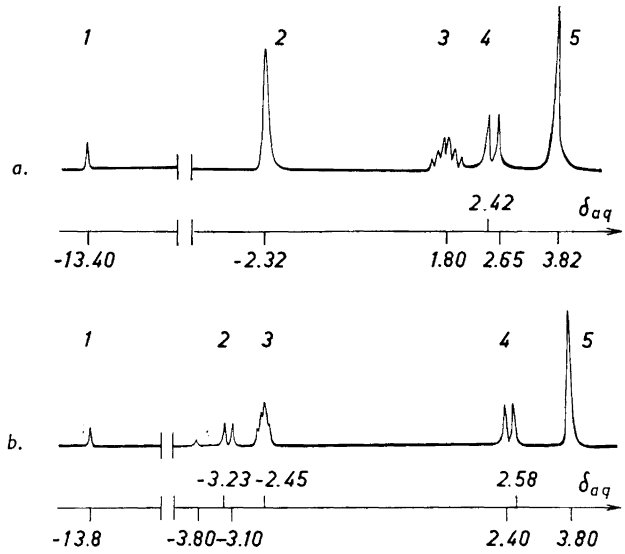


Fig. 3. PMR spectra of (*a*) hydrocinnamoyldimedone ($x = 0.05$) and (*b*) cinnamoyldimedone ($x = 0.04$) in carbon tetrachloride.

O-Methyl-3-acylfilicinic acids. It was shown previously [2] that ceroptene is a methyl ether of 3-cinnamoylfilicinic acid (III, R = Ph—CH = CH—).

3-Acylfilicinic acids could give several methyl ethers; only two are however capable of giving enols with strong intramolecular hydrogen bonds (IV and V). The ether indicated in formula IV in addition to the wholly ketonic form, might also give two different conjugated chelate enols (IV b and c). The ether indicated in V can only give one enol stabilised by strong intramolecular hydrogen bonding (V b). The other form (V c) is of the dimedone type and is unlikely to be present in appreciable amounts in dilute solutions in non-polar solvents. As discussed under triacetylmethane, the movement of protons along the hydrogen bond is very rapid and the corresponding additional forms need no separate discussion.

In previous work [2], it was shown that in ceroptene, dihydroceroptene and O-methyl-3-acetylfilicinic acid the methoxyl group has the same position and thus a choice has to be made between structures IV and V.

O-Methyl-3-acetylfilicinic acid (R = CH_3). The PMR spectrum is shown in Fig. 4. There are five main bands, four of which have small side bands. The ratio of the intensities of side and main bands was fairly constant — 1:6. In benzene solution, the splitting persisted although the side bands were smaller — the intensity ratio was 1:8. The most reasonable explanation for the splitting seems to be that two tautomeric forms are present and that these forms are very similar but not rapidly interconvertible. A change of solvent is likely to change the equilibrium proportions and the rate of interconversion but apparently this rate is low even in benzene. Assignments will be made without reference to the splitting.

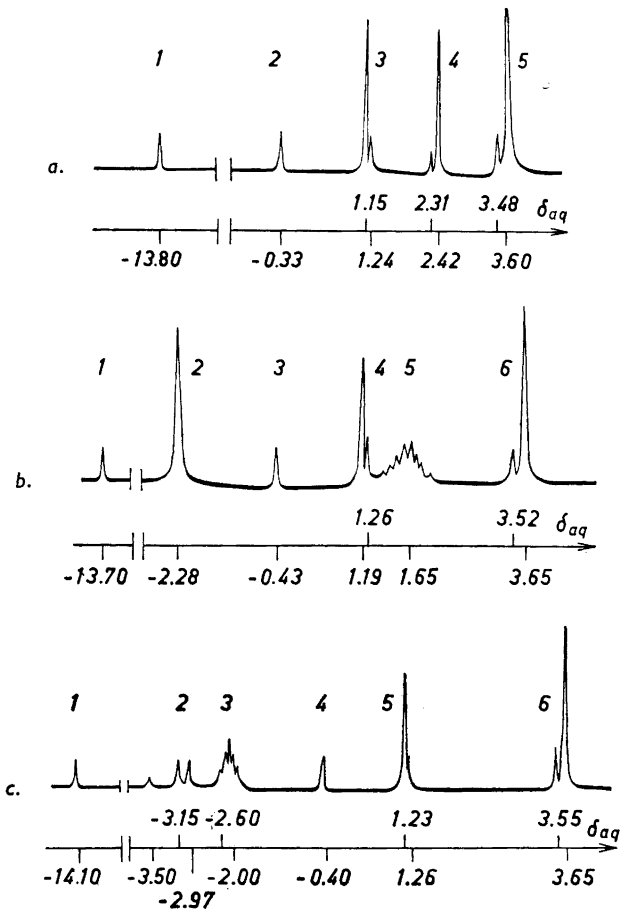


Fig. 4. PMR spectra of (*a*) O-methyl-3-acetylfilicinic acid ($x = 0.05$), (*b*) dihydroceroptene ($x = 0.06$) and (*c*) ceroptene ($x = 0.03$) in carbon tetrachloride.

Signal 1 was found at a very low field ($\delta_{aq} = -13.8$). It was undoubtedly due to the enolic proton. Signal 2 was assigned to the $-CH =$ group of the filicinic acid ring because of the chemical shift and the intensity. Signal 3 was considered to be due to the methoxyl protons. A change of solvent brings about a remarkable displacement of the resonance field ($\delta_{aq} = 1.05$ in carbon tetrachloride solution to $\delta_{aq} = 1.89$ in benzene solution; $\Delta\delta = 0.84$). Similar solvent-dependent shifts have, however, also been observed with aromatic methyl ethers, *e.g.* anisole and guaiacol ($\Delta\delta \approx 1.0$). Signal 4 was assigned to the acetyl group by analogy with acetyldimedone. Signal 5 was found to have approximately double the intensity of signals 3 or 4. Its position in the high field side of the spectrum provided further evidence that it was caused by the gem-dimethyl grouping.

Dihydroceroptene (R = Ph—CH_2—CH_2—). Apart from the "isolated" benzene ring, this compound is analogous to O-methyl-3-acetylfilicinic acid as shown by the almost identical ultraviolet spectra [2]. The similarities recur also in the PMR spectra (Fig. 4). The same splitting of the signals can be observed and the ratio of side band to main band is close to that for the previous compound (1:5). This indicates that the splitting is not due to impurities since the dihydroceroptene was obtained from the natural product whereas the O-methyl-3-acetylfilicinic acid was an independent synthetic product and the same amount and type of impurities cannot be expected to be present in both. In benzene solution, the intensity ratio was 1:4.5.

Signal 1 ($\delta_{aq} = -13.7$) was considered to be due to the enolic proton while signal 2 was assigned to the protons of the benzene ring and peak 3 to the —CH = group of the filicinic acid ring. Signal 4 was caused by the methoxyl group and showed the same solvent displacement as that discussed above. Signal 5 was composed by a number of peaks and undoubtedly was due to the methylene groups between the rings. Signal 6 was due to the gem-dimethyl group.

Ceroptene (R = Ph—CH = CH—). The splitting effects in this spectrum were slightly more marked than those in the two compounds above and the ratio was *ca.* 1:4. No splitting of signal 4 was observed but this was probably due to the low concentration. Ceroptene is not very soluble in carbon tetrachloride and this made it necessary to operate the spectrometer at the sensitivity limit. There was no appreciable splitting of the signals in benzene solution, which may indicate that one of the forms predominates.

Signal 1 was assigned to the enolic proton — it occurred at the lowest field yet reported for a proton resonance signal ($\delta_{aq} = -14.1$).* Signal group 3, consisting of two signals of high intensity and two of low intensity, was analogous to signal group 2 in the spectrum of cinnamoyldimedone. It was therefore attributed to the protons at the *trans*-ethylenic double bond. The spin-coupling constant, J, was 16 c/s. Signal 3 was assigned to the protons of the benzene ring and signal 4 to the proton of the filicinic acid ring. Signal 5 was due to the methoxyl group — it showed a strong solvent dependence. Signal 6 was assigned to the gem-dimethyl group.

Infrared measurements

The following list gives absorptions in the 1 500—1 700 cm^{-1} region with assignments and — in brackets — the approximate molecular extinction coefficients ($mole^{-1}$ 1 cm^{-1}).

All compounds investigated showed a very broad absorption in the region 3 200—1 900 cm^{-1}. It was however weak, ε *ca.* 20), and it was not possible to define a fixed hydroxyl stretching frequency. The chelate carbonyl absorptions were also rather broad and intensity comparisons could therefore be

* The proton involved in the symmetrical hydrogen bond in potassium hydrogen maleate has been found at a still lower field ($\delta_{aq} = -15.40$). Forsén, S. *J. Chem. Phys.* **31** (1959) 852.

misleading. It is not possible in these complicated spectra to make satisfactory measurements of the integrated absorption intensities.

All cinnamoyl derivatives gave absorption close to 1 310 and 970 cm^{-1} due to the *trans* ethylenic double bond.

The data for ceroptene and its transformation products have been taken in part from previous work [2].

Assignments: a conjugated, chelated carbonyl group; *b* conjugated carbonyl group (non- chelated); *c* conjugated aromatic ring; *d* ethylenic double bond; *e* enol ether double bond; *f* aromatic ring.

Triacetylmethane: 1 580 a (200), 1 678 b (300).

Acetyldimedone: 1 560 a (200), 1 660 b (500); *cf.* Refs. [3,13]

Hydrocinnamoyldimedone: 1 560 a (250), 1 600 (shoulder), 1 665 b (500).

Cinnamoyldimedone: 1 520 a (300), 1 578 c (250), 1 615 d (500), 1 665 b (500)

p-*Methoxyhydrocinnamoyldimedone:* 1 515 f (600), 1 563 a (250), 1 610 f (250), 1 665 b (500).

p-*Methoxycinnamoyldimedone:* 1 512 f (800), 1 525 a (shoulder *ca.* 400), 1 575 c (400), 1 603 f (700), 1 620 d (500), 1 660 b (500).

O-Methyl-3-acetylfilicinic acid: 1 525 a (500), 1 621 e (500), 1 660 b (500).

Dihydroceroptene: 1 525 a (500), 1 625 e (400), 1 665 b (500).

Ceroptene: 1 515 a (600), 1 580 c (250), 1 625 e, d (800), 1 655 b (400).

DISCUSSION

The concept of conjugated chelate systems was proposed by Rasmussen, Tunnicliff and Brattain [10] to cover the phenomenon of strong intramolecular hydrogen bonding and simultaneous conjugation between the electronegative atoms connected by the hydrogen bond. The term has been used mostly in connection with the interpretation of the infrared spectra of β-dicarbonyl compounds and related substances but has also been extended to cover some intermolecular hydrogen bonding phenomena, *e.g.* in the dimer of dimedone [10]. The applications have been discussed by Bellamy [18]. In the infrared spectra conjugate chelation gives rise to very broad and weak hydroxyl stretching absorptions at low frequencies (in general below 3 000 cm^{-1}) and to carbonyl absorption of unusual intensity shifted to a lower frequency than that for ordinary conjugated carbonyl compounds.

In the present investigation no evidence was obtained for the presence of non-enolised species of β-triketones in solution and it is evident that the β-triketones investigated are completely enolised in solution. The infrared spectra of the solid compounds in potassium bromide discs also indicated complete enolisation.

It is evident from the results obtained that the conjugated chelate system in the enolised β-triketones has rather exceptional properties, which are particularly marked in those compounds where further conjugation is present. Ceroptene appears to have the lowest proton resonance field and the lowest chelate carbonyl absorption frequency yet reported (*cf.* Reeves [14] and Bellamy [18]).

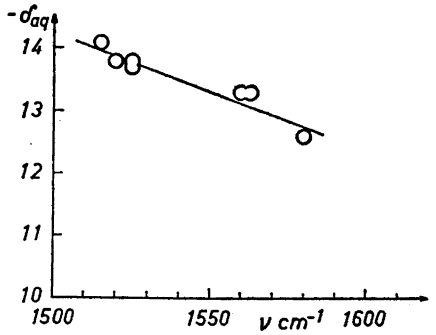


Fig. 5. Correlation of the PMR enol proton shift with the infrared chelate carbonyl frequency.

In Fig. 5, an attempt has been made to correlate the PMR enol proton resonance shifts with the infrared chelate carbonyl absorption frequencies. Although the number of measurements is limited, the general tendency is clear: a large negative enol proton PMR shift is accompanied by a low chelate carbonyl absorption frequency. This indicates a very strong interaction between the enol proton and the carbonyl group and may be taken as a proof that the assignment of the chelate carbonyl absorption is justified. The same conclusion can be drawn qualitatively from a study of the hydroxyl stretching absorption but, unfortunately, these bands are too weak for a quantitative study. Deuteration experiments have been started in order to investigate other hydroxyl absorptions. It has already been found that the rate of hydrogen exchange between acetyldimedone and heavy water is very low. This is in marked contrast to the behaviour of simple β-diketones, *e.g.* acetylacetone, and is an additional indication of the very strong intramolecular hydrogen bonding present.

In a discussion of the enolisation and acidity of carbonyl compounds, Hammond [19] points out that the formation of intramolecular hydrogen bonds and the separation in space of the oxygen atoms are important factors influencing the enolisation of β-dicarbonyl compounds. The first factor is well known and the second is exemplified by the pronounced enolisation of *trans*-fixed β-diketones which cannot form intramolecular hydrogen bonds although the enols may sometimes be stabilised by dimerisation [10].

In β-diketones, these tendencies, however, may work in the same direction since *trans*-conjugation to the non-chelated carbonyl group is possible. That this conjugation is very strong indeed is indicated by the infrared absorption of the non-chelated carbonyl group [3], which occurs at approximately the same frequency as that in dimedone methyl ether [18]. This conjugation effect has also been discussed by Schwarzenbach and Lutz in connection with the acidity of triacetylmethane [11].

The differences between the two unconjugated β-triketones, triacetylmethane and acetyldimedone, are to be expected by analogy with the well-known tendency of cyclic ketones to enolise more readily than the acyclic analogues [19].

The pronounced influence of external conjugation on the properties of the conjugated chelate system could be ascribed in part to steric factors but further experimental material is required for a discussion.

As far as the structure of ceroptene and the choice between structures IV and V for the O-methyl-3-acylfilicinic acids are concerned, the splitting found in the PMR spectra indicates that two enolic species are present in the solutions. The rate of interconversion is apparently low and the equilibrium proportions are dependent on the solvent. As calculated from the relative intensities of the split signals, the equilibrium constants are mostly of the order of 3—8 which means a free energy change of less than 1 kcal/mole. The tautomeric forms must therefore be very similar and the hydrogen bonds should be of approximately the same strength. Since only structure IV permits the existence of two tautomeric enols with intramolecular hydrogen bonds of similar properties (IV b and c), the results strongly indicate that ceroptene is actually 5-methoxy-2-cinnamoyl-6,6-dimethyl*cyclo*hex-4-ene-1,3-dione (IV, R = Ph—CH=CH—). Other structures, such as 5-methoxy-4-cinnamoyl-6,6-dimethyl-*cyclo*hex-4-ene-1,3-dione, which have not been discussed above, are ruled out by these considerations.

Acknowledgements. The authors would like to thank Professor H. Erdtman and Dr. E. Forslind for their kind encouragement and for valuable discussions. Thanks are also due to Miss Gurli Hammarberg for able assistance with the infrared measurements, to Mr. E. Pettersson for assistance with the preparative work and to Dr. D. Gillam and Dr. B. R. Thomas for linguistic criticism. *Atomkommittén, Statens naturvetenskapliga forskningsråd* and *Statens tekniska forskningsråd* have provided financial support for the NMR group. The cost of The NMR and the IR apparatus has been defrayed by grants from *Knut och Alice Wallenbergs Stiftelse.*

REFERENCES

1. Hassall, C. H. *Progr. in Org. Chem.* **4** (1958) 115.
2. Nilsson, M. *Acta Chem. Scand.* **13** (1959) 750.
3. Chan, W. R. and Hassall, C. H. *J. Chem. Soc.* **1956** 3495.
4. Wilmad Glass Co., Landisville, N.J., U.S.A.
5. Reilly, C. A., McConell, H. M. and Meisenheimer, R. G. *Phys. Rev.* **98** (1955) 264.
6. Allred, A. L. and Rochow, E. G. *J. Am. Chem. Soc.* **79** (1957) 5361.
7. Glick, R. E. and Kates, D. F. *J. Phys. Chem.* **62** (1958) 1469.
8. Nef, J. U. *Ann.* **277** (1893) 59.
9. Birckenbach, L., Kellermann, K. and Stein, W. *Ber.* **65** (1932) 1071.
10. Rasmussen, R. S., Tunnicliff, D. D. and Brattain, R. R. *J. Am. Chem. Soc.* **71** (1949) 1068.
11. Schwarzenbach, G. and Lutz, K. *Helv. Chim. Acta* **23** (1940) 1147.
12. Dieckmann, W. and Stein, R. *Ber.* **37** (1904) 3370.
13. Ukita, T., Tamura, T., Matsuda, R. and Kishiwabara, E. *Japan. J. Exptl. Med.* **20** (1949) 109.
14. Reeves, L. W. *Can. J. Chem.* **35** (1957) 1351.
15. Bernstein, H. J., Pople, J. A. and Schneider, W. G. *Can. J. Chem.* **35** (1957) 65.
16. Pople, J. A. *Chem. Soc. (London) Spec. Publ. No.* **12** (1958) 211.
17. Birch, A. J. *J. Chem. Soc.* **1951** 3026.
18. Bellamy, L. J. *The Infra-red Spectra of Complex Molecules.* 2nd ed., Methuen & Co., London 1958, p. 132.
19. Hammond, G. S. in Newman, M. S. (Ed.) *Steric Effects in Organic Chemistry.* Wiley & Sons, New York 1956, p. 425.

Received May 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1395–1396

The Chemistry of the Natural Order Cupressales XXIII *

L-Arabinoic Acid from the Heartwood of *Austrocedrus chilensis* (D.Don), Florin et Boutelje

ANDERS ASSARSSON, BENGT LINDBERG and HELMUT VORBRÜGGEN

Institutionerna för träkemi och organisk kemi, Kungl. Tekniska Högskolan, Stockholm, Sweden

L-Arabinoic acid was isolated in the form of the 1,4-lactone from the heartwood of *Austrocedrus chilensis*.

A water soluble carbohydrate fraction was isolated from the heartwood of *Austrocedrus chilensis* (natural order Cupressales) during the course of an investigation by one of us (H.V.) of the terpenoid and phenolic constituents **. A paper chromatographic investigation of this fraction revealed the presence of arabinose and galactose, which are generally found in these woods, and of smaller amounts of other reducing sugars, with both high R_F-values such as rhamnose and fucose, and with low R_F-values, similar to those of di- and trisaccharides. Indications were also found of the presence of several non-reducing carbohydrates, one of which seemed to occur in fairly large amount. This component was enriched by carbon column chromatography and obtained in a pure crystalline state by chromatography on a cellulose column; m.p. 97—99°, $[\alpha]_D$—72°. It gave a positive hydroxamate test for an ester, and the IR spectrum showed a strong peak at 1 760 cm^{-1}, the carbonyl frequency characteristic of a γ-lactone. This data agreed with that reported for L-arabino-1,4-lactone [1]. The m.p. was not depressed on admixture with an authentic sample of that substance and the IR spectra of the two substances were identical.

The 1,4-lactone is in equilibrium with the free acid and the 1,5-lactone, and spots due to these substances were also observed on paper chromatograms of the carbohydrate fraction. It is obvious that all three components occur in

* Part XXI, *Tetrahedron* 4 (1958) 361.
Part XXII, *Acta Chem. Scand.* 13 (1959) 1124.

** Sponsored by U.S. Army and Development Liaison Group under contract DA-91-508-ECU-288.

the wood, but the 1,4-lactone, being the most stable, was most easily isolated in a state of purity. The 1,4-lactone was isolated in a yield of about 0.01 % but the total percentage of L-arabinoic acid present both as lactones and as free acid is probably about twice this value.

L-Arabinoic acid has not been found in nature before. It is almost certainly formed by oxidation of L-arabinose, which is present in the wood in a fairly high concentration. Some bacteria [2], especially those of the genus *Pseudomonas*, are known to oxidise L-arabinose to L-arabinoic acid in the presence of air. The possibility that the L-arabinoic acid found in the heartwood of *Austrocedrus chilensis* is not a wood constituent but is formed by bacterial oxidation cannot be excluded but seems highly improbable since this extremely durable wood contains unusually large amounts of the highly toxic α-thujaplicin [3].

EXPERIMENTAL

Melting points are corrected.

Paper chromatography was done on Whatman No. 1 filter paper, using the solvent system (v/v): Ethyl acetate:acetic acid:water, 3:1:3 (upper phase).

The milled, airdried heartwood (40 kg), was extracted with acetone in a continuous extractor for two days. On concentration the extract separated into two layers, a brown oil and a yellow aqueous phase (1.5 l). On cooling, yellow crystals (52 g) separated from the aqueous phase, m.p. 240–241°, which were identified as taxifolin. The mother liquors were concentrated to about 200 ml, treated with decolourising carbon and concentrated to a thick syrup. On crystallisation from methanol this gave L-arabinose (70 g), m.p. 158°, $[\alpha]_D$ + 103°.

The mother liquors, which still contained some phenolic material, were concentrated to dryness. The product obtained was disolved in 50 % aqueous ethanol (1 l) and half of this solution was shaken with decolourising carbon (200 g) for two hours. The carbon was filtered off and washed with the same solvent. Extraction of the carbon with boiling ethanol gave phenolic material (1.5 g) which was not further investigated. The decolourised solution was concentrated and deionised by filtration through columns of IR 120 and IR 4B resins. The acid material recovered from the resin (3.45 g) was found by chromatography to be mainly L-arabinoic acid and its lactones.

The deionised solution was concentrated to a syrup (18.4 g), adsorbed on the top of a carbon-Celite solumn and then eluted with aqueous ethanol, using gradient elution. The first fractions contained traces of cyclitols, and the next contained the majority of the reducing sugars. The fractions following that contained the component that was later found to be the arabino-1,4-lactone. They were combined (7.6 g) and rechromatographed on a cellulose column, using butanol, saturated with water as eluant. The fraction containing the 1,4-lactone crystallised on concentration and was recrystallised from propanol, m.p. 97–99°, $[\alpha]_D^{20}$ −72° (water, *c*, 2).

Authentic L-arabino-1,4-lactone was isolated from the mixture of acid and lactones by chromatography on a cellulose column as above. It melted at 97–99°, alone or in admixture with the natural product.

The authors are indebted to Professor Holger Erdtman for his interest in this work.

REFERENCES

1. Fisher, E. and Piloty, B. *Ber.* **24** (1891) 4214.
2. Lockwood, L. B. and Nelson, G. E. N. *J. Bacteriol.* **52** (1946) 581.
3. Erdtman, H. and Pelchowicz, Z. *Acta Chem. Scand.* **9** (1955) 1728.

Received May 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1397–1399

Vibrational Mean-Square Amplitude Matrices

IV. Treatment of Tetrahedral X_4 Molecules with Application to Phosphorus

S. J. CYVIN

Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The theory of mean-square amplitude matrices previously evaluated, is applied to the tetrahedral X_4 molecular model. Numerical values of six mean-square amplitude matrix elements, including two interaction terms, are given for the phosphorus molecule at two temperatures.

In this paper the previously reported theory [1] of mean-square amplitude matrices will be applied to the tetrahedral X_4 molecular model, and numerical values of mean-square amplitude quantities for the phosphorus molecule will be given.

The vibrations of the considered model (Fig. 1) are described by the six interatomic distance deviations. The notations applied for these coordinates, as well as a set of symmetry coordinates, are given by Fig. 1 and the adherent legend. For the F and G matrices in Wilson's [2] notation, that will not be specified here, see Refs.[3,4] The symmetrized mean-square amplitude matrix in the case considered is specified in the following.

Symmetry species A_1: $[\sigma + 4\sigma' + \sigma'']$
Symmetry species E: $[\sigma - 2\sigma' + \sigma'']$
Symmetry species F_2: $[\sigma - \sigma'']$

The symbols here appearing are defined by:

$$\sigma = \overline{r_1^2}, \qquad \sigma' = \overline{r_1 r_2}, \qquad \sigma'' = \overline{r_1 r_4} \tag{1}$$

It should be noticed that σ' and σ'' are the so-called interaction mean-square amplitudes, corresponding to an adjacent and an opposite pair of bonds, respectively. The reported secular equations [1] may in the present case be

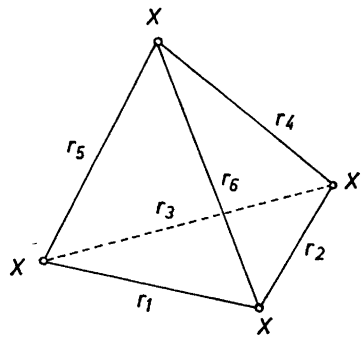


Fig. 1. Notation used for the interatomic distance deviations. Symmetry coordinates:

Symm.species A_1: $S_1 = (1/\sqrt{6})(r_1 + r_2 + r_3 + r_4 + r_5 + r_6)$

Symm.species E: $S_{2a} = (1/2\sqrt{3})(2r_1 - r_2 - r_3 + 2r_4 - r_5 - r_6)$; $S_{2b} = (1/2)(r_2 - r_3 + r_5 - r_6)$

Symm.species F_2: $S_{3a} = (1/\sqrt{2})(r_1 - r_4)$; $S_{3b} = (1/\sqrt{2})(r_2 - r_5)$; $S_{3c} = (1/\sqrt{2})(r_3 - r_6)$

factorized into linear equations, and the following result is found for the symmetrized mean-square amplitude elements.

$$\sigma + 4\sigma' + \sigma'' = 4\Delta_1\mu \quad (2)$$
$$\sigma - 2\sigma' + \sigma'' = \Delta_2\mu \quad (3)$$
$$\sigma - \sigma'' = 2\Delta_3\mu \quad (4)$$

These equations yield the following expressions for the quantities of eqn (1).

$$\sigma = (2\Delta_1 + \Delta_2 + 3\Delta_3)\mu \quad (5)$$
$$\sigma' = (4\Delta_1 - \Delta_2)\mu \quad (6)$$
$$\sigma'' = (2\Delta_1 + \Delta_2 - 3\Delta_3)\mu \quad (7)$$

In eqns (2)—(7) μ denotes the inverse atomic mass. The values of Δ_k ($k = 1, 2, 3$) are connected with the vibrational normal frequencies through the relation

$$\Delta_k = (h/8\pi^2\nu_k)\coth(h\nu_k/2kT);\ k = 1,\ 2,\ 3 \quad (8)$$

Here h is Planck's constant, k is Boltzmann's constant, and T the absolute temperature. ν_1, ν_2 and ν_3 are the normal frequencies belonging to the symmetry species A_1, E and F_2, respectively.

The equilibrium structure of the phosphorus molecule is definitely proved to be the tetrahedral one [5,6]. The evaluation of a complete set of harmonic force constants of this molecule has been given by Pistorius [3], who also made a semiquantitative anharmonicity correction of the observed fundamentals. His corrected values, *viz.* 604, 381 and 506 cm^{-1}, were inserted for ν_1, ν_2 and ν_3, respectively, in eqns (2)—(7). Table 1 shows the numerical values obtained

Table 1. Mean-square amplitude quantities in phosphorus.

Species	Mean-square amplitude (Å²) $T = 0$	298 °K
A_1	0.0036042	0.0040174
E	0.0014284	0.0019687
F_2	0.0021511	0.0025611
Symbol	**Mean-square amplitude (Å²) $T = 0$**	**298 °K**
σ	0.0021524	0.0026064
σ'	0.00036263	0.00034145
σ''	0.00000128	0.00004526

for the six mean-square amplitude quantities at the temperatures $T = 0$ and 298 °K. The mean amplitude of vibration for the interatomic distance is given by

$$u = \sqrt{\sigma} \tag{9}$$

in the present notation. For P_4 at $T = 0$ and 298 °K, Bakken [7] has reported values of u equal to 0.0478 Å and 0.0533 Å, respectively. These values [7,8] were based on vibrational frequencies without anharmonicty corrections, quoted by Herzberg [5]. From the present calculations, the corresponding numerical u-values are 0.0464 Å and 0.0511 Å, respectively.

Added in proof: In connection with the paper of Pistorius [3] it should be noted that Slater was the first one to give a discussion of the most general harmonic force field in phosphorus, including a set of calculated force constants; see: Slater, N.B., *Trans. Faraday Soc.* **50** (1954) 207.

REFERENCES

1. Cyvin, S. J. *Spectrochim. Acta* (Paper I of this series). *In press.*
2. Wilson, E. B. Jr. *J. Chem. Phys.* **7** (1939) 1047; **9** (1941) 76; Wilson, E. B. Jr., Decius, J. C. and Cross, P. C. *Molecular Vibrations*, McGraw-Hill Book Co., Inc., N.Y. 1955.
3. Pistorius, C. W. F. T. *J. Chem. Phys.* **29** (1958) 1421.
4. Cyvin, S. J. *Spectrochim. Acta* (1959). 341.
5. Herzberg, G. *Infrared and Raman Spectra of Polyatomic Molecules*, D. van Nostrand Co., Inc., N.Y. 1945.
6. Gutowsky, H. S. and Hoffman, C. J. *J. Am. Chem. Soc.* **72** (1950) 5751; Bernstein, H. J. and Powling, J. *J. Chem. Phys.* **18** (1950) 685.
7. Bakken, J. *Acta Chem. Scand.* **12** (1958) 594.
8. Bakken, J. *Teoretiske studier over molekylvibrasjoner*, Thesis, Trondheim, 1957.

Received May 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1400–1406

Vibrational Mean-Square Amplitude Matrices

V. Treatment of Bent Symmetrical XY_2 Molecules with Application to Nitrogen Dioxide

S. J. CYVIN

Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The theory of mean-square amplitude matrices is applied to the bent symmetrical XY_2 molecular model. Numerical computations for nitrogen dioxide are reported, including the calculation of ten mean-square amplitude quantities for $^{14}NO_2$ and for $^{15}NO_2$, both at the temperatures $T = 0$ and 298 °K. An application of the isotope rule for mean-square amplitude matrix elements is also given.

Spectroscopical studies of bent symmetrical XY_2 molecules have been the subject of many publications (see, *e.g.* Refs.[1–6]). In the present article, some of the theoretical results for this type of molecules will be summarized and supplied with the study of the mean-square amplitude matrix. Numerical calculations for nitrogen dioxide will be reported.

THEORETICAL TREATMENT

The harmonic vibrations of the considered molecular model are described by three internal coordinates, which may be chosen as a set of valence force coordinates (see Fig. 1). The following symmetry coordinates have been formed as normalized linear combinations of the valence force coordinates.

Symmetry species A_1: $\begin{cases} S_1 = (1/\sqrt{2})(r_1 + r_2) \\ S_2 = R\alpha \end{cases}$

Symmetry species B_1: $S_3 = (1/\sqrt{2})(r_1 - r_2)$

For the notations applied, see Fig. 1. It should be noticed that the angle displacement coordinate α is multiplied by R, designating the equilibrium X—Y distance.

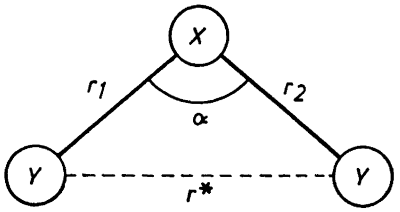


Fig. 1. Notation used for the bent symmetrical XY_2 molecular model. The symbols denote deviations from the equilibrium values.

The potential energy matrix in terms of the given symmetry coordinates may be written

$$F = \begin{bmatrix} F_1 & F_{12} & 0 \\ & F_2 & 0 \\ \text{(symm.)} & & F_3 \end{bmatrix}$$

where

$$F_1 = k + k', \qquad F_2 = f, \qquad F_{12} = \sqrt{2}\, g, \qquad F_3 = k - k' \tag{1}$$

The potential energy function (V) in terms of the valence force coordinates will be written down, in order to explain the symbols used in eqn (1).

$$V = \tfrac{1}{2}[k(r_1^2 + r_2^2) + 2k' r_1 r_2 + fR^2\alpha^2 + 2Rg(r_1 + r_2)\alpha] \tag{2}$$

The inverse kinetic energy matrix (G matrix) will not be given here. References are made to [7–10].

The symmetrized mean-square amplitude matrix [11,12] is given by

$$\Sigma = \begin{bmatrix} \Sigma_1 & \Sigma_{12} & 0 \\ & \Sigma_2 & 0 \\ \text{(symm.)} & & \Sigma_3 \end{bmatrix}$$

where

$$\Sigma_1 = \sigma + \sigma', \qquad \Sigma_2 = \tau, \qquad \Sigma_{12} = \sqrt{2}\,\varrho, \qquad \Sigma_3 = \sigma - \sigma' \tag{3}$$

The symbols introduced here are defind by:

$$\sigma = \overline{r_1^2} = \overline{r_2^2}, \qquad \sigma' = \overline{r_1 r_2}, \qquad \tau = R^2\,\overline{\alpha^2}$$
$$\varrho = R\,\overline{r_1\alpha} = R\,\overline{r_2\alpha} \tag{4}$$

The following equations exist for the vibrational normal frequencies ($\lambda_k = 4\pi^2\nu_k^2$).

$$\lambda_1 + \lambda_2 = F_1(2\mu_X\cos^2 A + \mu_Y) + 2F_2(2\mu_X\sin^2 A + \mu_Y)$$
$$2\sqrt{2}\, F_{12}\mu_X\sin 2A \tag{5}$$

$$\lambda_1\lambda_2 = 2(F_1F_2 - F_{12}^2)(2\mu_X + \mu_Y)\mu_Y \tag{6}$$

$$\lambda_3 = F_3(2\mu_X\sin^2 A + \mu_Y) \tag{7}$$

Here μ_X and μ_Y denote the inverse masses of the X and Y atoms, respectively. The equilibrium value of the inter-bond angle is denoted by $2A$. Another set of equations, containing the mean-square amplitude quantities instead of the force constants, has been evaluated as given below. The values of Δ are connected with the normal frequencies by $\Delta_k = (h/8\pi^2\nu_k)\coth(h\nu_k/2kT)$, where h is Plack's constant, k Boltzmann's constant, and T the absolute temperature.

$$\begin{aligned}\Delta_1 + \Delta_2 = [&\Sigma_1(2\mu_X\sin^2A + \mu_Y) + \tfrac{1}{2}\Sigma_2(2\mu_X\cos^2A + \mu_Y) \\ &+ \sqrt{2}\Sigma_{12}\mu_X\sin 2A](2\mu_X + \mu_Y)^{-1}\mu_Y^{-1}\end{aligned} \tag{8}$$

$$\Delta_1\Delta_2 = \tfrac{1}{2}(\Sigma_1\Sigma_2 - \Sigma_{12}^2)(2\mu_X + \mu_Y)^{-1}\mu_Y^{-1} \tag{9}$$

$$\Delta_3 = \Sigma_3(2\mu_X\sin^2A + \mu_Y)^{-1} \tag{10}$$

Additional mean-square amplitude quantities are to be taken into acconut when the non-bonded Y...Y distance is considered. If the corresponding distance deviations are identified by the symbol r^*, the following mean-square amplitude quantities are defined.

$$\tau^* = \overline{(r^*)^2}, \qquad \varrho^* = \overline{r_1 r^*} = \overline{r_2 r^*} \tag{11}$$

These quantities may be expressed in terms of those given by eqn (4) with the following result, deduced from geometrical considerations.

$$\begin{aligned}\tau^* &= 2(\sigma + \sigma')\sin^2A + \tau\cos^2A + 2\varrho\sin 2A \\ \varrho^* &= (\sigma + \sigma')\sin A + \varrho\cos A\end{aligned} \tag{12}$$

NUMERICAL COMPUTATIONS

Normal frequencies. The normal frequencies [13] applied in the present calculations are shown in Table 1. It should be pointed out that the three frequencies of a specific bent symmetrical XY_2 molecule, say $^{14}NO_2$, are not sufficient for the complete determination of the harmonic vibrational constants.

Table 1. Experimental vibrational frequencies of nitrogen dioxide molecules.

Species	No.	Normal frequencies in cm^{-1} units [a] $^{14}NO_2$	$^{15}NO_2$
	1	1 357.8 [b]	1 343.3 [b]
A_1	2	756.8	747.1
B_1	3	1 665.5	1 628.6

[a] From Ref. [13]; all values are corrected for anharmonicity. The frequencies of $^{15}NO_2$ were revised to fit accurately the product rule with the physical constants here applied, the discrepancies from the values in the cited paper being insignificant.

[b] Based on estimated fundamentals from vibrational analysis.

Table 2. Calculated force constants for nitrogen dioxide.

Symbol	mdyne/Å	Symbol	mdyne/Å
F_1	12.8754	k	10.8840
F_2	1.1290	k'	1.9914
F_{12}	0.51315	f	1.1290
F_3	8.8927	g	0.36285

The value of $2A = 134° 15'$ for the valence angle, quoted in Ref. [13], has also been adopted in the present work.

Force constants. The normal frequency 1 665.5 cm^{-1} (see Table 1) gives the value $F_3 = 8.8927$ mdyne/Å without ambiguity for the force constant of the species B_1. The force constants of the species A_1, *viz.* F_1, F_2 and F_{12}, are going to be discussed in the following. To obtain real values of the force constants consistent with a set of normal frequencies, only limited ranges of the constants are allowed. With the normal frequencies of $^{14}NO_2$ given in Table 1, it is found from eqns (5), (6) for the interaction constant F_{12} in mdyne/Å

$$-0.55788 \leq F_{12} \leq 4.5686$$

All the possible values of the force constants here considered have been represented graphically by an ellipse, this being a usual procedure (*see, e.g.* Refs [3,5,6,14-18]). The stippled curve (see Fig. 2) represents one of the alternative solutions for the force constants, arising from the quadratic secular equation.

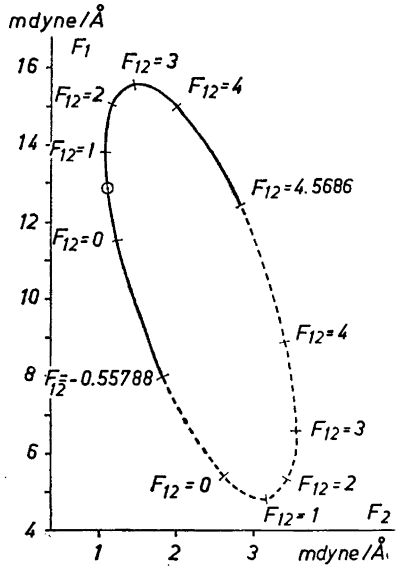


Fig. 2. Values of force constants for NO_2, consistent with $\omega_1 = 1357.8$ and $\omega_2 = 756.8$ cm^{-1}. ⊙ indicates the best calculated values (see the text).

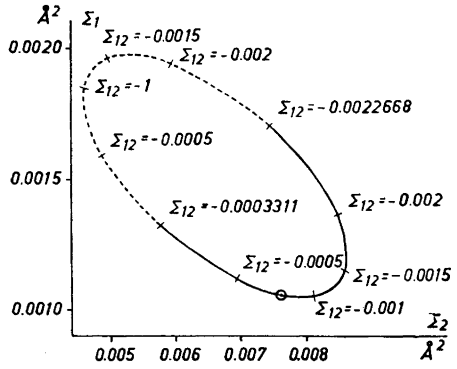


Fig. 3. Values of mean-square amplitude matrix elements at 298 °K for $^{14}NO_2$, consistent with $\omega_1 = 1357.8$ and $\omega_3 = 756.8$ cm^{-1}. ⊙ indicates the best calculated values (see the text).

With the additional knowledge of the normal frequencies of the $^{15}NO_2$ molecule, it is possible to calculate definite values of the force constants. The result of these calculations is given in Table 2, and the values of F_1 and F_2 in particular, are indicated on the curve of Fig. 2.

Mean-square amplitude quantities. An analogous procedure to that of the preceding paragraph is applied to the mean-square amplitude quantities of $^{14}NO_2$ at 298 °K. Eqn (10) gives the value 0.0018612 Å² for Σ_3. From eqns (8), (9), the remaining three mean-square amplitude matrix elements have been calculated and represented graphically in Fig. 3. The range for the interaction mean-quare amplitude in Å² is found to be

$$-0.0022668 \leq \Sigma_{12} \leq -0.0003311$$

The mean-square amplitudes of vibration of the two types of interatomic distances, *viz.* σ and τ^* in the present notation, are the most important ones.

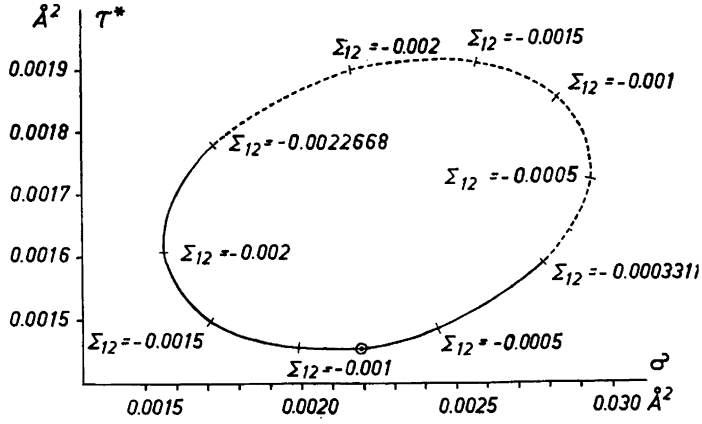


Fig. 4. Values of mean-square amplitudes of vibration at 298 °K for $^{14}NO_2$, consistent with $\omega_1 = 1357.8$ and $\omega_3 = 756.8$ cm^{-1}. ⊙ indicates the best calculated values (see the text).

Table 3. Calculated mean-square amplitude quantities for nitrogen dioxide molecules.

Symbol	Mean-square amplitudes in Å² units for $^{14}NO_2$ $T = 0$	298 °K
Σ_1	0.0010522	0.0010561
Σ_2	0.0072833	0.0076139
Σ_{12}	−0.0007502	−0.0007355
Σ_3	0.0018600	0.0018612
σ	0.0014561	0.0014586
σ'	−0.0004039	−0.0004025
τ	0.0072833	0.0076139
ϱ	−0.0005305	−0.0005201
τ^*	0.0021060	0.0021984
ϱ^*	0.0007632	0.0007709

Symbol	Mean-square amplitudes in Å² units for $^{15}NO_2$ $T = 0$	298 °K
Σ_1	0.0010438	0.0010477
Σ_2	0.0071348	0.0074809
Σ_{12}	−0.0007160	−0.0007026
Σ_3	0.0018187	0.0018201
σ	0.0014312	0.0014339
σ'	−0.0003875	−0.0003862
τ	0.0071348	0.0074809
ϱ	−0.0005063	−0.0004968
τ^*	0.0021249	0.0021975
ϱ^*	0.0007649	0.0007722

These quantities are observable from electron-diffraction (see Ref.[19] and references cited therein), the measurements for nitrogen dioxide being in progress [20]. In the present calculations the quantities in question are obtainable by means of eqns (3) and (12). Fig. 4 shows a graphical representation of all the possible real values of of σ and τ^*.

The best calculated values of mean-square amplitudes were obtained by including the normal frequencies for $^{15}NO_2$. The ten mean-square amplitude quantities defined in this paper were calculated for both $^{14}NO_2$ and $^{15}NO_2$ at the temperatures $T = 0$ and 298 °K. The numerical results are given in Table 3, and some of them indicated on Figs. 3 and 4.

Isotope rules. The normal frequencies given in Table 1 are adjusted to fit accurately the product rule. In consequence, also the isotope rules for mean-square amplitude quantities [11,12] will be fulfilled. In accordance with the theory of the cited papers, one has in the present case

$$\frac{\nu_1\nu_2}{\nu_1^*\nu_2^*} = \frac{\Sigma_1\Sigma_2-\Sigma_{12}^2}{\Sigma_1^*\Sigma_2^*-(\Sigma_{12}^*)^2} = \sqrt{\frac{(2\mu_X+\mu_Y)\mu_Y}{(2\mu_X^*+\mu_Y^*)\mu_Y^*}} \tag{13}$$

$$\frac{\nu_3}{\nu_3^*} = \frac{\Sigma_3}{\Sigma_3^*} = \sqrt{\frac{2\mu_X\sin^2A+\mu_Y}{2\mu_X^*\sin^2A+\mu_Y^*}} \tag{14}$$

Table 4. Mean amplitudes of vibration in nitrogen dioxide molecules.

Molecule	Distance	Mean amplitude of vibration (Å) $T = 0$	298 °K
$^{14}NO_2$	N−O	0.0382	0.0382
	O...O	0.0459	0.0469
$^{15}NO_2$	N−O	0.0378	0.0379
	O...O	0.0461	0.0469

Here the mean-square amplitude matrix elements are referred to the absolute zero. By inserting the appropriate atomic masses according to $\mu_X = 1/14.00754$, $\mu_X^* = 1/15.00489$ and $\mu_Y = \mu_Y^* = 1/16.00000$, and with the value $2A = 134°15'$, the numerical results 1.0239 and 1.0227 are obtained for the respective ratios of eqns (13) and (14). If the interaction mean-square amplitudes are neglected as an approximation, eqn (13) reduces to

$$\nu_1\nu_2/\nu_1^*\nu_2^* \approx \Sigma_1\Sigma_2/\Sigma_1^*\Sigma_2^* = 1.0290 \qquad (15)$$

Mean amplitudes of vibration. In the present notation, the square-roots of σ and τ^* represent the mean amplitudes of vibration for the two types of interatomic distances, such quantities being suitable as parameters in molecular structure studies [19]. In Table 4 the mean amplitudes of vibration from the present calculations for the nitrogen dioxide molecules are given.

REFERENCES

1. Darling, B. T. and Dennison, D. M. *Phys. Rev.* **57** (1940) 128.
2. Shaffer, W. H. and Newton, R. R. *J. Chem. Phys.* **10** (1942) 405.
3. Glockler, G. and Tung, J. *J. Chem. Phys.* **13** (1945) 388.
4. Heath, D. F. and Linnett, J. W. *Trans. Faraday Soc.* **44** (1948) 556.
5. Torkington, P. *J. Chem. Phys.* **17** (1949) 357.
6. Smith, S. and Linnett, J. W. *Trans. Faraday Soc.* **52** (1956) 891.
7. Meister, A. G. and Cleveland, F. F. *Am. J. Physics* **14** (1946) 13.
8. Decius, J. C. *J. Chem. Phys.* **16** (1948) 1025.
9. Wilson, E. B. Jr., Decius, J. C. and Cross, P. C. *Molecular Vibrations*, Mc-Graw-Hill Book Co., Inc., N.Y. 1955.
10. Meal, J. H. and Polo, S. R. *J. Chem. Phys.* **24** (1956) 1126.
11. Cyvin, S. J. *Acta Chem. Scand.* **13** (1959). *In press.*
12. Cyvin, S. J. *Spectrochim. Acta. In press.*
13. Arakawa, E. T. and Nielsen, A. H. *J. Molecular Spectroscopy* **2** (1958) 413.
14. Glockler, G. *Revs. Modern Phys.* **15** (1943) 111.
15. Torkington, P. *Proc. Phys. Soc. London* **A64** (1951) 32.
16. Duchesne, J. and Burnelle, L. *J. Chem. Phys.* **19** (1951) 1191.
17. Duchesne, J. and Nielsen, A. H. *J. Chem. Phys.* **20** (1952) 1968.
18. Linnett, J. W. and Heath, D. F. *Trans. Faraday Soc.* **48** (1952) 592.
19. Cyvin, S. J. *Kgl. Norske Videnskab. Selskabs Skrifter. In press.*
20. Hedberg, K. *Private communication.*

Received May 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1407–1421

Synthesis of Phosphopeptides

II.* O-Phosphorylated Dipeptides of L-Serine

GEORG FÖLSCH

Department of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden

Diphenylphosphoryl and dibenzylphosphoryl chlorides have been used for phosphorylation of six N-carbobenzoxy dipeptide benzyl esters of L-serine with amino acid sequences shown to occur in phosphoproteins. Catalytic hydrogenolysis of the dibenzylphosphoryl derivatives yields pure phosphopeptides, whereas the products obtained from the diphenyl analogs frequently contain partially hydrogenolysed derivatives.

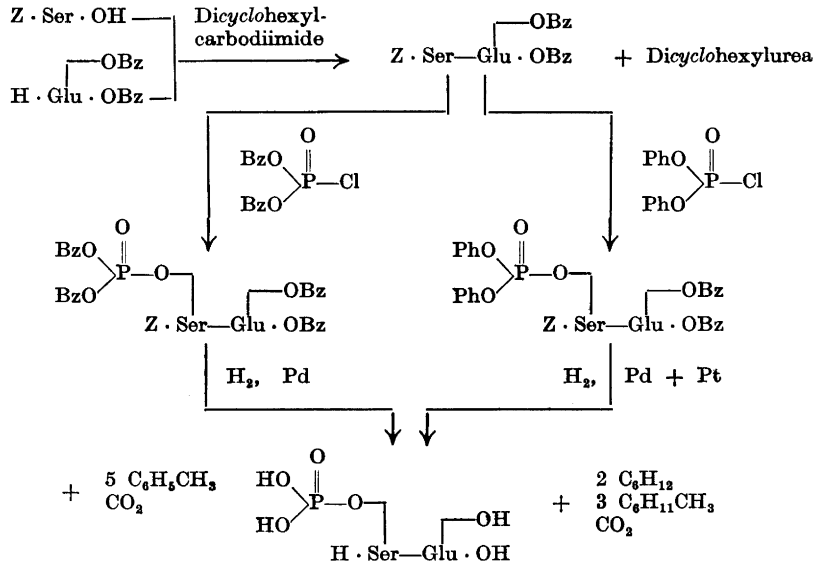
Phosphoproteins and phosphopeptide fractions obtained from phosphoproteins usually have a high content of the two dicarboxylic acids glutamic acid and aspartic acid [1-5], and several peptides have been isolated after degradation of such proteins in which O-phosphorylated serine residues were bound by peptide linkages to these acids. The peptide O-phosphoryl-L-seryl-L-glutamic acid was isolated from casein as early as 1933 [6-7]. Also the reverse sequence, Glu-SerP, seems to be present in casein as well as SerP-Asp apart from other sequences, such as Leu-SerP and SerP-SerP [8-9]. The last-mentioned sequence seems to build up a large portion of phosvitin **, whereas, in pepsin, the lone phosphorylserine residue, again, is bound to glutamic acid in the sequence Thr-SerP-Glu [10]. Finally, after acid hydrolysis, the two phosphorus atoms of ovalbumin are found in the fragments Glu-SerP-Ala and Asp-SerP-Glu, of which only the former is dephosphorylated by prostatic phosphatase [10].

For a more detailed study of the biological and chemical properties of phosphorylated peptides, *e. g.* their behaviour towards different phosphatases and proteolytic enzymes or their metal binding properties [11-17], synthetic preparation of these compounds seemed to be of interest. Synthetic preparation also permits considerable structural variations. In previous papers [17-21], a

* Paper I, see Ref.[20]. Abbreviations and symbols used as previously. The symbol SerP is used for an O-phosphorylserine residue, $-NH \cdot CH[CH_2OPO(OH)_2]CO-$; Z for carbobenzoxy, $C_6H_5CH_2OCO-$; Bz for benzyl, $C_6H_5CH_2$- and Ph for phenyl C_6H_5-groups (*cf.* also Goodman, M. and Kenner, G. W. *Advances in Protein Chem.* **12** (1957) 509–518).

** Mellander, O. and Strid, L. of this laboratory; *Personal communication.*

general route to O-phosphorylated serine peptides, applicable also to threonine peptides [22] has been reported. The synthesis of the L, D and DL forms of O-phosphorylserine [19] as well as of di and tri-peptides of this amino acid and glycine [18,20] were described. In the route followed, the peptide sequence in question is first synthesized by resorting to coupling methods which allow the use of unprotected serine hydroxyl groups. Protecting groups for the amino and carboxyl functions (N-carbobenzoxy groups [23]; benzyl esterification [24]) are selected, which are stable under the conditions of phosphorylation but which afterwards are eliminated by mild treatment (catalytic hydrogenolysis). The compounds so obtained, *i. e.* the N-carbobenzoxypeptide benzyl esters, are then phosphorylated with dibenzylphosphoryl [25] or diphenylphosphoryl [26,27] chloride, and the resulting triesters of phosphoric acid finally catalytically hydrogenolysed into free phosphopeptides, and into the volatile degradation products of the protecting groups, benzene (or *cyclo*hexane in platinum catalysed hydrogenation), toluene (methyl*cyclo*hexane) and carbon dioxide:



Such procedures are avoided as much as possible, that are suspected of causing racemization, hydrolysis of phosphate ester or peptide bonds, and acyl migrations [28-29] or β-elimination [22], *e. g.* treatment with strong acids or alkalies. The present paper describes the use of this procedure in the synthesis of six dipeptides of O-phosphorylated L-serine, combined with L-glutamic acid, L-aspartic acid and L-leucine.

DIPEPTIDE DERIVATIVES OF L-SERINE

The N-*carbobenzoxy dipeptide benzyl esters* required for the phosphorylation were prepared from N-carbobenzoxyamino acids [23] and amino acid benzyl

esters by the application of the carbodiimide procedure [30-31], which, besides its simplicity, allows the use of unprotected serine hydroxyl groups. The yields obtained by this procedure are frequently excellent, providing acylurea formation [30-32] can be avoided. For this purpose a mixture of tetrahydrofuran and acetonitril as recommended by Zahn and Diehl [32], was used as solvent in couplings with N-carbobenzoxy-L-serine, and in no case could a formation of N(N-carbobenzoxyseryl)-N,N'-di*cyclo*hexylurea [32] be observed. Couplings with more soluble carbobenzoxy derivatives were made in a methylene chloride solution. Although serine is considered to be one of the most easily racemizing α-amino acids [33], no signs of racemization were observed in the coupling reactions, a fact which supports the general view that N-acylamino acids, in contrast to N-acyl peptides, produce fully optically pure compounds when coupled by the carbodiimide or mixed anhydride procedure *. It should, however, be kept in mind, that minor quantities of diastereoisomers possibly formed may be eliminated in the isolation and purification of the reaction product, as the solubility of diastereoisomeric peptide derivatives is often sufficiently different to allow their separation (*cf.* the following paper [21]). The optical purity of two of the protected peptide derivatives was clearly demonstrated by hydrogenolysis forming the free peptides L-seryl-L-glutamic acid and L-seryl-L-serine, which had already been prepared by the *azide procedure* [38-39]. The additional four dipeptides of L-serine, obtained in the present work, had not been previously reported. The peptides and their protected derivatives are listed in Tables 2 and 3.

PHOSPHORYLATION OF PROTECTED L-SERINE PEPTIDES AND HYDROGENOLYSIS OF THE TRIESTERS OBTAINED

Among the various methods of phosphorylation reported in the literature, only those could be considered at the present time, in which a monofunctional phosphoryl chloride [25-27, 40-42] or a suitably protected pyrophosphate [43-45] was used for the phosphorylation of the free hydroxyl groups. Diphenylphosphoryl and dibenzylphosphoryl chlorides were used exclusively for the experiments, owing to their availability and simplicity in operation.

A. Peptide derivatives of diphenylphosphoric acid

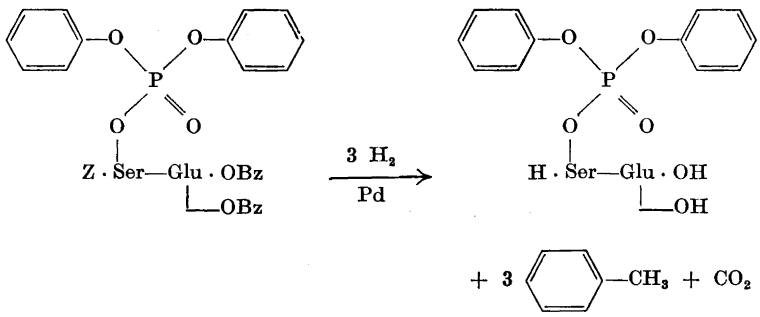
Diphenylphosphoryl chloride reacts readily with primary (serine) and secondary (threonine) alcohols, yielding 90 % or more of O-diphenylphosphoryl derivatives [17, 19-22]. For the conversion of such triesters into free O-phosphorylated amino acids and peptides, two different routes have been reported. Riley *et al.*[22] have used alkaline dephenylation with simultaneous saponification of carboxylic ester groups, the N-carbobenzoxy group having first been removed

* Partial racemization has been observed when N-acylated peptides have been coupled by these methods [34-36]. On the other hand, two tetrapeptide derivatives were recently condensed by carbodiimide, yielding an apparently optically pure octapeptide derivative [37].

with hydrogen bromide in glacial acetic acid. Keeping the well established sensitivity of serine peptide derivatives to alkali in mind * the low yields of the two phosphopeptides obtained by this process, H·SerP-Gly·OH and H·SerP-Glu·OH and the low melting point of the former and optical rotation of the latter as compared with the compounds prepared by hydrogenolysis may be explained. Furthermore, this route can be used for *N-terminal* O-phosphorylserine peptides only, as a free α-amino group at the phosphorylated serine residue is necessary to avoid the β-elimination reaction in alkaline solution [22].

A second method for the removal of the phenyl, benzyl and N-carbobenzoxy groups consists in applying catalytic hydrogenolysis. Although exceptionally mild conditions can be used in such a case, this route shows a disadvantage in the difficulty by which complete hydrogenolytic removal of the phenyl groups is obtained [17, 19–21].

The hydrogenolysis of diphenylphosphorylated derivatives of amino acids and peptides is usually performed in two stages, first using a palladium catalyst and then a platinum catalyst [19,49]. It has been stated [50], that only benzyl groups are eliminated by *palladium-catalyzed* hydrogenolysis:

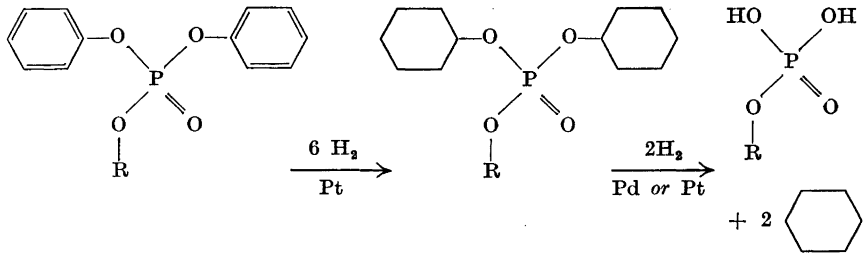


Mostert *et al.* [50] suggest that the claimed inertness of phenylic esters to H_2/Pd/C depends on the fact that the phenyl ring has to be converted to *cyclo*hexyl derivative prior to elimination, and that only platinum ** catalyzes this ring hydrogenation. (see p. 1411).

Our work does not confirm the *absolute* selectivity of the palladium (on charcoal) catalyst, but the cleavage of phenyl groups seems to be very slow, as indicated, for example, in the following experiment. Palladium catalyzed hydrogenolysis of O-diphenylphosphoryl-N-carbobenzoxy-DL-serine benzyl ester [19] in ethanol-water solution was followed by measuring the hydrogen

* Saponification of N-carbobenzoxypeptide esters containing serine frequently produces low yields of carbobenzoxypeptide owing to side reactions [46-47]. *Selective* racemization of the serine residue in N-carbobenzoxy-L-serylglycyl-L-alanine ethyl ester has been produced by treatment with triethylamine [48].

** The palladium catalyst of Kuhn and Haas, which catalyzes hydrogenolytic cleavage of N-aryl groups has been shown to catalyze ring hydrogenation of several aromatic compounds [51].



uptake and by paper chromatographic analysis, the results of which are shown in Table 1.

Table 1.

Reaction time in min	Hydrogen consumption ml (moles)	Derivatives of serine identified by paper chromatography		
		Diphenyl-phosphoryl	Monophenyl-phosphoryl	O-phosphoryl
10	200 (1.8)	++++	0	0
20	235 (2.05)	++++	Traces	0
60	245 (2.1)	+++	+	0
180	250 (2.2)	+++	++	Traces

From the final product 56 % of recrystallized O-diphenylphosphoryl-DL-serine was isolated. The appearance of monophenyl derivative after prolonged reaction time is very probably a result of hydrogenolytic cleavage and not of hydrolysis *, since diphenylphosphorylserine is comparatively stable in neutral solution. In alkaline solution **, however, phosphate is readily released (Fig. 1).

Results obtained by palladium catalyzed hydrogenolysis of peptide derivatives are very similar although hydrolysis forming unphosphorylated peptide and free amino acids thereby seems to occur to a somewhat greater extent as evidenced by paper chromatography.

Platinum catalysts have been successfully used for hydrogenolytic cleavage of numerous O-diphenylphosphorylated sugar derivatives. Complete removal of the phenyl groups of O-diphenylphosphorylated peptide derivatives, however, is very difficult ***. Appreciable amounts of O-monophenylphosph-

* Bevan *et al.*[52] obtained *O-monophenylphosphoryl* derivatives in similar experiments either with a palladium or platinum catalyst.

** Zervas and Katsoyannis [53] converted N-diphenylphosphorylated amino acid derivatives to N-monophenylphosphorylated compounds by treatment with barium hydroxide. Similar results have been obtained by Moffatt and Khorana [45].

*** Moffatt and Khorana [45] observed a similar difficulty in the hydrogenolysis of di-*p*-nitrophenylphosphorylated derivatives, the addition of a strong acid, such as hydrochloric acid, being necessary to eliminate the aryl group from the mono-*p-amino*-phenyl-phosphoryl derivatives initially formed.

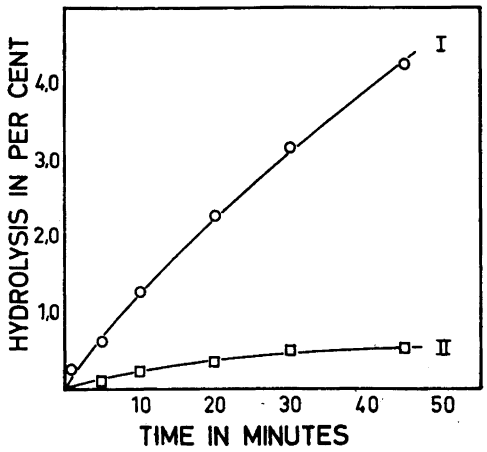


Fig. 1. Release of phosphoric acid from 0.01 M solutions of O-diphenylphosphoryl-DL-serine (I) and O-monophenylphosphoryl-DL-serine (II) at pH 9.5 (0.1 M ethanolamine — hydrochloric acid buffer) and 25°. Phosphorus determined according to Beerenblum and Chain, *Biochem. J.* **32** (1958) 295.

orylated peptide remain unaffected after hydrogenolysis with palladium on charcoal as catalyst, removal of this catalyst together with possibly occurring catalyst poisons and the repeated hydrogenolysis with platinum oxide as catalyst, regardless if acetic acid, ethanol-water or ethanol-water-hydrochloric acid is used as the solvent. This incomplete hydrogenolysis cannot be the result of insolubility [52], since several monophenylphosphorylated derivatives, *e.g.* those of serylglutamic and serylaspartic acid, are easily dissolved by the solvents used. Furthermore, isolated and purified monophenylphosphorylated derivatives are rapidly and completely hydrogenolysed to form free phosphopeptides in either of these solvents.

B. Derivatives of dibenzylphosphoric acid

In contrast to the diphenylphosphates, *dibenzylphosphorylated* derivatives [54] of peptides are rapidly and completely hydrogenolysed to form free phosphopeptides in a single stage, using a palladium catalyst. Although it has been stated to the contrary [55], dibenzylphosphoryl chloride does react with serine hydroxyl groups [18]. By careful working at low temperature the triester yield will be as high as 75 %. Some of the triesters have been obtained in a crystalline state (Table 5). They can rapidly be hydrogenolysed with the use of palladium on charcoal as catalyst, 1/2—2 h being sufficient. As a consequence of the rapid hydrogenolysis, very slight hydrolysis, if any, takes place. The yield of purified phosphopeptide is about 80 %, which corresponds to an overall yield of about 40 %, calculated from L-serine.

PURIFICATION AND PROPERTIES OF O-PHOSPHORYLATED PEPTIDES

The only by-products observed when preparing phosphopeptides *via* hydrogenolysis of dibenzylphosphorylated derivatives have been traces of unphosphorylated peptides and amino acids. By recrystallization, or by

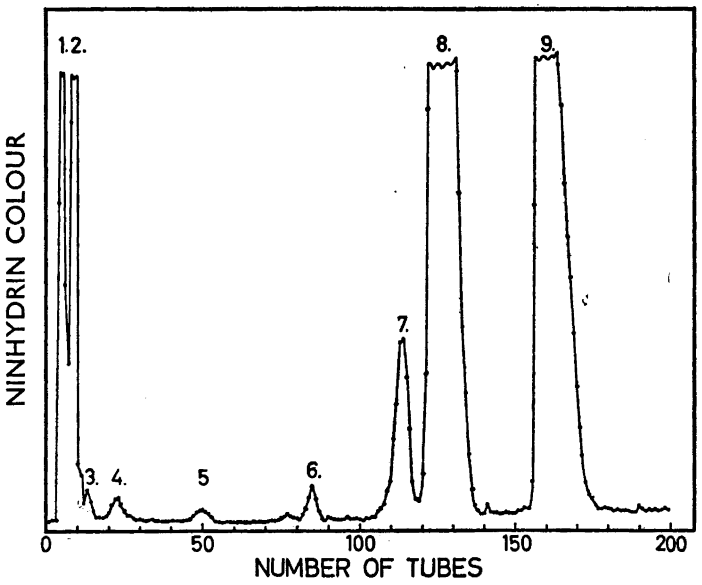


Fig. 2. Anion exchange chromatographic separation of the products obtained by platinum catalyzed hydrogenolysis of O-diphenylphosphoryl-N-carbobenzoxy-L-seryl-L-glutamic acid dibenzyl ester, as indicated by quantitative ninhydrin reaction. The two main products are: (8)O-phosphoryl-L-seryl-L-glutamic acid and (9) its monophenyl derivative. Fractions (5) and (6) are probably glutamic acid and O-phosphorylserine. Fraction 7 has not been identified.

treatment in water solution with a cation exchange resin (Amberlite IR 120 or Dowex 50, H^+-form), followed by evaporation and recrystallization (or precipitation), the phosphopeptides are easily obtained in a chromatographically and analytically pure state.

The reaction products obtained after hydrogenolysis of O-diphenylphosphorylated derivatives contain variable amounts of amino acids, unphosphorylated and monophenylphosphorylated peptides in addition to the desired phosphopeptides (Fig. 2). The latter have in several cases been successfully purified by fractional crystallization, with or without additional use of cation exchange resin [17, 19-20]. Several O-phosphorylated peptides, especially those of the more acidic type, containing glutamic and aspartic acid, however, are hygroscopic compounds and therefore difficult to crystallize. Separation of the reaction products is then most successfully accomplished by chromatographic procedures. Partition chromatography on cellulose has been applied to the reaction products obtained by hydrogenolysis of O-diphenylphosphoryl-N-carbobenzoxy-DL-serylglycine benzyl ester, using *n*-butanol-acetic acid-water as an eluent *. Another process [57], using anion exchange resin, has also been successfully used in several cases in this laboratory, (*cf.* Fig. 2).

* The author, unpublished experiments. Jones and Lipkin [56] have used acetone-acetic acid-water for similar purposes.

Purification of phosphorylated peptides may also be achieved, with the use of barium, lead and brucine salts [6,7,22].

Among the peptides reported in this paper, the O-phosphoryl-L-seryl-L-glutamic acid (compound 13, Table 4) may be of special interest, being the first phosphopeptide isolated from casein. The peptide isolated by Levene and Hill [6] and by Schmidt [7] was shown by Posternak and Pollaczek [11] to have the serine in N-terminal position, since this amino acid was absent in the hydrolysate of the peptide subsequent to deamination with nitrous acid. The analytical data of the barium and brucine salts, and the melting point of the latter, 171—172°, were reported. Riley *et al.* [22] have obtained this phosphopeptide by the alkaline dephenylation process mentioned above. Their peptide, isolated as the barium salt, $[\alpha]_D^{22}$ —1.3° (1 N HCl, *c* 3.12), was converted to free peptide, m.p. 145—147°, and to the brucine salt, m.p. 160—162°.

Our compound (13) which does not posess a sharply defined melting point but sinters at about 145° without decomposition has a much higher value of optical rotation, $[\alpha]_D^{21}$ —9.5°±0.5° (1 N HCl, *c* 4.2, *l* 1). Although this higher value, by no means, is proof of higher optical purity in view of the fact that two asymmetric centers are present, a comparison with the rotation of unphosphorylated L-seryl-L-glutamic acid (compound 7, Table 3) indicates that the value of rotation appears to have the correct magnitude since substitution of the β-hydroxyl group in serine peptides with phosphoric acid has little influence on the *molar* rotation. Compound (13) was also converted to its brucine salt, which, after a single recrystallization from water-acetone, melted at 171—173° with decomposition, thus confirming the results achieved by Levene and Hill. The action of intestinal alkaline phosphatase on O-phosphorylated peptides and their derivatives [16], and the acid hydrolysis [58] and acid ionization constants [17] of some of the phosphopeptides have been reported elsewhere.

EXPERIMENTAL

Where not otherwise stated, the compounds were dried at room temperature *in vacuo* over phosphorus pentoxide. The melting points were determined by the use of capillary tubes and are uncorrected. In the matter of paper chromatography on Whatman No. 1 paper, the following solvent systems were used: (I) Phenol-water (80/20 v/v), ascending, 6 h and (II) *n*-butanol-acetic acid-water (40/10/50/ v/v), descending, 18 h (cf. Table 6).

L-Serine benzyl ester

The racemic form of serine benzyl ester has been prepared by various procedures [22,32,55,59]. The pure optical antipodes have been obtained as hydrochlorides after separation of the dibenzoyl-D-tartrates [59]. The L-form was prepared in this laboratory several years ago by the process evolved by Cipera and Nicholls [60]:

a) *Benzenesulphonic acid salt.* A mixture of 4.2 g (40 mmoles) of L-serine, 8.0 g (44 mmoles) of recrystallized benzenesulphonic acid and 20 ml of benzyl alcohol was distilled with carbon tetrachloride on a water bath until no more water was formed (3—5 h). The ester salt was precipitated with dry ether and was, after some hours at +4°, filtered off, washed with ether and dried. The yield amounted to about 13.5 g, m. p. 85—90°. After recrystallization from *i*-propanol-ether, 10.2 g (72 %), m. p. 110—112° was obtained. (Found: N 4.1. Calc. for $C_{16}H_{19}O_6NS$ (353.4): N 4.0.) The reaction product is frequently difficult to crystallize, since no doubt by-products caused by, for example, selfesterification (forming O-peptide derivatives,) and O-benzylation [32] are formed.

b) p-*Toluenesulphonic acid salt.* With the use of the above process, but employing *p*-toluenesulphonic acid monohydrate instead, 11.0 g (75 %) of recrystallized L-serine benzyl ester *p*-toluenesulphonate, m. p. 94—95°, was obtained. (Found: N 3.8. Calc. for $C_{17}H_{21}O_6NS$ (367.4): N 3.8.)

c) *Hydrochloride,* prepared from a) and b). To a cold solution of 10 mmoles of a) (3.5 g) or b) (3.7 g) in 20 ml of chloroform and 1.4 ml (10 mmoles) of triethylamine, 100 ml of dry ether was added. The cooled and filtered solution was then saturated at 0° with hydrogen chloride. The precipitate was collected and recrystallized from *i*-propanol-ether, yielding 1.7 g (74 %), m. p. 172—174°, $[\alpha]_D^{21}$ −4.1° ± 0.5° (Methanol, *c* 4.4, *l* 1); reported [59] $[\alpha]_D^{20}$ −4.19° (Methanol, *c* 4.53). (Found: N 6.1. Calc. for $C_{10}H_{14}O_3NCl$ (231.7): N 6.1.)

N-Carbobenzoxy dipeptide benzyl esters

Procedure a) To a solution of 20 mmoles of an amino acid benzyl ester salt (L-serine benzyl ester benzenesulphonate, 7.1 g; L-leucine benzyl ester *p*-toluenesulphonate [61], 8.7 g; L-aspartic acid dibenzyl ester *p*-toluenesulphonate [61], 8.7 g ; L-glutamic acid dibenzyl ester *p*-toluenesulphonate [61], 9.0 g), 2.8 ml (20 mmoles) of triethylamine and 4.8 g (20 mmoles) of N-carbobenzoxy-L-serine [47] in 80—100 ml of tetrahydrofuran-acetonitril (1:1), was added 4.2 g (20 mmoles) of di*cyclo*hexylcarbodiimide. On the following day the precipitate of urea derivative was removed by filtration and the solution poured into 2 l of ice-cold water under vigorous stirring. After 4—12 h at +4° the precipitated peptide was collected, dried and recrystallized from ethyl acetate-light petroleum. The compounds obtained are listed in Table 2.

Table 2. N-Carbobenzoxy dipeptide benzyl esters. All compounds are of L—L configuration.

	Compound	Yield %	M. p. °C	Molecular formula	Analysis, % N Found	Calc.
1	Z · Ser-Glu · OBz	78	125	$C_{30}H_{32}O_8N_2$, 548.6	5.2	5.1
2	Z · Ser-Asp · OBz	89	128—129	$C_{29}H_{30}O_8N_2$, 534.5	5.2	5.2
3	Z · Ser-Leu · OBz	90	83—84	$C_{24}H_{30}O_6N_2$, 442.5	6.3	6.3
4	Z · Ser-Ser · OBz	71	138—139	$C_{21}H_{24}O_7N_2$, 416.2	6.5	6.7
5	Z · Glu-Ser · OBz	76	127	$C_{30}H_{32}O_8N_2$, 548.6	5.2	5.1
6	Z · Leu-Ser · OBz	86	122—123	$C_{24}H_{30}O_6N_2$, 442.5	6.1	6.3

Procedure b) To a solution of 7.1 g (20 mmoles) of L-serine benzyl ester benzenesulphonate and 2.8 ml (20 mmoles) of triethylamine in 100 ml of methylene chloride was added, first, 20 mmoles of a carbobenzoxy derivative (N-carbobenxoxy-L-leucine, 5.3 g; N-carbobenzoxy-L-glutamic acid γ-benzyl ester [62], 7.4 g) and then 4.2 g (20 mmoles) of di*cyclo*hexylcarbodiimide. On the next day the solution was filtered, washed with hydrochloric acid, water, sodium bicarbonate and water, and finally, after drying over sodium sulphate, evaporated to dryness *in vacuo*. The residue was then recrystallized from ethyl acetate-light petroleum (Table 2).

Peptides of L-serine

Catalytic hydrogenolysis of the protected peptides (compounds 1—6; 2—5 mmoles) in ethanol-water solution, using a 10 % palladium on charcoal catalyst, after filtration, evaporation to dryness and recrystallization from water-ethanol, resulted in the free peptides (compounds 7—12) listed in Table 3.

Phosphorylation with diphenylphosphoryl chloride

To a solution of 10 mmoles of an N-carbobenzoxypeptide benzyl ester (compounds 1—3, 5—6) in 10 ml of anhydrous pyridine was added 3.2 g (12 mmoles) of diphenylphosphoryl chloride, the temperature being kept below 40°. In the case of compound

Table 3. Dipeptides of L-serine

	Compound	Yield %	$[\alpha]_D^{21}$ (1 N HCl, *l* 1)	Molecular formula		Analysis Found C	H	N	Calc. C	H	N
7	H·Ser-Glu·OH	82	−10.8 (*c* 4.4) *	$C_8H_{14}O_6N_2$,	234.2	40.8	6.0	11.9	41.0	6.0	12.0
8	H·Ser-Asp·OH	78	+ 1.5 (*c* 4.0) **	$C_7H_{12}O_6N_2$,	220.2	37.9	5.4	12.7	38.2	5.5	12.7
9	H·Ser-Leu·OH	79	−18.8 (*c* 2.4)	$C_9H_{18}O_4N_2$, H_2O	236.3	45.9	8.4	11.9	45.7	8.5	11.9
10	H·Ser-Ser·OH	74	+14.8 (*c* 6.4) ***	$C_6H_{12}O_5N_2$,	192.2	37.1	6.3	14.5	37.5	6.3	14.6
11	H·Glu-Ser·OH	80	+29.0 (*c* 5.1)	$C_8H_{14}O_6N_2$,	234.2	40.7	6.2	11.8	41.0	6.0	12.0
12	H·Leu-Ser·OH	74	+27.8 (*c* 6.9)	$C_9H_{18}O_4N_2$,	218.3	48.6	8.3	12.6	49.6	8.3	12.8

* Reported [39] for the peptide prepared by the azide route $[\alpha]_D^{25}$ −9.4° (1 N HCl, *c* 6).

** Rotation in water solution $[\alpha]_D^{21}$ + 8.0 ± 0.6 (*c* 3.5, *l* 1).

*** Reported [39] for the peptide prepared by the azide route $[\alpha]_D^{25}$ +14.2° (1 N HCl, *c* 7).

4, only 5 mmoles (2.1 g) were used, the two equivalents of chloride reacting readily with strong heat evolution. After 4 h at room temperature, the excess of acid chloride was destroyed by adding 1 ml of water, and half an hour later the reaction mixture was taken up in 100 ml of water and 100 ml of ethyl acetate. The ethyl acetate layer was washed with cold 5 N sulphuric acid, water, bicarbonate solution and water, then dried over sodium sulphate and evaporated to dryness *in vacuo*. The triesters remained normally as colourless or slightly yellowish oils, of which those obtained from compounds 2 and 5 crystallized spontaneously (*c.f.* Table 5). The yield varied between 90 and 95 %.

Phosphorylation with dibenzylphosphoryl chloride

A solution of 10 mmoles of an N-carbobenzoxypeptide benzyl ester (compounds 1, 3 and corresponding derivatives of LD-serylglycine and glycyl-DL-serine) in 10 ml of anhydrous pyridine was cooled to just above the freezing point in an ethanol-carbon dioxide bath, and dibenzylphosphoryl chloride, prepared from 3.7 g (14 mmoles) of dibenzyl phosphite [25], was added. The mixture was shaken at this temperature for 15 min and then left over nigh at 0°. On the following day the almost colourless solution was worked up as above, yielding 75 % of triesters as colourless oils (if the oils are considered to be 100 % pure; they may contain some unphosphorylated neutral material). Two of them crystallized readily (Table 5).

In early experiments, phosphorylations were made at 0°. The yield of triester was then appreciably lower (about 50 %) and the product always more or less coloured.

Hydrogenolysis of O-diphenylphosphorylated derivatives of amino acids and peptides

$$\text{Z·Ser(OPO(OPh)}_2\text{)·OBz}$$

a) *Palladium catalyzed hydrogenolysis of Z·Ser·OBz.* To a solution of 2.8 g (5 mmoles) of O-diphenylphosphoryl-N-carbobenzoxy-DL-serine benzyl ester [19] in 75 ml of ethanol, water was added to slight turbidity, and hydrogenolysis started with 0.5 g 10 % palladium on charcoal. The shaking was stopped at intervals for the purpose of taking samples for paper chromatography, and then restarted. The results are shown in Table 1. After 3 h, the catalyst was filtered off and washed with hot acetic acid-water (1:1), the solution evaporated to dryness and the residue recrystallized from acetic acid-water, yielding, in the cold, 0.95 g (56 %) of O-diphenylphosphoryl-DL-serine, m.p. 130−131° (decomp.), reported [19] 129−130°.

b) *Palladium catalyzed hydrogenolysis of O-diphenylphosphorylated peptide derivatives.* In the hydrogenolytic conversion of the triesters obtained from diphenylphosphoryl

Table 4. Dipeptides of O-phosphoryl-L-serine.

Compound	Yield * %	$[\alpha]_D^{21}$ (1 N HCl, *l* 1)	Molecular formula	Analysis Found C	H	N	P	Calc. C	H	N	P
13 H · SerP-Glu · OH	53 (81)	− 9.5 (*c* 4.2) **	$C_8H_{15}O_9N_2P$	314.2	30.5	4.8	8.9	9.7	30.6	4.8	8.9	9.9
14 H · SerP-Asp · OH	62	− 2.2 (*c* 2.7)	$C_7H_{13}O_9N_2P$,	300.2	27.7	4.4	9.3	10.1	28.0	4.4	9.3	10.3
15 H · SerP-Leu · OH ***	64 (78)	−16.0 (*c* 4.0)	$C_9H_{19}O_7N_2P$,	298.2	36.1	6.4	9.4	10.3	36.2	6.4	9.3	10.3
16 H · SerP-SerP · OH	57	+ 8.2 (*c* 4.7)	$C_6H_{14}O_{11}N_2P_2$,	352.2	20.6	4.0	8.0	17.2	20.4	4.0	8.0	17.6
17 H · Glu-SerP · OH	54	+23.3 (*c* 4.4)	$C_8H_{15}O_9N_2P$,	314.2	30.7	4.8	8.8	9.8	30.6	4.8	8.9	9.9
18 H · Leu-SerP · OH	62	+24.5 (*c* 7.0)	$C_9H_{19}O_7N_2P$,	298.2	36.8	6.3	9.3	10.1	36.2	6.4	9.3	10.3

* The yields of pure crystalline phosphopeptides are calculated on basis of the amount of triester used for the hydrogenolysis, those figures within parenthesis referring to hydrogenolysis of *dibenzyl* derivatives.

** Reported [22] for the barium salt $[\alpha]_D^{22}$ −1.3° (1 N HCl, *c* 3.12).

*** For the D-seryl isomer, see Ref.[21]

Table 5. Dibenzyl, diphenyl and monophenyl phosphorylated derivatives.

	Compound *	Yield %	M. p.	Molecular formula		Analysis, % N Found	Calc.
19	Z · Ser-Gly · OBz └OPO(OBz)$_2$	75	104—105° **	$C_{34}H_{35}O_9N_2P$	646.6	4.3	4.3
20	Z · Gly-Ser · OBz └OPO(OBz)$_2$	76	81— 82°	$C_{34}H_{35}O_9N_2P$	646.6	4.4	4.3
21	Z · Ser-Asp(OBz)$_2$ └OPO(OPh)$_2$	93	62— 63°	$C_{41}H_{39}O_{11}N_2P$	766.7	3.6	3.7
22	Z · Glu-Ser · OBz BzO┘ └OPO(OPh)$_2$	90	75— 76°	$C_{42}H_{41}O_{11}N_2P$	780.7	3.7	3.6
23	H · Ser-Glu(OH)$_2$ └OPO(OPh)$_2$	48	182—185° (decomp.)	$C_{20}H_{23}O_9N_2P$	466.4	5.9	6.0
24	H · Ser-Glu · (OH)$_2$ *** └OPO(OPh)OH	21	174—178° (decomp.)	$C_{14}H_{19}O_9N_2P$	390.3	7.2	7.2
25	H · Ser-Asp · (OH)$_2$ └OPO(OPh)OH	28	167—171° (decomp.)	$C_{13}H_{17}O_9N_2P$	376.3	7.4	7.4
26	H · Ser-Leu · OH └OPO(OPh)OH	11	202—204° (decomp.)	$C_{15}H_{23}O_7N_2P,H_2O$	392.4	7.1	7.1

* Compounds 19 and 20 are derivatives of racemic serine. All the other are of L,L configuration.
Compounds 24 and 25 were isolated by anion exchange chromatography [57] (*cf.* Fig. 2).
** M. p. 90—93° when mixed with Z · Ser-Gly · OBz [19], m. p. 102—103°.
*** $[\alpha]_D^{21}$ −7.6° (1 N HCl, *c* 2.1, *l* 1).

chloride and compounds (1—6) to free phosphorylated peptides, palladium on charcoal was used in the first stage as per above. When the hydrogen uptake ceased, the catalyst was filtered off, and the solution and washings were evaporated to dryness. (The residue was then hydrogenolysed with a platinum catalyst as described below (c)). Paper chromatographic analysis of the residue showed at this stage the presence of large amounts of O-diphenylphosphorylated peptides, one of which was isolated and purified by recrystallization (comp. 23). Appreciable quantitites of other ninhydrinpositive compounds were also present.

c) *Platinum catalyzed hydrogenolysis.* The palladium-hydrogen treated product above (b) was dissolved in glacial acetic acid and rehydrogenolysed, now with Adams platinum oxide as catalyst. After an initial rapid hydrogen consumption lasting from 1/2 to 2 h, the reaction slowed down, and after 4—8 h no further hydrogen was consumed. The addition of a new catalyst or water had no marked effect. The filtered solution was evaporated to dryness and the residue analyzed by paper chromatography. In addition to the desired O-phosphorylated peptide, and its monophenylphosphoryl derivative, hydrolysis products were present. Re-hydrogenation with new catalyst in either acetic acid, ethanol-water or ethanol-water-hydrochloric acid did not increase the yield of phosphopeptide. Pure phosphopeptides (compounds 13—18, Table 4) were obtained by treatment in water solution with cation exchange resin (Dowex 50, H^+-form), evaporation to dryness and repeated recrystallization or precipitation from water with ethanol-ether. Compounds 13, 14, 16, 24 and 25, being difficult to obtain chromatographically pure by this method, were also obtained by separation of the products of hydrogenolysis by anion exchange column chromatography on a 2 × 30 cm column of Dowex 1, X 2 resin in formate form, using gradient elution with pyridinium formate [57]. The fractions emerging from the column were tested by quantitative ninhydrin reaction and the peaks

Table 6. R_{ser} values of peptides, O-phosphorylated peptides, and other ninhydrin-positive derivatives of L-serine.

Dipeptides			Phosphopeptides			Derivatives		
Compound No.	R_{ser} * I	II	Compound No.	R_{ser} I	II	Compound No.	R_{ser} I	II
7	0.6	1.2	13	0.3	0.4	23		3.5
8	0.5	1.1	14	0.2	0.3	24	1.4	1.6
9	2.2	3.3	15	0.8	1.8	25	1.3	1.4
10	0.6	1.0	16	0.1	0.1	26	1.6	3.3
11	0.6	1.2	17	0.2	0.3			
12	2.0	3.0	18	0.7	1.4			

* $R_{ser} = \frac{R_F \text{ for the compound}}{R_F \text{ for serine}}$. Solvent systems used for the paper chromatography are (I) phenol-water (80/20 v/v) and (II) *n*-butanol-acetic acid-water (40/10/50 v/v). R_F for serine is (I) 0.35; (II) 0.18.

thus obtained (Fig. 2) analyzed by paper chromatography. Those fractions containing phosphopeptide or monophenylphosphorylated peptide were evaporated to dryness *in vacuo*. The residues, probably being monopyridinium salts as indicated by elementary analysis, were dissolved in a small volume of water and converted to free acid by passage through a small column of Dowex 50 (H^+) resin and then lyophilized (Tables 4 and 5).

d) *Rehydrogenation of O-monophenylphosphorylated derivatives.* The solution of 1 mmole of compounds (24—26) in 15 ml of glacial acetic acid, water or ethanol-water was shaken with 0.1 g of platinum oxide in an atmosphere of pure hydrogen. Complete removal of the phenyl group was effected in 1/2—2 h, as shown by paper chromatography and by isolation of pure phosphopeptide in about 85 % yield by evaporation to dryness of the filtered solution.

When left for shaking in solvents containing water, appreciable hydrolysis into unphosphorylated peptide and into amino acids occurred, as found by paper chromatography.

Preparation of O-phosphorylated peptides from O-dibenzylphosphorylated derivatives

A solution of 5—10 mmoles of an (oily or solid) O-dibenzylphosphoryl-N-carbobenzoxy dipeptide benzyl ester in 75—100 ml of ethanol, water being added to slight turbidity, was shaken together with 0.5—1 g of 10 % palladium on charcoal in an atmosphere of pure hydrogen. In the first hour, at least 85 % of the theoretical amount of hydrogen (4—5 equiv. H_2) was absorbed, and the reaction was normally completed within a further hour, no more hydrogen being used. The catalyst was filtered off and washed with a small amount of hot distilled water, and the filtrate and washings evaporated to dryness. The crystalline residue was washed with acetone or ethanol-ether, and dried. Paper chromatography of the product showed the presence of traces of unphosphorylated peptide and (or) free amino acids. In the case of well crystallizing compounds such as H·SerP—Gly·OH [18, 20, 22] H·Gly—SerP·OH [20] and H·SerP—Leu·OH (compound 15), one or two recrystallizations from hot water-ethanol produced chromatographically and analytically pure phosphopeptides. Since H·SerP—Glu·OH (compound 13) did not crystallize well from solvents containing water * it was purified as follows. The solution

* No other solvents have been found sufficiently effective; although boiling methanol dissolves appreciable amounts of phosphopeptides, we cannot at the present time exclude the possibility of partial esterification of these strongly acidic compounds.

of 1.0 g in 40 ml of water was shaken with 2 ml of Dowex 50 cation exchange resin (H^+-form) for 10 min. The mixture was then filtered through a column, the resin washed with 5 ml of water and the solution evaporated to dryness, yielding 0.9 g. The compound was dissolved in 4 ml of water and precipitated with 25 ml of ethanol and 25 ml of ether, the yield of collected and dried precipitate of pure phosphopeptide then being 0.85 g (Table 4).

Brucine and barium salts of H·SerP-Glu·OH

a) *Brucine salt.* The solution of 0.95 g (3 mmoles) of compound 13 (Table 4) in 25 ml of water was treated with an excess of brucine (5 g) dissolved in 25 ml of methanol. Further 75 ml of water was added, and the excess of brucine extracted with 3 × 30 ml of chloroform. The water solution was filtered and evaporated to dryness, and the remaining crystalline product recrystallized by dissolving in 15 ml of water and adding aceton at the boiling point. After 4 h at +4°, the compound was collected and dried, the yield then being 2.3 g, m.p. 171—173° (decomp.), after a slight change in colour at 165°. (Found: N 7.9. Calculated, according to Levene and Hill [6], for the *mono*brucine salt, $C_{31}H_{41}O_{13}N_4P$ 708.7): N 7.9.)

b) *Barium salt.* The above brucine salt (2.0 g) was dissolved in 25 ml of water and neutralized (to phenolphthalein) with barium hydroxide solution. The precipitate of brucine was extracted with chloroform, the water solution concentrated *in vacuo* to 35 ml and the barium salt of compound 13 precipitated with 100 ml of ethanol. After centrifugation and reprecipitation from 50 ml of water with 50 ml of ethanol, the salt was collected by centrifugation and dried, yielding 0.9 g. (Found: N 5.4. Calculated for the tribarium salt $(C_3H_9O_{10}NPBa)_2Ba$ (1034.4): N 5.4.)

Acknowledgements. The author wishes to express his sincere thanks to Professor Olof Mellander for his interest and support in this work.

The work was supported by grants from *Swedish Medical Research Council* to Professor Olof Mellander.

REFERENCES

1. Mc Meekin, T. L. and Polis, B. D. *Advances in Protein Chem.* **5** (1949) 201.
2. Mellander, O. and deVerdier, C.-H. *Acta Soc.Med.Upsaliensis* **57** (1952) 218.
3. Österberg, R. *Arkiv Kemi* **13** (1959) 409.
4. Pantlitschko, M. and Gründig, E. *Monatsh.* **89** (1958) 274; 489.
5. Bennich, H., Johansson, B. and Österberg, R. *Acta Chem. Scand.* **13** (1959) 1171.
6. Levene, P. A. and Hill, D. W. *J. Biol. Chem.* **101** (1933) 711.
7. Schmidt, G. *Z.physiol. Chem. Hoppe-Seyler* **223** (1934) 86.
8. de Verdier, C.-H. *Acta Soc. Med. Upsaliensis* **60** (1955) 48.
9. Hipp, N. J., Groves, M. L. and Mc Meekin, T. L. *J. Am. Chem. Soc.* **79** (1957) 2559.
10. Flavin, M. *J. Biol. Chem.* **210** (1954) 771.
11. Posternak, T. and Pollaczek, H. *Helv. Chim. Acta* **24** (1941) 921.
12. Mellander, O. *Upsala Läkareförens. Förh.* **52** (1947) 107.
13. Mellander, O. *Acta Soc. Med. Upsaliensis* **55** (1950) 247.
14. Posternak, T. and Grafl, S. *Helv. Chim. Acta* **28** (1945) 1258.
15. Bennich, H., Johansson, B. and Mellander, O. *Acta Soc. Med. Upsaliensis* **62** (1957) 67.
16. Mellander, O., Bennich, H. and Fölsch, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 303.
17. Fölsch, G. and Österberg, R. *J. Biol. Chem.* **234** (1959) 2298
18. Fölsch, G. *Acta Chem. Scand.* **9** (1955) 1039.
19. Fölsch, G. and Mellander, O. *Acta Chem. Scand.* **11** (1957) 1232.
20. Fölsch, G. *Acta Chem. Scand.* **12** (1958) 561.
21. Fölsch, G. *Acta Chem. Scand.* **13** (1959) 1422.
22. Riley, G., Turnbull, T. H. and Wilson, W. *J. Chem. Soc.* **1957** 1373.
23. Bergmann, M. and Zervas, L. *Ber.* **65** (1932) 1192.
24. Bergmann, M., Zervas, L. and Ross, W. F. *J. Biol. Chem.* **111** (1935) 245.

25. Atherton, F. A., Openshaw, H. T. and Todd, A. R. *J. Chem. Soc.* **1945** 382.
26. Zeile, K. and Meyer, H. *Z. physiol. Chem. Hoppe-Seyler* **256** (1938) 131.
27. Brigl, P. and Müller, H. *Ber.* **72** (1939) 2121.
28. Plapinger, R. E. and Wagner-Jauregg, T. *J. Am. Chem. Soc.* **75** (1953) 5755.
29. Josefsson, L. and Edman, P. *Biochim. et Biophys. Acta* **25** (1957) 614.
30. Sheehan, J. C., Goodman, M. and Hess, G. P. *J. Am. Chem. Soc.* **78** (1956) 1367.
31. Khorana, H. G. *Chem. & Ind. London* **1955** 1087.
32. Zahn, H. and Diehl, J. F. *Z. Naturforsch.* **12 b** (1957) 85.
33. Daft, F. S. and Coghill, R. D. *J. Biol. Chem.* **99** (1931) 213.
34. Hofmann, K., Woolner, M. E., Spühler, G. and Schwartz, E. T. *J. Am. Chem. Soc.* **80** (1958) 1486.
35. Anderson, G. W. and Callahan, F. M. *J. Am. Chem. Soc.* **80** (1958) 2902.
36. Schwarz, H. and Bumpus, F. M. *J. Am. Chem. Soc.* **81** (1959) 890.
37. Boissonas, R. A., Guttmann, St., Huguenin, R. L., Jaquenoud, P.-A. and Sandrin, Ed. *Helv. Chim. Acta* **41** (1958) 1867.
38. Curtius, T. *Ber.* **35** (1902) 3226.
39. Fruton, J. S. *J. Biol. Chem.* **146** (1942) 463.
40. Baer, E. *Biochem. Preparations* **1** (1949) 51; **2** (1952) 96.
41. Zervas, L. and Dilaris, I. *J. Am. Chem. Soc.* **77** (1955) 5354.
42. Miyano, M. and Funahashi, S. *J. Am. Chem. Soc.* **77** (1955) 3522.
43. Atherton, F. A. and Todd, A. R. *J. Chem. Soc.* **1947** 674.
44. Zervas, L. and Dilaris, I. *Chem. Ber.* **89** (1956) 925.
45. Moffatt, J. G. and Khorana, H. G. *J. Am. Chem. Soc.* **79** (1957) 3741.
46. Harris, J. I. and Fruton, J. S. *J. Biol. Chem.* **191** (1951) 143.
47. Guttmann, St. and Boissonas, R. A. *Helv. Chim. Acta* **41** (1958) 1852.
48. Schnabel, E. *Z. physiol. Chem. Hoppe-Seyler* **314** (1959) 114.
49. Baer, E. and Stancer, H. C. *Can. J. Chem.* **34** (1956) 436.
50. Mostert, Pzn, S., Stegerhoek, L. J. and Verkade, P. E. *Rec. trav. chim.* **77** (1958) 133.
51. Kuhn, R. and Haas, H. J. *Ann.* **611** (1958) 57.
52. Bevan, T. H., Malkin, T. and Tiplady, J. M. *J. Chem. Soc.* **1957** 3086.
53. Zervas, L. and Katsoyannis, P. G. *J. Am. Chem. Soc.* **77** (1955) 5351.
54. Zervas, L. *Naturwiss.* **27** (1939) 317.
55. Li, S. O. and Eakin, R. E. *J. Am. Chem. Soc.* **77** (1955) 1866.
56. Jones, E. E. and Lipkin, D. *J. Am. Chem. Soc.* **78** (1956) 2408.
57. Strid, L. *Acta Chem. Scand.* **13** (1959). *In press.*
58. Fölsch, G., Mellander, O. and Strid, L. *Acta Chem. Scand. In press.*
59. Losse, G. and Augustin, M. *Chem. Ber.* **91** (1958) 157.
60. Cipera, J. D. and Nicholls, R. V. V. *Chem. & Ind. London* **1955** 16.
61. Zervas, L., Winitz, M. and Greenstein, J. P. *J. Org. Chem.* **22** (1957) 1515.
62. Hanby, W. E., Waley, S. G. and Watson, J. *J. Chem. Soc.* **1950** 3239.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1422–1424

Synthesis of Phosphopeptides

III *. Derivatives of D-Seryl-L-leucine Obtained by Separation from a DL-L Diastereoisomeric Mixture

GEORG FÖLSCH

Department of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden

The two diastereoisomers of N-carbobenzoxy-DL-seryl-L-leucine benzyl ester, were separated by fractional crystallisation. The D-seryl derivative was converted to D-seryl-L-leucine, O-phosphoryl-D-seryl-L-leucine and O-monophenylphosphoryl-D-seryl-L-leucine.

The use of racemic amino acids in peptide synthesis yields mixtures of diastereoisomers when α-amino acids other than glycine are used, as all other α-amino acids contain at least one asymmetric center. The components of such diastereoisomeric mixtures are sometimes separated by fractional crystallisation [2-3]. For large scale preparation of dipeptides containing a difficultly accessible antipode of an amino acid, such procedures may be of value, as the amino acid in question can be used in the generally more easily accessible racemic form.

In this paper the resolution of the carbobenzoxy peptide benzyl ester (I) obtained from DL-serine and L-leucine is described. The difference in solubility of the two isomers made it comparatively easy to isolate the higher melting isomer, *i.e.* N-carbobenzoxy-D-seryl-L-leucine benzyl ester (II). After two recrystallizations, the m.p. and optical rotation had the values of an authentic sample, prepared from pure D-serine. The isolation of pure L-L derivative (III) was, however, somewhat more difficult since this isomer, in none of the solvent systems tried, was less soluble than compound (II), and five recrystallizations were necessary to obtain the m.p. and rotation values of a previously prepared [1] sample of (III).

Compound (II) was converted to D-seryl-L-leucine by hydrogenolysis, and to O-phosphoryl-D-seryl-L-leucine and O-monophenylphosphoryl-D-seryl-L-leucine by phosphorylation with diphenylphosphoryl chloride followed by hydrogenolysis. The phosphopeptides may be of considerable interest in

* For part II, see Ref.[1]

current studies on the action of phosphatases [4] and proteolytic enzymes on phosphorylated peptides, as it has been shown, that the enzyme phosphoserine phosphatase acts on O-phosphoryl-D-serine as well as on the L-isomer [5-7].

EXPERIMENTAL

*N-Carbobenzoxy-*DL*-seryl-*L*-leucine benzyl ester* (I). To a solution of 12.0 g (50 mmoles) of N-carbobenzoxy-DL-serine [8], 13.0 g (50 mmoles) of L-leucine benzyl ester hydrochloride [9] and 7.2 ml (50 mmoles) of triethylamine in 400 ml of tetrahydrofuran-acetonitrile (1:1) was added 10.5 g (50 mmoles) of N,N'-di*cyclo*hexylcarbodiimide. The mixture was shaken for 2 h and then left over night. After filtration, the solvent was distilled off *in vacuo* and the residue dissolved in ethyl acetate. After washing with water, 1 N hydrochloric acid, water, saturated bicarbonate solution, and water, followed by drying over sodium sulphate, the ethyl acetate was distilled off *in vacuo* and the residue brought to solid state by addition of light petroleum. The product was collected and then dried. Yield 20.0 g (90 %) of crude (I).

Separation of isomers. The above mixture of diastereoisomers was dissolved in 200 ml of ethyl acetate, and light petroleum was added to slight turbidity. When left for 24 h at +4°, crystals were formed which, after filtration, washing with ethyl acetate-light petroleum (1:1) and drying weighed 8.1 g (73 % of theory). M. p. 110–111°. After one additional crystallization from the same solvent system, 7.1 g (64 %) of a compound (II) with m. p. 115° $[\alpha]_D^{21}$ −19.9° (ethanol, *c* 6.7, *l* 1); $[\alpha]_D^{21}$ −4.0° (chloroform, *c* 11, *l* 1) was obtained. For the L–L derivative, a m. p. of 83–84° has been reported [1]. Therefore, the actual compound had to be the D–L isomer, and this was proved by comparison with an autenthic sample of N-carbobenzoxy-D-seryl-L-leucine (III). This was synthesized by the carbodiimide procedure from 0.6 g (2.5 mmoles) of N-carbobenzoxy-D-serine [8] as described above, whereby a yield of 0.95 g (86 %) was obtained after recrystallization from ethyl acetate-light petroleum. M. p. 114 *; mixed with II, m. p. 114°. $[\alpha]_D^{21}$ −20.2° (ethanol, *c* 3.4, *l* 1); $[\alpha]_D^{21}$ −4.2° (chloroform, *c* 12, *l* 1).

The mother liquors and washings from compound (II) were evaporated to dryness and the residue repeatedly crystallized from methanol-water. After five crystallizations, 4.6 g (42 %) were collected (IV). M. p. 83–84°, mixed with pure N-carbobenzoxy-L-seryl-L-leucine benzyl ester, m. p. 83–84°; $[\alpha]_D^{21}$ −26.5° (ethanol, *c* 6.1, *l* 1).

D-*Seryl-*L*-leucine* (V). By catalytic hydrogenolysis of 2.2 g of compound (II) in ethanol-water, using palladium catalyst, followed by filtration, evaporation to dryness and recrystallization from water-ethanol-ether, a yield of 0.9 g (76 %) of the free peptide as the monohydrate was obtained. $[\alpha]_D^{21}$ −38° (1 N HCl, *c* 6.7, *l* 1). (Found: C 45.9; H 8.3; N 11.8. Calc. for $C_9H_{18}N_2O_4$, H_2O (236.3): C 45.7; H 8.5; N 11.8.)

*O-Phosphoryl-*D*-seryl-*L*-leucine* (VI). To 4.4 g (10 mmoles) of compound II in 10 ml of anhydrous pyridine was added 3.2 g (12 mmoles) of diphenylphosphoryl chloride, the temperature being kept below 40°. After 4 h, the excess of acid chloride was destroyed by addition of water, and the product taken up in 100 ml of water and 100 ml of ether. The ether layer was washed with 4 N sulphuric acid, water, saturated sodium bicarbonate and water and was then dried over sodium sulphate. After the ether had been distilled off *in vacuo*, 6.2 g (92 %) of oily O-diphenyl-phosphoryl-N-carbobenzoxy-D-seryl-L-leucine benzyl ester was obtained. This oil was dissolved in 100 ml of ethanol and the solution, after addition of water to slight turbidity was then shaken in an atmosphere of pure hydrogen, 1.0 g of 10 % palladium on charcoal being used as catalyst. In 4 h, 480 ml of hydrogen was used. The catalyst was filtered off and was well washed with a small quantity of hot acetic acid. The filtrate and washings were evaporated to dryness and

* In a study of O → N acyl shifts in serine derivatives, Josefsson and Edman [10] reported the preparation of compound (I) *via* the mixed chlorocarbonic anhydride of N-carbobenzoxy-DL-serine. The isolated compound was obviously practically pure D-seryl-L-leucine derivative and had a m. p. 107°.

the residue again hydrogenolysed, but this time in glacial acetic acid solution and with 1.0 g of Adams platinum oxide as catalyst. The hydrogen uptake was completed in 8 h. After filtration and evaporation *in vacuo*, 2.8 g of crystalline residue was obtained. This residue was recrystallized from boiling water. Insoluble material (VII) was filtered off, and in the cold room the aqueous solution gave nicely crystallizing O-phosphoryl-D-seryl-L-leucine as monohydrate, 2.1 g (66 %). M. p. 138—140° (decomp.); $[\alpha]_D^{21}$ −28.1 (1 N HCl, *c* 3.6, *l* 1). (Found: C 33.8; H 6.9; N 8.6; P 9.6. Calc. for $C_9H_{19}O_7N_2P$, H_2O (316.2): C 34.2; H 6.7; N 8.9; P 9.8.) A sample dried to constant weight at 65° and 0.05 mm Hg lost 7.7 % of weight and the compound then analyzed correctly for anhydrous phosphopeptide. (Found: C 36.3; H 6.3; N 9.4; P 10.4. Calc. for $C_9H_{19}O_7N_2P$ (298.2): C. 36.2; H 6.4; N 9.4; P 10.4.)

*O-Monophenylphosphoryl-*D*-seryl-*L*-leucine* (VII). The water-insoluble material from the recrystallization of (VI) was monophenylphosphoryl-D-seryl-L-leucine and crystallized from acetic acid-water, as monohydrate at a yield of 0.35 g (9 %). M. p. 213—215° (decomp.). (Found: C 45.8; H 6.4; N 7.1; P 7.8. Calc. for $C_{15}H_{23}O_7N_2P$, H_2O (392.4): C 45.9; H 6.4; N 7.1; P 7.9.)

REFERENCES

1. Fölsch, G. *Acta Chem. Scand.* **13** (1959) 1407.
2. Polglase, W. J. and Smith, E. L. *J. Am. Chem. Soc.* **71** (1949) 3081.
3. Schnabel, E. and Zahn, H. *Ann.* **614** (1948) 141.
4. Mellander, O., Bennich, H. and Fölsch, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 303.
5. Borkenhagen, L. F. and Kennedy, E. P. *Biochim et Biophys. Acta* **28** (1958) 222.
6. Schramm, M. *J. Biol. Chem.* **233** (1958) 1169.
7. Neuhaus, F. C. and Byrne, W. L. *J. Biol. Chem.* **234** (1959) 113.
8. Baer, E. and Maurukas, J. *J. Biol. Chem.* **212** (1955) 25.
9. Miller, H. K. and Waelsch, H. *J. Am. Chem. Soc.* **74** (1952) 1092.
10. Josefsson, L. and Edman, P. *Biochim. Biophys. Acta* **25** (1957) 614.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1425–1436

Phosphorylmuramic Acid in *Lactobacillus casei*. Its Presence in Protein and in a Nucleoside-pyrophosphate Fraction

CARL-HENRIC de VERDIER and GUNNAR ÅGREN

Institute of Medical Chemistry, University of Uppsala, Sweden

A method has been developed for the isolation of nucleotide bound muramic acid peptides from *Lactobacillus casei*. The amino acid composition of these peptides has been studied and compared with acid resistant phosphopeptides from hydrolyzed phosphoproteins. A closely related amino acid composition has been found. Evidence for the existence of nuleotide bound phosphorylmuramic acid is given and the specific activity for such a compound is consistent with the view that it could be a precursor in the synthesis of phosphoproteins in *Lactobacillus casei*.

In 1955 a new phosphorylated amino acid was isolated from a hydrolysate of the protein residue of *L. casei* [1,2]. Recently it was identified as a phosphorylated derivative of 3-O-α-carboxyethyl-D-glucosamine [3] which has also been named muramic acid *. In one of the earlier communications [2] we also reported the presence of several UDP-derivatives in a cold 10 % TCA extract of the microorganism.

Park and Johnson [4] in 1949 observed that uridine nucleotides accumulated in *Staphylococcus aureus* which was inhibited by penicillin. Later they were identified as UDP linked to Mur or Mur-peptides [5]. Uridine nucleotide accumulation was also found in a penicillin-sensitive *Lactobacillus helveticus* [6] where the effect of penicillin was far less conspicuous. Evidence has also been obtained which suggests that the UDP-Mur-peptides are precursors of the bacterial cell wall in a metabolic pathway inhibited by penicillin.

* The following abbreviations are used: Mur = muramic acid; MurP = O-phosphorylmuramic acid; P = inorganic phosphate; ^{32}P = radioactive inorganic phosphate; cpm = counts per minute; cps = counts per second; AMP = adenylic acid; ADP = adenosine diphosphate; ATP = adenosine triphosphate; $AT^{32}P$ = radioactive adenosine triphosphate; CMP = cytidylic acid; GTP = guanosine triphosphate; UMP = uridylic acid; UDP = uridine diphosphate; UTP = uridine triphosphate; DPN = diphosphopyridine nucleotide; TCA = trichloroacetic acid; Ala = alanine; Glu = glutamic acid; Leu = leucine; UV = ultra violet; E_{260} = optical density at 260 mμ.

The previous observations [2,3] may also throw some light on the mechanism for the synthesis of protein bound MurP where the phosphoryl group most probably is bound to the sixth carbon atom of the glucosamine moiety. The UDP-linked MurP reported in this paper may be an intermediate in the biosynthesis of protein bound MurP.

MATERIALS AND METHODS

Lactobacillus casei (7469) was grown in the medium previously described [3]. Bacterial growth was estimated by measuring the turbidity of suspensions with filter No. 59 in the Klett-Summerson photometer. The growth of the microorganism was inhibited by penicillin as shown in Table 1.

Chromatographic separation of acid soluble nucleotides. The cultivations were made with the suspension of microorganisms of step 5 according to previous description [3] grown for 18 h and diluted with fresh medium 1:30. Most of the experiments were made with 90 liter cultures and with the addition of 10 μg of benzylpenicillin per ml when one-half the maximum of growth was reached. The cultivation technique and the extraction methods were the same as in the preceding paper [3]. In all radioactivity experiments the medium contained 0.33 mC ^{32}P per liter from the time of incubation. The cold TCA extract from each experiment was shaken with ether to remove the TCA and then neutralized to pH 6 to 7 by the addition of a few drops of 1 N NaOH. The solution was evaporated *in vacuo* to a volume of about 30 ml and mostly run on two 70 × 3 cm Dowex 1 formate (2 % DVB) columns and eluted mainly according to Hurlbert *et al.*[7] The eluate was collected in fractions of about 30 to 40 ml every 15 min.

The elution was started with water and about 25 fractions were collected. In the system, which followed, a 600 ml mixing volume was used. The four elution ranges which approximated 1 N formic acid, 4 N formic acid, 0.2 M ammonium formate in 4 N formic acid and 0.4 M ammonium formate in 4 N formic acid were obtained by changing the reservoir to contain these solutions at tubes 25, 100, 250 and 350. Altogether 500 to 600 fractions were collected in each series. In two experiments the TCA extract was run with the elution system of Strominger [6].

Partial hydrolysis of the Schneider protein residue was performed with 11 N HCl at 37°C during 3 days according to Flavin [8]. The hydrolysate was evaporated *in vacuo*

Table 1. Growth-inhibition of *Lactobacillus casei* by penicillin given as photometric readings of the growth culture. Penicillin was added after 3.5 h of growth.

Incubation time, h	Control culture	Cultures with penicillin		
		1 000 μg/ml	100 μg/ml	10 μg/ml
0	25			
0.5	27			
1	30			
1.5	42			
2	55			
2.5	85			
3	112			
3.5	152			
4	184			
4.5	230	210	210	206
5.5	290	230	240	230
24	420	235	245	250

several times to remove excess of HCl and applied to a 7 × 50 cm Dowex 50 (8 % DVB) column. Elution with 0.01 N HCl was used as for the elution of MurP [3]. Excess of HCl was then removed from the different fractions separated on the Dowex 50 column as described above. The residues were dissolved in small volumes of water and applied to Dowex 1 formate columns (dimensions 2 × 45 cm). Elution was carried out with a gradient 0 → 1 M formic acid and with a volume of 200 ml in the mixer.

Analytical methods. The nucleotides were assayed by optical density determinations of suitable dilutions from each tube using a 1 cm quartz cuvette in a Beckman model DU spectrophotometer at 250, 260 and 280 mμ. The ratios used for identifying the UDP-derivatives were the following [9]: 250/260 = 0.73 and 280/260 = 0.39.

The radioactivity of the different fractions from the column chromatography of the TCA soluble nucleotides and protein residue was measured in glass cups with a L.K.B. Robot Scaler. Scanning of the paper chromatograms of nucleotides and MurP-peptides was made according to Ågren *et al.*[10] Determinations of specific activity values were made on stainless steel planchettes with the Tracerlab Superscaler. At least 1 000 counts were measured. The specific activities of the different nucleotides and of MurP were calculated as counts per min per μmole of compound. All activity values were recalculated to the time of incubation.

The amino acid composition of the UDP-peptides and the peptides isolated by partial hydrolysis of phosphoproteins was determined by two dimensional paper chromatography [11]. The UDP-peptides were hydrolyzed with 1 N HCl for 3 h at 100°C and the peptides from phosphoproteins with 2 N HCl for 20 h at 120°C in sealed tubes. In some cases the solvents used for the identification of Mur were suitable, as listed in a preceding paper [1]. In chromatography Whatman No. 1 or Munktell OB papers were used, and for ionophoresis Whatman No. 3 where the buffer solutions were either a 0.1 M solution of pyridine-acetic acid at pH 5 or 1 N acetic acid.

RESULTS

Isolation of UDP-linked Mur-peptides. In Fig. 1 an elution diagram is presented for the TCA extract prepared from a 45 liter culture passing through a Dowex 1 column with the formic acid system. 15 mC radioactive phosphate had been added at the inoculation and penicillin after 3.5 h of growth. The culture was harvested after 4.5 h of growth in the middle of the log phase. In several points the diagram was similar to that obtained with TCA extracts of yeast [12,13]. The peaks corresponding to CMP, DPN and AMP appeared in the same position. UMP and ADP were found in the expected position after the inorganic phosphate. After ADP eight or sometimes nine E_{260}-absorbing peaks appeared, in Fig. 1, Nos. 1—8. The two first peaks after ADP generally coincided with the elution of two yellow coloured fractions from the columns. The first fraction appeared in the same position as a UDP-linked peptide containing Mur and up to four other amino acids. The 280/260 ratio of the eight different UV peaks were 0.40, 0.50, 0.40, 0.51, 0.37, 0.40, 0.38 and 0.39, respectively.

Only peak 1 and 7 seemed to contain a muramic acid peptide linked to UDP. Paper chromatograms of the hydrolyzed peptides showed the presence mainly of muramic acid, alanine, glycine, glutamic acid and another ninhydrin positive substance which so far has not been identified. The total yield of UDP-linked Mur-peptides was about 10 μmoles which is much less than Strominger [6] obtained from cultures of *L. helveticus*. This yield was not increased to any greater extent when penicillin was present in the log phase cultures. Of interest is the observation that ATP, GTP and UTP were not present in

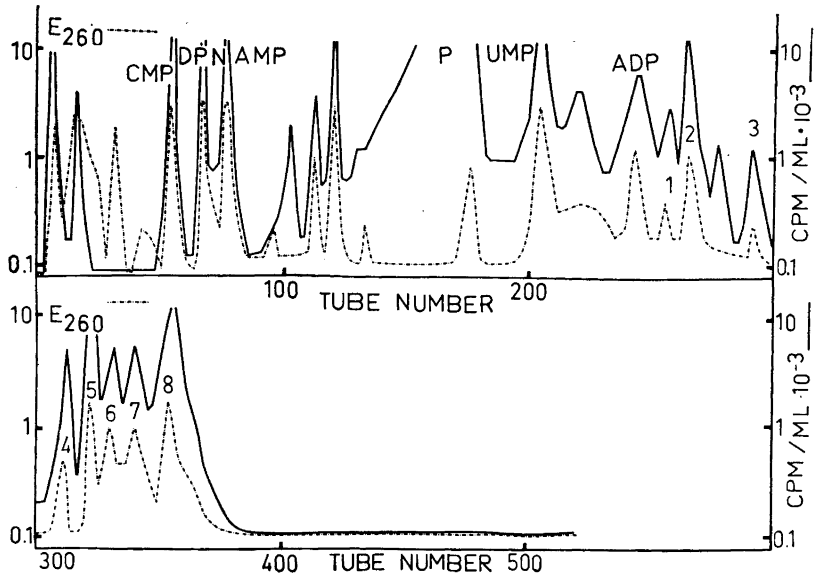


Fig. 1. Acid-soluble nucleotides from 80 g of wet cells after 4 h of incubation with 15 mC of radioactive inorganic phosphate. The nucleotides were separated by gradient elution with the reservoir content changed at the tubes numbered as follows: *25*, 1 N formic acid; *100*, 4 N formic acid; *250*, 0.2 M ammonium formate + 4 N formic acid; *350*, 0.4 M ammonium formate + 4 N formic acid; column dimensions 3 × 70 cm; Dowex 1 formate form. The continuous line represents radioactivity values, the broken line represents E_{260} values.

our TCA extracts of *L. casei*. The elution system used by Strominger [6] has also been used but according to our experience has no advantage over that used by us. It is worth mentioning that in the region between peaks C and D in the Strominger system there was no Mur-containing material in the TCA extracts of *L. casei* while Strominger found considerable amounts in extracts from *S. aureus*. The stepwise elution system used by Strominger we also found more laborious.

Isolation of UDP-linked MurP. It has been previously mentioned in this paper that UDP-linked MurP could be an intermediate in the biosynthesis of protein bound MurP. Several experiments were carried out to isolate such a compound in the TCA extracts of *L. casei*. As it could be supposed that the yield of such a compound should be very low no attempts were made to isolate a separate fraction, containing this material. Instead, all substances emerging from the columns after the ADP peak had appeared were pooled. Ammonium ions were removed by rapidly passing the eluate through a short cooled Dowex 50 filter which was afterwards washed with an equal volume of cold water. The formic acid eluate was lyophilized and extracted with a small volume of water. Hydrolysis was carried out with 0.1 N HCl at 100°C for 7 min which is far in excess of that used by Caputto *et al.*[14] for the liberation of glucose

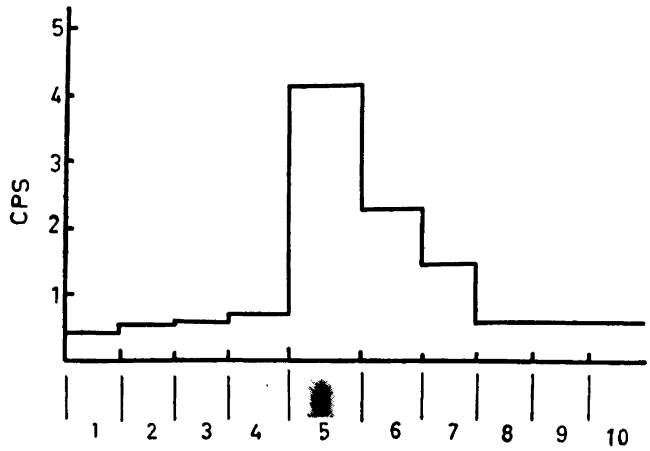


Fig. 2. Scanning curve (upper part of the figure) from a paper chromatographic strip of a hydrolysate of radioactive nucleotide bound MurP run parallelly with unlabeled MurP giving the ninhydrin spot on the lower strip. The paper strip with the chromatographically purified radioactive hydrolysate was cut in 10 equal pieces and the time for 1 000 counts was measured for each piece of paper.

from UDP-glucose. HCl was removed from the hydrolysate which was afterwards concentrated to a small volume and put on a Dowex 50 column and eluted with 0.01 N HCl. In the expected position for MurP a radioactive peak appeared. The identity of the material in this peak was checked by ionophoresis in a pyridine-acetate buffer at pH 5 and in paper chromatography (Fig. 2) and with a *iso*butyric acid-ammonia system [15]. In both cases radioactivity run parallel with added MurP. From Fig. 2 it is also obvious that the presence in this material of MurP-peptides could not be excluded. As described below such peptides leave the Dowex 50 column in about the same column volume as MurP.

It could not be excluded that the isolated MurP had been formed by splitting of UDP-linked Mur by a migration of the phosphoryl group from 1- to

Table 2. 25 mg sodium salt of UDP-acetyl glucosamine corresponding to 39.8 μmoles was hydrolyzed. The following products were obtained:

	μmoles	yield, %
Glucosamine-6-phosphate	0.01	0.025
Uridine-monophosphate	15	38 *
Inorganic phosphate	39	98
Glucosamine	23	58

* Calculated per uridine residue.

6-position. However, a more likely explanation seemed to be the existence of a UDP-linked MurP. As an example of a closely connected substance may be mentioned the recently isolated UDP-acetylglucosamine phosphate from the oviduct of laying hen [16]. The possibility of the postulated migration was tested in a model experiment with UDP-glucosamine. 25 mg of a commercially available substance (Sigma, 95—100 % pure, from yeast) was hydrolysed under the same conditions as the rest protein fraction (2 N HCl, 20 h and at 100°C).[15] The different components of the hydrolysate were separated on a Dowex 1 (2 % DVB) column by gradient elution with formic acid and the results are given in Table 2.

Glucosamine was determined in the fraction eluted with water according to Blix' modification of the Elson-Morgan reaction [17]. To detect the phosphate a small amount of carrier free ^{32}P-labeled orthophosphate was added before the separation. Phophorus was determined according to an earlier described method [10]. Glucosamine-6-phosphate was essayed by comparison on the paper chromatogram strips with known amounts of the pure substance prepared mainly according to Brown [18]. For development of the chromatograms *iso*-butyric acid-0.5 N ammonia (10:6) and propionic acid-benzene-water (58:25:25) were used. UMP was determined from UV absorption data at 260 and 280 mμ of a aliquot of the collected UMP peak.

The extremely low yield of glucosamine phosphate obtained in this experiment seemed to be insignificant when considering a possible migration of the phosphoryl group from 1- to 6-position as a source of error. The small amount found may be due to an impurity in the UDP-glucosamine preparation obtained from yeast.

Isolation of phosphorylated peptides from the cell protein residue. If UDP-linked Mur or rather, similarly bound MurP or MurP-peptides, were intermediates in the biosynthesis of protein bound MurP this hypothesis would be supported by the isolation of phosphopeptides with the same amino acid content as in the UDP-linked peptides. Attempts were accordingly made to isolate such peptides from the partially hydrolyzed protein residues (11 N HCl at 37° during 3 days). In Fig. 3 a typical elution curve from a Dowex 50 column is presented for the protein hydrolysate from a 90 l culture of *L. casei* cultivated in the presence of penicillin. The peak after inorganic phosphate eluted between

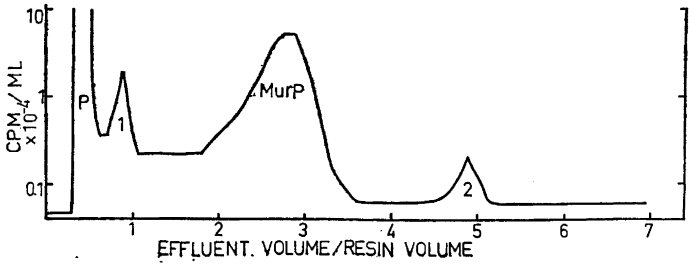


Fig. 3. Radioactivity curve from a hydrolysate of the protein residue from *L. casei* incubated for 4 h in a ^{32}P-containing medium. The curve is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube.

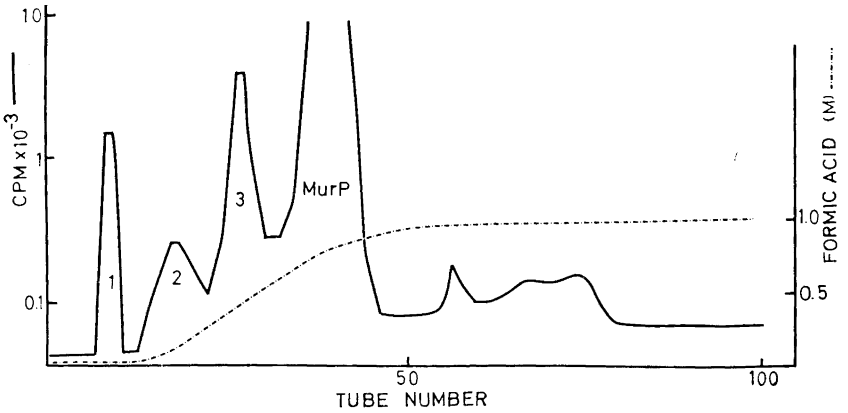


Fig. 4. Radioactivity curve from a mixture of MurP and MurP-peptides separated on a Dowex 1 column. The radioactivity curve (the continuous line) is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

0.72 to 0.96 column volumes (effluent volume/resin volume) and the MurP peak appearing between 1.9 to 3.3 column volumes were separately rechromatographed on Dowex 1 formate columns after removal of excess HCl (column dimensions 0.9 × 50 cm and 2 × 40 cm, respectively). A few times the peak emerging from the Dowex 50 column between 4.9 and 5.1 column volumes was also rechromatographed on the smaller type of Dowex 1 column. Fig. 4 gives a typical elution curve for the rechromatography of the MurP peak from the Dowex 50 column on a Dowex 1 formate column. Elution was carried out with the gradient 0 → 1 M formic acid with a volume of 200 ml in the mixer. The large peak appearing when the acidity of the eluate reached a value which corresponded to about 0.60 M formic acid was practically pure MurP. The peak 3 eluted somewhat earlier at about 0.40 M formic acid was homogeneous by analysis with inophoresis and chromatographic procedures. After hydrolysis of a small part of the material paper chromatographic analysis showed three strong spots corresponding to Mur, alanine and glutamic acid and five weaker spots. After rechromatography of the phosphopeptide on a small Dowex 50 (4 % DVB) column and hydrolysis the peptide contained only alanine and Mur. The first peak in Fig. 4 appearing with water was also homogeneous when analyzed with ionophoretic and chromatographic methods. After hydrolysis paper chromatographic analysis showed spots for Mur and alanine and possibly for glutamic acid. Compared with peak 3 there was less alanine in relation to Mur in the material from peak 1. The small amounts of material available did not permit rechromatography on a second Dowex 50 column. A peak in the same position as 1 could also be eluted from the MurP fractions obtained by hydrolysis in the usual way [10] and then separated on a Dowex 50 column. This material was not homogeneous when analyzed by paper chromatography or ionophoresis in 1 M acetic acid. Three different

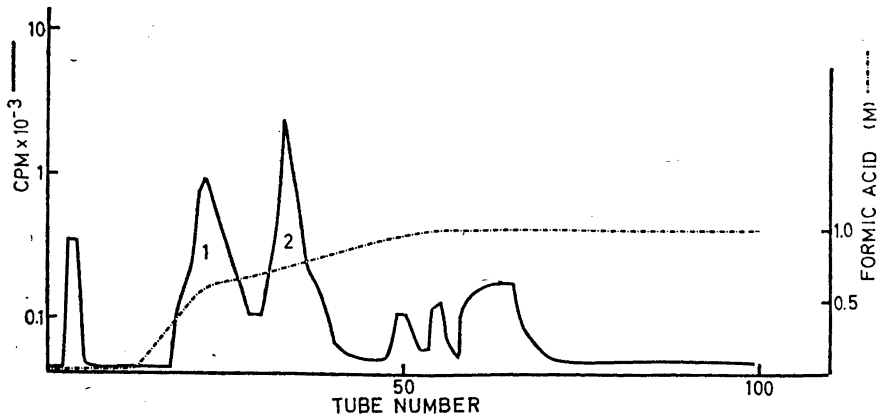


Fig. 5. Radioactivity curve from a mixture of MurP-peptides separated on a Dowex 1 column. The radioactivity curve is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

fractions were obtained. They all contained Mur, alanine and glutamic acid with about the same relation of Mur to alanine as in peak 1 (Fig. 4).

Fig. 5 demonstrates a typical Dowex 1 elution curve at the rechromatography of the material from peak No. 1 of the Dowex 50 curve (Fig. 3). The material corresponding to the two larger peaks 1 and 2 were homogeneous when analyzed with ionophoretic and paper chromatographic methods. After hydrolysis fraction 1 showed paper chromatographic spots corresponding to glutamic acid, alanine, Mur, valine, leucine and a spot in the position of γ-amino butyric acid. The amount of material corresponding to peak 1 allowed a rechromatography on a second Dowex 50 (4 % DVB) column. Only one radioactive fraction was obtained by elution with 0.01 N HCl. After hydrolysis of this material paper chromatographic analysis showed the presence of glutamic acid in reduced amounts, alanine, Mur and leucine. Fraction 2 from the Dowex 1 column (Fig. 5) was also hydrolyzed and analyzed by paper chromatography. Spots were obtained corresponding to the position of Mur, alanine and glutamic acid. Available amounts did not allow a rechromatography on a second Dowex 50 column.

The material from peak 2 on the Dowex 50 column (Fig. 3) was also rechromatographed on Dowex 1 columns. At least eight small radioactive fractions were obtained. The amounts of material in each fraction were extremely small and not further analyzed. A summary of the phosphopeptide compositions is given in Table 3. While a detailed sequence so far has not been established a certain agreement in amino acid composition between the different phosphopeptides can be noted.

Specific activity values of protein bound MurP. In Table 4 a summary is given of the MurP values obtained from different types of experiments. 30 mC ^{32}P per 90 liter of culture were used in experiments Nos. 1 to 6, otherwise 40 mC except in experiment Nos. 13 and 14 where 60 and 100 mC, respecti-

Table 3. ^{32}P-Phosphopeptides isolated from the protein residue of *Lactobacillus casei.*

Phosphopeptide obtained by separation on a Dowex 50 column (Fig. 3)	Further separation on Dowex 1 columns (Figs. 4 and 5)	Amino acid composition
Peak 1	Peak 1	Glu, Mur, Ala, Leu
	Peak 2	Glu, Mur, Ala
MurP	Peak 1	Mur, Ala
	Peak 3	Mur, Ala

vely, were used. In the series marked a and b each represents comparable 45 liter cultures. Bactopeptone and liver furnished sufficient total phosphorus (0.057 %) for normal growth. The corresponding value for inorganic phosphate was 0.024 % and the specific activity of inorganic phosphate in the medium

Table 4. Specific activities of protein bound MurP.

Expt. No.*	^{32}P added after **	Penicillin added after **	Time for harvesting **	mC ^{32}P added per litre of culture	Specific activity ***	Dry cell weight in g
1 a	0	3	4	0.33	4 700	20.5
1 b	2.5	3	4	0.33	2 600	25.0
2 a	0	3	4	0.33	2 320	21.7
2 b	3	3	4	0.35	1 700	26.0
3 a	0	3	4	0.33	2 320	13.7
3 b	0	—	4	0.33	1 700	13.0
4	0	2	3	0.33	2 120	40.5
5 a	0	3	4	0.33	2 030	21.5
5 b	0	—	4	0.33	2 900	23.5
6 a	0	0	0.25	0.33	15	7.1
6 b	0	—	0.25	0.33	32	7.2
6 c	0	0	1	0.33	32	7.2
6 d	0	—	1	0.33	2 290	7.3
6 e	0	0	2	0.33	98	7.2
6 f	0	—	2	0.33	1 820	10.5
7	0	—	18	0.44	3 640	78
8	0	—	18	0.44	3 930	72
9	0	—	18	0.44	4 080	75
10	0	—	18	0.44	2 900	70
11	0	—	18	0.44	4 620	73
12	0	—	18	0.44	4 400	98
13	0	—	18	0.66	6 730	114
14	0	—	4	1.1	14 400	42

* Experiments with the same figures are run in parallels and from the same batch of cells.
** All times are given in hours.
*** Counts per min and μg of phosphorus.

at the time of inoculations was 5 010 in one experiment where 1.1 mC ^{32}P per liter of medium was used.

In order to increase the utilization of isotopic phosphate added to the medium inorganic phosphate was excluded in all experiments. Experiments Nos. 3 and 5 are comparable pairs and demonstrate that penicillin in these series had no significant influence on specific activity values of MurP when added in the log phase of growth. A comparison with the corresponding TCA extracts also seem to show rather similar amounts of UDP-linked Mur in the presence or absence of penicillin. Likewise experiments 1 and 2 are comparable pairs. Since penicillin had no influence on specific activity values in experiments Nos. 3 and 5 these series seem to show a dilution of radioactive MurP with already synthesized unlabeled or slightly labeled material. Whether MurP-phosphoproteins take an active part in exchange and transfer reactions or MurP represents structurally bound MurP cannot be decided from these figures only [19].

The experiments in series No. 6 were carried out in the same manner as by Strominger [6] with material from 6 comparable 15 liter cultures. The effect of penicillin on the specific activity is rather striking. The weight figures in Table 4 seem to indicate that when penicillin is present there is no growth and only a wery small incorporation of radioactivity in MurP. In the controls the weight figures and the photometer readings in Table 1 seem to indicate that as usual with the amount of inocula used the limit between lag phase and log phase is found after 1 h of incubation. Of special interest is the high specific activity in MurP at this time when it is questionable whether any new cell walls have been formed. The results are not in agreement with those obtained by Trucco and Pardee [20]. They studied the incorporation of uniformly labeled ^{14}C glucose in different cell fractions of *E. coli* up to 180 min of incubation and did not find any differences between specific activity values in controls and penicillin cultures for cell walls, cytoplasm or unbroken cells.

The specific activity of MurP from UDP-linked MurP was determined in experiment No. 5. To obtain sufficient amount of material all UDP peaks after ADP from 5a and 5b (Fig. 1) were pooled and MurP was isolated as previously described. The specific activity of this MurP phosphorus was 5 070 as compared with the mean value of 2 470 of the protein bound MurP phosphorus. This means that UDP-linked MurP could be a precursor to protein bound MurP.

DISCUSSION

With the method described in this paper it has been possible to isolate also from penicillin inhibited *L. casei* UDP-linked Mur-peptides of similar composition as previously found by Park and Strominger [5] in *S. aureus* and *L. helveticus*. Nucleotides of this type isolated from normal *L. casei* seemed to be identical with or closely related to the nucleotides from penicillin inhibited cultures. These observations suggest that the nucleotide bound peptides are normal metabolites and that they are themselves not toxic to the cell. The occurrence of these compounds in several species also suggests that they may have some general significance in bacterial metabolism.

The amino acids present in the UDP-linked Mur-peptides were of considerable interest. So far only glutamic acid, alanine, and Mur have been identified while a fourth amino acid also present is still of unknown character. According to Cummins and Harris [21] a hydrolysate of the bacterial cell wall of *L. casei* contained the following amino acids: aspartic acid, glutamic acid, alanine, Mur, and lysine. The unknown amino acid in our UDP-linked Mur-peptide was not identical with diaminopimelic acid or lysine which was found by Park [22] in his UDP-linked peptide by side of alanine, muramic acid and glutamic acid, also present in our UDP-linked peptide.

Under our experimental conditions there was usually no accumulation of UDP-linked peptides as a consequence of penicillin inhibition. That may be due to the fact that penicillin was added rather late in the log phase of growth when the effect of penicillin could be expected to act only for a comparatively short time. The conditions of experiment 6 might have been more favourable for studying an accumulation of UDP-linked peptides.

Park and Strominger [5] have suggested that the UDP-linked Mur-peptides are biosynthetic precursors of the bacterial cell wall and accumulate in penicillin treated *S. aureus* as a conseuqence of the interference by penicillin with the biosynthesis of the cell wall. From our point of view the biosynthesis of phosphoproteins is of general interest and with regard to the cell wall composition it has previously been demonstrated that about 80 % of the phosphoproteins in *E. coli* are located in the cell wall protein fraction [23]. It has previously been pointed out that UDP-linked MurP could be an intermediate in the biosynthesis of protein bound MurP. Of considerable interest was therefore the isolation of UDP-linked MurP with higher specific activity than protein bound MurP.

If it is assumed that UDP-linked Mur-peptides are in equilibrium with protein bound MurP-peptides it could be expected that phosphopeptides with a similar composition should be isolated from the hydrolyzed cell protein residue, containing the phosphoproteins. This actually was the case. One phosphopeptide containing Mur and alanine was found and a second one contained Mur, alanine and glutamic acid.

Some preliminary experiments have been carried out in an attempt to elucidate how a phosphorylation of Mur could be effected. Lyophilized cells of *L. casei* were disintegrated with a Raytheon magnetostriction oscillator and different fragment fractions were separated by differential centrifugation at 2 C°. Media containing the different fragment fractions, Mur, AT^{32}P, and magnesium ions were incubated for different times, but so far Mur^{32}P could not be isolated. There may be two explanations of the failure to find a muramic acid kinase. Either the phosphorylation may only take place when Mur is activated by a nucleotide linkage or the phosphorylation may occur before the muramic acid moities are fused together.

This investigation was supported by grants from the *Swedish Medical Research Council.*

REFERENCES

1. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Soc. Med. Upsaliensis* **60** (1955) 30.
2. Ågren, G., de Verdier, C.-H. and Glomset, J. *Rev. fermentations et inds. aliment.* **11** (1956) 234.
3. Ågren, G. and de Verdier, C.-H. *Acta Chem. Scand.* **12** (1958) 1927.
4. Park, J. T. and Johnson, M. J. *J. Biol. Chem.* **179** (1949) 585.
5. Park, J. T. and Strominger, J. L. *Science* **125** (1957) 99.
6. Strominger, J. L. *J. Biol. Chem.* **224** (1957) 509.
7. Hurlbert, P. B., Schmitz, H., Brumm, A. F. and Potter, V. R. *J. Biol. Chem.* **209** (1954) 23.
8. Flavin, M. *J. Biol. Chem.* **210** (1954) 771.
9. *Pabst Laboratories Circular* OR-10, Milwaukee 1956.
10. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1570.
11. de Verdier, C.-H. and Ågren, G. *Acta Chem. Scand.* **2** (1948) 783.
12. Engström, L. *Acta Soc. Med. Upsaliensis* **63** (1958) 128.
13. Ågren, G. *Acta Soc. Med. Upsaliensis* **63** (1958) 137.
14. Caputto, R., Leloir, L. F., Cardini, C. E. and Paladini, A. C. *J. Biol. Chem.* **184** (1950) 333.
15. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 503.
16. Strominger, J. L. *Biochim et Biophys. Acta* **17** (1955) 283.
17. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
18. Brown, D. H. *Methods of Enzymology*, New York 1957, Vol. 3, p. 158.
19. Ågren, G. *Acta Univ. Upsaliensis* **1958** No. 5, p. 1
20. Trucco, R. E. and Pardee, A. B. *J. Biol. Chem.* **230** (1958) 434.
21. Cummins, C. S. and Harris, H. *J. Gen. Microbiol.* **14** (1956) 5831.
22. Park, J. T. *J. Biol. Chem.* **194** (1952) 897.
23. Ågren, G. *Acta Chem. Scand.* **10** (1956) 152.

Received May 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1437–1452

On the Complex Chemistry of the Tervalent Rare-Earth Ions

IV. Ion-Exchange Studies of the Gadolinium Acetate and Glycolate Systems

ARTUR SONESSON

Department of Inorganic and Physical Chemistry, Chemical Institute, University of Lund Lund, Sweden

The gadolinium acetate and glycolate systems have been investigated by means of both cation and anion exchangers. The measurements have been performed at 20°C using the radioactive isotope ^{153}Gd. For the investigation with cation exchanger solutions with the constant ionic strength $I = 1.0$ M have been used ($NaClO_4$ was added). The solutions used for the investigation with the anionic exchanger had varying ionic strength.

The complexity constants obtained for both the acetate system and the glycolate system by means of the cation-exchange method are in good agreement with the constants obtained potentiometrically (pages 1444 and 1446). Anionic complexes, however, could not be proved by the cation-exchange method.

The investigation of the glycolate system by means of an anion exchanger confirms the existence of anionic glycolate complexes. The maximum point of the φ-curve (Fig. 3) indicates that $\bar{n} = 3$ in a water solution having $[AcOH^-] \approx 100$ mM, which is quite in accordance with the potentiometric measurements. The ligand numbers computed from data obtained by the anion exchange method are in good agreement with the ligand numbers obtained potentiometrically in the whole region investigated. Attempts have also been made to compute the stability constants.

According to the measurements with the anion exchanger no anionic complexes should be formed in the gadolinium acetate system for $[Ac^-] < 800$ mM (Fig. 3). However, the low φ-values may also be caused by steric effects which depress the sorption of the acetate complexes into the exchanger below measurable values.

In the Parts I, II, and III (Sonesson [1–3]) we have given an account of our investigations of the complex formation in acetate and glycolate solutions of some rare earths. The complexity constants have been computed from the hydrogen-ion concentration of the complex solutions. It is not

possible in investigations of this kind to eliminate completely the errors caused by the variation of the activity factors when the perchlorate ions of the initial solution are replaced by acetate or glycolate ions. The constants computed from data obtained at high ligand concentrations are especially uncertain. Therefore it is necessary to check the results obtained potentiometrically by new investigations, which are independent of the potentiometric measurements.

The erbium acetate system and the neodymium glycolate system have been examined extinctiometrically as well as potentiometrically. The accordance between the complexity constants obtained potentiometrically and those obtained extinctiometrically is good, but the constants obtained by the latter method must be considered as relatively uncertain. Therefore investigations by means of ion exchangers have also been performed.

Fronæus [4] has used a cation exchanger for an examination of the cerium acetate system. For the first three complexity constants the accordance between the values obtained by the cation-exchange method and those obtained by the potentiometric method was very good. The present author has used the method given by Fronæus for the examination of the acetate and the glycolate systems of gadolinium.

The formation of anionic complexes in solutions containing cerium and acetate ions has also been examined by Fronæus [5] by means of anion exchange. According to the potentiometric measurements the ligand number exceeds three when $[Ac^-] > 500$ mM. In the examination with the anion exchanger anionic complexes could not be proved, although $[Ac^-]$ was increased to about 1 000 mM. Now both the acetate and the glycolate systems of gadolinium have been examined by means of an anion exchanger.

A. INVESTIGATION BY MEANS OF A CATION EXCHANGER

Theoretical

The method employed in the present investigation has been developed by Fronæus [4].

The same notations as in the previous Parts I, II and III have been used as much as possible, but in some cases the definitions of the symbols have been altered in some respects.

C'_M, C'_A = the total concentrations of the rare earth and the ligand, respectively, in the solution before the exchanger is added.

C_M = the total concentration of the rare earth in the solution at equilibrium with the exchanger.

$[M^{3+}]$, $[A^-]$ = the concentration of free central ion and free ligand, respectively, in the solution at equilibrium.

Then, as earlier, we have

$$C_M = [M^{3+}](1 + \sum_{j=1}^{N} \beta_j \cdot [A^-]^j) = [M^{3+}] \cdot X \qquad (1)$$

In addition the following notations are introduced:

$[M^{3+}]_R$, $[MA^{2+}]_R$, etc. = the concentrations in the resin phase at equilibrium with the solution. The concentrations are expressed in moles per gram of the air-dried resin.

β_{jR} = the complexity constants for the complexes MA_{jR}^{3-j} in the resin.

C_{MR} = $[M^{3+}]_R + [MA^{2+}]_R + [MA_2^+]_R$ =

$$= [M^{3+}]_R(1 + \beta_{1R} \cdot [A^-]_R + \beta_{2R} \cdot [A^-]_R^2) \quad (2)$$

$\bar{n}_R$ = the ligand number in the resin. For the cation exchanger we have

$$\bar{n}_R = ([MA^{2+}]_R + 2\ [MA_2^+]_R) \cdot C_{MR}^{-1} \quad (3)$$

m = the weight in grams of the air-dried resin.

v = the volume of the solution before the resin is added.

$v \cdot \delta$ = the volume at equilibrium with the resin. δ is a factor caused by the swelling of the resin.

The following equilibrium is obtained between a cation exchanger in the sodium form and a solution containing rare-earth ions M^{3+} and complex ions MA_j^{3-j}:

$$(3\text{-}j)\ Na_R^+ + MA_j^{3-j} \rightleftharpoons (3-j)\ \overline{Na}^+ + MA_{jR}^{3-j};\ (j = 0,\ 1,\ 2)$$

If we apply the law of mass action to this exchange equilibrium, we get

$$\frac{[MA_j^{3-j}]_R}{[MA_j^{3-j}]} = k_j \frac{[Na^+]_R^{3-j}}{[Na^+]^{3-j}} = l_j;\ (j = 0,\ 1,\ 2) \quad (4)$$

In the cation-exchange investigations described in this paper the total concentration of the rare earth is very low ($C_M \leq 3.0$ mM, $C_{MR} < 0.07$ mmole·g^{-1}). Therefore both $[Na^+]$ and $[Na^+]_R$ have been approximately constant. The load C_{MR} has been very low compared with the total exchange capacity (≈ 3.85 mequiv. g^{-1}) and the ionic strength of both the exchanger and the water phase has been nearly constant. In the calculations all values have been referred to a constant value of C_{MR} and then both k_j and l_j may be considered approximately constant.

If we denote by φ the distribution of the rare earth between the resin phase and the solution, then

$$\varphi = \frac{C_{MR}}{C_M} = l_o \frac{1 + l_1' \cdot [A^-] + l_2' \cdot [A^-]^2}{X} \quad (5)$$

$$l_o = \frac{[M^{3+}]_R}{[M^{3+}]}\ ;\ l_j' = \beta_j \frac{l_j}{l_o}\quad (j = 1,\ 2) \quad (6)$$

To facilitate the calculations Fronæus introduced two new parameters

$$\varphi_1 = \frac{l_o \cdot \varphi^{-1} - 1}{[A^-]} \quad (7)$$

$$f = \frac{l_o \cdot \varphi^{-1}[(\beta_1 - l_1')\ [A^-] - 1] + 1}{[A^-]^2} \quad (8)$$

It can be shown by combining eqns. (5), (7) and (8) that

$$f = \beta_1 \cdot \varphi_1 - X_2 + l_2' \cdot l_o \cdot \varphi^{-1} \tag{9}$$

After having introduced the value of φ from eqn. (5) into eqns. (7) and (8), we get

$$\lim_{[A^-]\to 0} \varphi_1 = \varphi_1^\circ = \beta_1 - l_1' \tag{10}$$

$$\lim_{[A^-]\to 0} f = f^\circ = \beta_1 \cdot \varphi_1^\circ - (\beta_2 - l_2') \tag{11}$$

From eqns. (9), (10) and (11) it can be deduced that

$$\left.\begin{array}{c} \Delta f \cdot [A^-]^{-1} = \beta_1 \cdot \Delta\varphi_1 [A^-]^{-1} - \beta_3 - X_4[A^-] + l_2'\varphi_1 \\ \Delta f = f - f^\circ \text{ and } \Delta\varphi_1 = \varphi_1 - \varphi_1^\circ \end{array}\right\} \tag{12}$$

The last two terms of (12) vary with $[A^-]$. At low values of $[A^-]$, however, they are only correction terms and we can calculate an approximate value of β_1 by plotting $\Delta f \cdot [A^-]^{-1}$ as a function of $\Delta\varphi_1 \cdot [A^-]^{-1}$.

Now β_1 being known, an approximate value of $\beta_2 - l_2'$ can be calculated by means of eqn. (11). Then a new parameter g is introduced

$$g = \frac{f - \beta_1\varphi_1 + l_o \cdot \varphi^{-1}(\beta_2 - l_2')}{[A^-]} = \beta_2\varphi_1 - \beta_3 - X_4 \cdot [A^-] \tag{13}$$

At so low $[A^-]$ that X_4 can be neglected, g is a linear function of φ_1. The slope of the line gives β_2, and the intercept an approximate value of β_3.

X_2 can be computed according to eqn. (9), but we must know l_2'. As l_2' seems to be very small compared with β_2, we get only a very rough value of l_2' from β_2 and $\beta_2 - l_2'$. Another way of computing l_2' is shown by Frønæus [4, p. 20]. With the assumption that $\beta_{1R}^2 \cdot \beta_{2R}^{-1}$ has about the same value as $\beta_1^2 \cdot \beta_2^{-1}$ it can be deduced that

$$\frac{\beta_1^2}{\beta_2} = \frac{(l_1')^2}{l_2'} \tag{14}$$

For the glycolate system eqn. (12) could not be used for the computation of β_1, as $\Delta\varphi_1 \cdot [A^-]^{-1}$ and $\Delta f \cdot [A^-]^{-1}$ are approximately constant. However, if only four complexes are formed, eqn. (12) can be written

$$\beta_1 = \frac{\Delta f}{\Delta\varphi_1} + \beta_3 \cdot \frac{[A^-]}{\Delta\varphi_1} + \beta_4 \cdot [A^-] \cdot \frac{[A^-]}{\Delta\varphi_1} - l_2'\varphi_1 \cdot \frac{[A^-]}{\Delta\varphi_1} \tag{15}$$

The first term, $\Delta f \cdot \Delta\varphi_1^{-1}$, dominates. Then, an approximate value of β_1 can be calculated if rough values of β_3, β_4 and l_2' are known.

All the equations above have been deduced on the assumption that only mononuclear complexes are formed both in the solution and in the resin. The

potentiometric measurements on the $Gd^{3+}-Ac^{-}$-system reported in Part I showed a slight tendency for formation of polynuclear complexes. However, the tendency is so slight that it may be neglected in the present investigation, as both C_M and C_{MR} are very low.

Experimental

Chemicals used. A known amount of radioactive Gd_2O_3 (from A.E.R.E., Harwell, England) was dissolved in perchloric acid. After the addition of a known amount of inactive gadolinium perchlorate, the solution was evaporated to remove the excess perchloric acid.

The concentration of the stock solution of radioactive gadolinium perchlorate was calculated from the known amounts of radioactive and inactive materials. The stock solution contained $NaClO_4$ to make the ionic strength 1.0 M.

The cation exchanger was Dowex 50 converted into the sodium form and air-dried. The same batch was used for all of the measurements. The exchange capacity was 3.85 mequiv. g^{-1}.

The value of the factor δ was determined by shaking a solution with a known concentration of $NaClO_4$ and a known amount of the resin until equilibrium was reached. Then the value of δ was given by the ratio between the initial and the equilibrium sodium-ion concentrations, which were determined by means of a cation-exchanger column in the hydrogen state. δ was determined to be 0.985.

Other chemicals were of analytical grade as in Parts I, II, and III.

The apparatus. The total gadolinium concentration of the solutions was determined by measurement of the gamma activity of ^{153}Gd. A scintillation detector type P-20 from Tracerlab was used. The scintillation tube was immersed to a fixed depth in the centre of a lead shielding. A glass cup containing 10 ml of the solution to be measured was placed in a fixed place in the lead shielding. A satisfactory reproducibility of the geometry was assured. A solution of radioactive $Gd(ClO_4)_3$ was diluted with varying amounts of 1 M $NaClO_4$ and the solutions were counted. It was found that the relation between the cpm and the gadolinium concentration was linear within the limits of the statistical errors ($\sim$ 1 %).

The gadolinium acetate system

The complex solutions to be measured were prepared by mixing two solutions, S_1 and S_2, in varying proportions.

$$S_1 = \begin{cases} C_M^\circ \text{ mM } Gd(ClO_4)_3 \\ C_H^\circ \text{ mM } HClO_4 \\ 1000 \text{ mM } NaClO_4 \end{cases} \quad \text{and} \quad S_2 = \begin{cases} C_{HA}^\circ \text{ mM HAc} \\ C_A^\circ \text{ mM NaAc} \\ (1\,000 - C_A^\circ) \text{ mM } NaClO_4. \end{cases}$$

Four S_1 solutions were used with C_M° = 0.566, 1.010, 2.020 and 3.010 mM, respectively. All solutions had $C_H^\circ \approx 0.03$ mM. At this pH the hydrolysis of the gadolinium ions can be neglected, as shown in Part I.

One buffer solution had $C_{HA}^\circ = C_A^\circ = 500$ mM, and the other $C_{HA}^\circ = C_A^\circ = 1\,000$ mM.

A fixed volume (v liters) of the complex solution was shaken (about 20 h) in a thermostat at 20°C with a fixed amount (m gram) of the dried resin. The value of $v \cdot m^{-1}$ was kept at 0.025 l · g^{-1}. After exchange equilibrium was reached, the resin phase was separated from the solution. The equilibrium concentration C_M was computed by comparing the radioactivity of 10 ml of the complex solution with the radioactivity of 10 ml of solution S_1.

Table 1. Cation-exchange measurements on the acetate system.

$C'_A \cdot \delta^{-1}$ mM	C'_M mM	$C_{MR} \times 10^3$ mmole·g^{-1}	$\varphi \times 10^3$ l·g^{-1}	C'_M mM	$C_{MR} \times 10^3$ mmole·g^{-1}	$\varphi \times 10^3$ l·g^{-1}	C'_M mM	$C_{MR} \times 10^3$ mmole·g^{-1}	$\varphi \times 10^3$ l·g^{-1}	C'_M mM	$C_{MR} \times 10^3$ mmole·g^{-1}	$\varphi \times 10^3$ l·g^{-1}
0	0.566	13.1	320	1.010	23.4	314	2.020	46.6	293	3.010	69.1	277
8.32	0.557	12.4	207	0.995	22.2	204	1.985	44.1	191			
16.00	0.557	12.0	148	0.995	21.2	143	1.990	42.1	134			
22.60	0.541	11.1	114.5	0.965	19.8	112	1.930	39.2	107.5			
32.75	0.548	10.5	81.0	0.975	18.4	79.8	1.955	37.0	76.6			
49.1	0.511	8.65	51.5	0.910	15.3	50.4	1.825	30.4	49.1			
67.7	0.528	7.65	34.0	0.945	13.5	33.0	1.885	27.0	32.9			
101.5	0.453	4.78	18.0	0.810	8.55	18.1	1.615	16.9	17.8	2.410	26.0	18.6
135.3				0.875	6.50	10.4	1.750	13.5	11.0	2.610	19.6	10.6
169.2				0.675	3.72	7.0	1.345	7.70	7.3	2.005	11.25	7.2
217.5				0.795	2.80	4.0	1.585	6.00	4.4	2.365	11.15	5.5

The results for the four series are shown in Table 1. C_{MR}, the total equilibrium concentration of gadolinium in the resin phase, has been calculated according to the equation

$$C_{MR} = v \cdot m^{-1}(C'_M - \delta \cdot C_M) \qquad (16)$$

As φ varies with C_{MR}, all φ-values used in the calculations must be referred to a constant value of C_{MR}. The relation between φ and C_{MR} at a constant value of $C'_A \cdot \delta^{-1}$ proves to be practically linear. Then φ in Table 2 has been determined by graphic interpolation for $C_{MR} = 10 \times 10^{-3}$ mmole . g^{-1}.

[A$^-$], the equilibrium concentration of the free ligand in the solution, must be known before the functions φ_1 and f can be computed. We know the total concentration C'_A before the resin is added and must, when computing [A$^-$], correct for:

1) ligand ions bound in the complexes MA_j^{3-j} in the solution (correction term: $a_1 = \bar{n} \cdot C_M$),
2) ligand ions bound in the complexes MA_R^{2+} and MA_{2R}^{+} in the resin phase (correction term: $a_2 = \bar{n}_R \cdot C_{MR} \cdot m \cdot v^{-1} \cdot \delta^{-1}$),
3) ligand ions produced by the protolysis of the acid HA (the correction term a_3 can be neglected in this investigation of the acetate system as the starting pH of the solutions to be measured is about 4.5),
4) ligand ions produced when hydrogen ions replace sodium ions in the resin (as the acetate buffers have pH $\approx$ 4.8, this correction term, a_4, can be neglected in the investigation of the acetate system).

Then we have

$$[A^-] + \bar{n} \cdot C_M + \bar{n}_R \cdot C_{MR} \cdot m \cdot v^{-1} \cdot \delta^{-1} = C'_A \cdot \delta^{-1} + a_3 + a_4 \qquad (17)$$

As both C_M and C_{MR} are low, the terms a_1 and a_2 are small compared with [A$^-$]. Then it may be allowed as a first approximation to consider $[A^-] = C'_A \cdot \delta^{-1}$.

However, as we have already examined the gadolinium system potentiometrically (Part I), we can use the potentiometrically obtained values of $\bar{n}$ for the computation of $\bar{n} \cdot C_M$.

In the second correction term $\bar{n}_R$ can be computed according to Fronæus [6] by means of the formula

$$\bar{n} - \bar{n}_R = - \frac{[A^-]}{\varphi} \left(\frac{\partial \varphi}{\partial [A^-]} \right) C_{MR} \approx - \frac{C'_A}{\varphi} \left(\frac{\partial \varphi}{\partial C'_A} \right) C_{MR} \qquad (18)$$

The second approximate expression has been used for the computation of $\bar{n}_R$.

Table 2. The cation-exchange measurements on the acetate system. The corresponding values of φ, $[A^-]$ and the functions formed from these quantities are valid for the constant load on the resin of $C_{MR} = 10 \times 10^{-3}$ mmole·g^{-1}. (4), (18), *etc.* refer to the equations with these numbers

$C'_A \cdot \delta^{-1}$ mM	$\varphi \times 10^3$ l.g^{-1}	(4) C_M mM	$\bar{n}_{pot}$	(18) $\bar{n}_R$	(17) $[A^-]$ mM	(7) φ_1 M^{-1}	$\Delta\varphi_1$ / $[A^-]$ M^{-2}	(8) f M^{-2}	Δf / $[A^-]$ 10^{-2} M^{-3}	(13) $g \times 10^{-3}$ M^{-3}	$X_3 \times 10^{-3}$ M^{-3}
0	323	0.0310	0	0	0	*58.9*		*2 530*			~ *9*
8.32	208	0.0480	0.45	0.08	8.27	66.9	965	2 975	540	77.4	
16.00	148	0.0675	0.75	0.11	15.90	74.4	975	3 410	555	89.6	
22.60	114.5	0.0875	0.94	0.12	22.45	81.1	990	3 790	560	98.9	
32.75	81.0	0.1235	1.17	0.15	32.55	91.8	1 010	4 400	575	113.3	
49.1	51.2	0.1955	1.43	0.22	48.8	108.8	1 025	5 385	585	136.5	10.4
67.7	33.5	0.299	1.65	0.24	67.1	129	1 045	6 545	600	163	11.0
101.5	18.1	0.552	1.91	0.25	100.3	168	1 085	8 805	625	215.5	11.2
135.3	11.0	0.909	2.12	0.34	133.1	213	1 155	11 400	670	275.5	11.9
169.2	7.2	1.39	2.27	0.4	166	264	1 235	14 330	710	343.5	12.9
217.5	5	2.0	2.43	0.8	212	300	1 135	16 540	660	410	

φ_1° and f° in Table 2 have been determined by graphic extrapolations of φ_1 and f, respectively, to $[A^-] = 0$. The extrapolations could be performed with rather great accuracy.

As seen in Fig. 1, $\Delta f \,.\, [A^-]^{-1}$ is approximately a linear function of $\Delta\varphi_1 \cdot [A^-]^{-1}$. The slope of the line gives $\beta_1 = (66 \pm 3)$ M^{-1}.

According to eqn. (11) we obtain $\beta_2 - l'_2 \approx 1\,350$. Then g is computed by means of eqn. (13). The slope of the line in Fig. 2 gives $\beta_2 = 1\,347$ and the intercept gives $\beta_3 \sim 9\,000$. Then X_3 is computed from the expression $X_3 = \beta_2 \,.\, \varphi_1 - g$. The extrapolation of X_3 to $[A^-] = 0$ gives also $\beta_3 \sim 9\,000$.

X_3 seems to increase somewhat with increasing values of $[A^-]$, which should indicate the formation of a fourth complex. However g is a quite linear function of φ_1 in whole the region, indicating that X_4 is negligible. Therefore, the formation of anionic complexes is very uncertain.

The accordance is good between the complexity constants obtained by the potentiometric method and those obtained by the cation-exchange method.

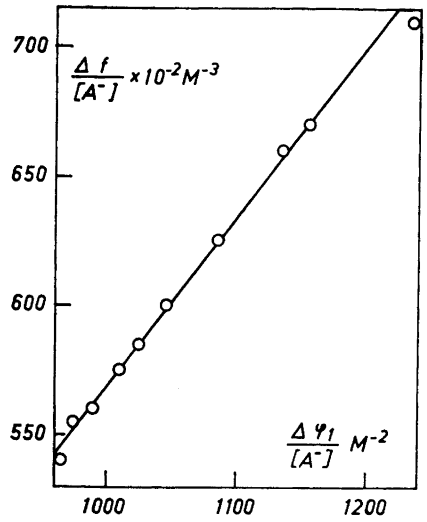


Fig. 1. The cation-exchange measurements on the acetate system. $\Delta f \cdot [A^-]^{-1}$ is represented as a function of $\Delta\varphi_1 \cdot [A^-]^{-1}$ for acetate-ion concentrations between 8 and 218 mM. The slope of the line gives β_1.

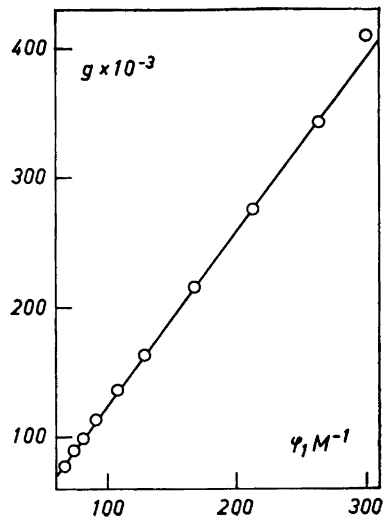


Fig. 2. The cation-exchange measurements on the acetate system. g is represented as a function of φ_1 for acetate concentrations between 8 and 218 mM. The slope of the line gives β_2 and the intercept for $\varphi_1 = 0$ gives $-\beta_3$.

	Potentiometric measurements. I = 2.0 M.	Cation-exchange measurements. I = 1.0 M	
β_1:	69 ± 2	66 ± 3	M^{-1}
β_2:	1 320 ± 150	1 350 ± 250	M^{-2}
β_3:	5 200 ± 1 000	9 000 ± 3 000	M^{-3}
β_4:	~ 5 000	— — —	M^{-4}

The gadolinium glycolate system

The complex solutions were obtained by mixing the solutions S_3 and S_4 in varying proportions:

$$S_3 = \begin{cases} C_M^\circ \text{ mM } Gd(ClO_4)_3 \\ C_H^\circ \text{ mM } HClO_4 \\ 1\,000 \text{ mM } NaClO_4 \end{cases} \quad \text{and} \quad S_4 = \begin{cases} C_{HA}^\circ \text{ mM HAcOH} \\ C_A^\circ \text{ mM NaAcOH} \\ (1\,000 - C_A^\circ) \text{ mM } NaClO_4 \end{cases}$$

The measurements were performed with three S_3 solutions having C_M° = 0.550, 1.000 and 2.000 mM and C_H° = 0.03 mM. The buffer solution S_4 had $C_{HA}^\circ = C_A^\circ$ = 300 mM. The investigation was performed exactly in the same way as for the acetate system.

The results of the measurements on the glycolate system are shown in Table 3. For the calculation of $[A^-]$ at equilibrium eqn. (17) was used. The potentiometrically obtained values of $\bar{n}$ were used in the computation of the

Table 3. The cation-exchange measurements on the glycolate system.

$C_M^\circ \rightarrow$	0.550 mM		1.000 mM		2.000 mM		$C_{MR} = 10 \times 10^{-3}$ mM				
$C'_A \cdot \delta^{-1}$ mM	$C_{MR} \times 10^3$ mmole · g^{-1}	$\varphi \times 10^3$ $l \cdot g^{-1}$	$C_{MR} \times 10^3$ mmole · g^{-1}	$\varphi \times 10^3$ $l \cdot g^{-1}$	$C_{MR} \times 10^3$ mmole · g^{-1}	$\varphi \times 10^3$ $l \cdot g^{-1}$	$\varphi \times 10^3$ $l \cdot g^{-1}$	(4) C_M mM	$\bar{n}_{pot}$	(18) $\bar{n}_R$	(17) $[A^-]$ mM
0	12.75	322					323	0.0310			0
1.213	12.30	216	22.3	215			216	0.0465	0.35	0.02	1.380
2.815	11.75	151.5	21.4	152.5			151	0.0660	0.66	0.06	2.97
4.995	10.90	102.5	19.9	106			102	0.0980	0.96	0.08	5.10
7.14	10.05	73.1	18.4	75.3			73.0	0.1370	1.18	0.12	7.17
9.83	9.00	52.7	16.7	54.9			53.0	0.189	1.40	0.25	9.70
14.51	7.50	32.7	13.80	33.9			33.2	0.301	1.66	0.30	14.15
20.30	5.75	20.0	10.80	21.4	22.5	22.9	21.0	0.475	1.90	0.40	19.50
29.60	3.85	11.1	7.35	11.9	15.95	13.5	12.4	0.805	2.13	0.55	27.90
40.6			4.90	7.2	10.35	7.7	7.7	1.30	2.33	0.75	37.5
50.8			3.15	4.4	7.05	5.0	5.5	1.82	2.46	0.9	46.2
60.9			2.25	3.1	5.05	3.6	4.3	2.33	2.60	1.0	54.7
71.0			1.60	2.3	3.90	2.8					

correction term a_1. An approximate value of $\bar{n}_R$ has been determined according to eqn. (18). For the glycolate system a_3 and a_4 cannot be neglected for the lowest values of C'_A. For the ionic strength $I = 1.0$ M the acid constant of glycolic acid is 2.4×10^{-4} M (*cf.* Fronæus [7, p.67]). Using this value we get $a_3 = 0.16$ mM for $[A^-] = 1.4$ mM and $a_3 = 0.20$ mM for $[A^-] = 28$ mM. The correction term a_4 has been computed on the assumption that $[H^+]_R \cdot [H^+]^{-1} \approx [Na^+]_R \cdot [Na^+]^{-1}$. Then we get $a_4 \approx 0.03$ mM.

β_1 for the glycolate system has been computed by means of eqn. (15) instead of eqn. (12) as both $\Delta f \,.\, [A^-]^{-1}$ and $\Delta\varphi_1 \,.\, [A^-]^{-1}$ are approximately constant. For β_3 and β_4 the potentiometrically obtained constants have been used. A probable value of l'_2 has been calculated by means of eqn. (14) in the following way. We know that $\varphi_1^\circ = \beta_1 - l'_1 = 322$ M^{-1}. In most cases l'_1 is about 10 % of β_1. Then we have assumed that $l'_1 \approx 30$ M^{-1} and $\beta_1 \approx 350$ M^{-1}. As $f^\circ = \beta_1 \cdot \varphi_1^\circ - (\beta_2 - l'_2) = 82\,500$ we can write $\beta_2 \approx \beta_2 - l'_2 \approx 30\,000$ M^{-2} (in most cases $l'_2 \ll \beta_2$). This value of β_2 is in good accordance with the potentiometric value. Then eqn. (14) gives $l'_2 \approx 200$ M^{-2}.

As seen in Table 4 the β_1-values calculated by means of eqn. (15) are very close to 350. It should be emphasized that l'_2 has only a slight influence on β_1. Therefore the values of β_1 calculated from eqn. (15) are practically independent of the value of l'_1 assumed above.

$\beta_1 = 350$ M^{-1} gives $\beta_2 - l'_2 = 29.5 \times 10^3$ M^{-2}. Then β_2 can be determined by means of eqn. (13). The function $g(\varphi_1)$ seems to be linear over the whole region and therefore X_4 must be negligible for $[A^-] < 55$ mM. The slope of

Table 4. The cation-exchange measurements on the glycolate system. The values of $[A^-]$, φ and the various functions are valid for $C_{MR} = 10 \times 10^{-3}$ mmole.g^{-1}. In calculating β_1 the potentiometric obtained values $\beta_3 = 62 \times 10^4$ and $\beta_4 = 26 \times 10^5$ have been used.

$[A^-]$ mM	$\varphi \times 10^3$ l . g^{-1}	φ_1 M^{-1}	$f \times 10^{-2}$ M^{-2}	β_1 from eqn. (15)	(13) $g \times 10^{-4}$ M^{-3}
0	323	*322*	*825*		
1.40	216	353	915		
3.00	150	385	1 030	325 + 31 + 0 − 4 = 352	1 060
5.00	103.5	424	1 165	333 + 32 + 1 − 4 = 362	1 205
7.00	75.5	468	1 300	325 + 31 + 1 − 5 = 352	1 320
10.00	51.2	531	1 500	323 + 31 + 1 − 5 = 350	1 505
14.00	33.5	617	1 775	322 + 31 + 2 − 6 = 349	1 755
20.0	20.3	746	2 190	322 + 31 + 3 − 7 = 349	2 135
28.0	12.3	902	2 700	323 + 31 + 4 − 9 = 349	2 605
38.0	7.5	1 107	3 360	323 + 31 + 5 − 11 = 348	3 210
46.0	5.5	1 255	3 840	323 + 32 + 6 − 12 = 349	3 645
55.0	4.2	1 380	4 265	325 + 34 + 7 − 14 = 352	4 025

the line gives $\beta_2 = 29.5 \times 10^3$ M^{-2}, and the intercept for $\varphi_1 = 0$ gives $\beta_3 \approx 70 \times 10^4$ M^{-3}.

The values of β_1, β_2 and β_3 obtained in this investigation are in good accordance with those obtained potentiometrically.

	Potentiometric measurements. $I = 2.0$ M.	Cation-exchange measurements. $I = 1.0$ M.	
β_1:	300 ± 15	350 ± 20	M^{-1}
β_2:	$(27 \pm 4) \times 10^3$	$(30 \pm 4) \times 10^3$	M^{-2}
β_3:	$(62 \pm 8) \times 10^4$	$(70 \pm 20) \times 10^4$	M^{-3}
β_4:	$(26 \pm 5) \times 10^5$	— — —	M^{-4}

B. INVESTIGATION BY MEANS OF AN ANION EXCHANGER

Theoretical

Fronæus [5] has given a theory for the equilibrium distribution of the central ion M^{3+} between a solution containing a complexing ligand A^- and an anion exchanger saturated with A^-.

In the anion-exchange investigation the ionic strength of the outer solution is not kept constant by addition of sodium perchlorate as perchlorate ions would displace ligand ions in the resin. Then the activity coefficients will vary when the buffer solution is added.

For the distribution of the ions M^{3+} and A^- between the resin and the solution, we have according to the Donnan equation

$$[M^{3+}]_R \cdot [A^-]_R^3 = K \cdot [M^{3+}] \cdot [A^-]^3 \qquad (19)$$

The factor K includes the activity coefficients of the two ion species in both phases.

For the load on the exchanger we have the equation

$$C_{MR} = [M^{3+}]_R(1 + \sum_{j=1}^{N} \beta_{jR} \cdot [A^-]_R^j) = [M^{3+}]_R \cdot X([A^-]_R) \quad (20)$$

Then we get according to eqn. (19)

$$\varphi = \frac{C_{MR}}{C_M} = K \frac{X([A^-]_R)}{[A^-]_R^3} \cdot \frac{[A^-]^3}{X[(A^-])} = l_3 \cdot \frac{\alpha_3}{\alpha_{3R}} \quad (21)$$

with $l_3 = K \cdot \beta_{3R} \cdot \beta_3^{-1}$

Here l_3 is the Nernst distribution coefficient for the third uncharged complex and can be assumed to be approximately constant. α_3 and α_{3R} denote the fractions $[MA_3] \cdot C_M^{-1}$ and $[MA_3]_R \cdot C_{MR}^{-1}$, respectively.

As shown by Fronæus [5] the function $\varphi([A^-])$ shows a maximum for a ligand concentration in the outer solution corresponding to $\bar{n} = 3$.

Then, if anionic complexes are formed, φ will to begin with increase with increasing $[A^-]$. After the maximum point has been reached at $\bar{n} = 3$, φ will decrease with increasing values of $[A^-]$.

If anionic complexes are not formed, φ ought to increase uniformly for increasing $[A^-]$.

However, the possibility must be taken into consideration that a part of C_{MR} is due to gadolinium ions or complexes adsorbed on the surface of the resin particles. This has been discussed by Fronæus [8]. In addition to the quaternary ammonium groups the anion exchanger possibly contains amine groups, which might form complexes with the central ion M^{3+} and, most likely to a lesser extent, with the complexes MA_j^{3-j}. Then the presumed amine complexes ought to be found preferably on the surface of the resin. The contribution of the surface adsorption to φ is denoted by $\Delta\varphi^s$. As $[M^{3+}]$ decreases when $[A^-]$ increases, this surface adsorption ought to decrease with increasing $[A^-]$ — that is, $\Delta\varphi^s$ decreases when $[A^-]$ increases.

Experimental

Chemicals used. Dowex 1 × 4, an anion-exchanger containing fixed quaternary ammonium groups, was used. It was converted to the acetate or the glycolate form and air-dried. The swelling factor δ was determined to be 0.96.

Method of investigation. The complex solutions had the initial composition

$$C'_M \text{ mM } Gd(ClO_4)_3 + C'_{HA} \text{ mM HA} + C'_A \text{ mM NaA}$$

The gadolinium perchlorate contained the radioactive isotope ^{153}Gd. C' was kept constant in a series (0.0730 mM and 0.0630 mM in the investigations of the acetate and the glycolate systems, respectively).

The quotient $C'_{HA} : C'_A$ was 2.0 for the acetate buffer and 1.0 for the glycolate buffer. The pH of the initial solution of $Gd(ClO_4)_3$ was about 4.5.

The complex solution (v liters) together with m grams of air-dried exchanger was shaken during 20 h in a thermostat at 20°C. The value of $v \cdot m^{-1}$ was always 0.025 l · g^{-1}.

As the measurements of the equilibrium concentration C_M of the water phase showed that the sorption was very slight at all concentrations of acetate and glycolate ions, C_{MR} could not be calculated indirectly by means of eqn. (16) from the difference $C'_M - \delta \times C_M$. For this reason C_{MR} was determined directly by eluting the resin, which had been carefully separated from the solution, with v_1 liters of 2 M $HClO_4$ in small portions. After having determined the gadolinium concentration C''_M of this solution, it was possible to calculate C_{MR} from the relation $C_{MR} = v_1 \cdot m^{-1} \cdot C''_M$. Here $v_1 \cdot m^{-1}$ had the value 0.042 1 . g^{-1}. However, a certain amount of radioactive solution adhered to the particles of the separated resin, which could not be washed before the elution. Thus the C_{MR}-value included a quantity C^a_{MR} caused by this adherent solution. As $v \cdot m^{-1}$ had the same value for all the complex solutions and as the experiments were performed in quite the same manner, we have $C^a_{MR} \approx k \cdot C_M$, where k is a constant. The C^a_{MR} contributes to φ in the term $\Delta\varphi^a = C^a_{MR} \cdot C_M^{-1}$ — that is, all φ-values differ from the true φ-values by the constant term $\Delta\varphi^a = k$.

The gadolinium acetate system. The data from the measurements on the Gd^{3+}—Ac^--system are collected in Table 5 a. Curve *a* in Fig. 3 shows φ plotted against $[Ac^-]$. In the calculation of $[Ac^-]$ it is supposed that the perchlorate ions in the initial solution are nearly completely replaced by an equivalent amount of acetate ions from the resin.

According to the potentiometric measurements anionic complexes are formed in the Gd^{3+}-Ac^--system and the ligand number $\bar{n} = 3$ is reached at $[Ac^-] \approx 500$ mM. However, the function $\varphi([Ac^-])$ does not show any maximum at this acetate concentration. For 100 mM $< [Ac^-] <$ 800 mM, φ has a very low value and is approximately constant. At low $[Ac^-]$, φ is somewhat higher,

Table 5. The anion-exchange measurements on the acetate and glycolate systems.

a. The acetate system $C'_M = 73.0 \times 10^{-3}$ mM				b. The glycolate system $C'_M = 63.0 \times 10^{-3}$ mM			
$[A^-]$ mM	$C_M \times 10^3$ mM	$C_{MR} \times 10^4$ mmole · g^{-1}	$\varphi \times 10^3$ l · g^{-1}	$[A^-]$ mM	$C_M \times 10^3$ mM	$C_{MR} \times 10^4$ mmole · g^{-1}	$\varphi \times 10^3$ l . g^{-1}
0.2	72.0	1.05	1.45	0.2	63.0	1.00	1.60
11.7	71.5	1.23	1.70	12.5	60.0	1.52	2.50
104	75.0	0.44	0.59	26	57.5	2.08	3.60
208	75.0	0.39	0.51	45	58.0	2.19	3.80
347	76.0	0.38	0.50	64	57.5	2.22	3.85
485	74.5	0.38	0.52	128	56.0	2.25	4.00
625	74.0	0.39	0.52	174	57.0	2.13	3.75
830	73.5	0.48	0.65	243	58.0	2.10	3.60
				347	58.0	1.85	3.20
				555	61.0	1.48	2.40

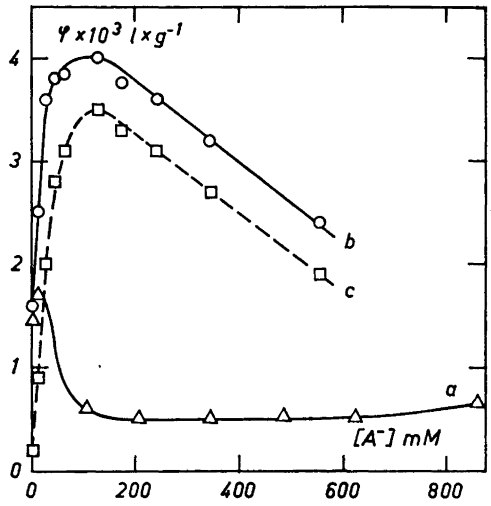


Fig. 3. The anion-exchange measurements on the acetate and the glycolate systems. Curve *a* represents the function $\varphi([Ac^-])$ and curve *b* the function $\varphi([AcOH^-])$. Curve *c* is the function $\varphi_c([AcOH^-])$, where φ_c means the φ-values of the glycolate system reduced by the φ-values of the acetate system.

certainly depending on the surface adsorption, which increases for decreasing $[Ac^-]$.

Then, according to these measurements anionic gadolinium acetate complexes cannot be formed by appreciable amounts in the resin phase. However, this does not necessarily mean that anionic complexes are not formed in the water phase.

The gadolinium glycolate system. The results from the measurement on the Gd^{3+}—$AcOH^-$-system are collected in Table 5 b. As shown in Fig. 3 (curve *b*) the function $\varphi([AcOH^-])$ has a distinct maximum for $[AcOH^-] \approx 100$ mM. This agrees with the potentiometric measurements as $\bar{n}_{pot} = 3$ for $[AcOH^-] \approx 100$ mM. Thus it is very probable that anionic glycolate complexes are formed both in the resin phase and in the water phase.

We may assume that the φ-values of the acetate system are due to surface adsorption and to solution adherent to the resin particles — that is, $\varphi_{Ac} \approx \Delta\varphi^s_{Ac} + \Delta\varphi^a_{Ac}$. We may also assume that these two $\Delta\varphi$-values have about the same values for the acetate and the glycolate systems. Then the true φ-values for the glycolate system should be $\varphi_c = \varphi - \varphi_{Ac}$. Curve *c* in Fig. 3 shows φ_c as a function of $[AcOH^-]$.

If we take the logarithm of eqn. (21), derivate $\ln\varphi$ with respect to $\ln[A^-]$ and introduce the ligand numbers $\bar{n}_R$ and $\bar{n}$ according to the definitions ($\bar{n} = d\ln X/d\ln[A^-]$), we get

$$\frac{d\ln\varphi}{d\ln[A^-]} = \frac{d\ln[A^-]_R}{d\ln[A^-]}(\bar{n}_R - 3) - (\bar{n} - 3) \qquad (22)$$

In this investigation we may consider $[A^-]_R$ as constant. Then we get

$$\frac{d\ln\varphi}{d\ln[A^-]} = 3 - \bar{n} \qquad (23)$$

Table 6. The anion-exchange measurements on the gadolinium glycolate system. The function $\frac{1}{k} \cdot X$ is computed according to eqn. (24) and the functions $\frac{1}{k} \cdot X_n$ according to eqn. (8) in paper II.

$[A^-]$ mM	$\varphi_c \times 10^3$ $l \cdot g^{-1}$	(23) $\bar{n}$	$\bar{n}_{pot}$	$\frac{[A^-]^3}{\varphi_c} = \frac{1}{k} \cdot X$ $M^3 \cdot g \cdot l^{-1}$	$\frac{1}{k} \cdot X_1$ $M^2 \cdot g \cdot l^{-1}$	$\frac{1}{k} \cdot X_2$ $M \cdot g \cdot l^{-1}$	$\frac{1}{k} \cdot X_3$ $g \cdot l^{-1}$	$\frac{1}{k} \cdot X_4$ $M^{-1} \cdot g \cdot l^{-1}$
0	0			—	*0.10*	*4.0*	*200*	*~500*
0.2	0.1	—	0.1	$0.1 \cdot 10^{-6}$	—	—	—	
12.5	0.9	1.5	1.6	0.0022	0.18	6.5	200	
26	2.0	2.3	2.1	0.0088	0.34	9.2	200	
45	2.8	2.6	2.5	0.032	0.72	13.8	220	
64	3.1	2.7	2.7	0.085	1.32	19.0	230	
128	3.5	3.0	3.1	0.60	4.7	36	250	400
174	3.3	3.2	3.3	1.60	9.2	52	270	400
243	3.1	3.3	3.5	4.6	19.0	78	300	400
347	2.7	3.6	3.7	15.5	45	130	360	500
555	1.9	4.0	3.9	90	162	290	510	600

The values of $\bar{n}$ in Table 6 are calculated from the corrected φ_c-values by means of this equation. The accordance with the ligand numbers obtained potentiometrically is good.

Attempts have also been made to calculate approximate values of the complexity constants from the anion-exchange measurements. The φ_c-values have been used. Eqn. (21) can be transformed to

$$\frac{[A^-]^3}{\varphi} = \frac{1}{k} \cdot X = \frac{1}{k} + \frac{\beta_1}{k}[A^-] + \frac{\beta_2}{k}[A^-]^2 + \ldots . \qquad (24)$$

where

$$k = l_3 \cdot \beta_3 \cdot \alpha_{3R}^{-1}$$

α_{3R} is assumed to be constant as the ligand concentration of the resin is approximately constant.

Theoretically, the value of $1/k$ and the complexity constants can be calculated by means of the X/k-functions by extrapolation to $[A^-] = 0$.

However, the X-function decreases so rapidly with decreasing $[A^-]$ that it is impossible to obtain even an approximate value of $1/k$ (*cf.* Table 6), but for the X_n/k-funtions we have obtained the following approximate values for $[A^-] = 0$:

$$\frac{\beta_1}{k} = 0.10; \qquad \frac{\beta_2}{k} = 4.0; \qquad \frac{\beta_3}{k} = 200; \qquad \frac{\beta_4}{k} = 500$$

As we have no value of $1/k$ we can compute only the stability constants $b_n = \beta_{n+1} \cdot \beta_n^{-1}$. Thus $b_2 = 40$, $b_3 = 50$, $b_4 = 2.5$. These values are of the same order of magnitude as the potentiometrically obtained values $b_2 = 90$, $b_3 = 20$, $b_4 = 4$, but there are considerable differences between the two

sets of constants. One of the reasons may be that the potentiometric measurements are performed at the constant ionic strength 2.0 M whereas the anion-exchange experiments have been performed with solutions having an ionic strength increasing from about 0.01 M for the lowest value of $[A^-]$ (12.5 mM) to about 0.5 M for the highest value of $[A^-]$. In this region the activity coefficients are, of course, not constant. On the whole the stability constants obtained from the anion-exchange measurements are very uncertain. The cause of the too low value of b_2 may be a too high value of $\beta_1 \cdot k^{-1}$, because we have used the same value of $\Delta\varphi^s$ for the glycolate system as for the acetate system. If especially non-complex gadolinium ions are adsorbed on the surface of the resin, $\Delta\varphi^s$ ought to decrease more rapidly for the glycolate system than for the acetate system.

DISCUSSION

The investigations by use of the cation exchanger confirm the potentiometrically obtained values for the first three complexity constants for both the acetate and the glycolate system. It is true that the constants obtained for the glycolate system by the cation-exchange method are not quite independent of those obtained potentiometrically, but the potentiometric influence is so slight that it falls for the most part within the limits of the maximum random errors.

The question whether anionic complexes are formed can be answered definitely only for the glycolate system. The φ-curve obtained in the anion-exchange investigation of this system shows a rather pronounced maximum for $[AcOH^-] \approx 100$ mM, and, according to the potentiometric measurements, we should have $\bar{n} \approx 3$ for just this concentration of free glycolate ions. We may then conclude that anionic glycolate complexes are formed.

The φ-function obtained for the acetate system by means of the anion exchanger has an aspect quite different from that of the φ-curve of the glycolate system. We must conclude that anionic acetate complexes are not formed in the resin phase (*cf.* Fronæus [5]), but this does not necessarily mean that anionic complexes are not formed in the water phase. The low φ-values at the maximum point for the glycolate system indicates that it is very difficult for the complexes to be formed in the anionic resin phase. There may be a steric hindrance reducing the tendency to form complexes. This may be the reason why the considerably weaker acetate complexes cannot be formed in the resin phase. The relation between the heights of φ_{max} of systems of equal type should, according to Fronæus [5, p.3], give a qualitative measure of the difference in strength of the anionic complexes. However, it is also possible that the potentiometric measurements have given too high values for the complexity at high ligand concentrations, so that anionic complexes are not really formed to a measureable extent at the concentrations used in the anion-exchange measurements.

As seen in Fig. 3 the φ-values of the acetate system increase slightly at high ligand concentrations. This is probably due to the presence of a non-exchange electrolyte in the resin. Calculations show that this ought to increase the φ-values appreciably when $[A^-] > 0.7$ M. As $[A^-]_R \approx 3$ M for $[A^-] = 0$,

the increase in $[A^-]_R$ ought to be ~ 5 % at $[A^-] \approx 0.7$ M (*cf.* Fronæus [8, p.1181]). The presence of a non-exchange electrolyte probably does not influence the calculations for the glycolate system at lower ligand concentrations.

The sorption of the complex ions in the cation exchanger is very low compared with that of the non-complex gadolinium ions. For the acetate system we get $l_1' = (7 \pm 3)$ M^{-1} and $l_2' \approx 15$ M^{-2} (according to eqn. (14)). Eqns. (6) and (4) give for the distribution of the non-complex gadolinium ions and the acetate complexes between the resin phase and the water phase $l_o = 0.323$ $l \cdot g^{-1}$, $l_1 = 0.035$ $l \cdot g^{-1}$ and $l_2 = 0.0035$ $l \cdot g^{-1}$. For the glycolate system the corresponding values are $l_o = 0.323$ $l \cdot g^{-1}$, $l_1 = 0.025$ $l \cdot g^{-1}$ and $l_2 = 0.002$ $l \cdot g^{-1}$. This shows that the equilibrium concentration of $Gd(AcOH)_2^+$ is only about twice as high in the resin phase as in the water phase. Then, when $Gd(AcOH)_2^+$ is tranformed to higher complexes, the absolute decrease of φ will be rather small (relatively however, the decrease may be large). Therefore the random errors will seriously affect the determination of β_3 and the higher complexity constants, and this is probably the reason why the existence of the anionic glycolate complexes could not be detected by the cation-exchange method.

The author wishes to express his sincere gratitude to Professor S. Bodforss and to Professor S. Fronæus for their kind interest that has promoted the work.

This investigation has been supported by a grant from the *Swedish Natural Science Research Council* which is gratefully acknowledged.

REFERENCES

1. Sonesson, A. *Acta Chem. Scand.* **12** (1958) 165.
2. Sonesson, A. *Acta Chem. Scand.* **12** (1958) 1937.
3. Sonesson, A. *Acta Chem. Scand.* **13** (1959) 998.
4. Fronæus, S. *Svensk Kem. Tidskr.* **65** (1953) 19.
5. Fronæus, S. *Svensk Kem. Tidskr.* **65** (1953) 1.
6. Fronæus, S. *Svensk Kem. Tidskr.* **64** (1952) 64.
7. Fronæus, S. *Komplexsystem hos koppar* (Diss.) Lund 1948.
8. Fronæus, S. *Acta Chem. Scand.* **8** (1954) 1174.

Received May 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1453–1459

On Vitamins in Sewage Sludge

IX. Production of Vitamin B_{12} by Methane Bacteria

HALINA Y. NEUJAHR and DANLEY A. CALLIERI

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

Twelve different enrichment cultures of methane-producing bacteria have been prepared and their ability to produce vitamin B_{12} has been studied.

Most of the cultures produced small amounts of vitamin B_{12}. The enrichment cultures grown in the media containing either methanol, acetate or butyrate were shown to produce considerably larger amounts of vitamin B_{12} than those grown in the other media. The amount of vitamin B_{12} produced in the methanol-containing medium seems to be higher than that reported in the literature for many other microorganisms.

It was shown that the kind of factors produced by the enrichment cultures depends upon the composition of the medium. The formation of cyanocobalamin depends upon the supply of 5,6-dimethyl benziminazole.

In the two preceding papers [1,2], the authors have reported investigations on the ability of an enrichment culture of *Methanobacterium omelianskii* to produce vitamin B_{12}. This culture proved to have a rather limited synthetic ability with respect to vitamin B_{12}. The greatest yield of cyanocobalamin obtained using certain modifications of a simple synthetic medium did not exceed 100 mμg/ml. Since the high contents of vitamin B_{12} in sewage sludge evidently could not be explained by only the activity of *Methanobacterium omelianskii*, the synthetic ability of other methane bacteria, active during the digestion of sewage sludge, was investigated. Enrichment cultures of methane-producing bacteria were successfully prepared by many workers already in the beginning of this century (*cf.* Barker: *Bacterial Fermentations*, 1956 [9]). Such cultures are usually characterized by a high degree of morphological and physiological homogeneity. Wikén [3] gives a review of the earlier works and describes the preparation of several enrichment cultures as well as the isolation of some pure strains of methane bacteria. Using the technique described by Wikén, the authors prepared twelve enrichment cultures from digesting sewage sludge and investigated their ability to produce vitamin B_{12}, both in

a simple synthetic medium containing only one low molecular organic compound and also in the same medium modified by the addition of Co^{2+}, 5,6-dimethyl benziminazole and Bacto Peptone.

EXPERIMENTAL

Basal medium. The basal medium used was that described by Wikén [3] — it had the following composition: NH_4Cl, 1.0 g; K_2HPO_4, 0.4 g; $MgCl_2 . 6H_2O$, 0.1 g; tap water, 1 000 ml. To each 100 ml of this medium, a separately sterilized solution consisting of 2 ml of 1 % $Na_2S . 9H_2O$ solution + 8 ml of 5 % $Na_2CO_3 . 10H_2O$ solution was added. The pH of the medium was adjusted with separately sterilized HCl (1:5) to 6.5—7.5.

Elective media. Twelve different elective media were used to obtain the various enrichment cultures. These media were prepared by supplementing twelve portions of the basal medium with the following organic compounds (one compound only was added to each portion of the basal medium): methanol 1 %, ethanol 2 %, *n*-propanol 1 %, *iso*-propanol 1 %, *iso*amyl alcohol 0.5 %, acetone 0.5 %, calcium formate 2 %, calcium acetate 2 %, calcium propionate 2 %, and calcium *n*-butyrate 2 %. The media containing alcohols were further supplemented with 2 % of $CaCO_3$ in order to neutralize the acid formed during the fermentation and to form a sediment which, according to many workers, is very favourable for the growth of methane bacteria. The media containing acids (as salts) were instead supplemented with 2 % fine asbestos powder.

The preparation of the enrichment cultures was carried out by inoculating a few milliliters digesting sludge into the appropriate media. When a vigorous gas evolution was established, a reinoculation was performed into an identical medium. This procedure was repeated until altogether 10 reinoculations had been made in the same medium. To the last (the tenth) reinoculation, Co^{2+} was added (2 500 mμg/ml). After two further reinoculations into Co^{2+}-containing media, a reinoculation was made into a medium containing both Co^{2+} (2 500 mμg/ml) and 5,6-dimethyl benziminazole (1 000 mμg/ml), see Table 1. Finally, the media were also supplemented with Bacto Peptone (0.25 %) as seen in Table 1.

All cultures were grown in conical flasks of 100—300 ml capacity and incubated at 37°C.

Anaerobic conditions. All manipulations with the media and cultures were made in the carbon dioxide chamber described in a preceding paper [1]. The cultures were always covered with a layer of paraffin oil.

Samples were taken from the cultures at the end of the fermentation just before each reinoculation and were analyzed for vitamin B_{12} activity using *E. coli* 113—3 as test organism in a plate assay and by bioautography as described elsewhere [4].

RESULTS AND DISCUSSION

The results of the investigation can be seen in Table 1. The enrichment cultures grown in the media containing methanol, ethanol, *n*-propanol, *n*-butanol, *iso*butanol, *iso*amyl alcohol, acetate or butyrate grew relatively easily and considerable gas evolution usually occurred after incubation at 37°C for 5—15 days. The cultures grown in media containing methanol, acetate or butyrate were especially active — the most intensive gas evolution occurring only 1—2 days after the inoculation. The remaining four media produced much poorer growths and culturing times up to several months were required before a subsequent reinoculation could be made. An examination of Table 1 reveals that, apart from media containing methanol, acetate or butyrate, only small amounts of vitamin B_{12} activity (comparable with those produced in the ethanol-containing medium in which *Methanobacterium omelianskii* is dominant) were produced.

The cultures which were grown in the media containing methanol, acetate or butyrate (especially the first named) provide, however, a striking exception. When supplied with Co^{2+}, 5,6-dimethyl benziminazole and Bacto Peptone, the methanol culture produced vitamin B_{12} activities up to 6 000 mμg/ml estimated as *E. coli* activity in the cup plate assay and calculated as cyanocobalamin. The amounts of *E. coli* activity produced by this culture are thus 80—300 times greater than those produced by the cultures developed in most of the other media and even ~3 resp. 6 times greater than the amounts produced by the butyrate resp. acetate cultures. The yields of vitamin B_{12} activity obtained with the methanol culture seem to be even higher than those reported for *Streptomyces olivaceus* [5].

All three cultures produce sufficient amounts of *E. coli* activity to explain the high vitamin B_{12} activity of digested sewage sludge. Moreover, it seems as if the amount of vitamin B_{12} activity which can be obtained with the three most active cultures, especially when calculated on a dry solids basis, is even higher than the vitamin B_{12} activity of digested sludge. A possible explanation of this phenomenon is that digested sludge, as suggested in a previous work [6], probably contains organisms which consume vitamin B_{12}. Furthermore, the essential substrates, *viz.* methanol, acetate or butyrate may be present in the sludge only in limited quantities or the rate of their formation may not be rapid enough to provide the appropriate organisms with adequate quantities of the substrates. This may also be true for 5,6-dimethyl benziminazole. However, it has been shown in a previous work [1] that an increase above 1 000 mμg/ml in the content of 5,6-dimethyl-benziminazole in the medium fermented by *Mb. omelianskii* does not produce any increase in the yield of vitamin B_{12} and that the addition of 10 000 mμg/ml of benziminazole, *i.e.* a tenfold increase, seems instead to bring about an inhibition of the growth. Similar observations have also been made with the methanol culture — more detailed results will be reported in a separate communication. It can further be seen in Table 1 that the addition of Co^{2+} may stimulate the growth of certain cultures (as judged by the gas evolution), *e.g.* the cultures grown in the media containing methanol, ethanol, acetate or butyrate. This effect was most pronounced in the last two cases — the addition of Co^{2+} seemed to be essential for the production of any appreciable growth. In most cultures, even those in which the Co^{2+} addition had no effect on the gas evolution, it was found that the formation of *E. coli* activity was stimulated by this addition. An exception in this respect was the culture grown on *iso*butanol.

The *E. coli* activity produced by the different cultures could in most cases be separated into at least two spots by the usual chromatographic-bioautographic technique. One of these spots corresponded to cyanocobalamin, R_c (= R_F relative to R_F of cyanocobalamin) = 1.0. Although cyanocobalamin was formed in most cultures, its formation in some cultures was dependent upon the addition of Co^{2+} alone (*e.g.* cultures on ethanol, *n*-propanol, *iso*propanol, acetone, butyrate) while, in others, both Co^{2+} and 5,6-dimethyl benziminazole had to be added in order to obtain the formation of cyanocobalamin (*e.g.* cultures on methanol, *n*-butanol, *iso*amyl alcohol and acetate).

Even in those cultures in which the addition of Co^{2+} alone stimulated the synthesis of cyanocobalamin, a simultaneous addition of 5,6-dimethyl benzi-

Table 1. Production of vitamin B_{12} by enrichment cultures of methane bacteria. Co — addition of Co^{2+}, 2 500 mμg/ml. DMB — addition of 5,6-dimethyl benziminazole, 1 000 mμg/ml. BP — addition of Bacto Peptone (Difco), 0.25 %. + slight gas evolution, ++ medium gas evolution, +++ strong gas evolution, ++++ very strong gas evolution.

Enrichment culture grown in the basal medium, supplemented with	Further additions to the medium	Gas-evolution	*E. coli* activity produced	
			mμg/ml **	chromatographic spots corresponding to factors ***
Methanol 1 %	none	+	traces	not identified
» »	Co	+++	500 — 900	B; W (or III) *; Z
» »	Co + DMB	+++	600 — 2 000	B; Cy; W (or III) *; Z
» »	Co + DMB + BP	++++	4 000 — 6 000	B; Cy; W (or III) *; Z
Ethanol 2 %	none	++	1 — 3	Met
» »	Co	+++	60 — 130	B; Cy; Met
» »	Co + DMB	+++	15 — 30	Cy;
» »	Co + DMB + BP	++++	50 — 70	Cy;
n-Propanol	none	++	1 — 3	not identified
»	Co	++	40 — 80	B; Cy; W (or III) *
»	Co + DMB	++	20 — 30	B; Cy
»	Co + DMB + BP	++	20 — 30	Cy; W (or III) *
*Iso*propanol 1 %	none	++	1 — 3	not identified
» »	Co	++	4 — 6	Cy; W (or III) *
» »	Co + DMB	++	10 — 20	Cy; W (or III) *
» »	Co + DMB + BP	++	40 — 60	Cy; W (or III) *
n-Butanol 1 %	none	++	1 — 3	not identified
» »	Co	++	10 — 30	B; W (or III) *
» »	Co + DMB	++	10 — 20	Cy
» »	Co + DMB + BP	++	10 — 20	Cy; W (or III) *
*Iso*butanol 1 %	none	++	1 — 3	not identified
» »	Co	++	1 — 3	not identified
» »	Co + DMB	++	4 — 6	Cy; W (or III) *
» »	Co + DMB + BP	++	20 — 30	Cy; W (or III) *

minazole directed the synthesis towards the formation of cyanocobalamin at the expense of the other factors. In two cases (ethanol and *iso*amyl alcohol), such a simultaneous addition resulted in the formation of cyanocobalamin as the only factor, although in rather smaller amounts. In these two and certain other cultures, the addition of 5,6-dimethyl benziminazole seemed to decrease the *E. coli* activity estimated in the cup plate assay as compared with a corresponding culture grown with the addition of Co^{2+} only. It cannot, however, be concluded whether this was a real decrease in the vitamin B_{12} activity or merely a reflection of the differences in activity towards *E. coli* in the cup plate assay between the other factors and the cyanocobalamin to which they had been converted due to the addition.

Table 1. Contd.

Enrichment culture grown in the basal medium, supplemented with	Further additions	Gas-evolution	*E. coli* activity produced	
			mμg/ml **	chromatographic spots corresponding to factors ***
Iso-amyl alcohol 0.5 %	none	++	1— 3	not identified
» » »	Co	++	30— 130	W (or III) *
» » »	Co + DMB	++	2— 4	Cy
» » »	Co + DMB + BP	++	10— 20	Cy
Acetone 0.5 %	none	+	traces	not identified
» »	Co	+	»	Cy; W (or III) *
» »	Co + DMB	+	»	not identified
» »	Co + DMB + BP	+	»	not identified
Calcium formate 2 %	none	+	traces	not identified
» » » »	Co	+	»	not identified
» » » »	extremely slow growth			
» » » »				
Calcium acetate 2 %	none	+	traces	not identified
» » » »	Co	++++	50— 250	W (or III) *
» » » »	Co + DMB	++++	600— 700	Cy; W (or III) *
» » » »	Co + DMB + BP	++++	800—1 000	Cy; W (or III) *
Calcium propionate 2 %	none	+	traces	not identified
» » » »	Co	+	2— 3	not identified
» » » »	extremely slow growth			
» » » »				
Calcium *n*-butyrate 2 %	none	+	5— 15	not identified
» » » »	Co	++++	100—1 000	Cy; W (or III) *
» » » »	Co + DMB		not determined	
» » » »	Co + DMB + BP	++++	1 000—2 200	Cy; W (or III) *

* and/or possibly some incomplete factor with R_c-value (R_F-value relative to that of cyanocobalamin) in the range 0.6—0.7; *cf.* discussion and Ref [7].

** calculated as cyanocobalamin in plate assay.

*** identified by paper chromatography in solvent system I [7].

The second chromatographic spot of *E. coli* activity obtained in most cultures had an R_c-value in the range 0.6—0.7. It has been reported earlier [7] that the presence of at least 8 different vitamin B_{12} factors can be attributed to such a spot, *viz.* factor III, (Bernhauer) [8], factor A (Ford, Kon and Porter)[11], pseudovitamin B_{12} (Pfiffner) [12] and the factors X1, X2, X3, X4, and W isolated from sewage sludge in this laboratory [7].

More accurate chromatographic and electrophoretic studies, however, indicated that it was improbable that factor A or (and) pseudovitamin B_{12} were present in the spot.

It has also been shown in a previous work [1] that one of the methane bacteria, *viz. Mb. omelianskii*, cannot utilize adenine in the presence of Co^{2+} or even in the presence of added factor B to produce pseudovitamin B_{12}. It is therefore probable that neither pseudovitamin B_{12} nor factor A was formed by the various enrichment cultures. The ever increasing number of vitamin B_{12} factors reported in the literature makes the identification of any factor by the chromatographic-bioautographic technique quite uncertain. However, certain interesting observations can be made concerning the occurrence of certain factors (chromatographic spots) in certain cultures while not in others. It has been found in an earlier study and confirmed by the present investigation that the enrichment culture grown in the ethanol-containing, but not further supplemented, basal medium produces only one vitamin B_{12} factor, *viz.* factor Met. This factor has an R_c-value ($\sim$ 0.7) close to that for the eight factors mentioned above and its occurrence in sewage sludge has never before been noticed (probably partly due to its very low concentration in this material). It is interesting to note that factor Met was not formed by any other enrichment culture. It seems therefore to be in some way characteristic for the enrichment culture of *Mb. omelianskii* prepared in the ethanol-containing medium. As far as the remaining factors which may be present in the second chromatographic spot (R_c = 0.6—0.7) of *E. coli* activity are concerned, no conclusions could be made about which of these factors were formed by the respective cultures. The results of paper electrophoretic studies indicated that, in most cultures, the spot with R_c = 0.6—0.7 corresponded mainly to factor W (and/or factor III) possibly accompanied by minor quantities of the incomplete factors X4 and (or) X3. In the culture grown on butyrate, these last two factors were formed in considerably greater amounts than in the other cultures.

The culture grown in the methanol-containing medium produced the greatest number of vitamin B_{12} factors. As pointed out above, this culture also produced the largest amount of *E. coli* activity. A chromatographic separation of the activity gave 4 spots when both Co^{2+} and 5,6-dimethyl benziminazole were added to the medium, but only 3 spots when the benziminazole was omitted. The four spots corresponded to factors: B (R_c = 1.3—1.5), Cy (R_c = 1.0), W and (or) factor III + minor quantities of X4 and (or) X3 (R_c = 0.6—0.7) and finally some of the factors Z. When 5,6-dimethyl benziminazole was lacking, but Co^{2+} present, in the medium, the spot corresponding to cyanocobalamin was always absent after chromatographic separation.

It can, in addition, be seen in Table 1 that the enrichment culture grown in the methanol-containing medium was the only one which produced factors Z [7]. This culture was exceptional also with respect to the occurrence of factor B since the chromatographic spot corresponding to this factor was not (or not completely) converted to the cyanocobalamin-spot in the presence of added 5,6-dimethyl benziminazole and Bacto Peptone. In a preliminary study of this problem, we investigated whether greater additions of 5,6-dimethyl benziminazole to this culture cause a more complete conversion of factor B (and possibly also of factors Z) to cyanocobalamin. However, the results indicated that greater additions of this compound ($>$ 1 μg/ml medium) may have an inhibiting effect on the growth of the culture.

It is possible that the spots corresponding to factors B and Z contained also some other factors which could not be converted to cyanacobalamin by the culture.

Microscopic examinations of the enrichment culture grown in the basal medium supplemented with methanol revealed the presence of at least two kinds of organisms. One of them was a coccus of about 1.5 μ diameter, the second was a rod-shaped organism the size of which was greater than that of *Methanobacterium omelianskii*. Both were Gram variable. The coccoidal form was the dominant one. A rough estimation indicated that the coccoidal form was 1 000 times more abundant than the rod shaped one.

The enrichment culture grown in the butyrate-containing medium consisted of rods 3—6 μ long and 1 μ thick, whereas the culture obtained in the acetate-containing medium contained almost exclusively coccoidal forms of 1.5—2 μ diameter. In agreement with our previous experience, the culture grown in the ethanol-containing medium was composed of rods, sometimes slightly bent, which corresponded morphologically to the *Mb. omelianskii* described by Barker [10]. All those forms were Gram variable. Attempts to isolate pure strains form the enrichment cultures have so far failed. As soon as a single colony form a semi-solid medium was transferred to a fresh medium, the growth ceased in spite of great precautions for the maintenance of anaerobiosis.

Acknowledgements. The authors are very grateful to Professor H. Lundin, Head of the Division, for his encouraging interest in and valuable advice concerning the execution of this work.

REFERENCES

1. Neujahr, H. Y. and Callieri, D. A. *Acta Chem. Scand.* **12** (1958) 1153.
2. Neujahr, H. Y. and Callieri, D. A. *Acta Chem. Scand.* **12** (1958) 1167.
3. Wikén, T. *IVA* **1942**:3.
4. Neujahr, H. Y. *Acta Chem. Scand.* **11** (1957) 1191.
5. Hall, H. H., Benedict, R. G., Wiesen, C. F., Smith, C. E. and Jackson, R. W. *Applied Microbiology* **1** (1953) 124.
6. Neujahr, H. Y. *Acta Chem. Scand.* **9** (1955) 622.
7. Neujahr, H. Y. *Acta Chem. Scand.* **10** (1956) 917.
8. Bernhauer, K. und Friedrich, W. *Angew. Chem.* **66** (1954) 776.
9. Barker, H. A. *Bacterial Fermentations*, John Wiley, 1956, p. 6.
10. Barker, H. A. *Antonie van Leeuwenhoek* **6** (1939/40) 201.
11. Ford, J. E., Kon, S. K. and Porter, J. W. G. *Biochem. J.* **50** (1951) IX.
12. Pfiffner, J. J., Dion, H. W. and Calkins, D. G. *Federation Proc.* **11** (1952) 269.

Received April 17, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1460–1462

Studies Related to Naturally Occurring Acetylene Compounds, XXVI. The Synthesis of 5-(1-propynyl)-2-formylthiophene, Junipal, and *trans* Methyl 5-(1-propynyl)-2-thienylacrylate

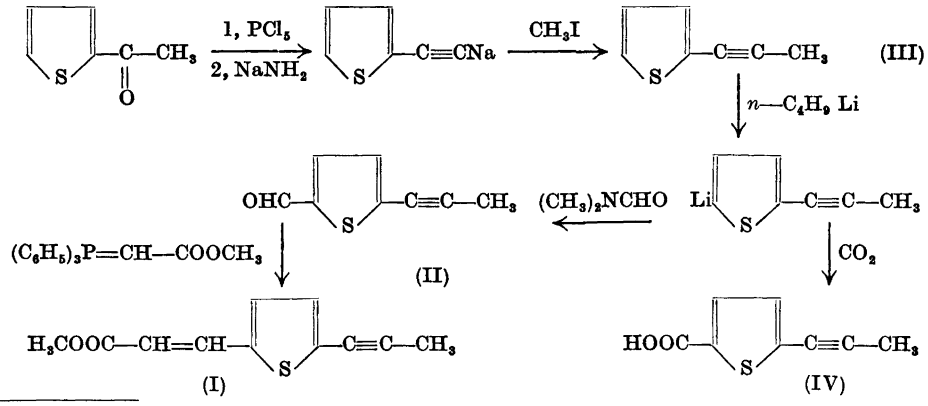
LARS SKATTEBÖL*

SINTEF, Norges Tekniske Högskole, Trondheim, Norway

The synthesis is described of junipal (II), a metabolic product from a wood-rotting fungus, and also of *trans* methyl 5-(1-propynyl)-2-thienylacrylate (I), the steric isomer of a naturally occurring substance isolated from a *Compositae* plant.

In the previous communication of this series [1] Guddal and Sörensen described the isolation of a naturally occurring thiophene derivative from the root of Tancy (= *Chrysanthemum vulgare* Bernh.) of the *Compositae* family. The compound was deduced as *cis* methyl 5-(1-propynyl)-2-thienylacrylate (I) on the basis of analysis, characterisation of the hydrogenation product and evidence obtained from ultraviolet- and infrared spectra.

In order to confirm this structure the synthesis of (I) was undertaken through the following route:



Present address: European Research Associates, 95, rue Gatti de Gamond, Bruxelles 18, Belgium.

An intermediate in this synthesis, *viz.* 5-(1-propynyl)-2-formylthiophene (II), has the same structure as that proposed for junipal, a metabolic product isolated by Birkinshaw and Chaplen [2] from the wood-rotting fungus *Daedalea juniperina* Murr.

2-Thienylacetylene was prepared from 2-acetylthiophene by chlorination with phosphorus pentachloride and subsequent dehydrochlorination with sodium amide in liquid ammonia, in the way described by Vaitiekunas and Nord [3]. The ethynyl compound was not isolated but reacted further, as the sodium salt, with methyl iodide to give 2-(1-propynyl)-thiophene (III) in 30 % overall yield. The compound turned dark brown fairly rapidly, when stored at room temperature. With butyl lithium in ether (III) readily formed a lithium derivative. The lithium is most probably substituted in the 5-position. This is analogous to previous results from this reaction with thiophenes [4], and it is also in agreement with kinetic measurements [5,6]. Reaction of the lithium compound with N-dimethylformamide [7] gave the corresponding aldehyde (II) in 21 % yield, as almost colourless crystals, m.p. 81—82°. A good deal of polymeric material was formed. The ultraviolet- and infrared spectra were in agreement with those published for junipal [2], and a mixed melting point determination with the natural substance, kindly provided by Professor J.H. Birkinshaw, showed no depression.

Carbonation of the lithium derivative yielded 2-(1-propynyl)-thiophene-5-carboxylic acid (IV), = junipic acid [2].

The aldehyde (II) readily underwent a Wittig reaction [8] with methylcarbethoxytriphenylphosphonium chloride. From the reaction mixture the wanted ester (I) was obtained in 55 % yield as colourless needles, m.p. 77°. The band at 962 cm^{-1} in the infrared spectrum strongly indicated that the configuration about the double bond was *trans*.[9] The compound was found to be in all respects identical with the photo-isomerised naturally occurring substance [1]. Theoretically the *cis*-isomer should also be formed in the Wittig reaction, and this has been confirmed in some instances [8,10]. We were not able, however, to isolate any of the *cis*-isomer from our reaction mixture.

Birkinshaw and Chaplen [2] also reported the isolation of a second thiophene derivative, accompanying junipal. They were only able to obtain it pure as the 2,4-dinitrophenylhydrazone. The analysis and ultraviolet spectrum pointed to a structure similar to junipal, but with an additional double bond. It was suggested [11] that the substance was the aldehyde corresponding to the ester(I), *viz.* 2-(1-propynyl)-5-thienylacraldehyde. The synthesis of this aldehyde, however, showed that this unfortunately was not so. Further work on the structure of this metabolic product is in progress.

EXPERIMENTAL

All melting points are uncorrected. The carbon-hydrogen analyses were carried out by Alfred Bernhardt, Mühlheim, Ruhr, Germany.

2-(1-Propynyl) thiophene (III). The sodium salt of 2-ethynylthiophene in liquid ammonia was prepared from 75 g of 2-acetylthiophene according to the method of Vaitiekunas and Nord [3]. Methyl iodide (70 g) was then added over a period of 25 min and the mixture was stirred overnight. 400 ml of water were carefully added and the dark brown solution extracted with ether. The extract was washed with water and dried over anhydrous sodium sulphate. The ether was removed under vacuum, and the residue purified by chromatography on deactivated alumina (1 % water). A yellow oil was eluted

with light petroleum and fractionation of this gave 22.0 g (30 % from acetylthiophene) of 2-(1-propynyl) thiophene as a colourless liquid, b. p. 65—66°/7 mm, n_D^{17} 1.5950. (Found: C 69.05; H 4.75. Calc. for C_7H_6S: C 68.81; H 4.95.) Ultraviolet absorption, in ethanol: λ_{max} 267.5 mμ; log ε 3.82.

5-(1-Propynyl)-2-formylthiophene, junipal (II). 2-(1-propynyl) thiophene (III) (1.3 g) was dissolved in 10 ml of dry ether, and 9.6 ml of a 1.1 N butyl lithium solution in ether was added with stirring. After 15 min at room temperature the red-coloured solution was cooled to −10° and 0.8 g of N-dimethylformamide in 2 ml of dry ether was added with stirring. The mixture was allowed to attain room temperature (15 min) and then decomposed with a saturated aqueous solution of ammonium chloride. Extraction with ether, drying and evaporation of the solvent under vacuum gave a crystalline residue. Recrystallisation from light petroleum-ether yielded the aldehyde (330 mg, 21 %), m. p. 79—81° as slightly yellow-coloured needles. Almost colourless crystals were obtained by sublimation at 50°/0.4 mm, m. p. 81—82°. (Found: C 63.87; H 4.11. Calc. for C_8H_6OS: C 63.97; H 4.03.) Ultraviolet absorption, in ethanol: λ_{max} 286 and 320 mμ; log ε 3.91 and 4.27, respectively. Birkinshaw and Chaplen [2] report m. p. 80°. Ultraviolet absorption in ethanol: λ_{max} 216.5, 286.5 and 320 mμ; log ε 3.73, 3.95 and 4.29, respectively. A 1:1 mixture with the natural substance melted at 80—81.5°.

5-(Propynyl) thiophene-5-carboxylic acid (IV). 2-(1-Propynyl) thiophene(III) (2.0 g) was dissolved in 10 ml of dry ether, and 14.5 ml of a 1.13 N solution of butyl lithium in ether was added with stirring. After 15 min at room temperature the solution was poured into a stirred slurry of solid carbon dioxide and dry ether. When the excess carbon dioxide had evaporated, water was added and the organic phase separated. The aqueous phase was acidified with dilute hydrochloric acid and extracted with ether. Evaporation of the solvent and recrystallisation of the residue from light petroleum-ether gave the acid (1.1 g; 37 %) as yellow needles, m. p. 175—177°. Further purification by sublimation and recrystallisation raised the melting point to 176.5—177.5°. (Found: C 57.65; H 4.20. Calc. for $C_8H_6O_2S$: C 57.80; H 3.60.) Ultraviolet absorbtion, in ethanol: λ_{max} 293 mμ; log ε 4.02. Birkinshaw and Chaplen [2] record m. p. 180°.

Methyl 5-(1-propynyl)-2-thienylacrylate (I). Methylcarbethoxy-triphenyl-phosphonium chloride (550 mg) was dissolved in 2 ml absolute methanol, and 8.0 ml of a 0.185 N solution of sodium methoxide was added with stirring. After 30 min at room temperature, 215 mg of 5-(1-propynyl)-2-formylthiophene (II) was added with stirring. The solution was left at room temperature for 40 h. The methanol was distilled under vacuum, and the residue extracted with ether. The extract was washed with 2 N hydrochloric acid, then with water and finally dried over magnesium sulphate. Chromatography on neutral alumina and subsequent recrystallisation from light petroleum yielded the ester (I) (156 mg; 55 %) as colourless needles, m. p. 77°. (Found: C 64.19; H 5.09. Calc. for $C_{11}H_{10}O_2S$: C 64.05; H 4.89.) Ultraviolet absorption, in ethanol: λ_{max} 235 and 341.5 mμ; log ε 3.98 and 4.50, respectively.

Acknowledgements. I am indebted to Professor N. A. Sörensen and lic.techn. E. Guddal for their interest in this work. I also wish to thank Professor J. H. Birkinshaw for a sample of natural junipal.

REFERENCES

1. Guddal, E. and Sörensen, N. A. *Acta Chem. Scand.* **13** (1959). 1185.
2. Birkinshaw, J. H. and Chaplen, P. *Biochem. J.* **60** (1955) 255.
3. Vaitiekunas, A. and Nord, F. F. *J. Org. Chem.* **19** (1954) 902.
4. Gilman, H. and Morton, J. W. *Org. Reactions.* **8** (1954) 258.
5. Gronowitz, S. and Halvarson, K. *Arkiv Kemi* **8** (1955) 343.
6. Halvarson, K. and Melander, L. *Ibid* **8** (1955) 29.
7. Evans, E. A. *J. Chem. Soc.* **1956** 4691; *Chem. & Ind. London* **1957** 1596.
8. Wittig, G. and Schöllkopf, U. *Chem. Ber.* **87** (1954) 1318; Wittig, G. and Haag, W. *Ibid.* **88** (1955) 1654.
9. Allan, J. L. H., Meakins, G. D. and Whiting, M. C. *J. Chem. Soc.* **1955** 1874.
10. Bohlmann, F., Inhoffen, E. and Herbst, P. *Chem. Ber.* **90** (1957) 1661; Wailes, P. C. *Chem. & Ind. London* **1958** 1086.
11. *Private communication* from Professor J. H. Birkinshaw.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1463–1480

Short Communications

Studies on the O,N-Acyl Shift

JOSEPH L. RABINOWITZ*

Carlsberg Laboratorium, Chemical Department, Copenhagen, Denmark

Elliott [1], Lucas [2], and others demonstrated the occurrence of an O,N-acyl shift [3] in various proteins and model peptides [4] under the influence of concentrated sulfuric acid, anhydrous phosphoric acid and other strong reagents. Josefsson and Edman [5] reported the reversible inactivation of lysozyme and ribonuclease due to an O,N-acyl shift induced by the mild action of "100 % formic acid" at 22° for 24 h. This preliminary note presents the results of studies of the action of anhydrous formic acid on various model compounds.

The infrared spectra of the above compounds were determined by the use of the Scheidt method [6]. The infrared spectra were also determined on the recovered materials after treatment with anhydrous formic acid and with concentrated sulfuric acid. The differences between the spectra of the original compounds and those of the formic acid treated peptides were not extensive, whereas the spectra of materials recovered after sulfuric acid treatment differed greatly from both those of the formic acid treated material and the original. Due to its waxy appearance, however, the sulfuric acid treated material gave poor infrared spectra by the Scheidt technique.

Table 1 shows the results obtained from the incubation of ^{14}C-formic acid with various substrates at 22° for 24 h.

Contrary to the findings of Josefsson and Edman [5] these results clearly indicate that formylation occurred in the serine polypeptide during the action of the formic acid, yet this data do not show if an O,N-acyl shift occurred.

Table 1. Radioactivity recovered in c.p.m. per mg carbon.

		Ext 1	Ext 2
1	Anhydrous ^{14}C—formic acid used	6 050	68 000
2	Poly-DL-serine after treatment	1 095	19 500
3	The above (2) after repeated washings	1 165	19 400
4	The recovered formate hydrolysis of the washed material (3)	5 310	63 000
5	The recovered poly-DL-serine after hydrolysis of the washed material (3)	4	11
6	Poly-DL-alanine after ^{14}C-formic acid treatment	4	—

Poly-DL-serine incubated at 22° for 24 h with anhydrous acetic acid produced a compound that by infrared spectra and independent synthesis [7] was found to be poly-O-acetyl-DL-serine. Poly-DL-serine is exceedingly water soluble and deliquescent; both the prepared poly-O-acetyl-DL-serine, as well as the recovered product after treatment with formic acid, were water insoluble. The Biuret test [8] was found to be positive for poly-DL-serine as well as for its

* Present address: Dept. Biochem., University of Pennsylvania, and Radioisotope Dept., Veteran Administration Hospital, Philadelphia, Penna., USA.

formic acid product and for poly-O-acetyl-DL-serine, but not for the material obtained after poly-DL-serine underwent treatment with concentrated sulfuric acid. These tests are suggestive of the absence of peptide-bond alterations for the formic acid treated materials and for elimination of all peptide bonds in the sulfuric acid treated materials.

The presented results as well as the additional evidence obtained by the use of model compounds indicate that formic acid under the conditions used is undoubtedly an excellent formylating agent. Recently Narita [9] and Neurath [10] have reported similar conclusions on the use of formic acid on various proteins. A more extensive article is in preparation for *Compt. Rend. trav. lab. Carlsberg.*

1. Elliott, D. F. *Biochim. J.* **50** (1952) 542.
2. Lucas, F., Shaw, J. T. B. and Smith, S. G. *Biochem. J.* **66** (1957) 468.
3. Bergmann, M., Brand, E. and Dreyer, F. *Ber.* **54** (1921) 936.
4. Lindstrøm-Lang, K. and Jacobsen, C. F. *J. Biol. Chem.* **137** (1941) 443.
5. Josefsson, L. and Edman, P. *Biochim. et Biophys. Acta* **25** (1957) 614.
6. Scheidt, U. and Rhinewine, H. *Z. Naturforsch.* **7b** (1952) 270.
7. Frankel, M., Cordova, S. and Brewer, M. *J. Chem. Soc.* **1953** 1991.
8. Gornall, A. G., Bardawill, C. J. and David, M. M. *J. Biol. Chem.* **177** (1949) 751.
9. Narita, K. *J. Am. Chem. Soc.* **81** (1959) 1751.
10. Smillie, L. B. and Neurath, H., *J. Biol. Chem.* **234** (1959) 355.

Received July 1, 1959.

Effect of Lysine on the Purification of Human Plasminogen on Cellulose Ion Exchangers

PER WALLÉN and KURT BERGSTRÖM

Kemiska Institutionen II, Karolinska Institutet, Stockholm, Sweden

Plasminogen, the precursor of the proteolytic enzyme plasmin, is present in mammalian blood plasma. Experience has shown that it is rather difficult to purify it. One of the reasons seems to be that the plasminogen has a marked tendency to coprecipitate with other proteins (Remmert and Cohen [1]). The purest plasminogen preparations have been prepared according to the method of Kline [2]. This method is based on the resistance of plasminogen to acid and alkali denaturation. In the present work an alternative method is outlined.

According to Müllertz and Lassen [3,4] there are in human plasma two different factors concerned in the fibrinolytic system when streptokinase is used as activator, the plasminogen and the proactivator. After activation with streptokinase the former gets proteolytic and the latter plasminogen activating activity. Test methods, in which bovine fibrin containing plasminogen is used as substrate measure mainly the activator activity whereas those methods in which casein or another protein is used as substrate measure the proteolytic activity.

The preparations and fractions obtained in this work have been tested with the caseinolytic method of Müllertz [4] and the fibrinolytic method of Christensen [5]. As no significant separation between the two plasma factors has been obtained and because the caseinolytic method seems to be the more reliable one of the two methods, the figures in the text concerning yield and specific activity refer to this method.

Attempts have been made to reproduce the method of Kline. The proteolytic potency of the preparations obtained has varied considerably. The activity per mg substance has been 5 to 10 times that of Fraction III (Cohn) as prepared by Oncley *et al.*[6] According to our calculations this should mean an increase in activity of about 100 to 200 times as compared to plasma on a protein basis, which is 2 to 4 times lower than the result published by Kline. The yields have been low, only 5 to 15 % from Fraction III.

In order to avoid this rough handling and the accompanying low yields we have tried other, milder forms of separation. A partial purification of plasminogen has been obtained by dialysing a suspension of Fraction III paste against a 0.02 M sodium phosphate buffer of pH 7.0 during 20 h at +5°C. A precipitate is obtained, which contains 12 to 15 % of the protein in the starting material. The precipitate has a specific caseinolytic and fibrinolytic activity which is 4 to 5 times that of Fraction III. The yield of plasminogen is about

formic acid product and for poly-O-acetyl-DL-serine, but not for the material obtained after poly-DL-serine underwent treatment with concentrated sulfuric acid. These tests are suggestive of the absence of peptide-bond alterations for the formic acid treated materials and for elimination of all peptide bonds in the sulfuric acid treated materials.

The presented results as well as the additional evidence obtained by the use of model compounds indicate that formic acid under the conditions used is undoubtedly an excellent formylating agent. Recently Narita [9] and Neurath [10] have reported similar conclusions on the use of formic acid on various proteins. A more extensive article is in preparation for *Compt. Rend. trav. lab. Carlsberg.*

1. Elliott, D. F. *Biochim. J.* **50** (1952) 542.
2. Lucas, F., Shaw, J. T. B. and Smith, S. G. *Biochem. J.* **66** (1957) 468.
3. Bergmann, M., Brand, E. and Dreyer, F. *Ber.* **54** (1921) 936.
4. Lindstrøm-Lang, K. and Jacobsen, C. F. *J. Biol. Chem.* **137** (1941) 443.
5. Josefsson, L. and Edman, P. *Biochim. et Biophys. Acta* **25** (1957) 614.
6. Scheidt, U. and Rhinewine, H. *Z. Naturforsch.* **7b** (1952) 270.
7. Frankel, M., Cordova, S. and Brewer, M. *J. Chem. Soc.* **1953** 1991.
8. Gornall, A. G., Bardawill, C. J. and David, M. M. *J. Biol. Chem.* **177** (1949) 751.
9. Narita, K. *J. Am. Chem. Soc.* **81** (1959) 1751.
10. Smillie, L. B. and Neurath, H., *J. Biol. Chem.* **234** (1959) 355.

Received July 1, 1959.

Effect of Lysine on the Purification of Human Plasminogen on Cellulose Ion Exchangers

PER WALLÉN and KURT BERGSTRÖM

Kemiska Institutionen II, Karolinska Institutet, Stockholm, Sweden

Plasminogen, the precursor of the proteolytic enzyme plasmin, is present in mammalian blood plasma. Experience has shown that it is rather difficult to purify it. One of the reasons seems to be that the plasminogen has a marked tendency to coprecipitate with other proteins (Remmert and Cohen [1]). The purest plasminogen preparations have been prepared according to the method of Kline [2]. This method is based on the resistance of plasminogen to acid and alkali denaturation. In the present work an alternative method is outlined.

According to Müllertz and Lassen [3,4] there are in human plasma two different factors concerned in the fibrinolytic system when streptokinase is used as activator, the plasminogen and the proactivator. After activation with streptokinase the former gets proteolytic and the latter plasminogen activating activity. Test methods, in which bovine fibrin containing plasminogen is used as substrate measure mainly the activator activity whereas those methods in which casein or another protein is used as substrate measure the proteolytic activity.

The preparations and fractions obtained in this work have been tested with the caseinolytic method of Müllertz [4] and the fibrinolytic method of Christensen [5]. As no significant separation between the two plasma factors has been obtained and because the caseinolytic method seems to be the more reliable one of the two methods, the figures in the text concerning yield and specific activity refer to this method.

Attempts have been made to reproduce the method of Kline. The proteolytic potency of the preparations obtained has varied considerably. The activity per mg substance has been 5 to 10 times that of Fraction III (Cohn) as prepared by Oncley *et al.*[6] According to our calculations this should mean an increase in activity of about 100 to 200 times as compared to plasma on a protein basis, which is 2 to 4 times lower than the result published by Kline. The yields have been low, only 5 to 15 % from Fraction III.

In order to avoid this rough handling and the accompanying low yields we have tried other, milder forms of separation. A partial purification of plasminogen has been obtained by dialysing a suspension of Fraction III paste against a 0.02 M sodium phosphate buffer of pH 7.0 during 20 h at +5°C. A precipitate is obtained, which contains 12 to 15 % of the protein in the starting material. The precipitate has a specific caseinolytic and fibrinolytic activity which is 4 to 5 times that of Fraction III. The yield of plasminogen is about

60 %. The precipitate is very slightly soluble in buffers of neutral reaction.

The preparations were purified chromatographically on DEAE-cellulose of the Sober and Peterson type [7]. They were dissolved in 0.02 M ammonium acetate buffer pH 9.0 and applied to DEAE-cellulose columns previously equilibrated with the same buffer. Under these conditions most of the protein as well as the plasminogen is adsorbed on the DEAE-cellulose. The elution was performed stepwise with buffers of increasing acidity, from pH 9.0 to pH 4.0. Plasminogen appeared in several of the fractions but no or very slight increase in specific activity was obtained. The yields were very low.

Recently Alkjaersig, Fletcher and Sherry [8] reported that ε-aminocaproic acid, lysine and certain other basic amino acids in low concentrations increased the solubility of plasminogen and plasmin at neutral pH. This effect of lysine may depend on a specific interaction between plasminogen and lysine. To see if an addition of lysine to the buffers used for elution would give a better separation of plasminogen at the chromatography on DEAE-cellulose the following experiment was performed.

About 5 g of DEAE-cellulose (Brown Comp. New Hampshire, USA) were suspended in 0.02 M ammonium acetate buffer, pH 8.9 to 9.0, containing lysine to a concentration of 0.01 M. The slurry was poured in a column with a diameter of 2 cm and allowed to settle under a slight hydrostatic pressure. The column was equilibrated with the same buffer. 125 mg of the partially purified plasminogen preparation obtained through dialysis against phosphate buffer were dissolved in 12.5 ml of the equilibration buffer at pH 8.9. Some of the material did not dissolve and was removed by centrifugation. It contained about 10 % of the protein and 3 % of the plasminogen activity. The opalescent supernatant was applied to the column, which was then developed with about 200 ml of the equilibration buffer. The elution was performed stepwise with the following buffers: 0.02 M sodium phosphate pH 6.5, 0.02 M sodium citrate pH 5.5, 5.0 and 4.0. All buffers contained lysine to a concentration of 0.01 M. The effluent was collected in fractions of 10 ml/h. The protein content of each fraction was estimated with the color reaction of Herriott-Lowry [9,10]. The fractions corresponding to each peak in the chromatogram were pooled, lyophilized and redissolved in 0.15 M sodium phosphate buffer pH 7.3. After dialysis against the same phosphate buffer the volumes were made up to 50 ml and the fractions analysed in respect to protein concentration, caseinolytic and fibrinolytic activity. For comparison another chromatogram was made at the same time and in exactly the same way but with the exception that lysine was replaced by sodium chloride. The results are shown in Fig. 1.

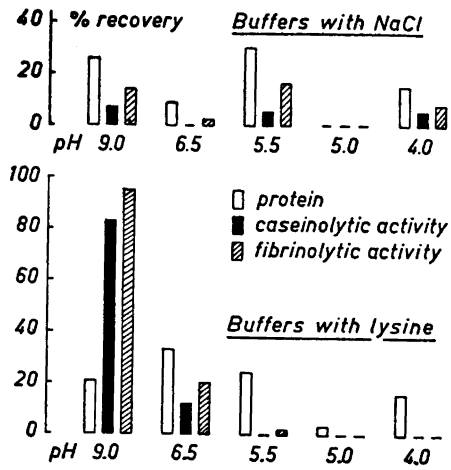


Fig. 1. Chromatography of partially purified plasminogen on DEAE-cellulose. The diagrams show the distribution of protein, caseinolytic and fibrinolytic activity among the fractions that appear at different pH. Upper diagram shows the pattern obtained when 0.01 M NaCl is added to the buffers. Lower diagram shows the pattern obtained when 0.01 M lysine is added to the buffers.

The upper part of Fig. 1 is an example of the pattern obtained in the chromatography without lysine. About 10 % of the plasminogen appears with the front. In the subsequent eluates the activity appears in the fractions containing most protein (pH 5.5 and 4.0). The yield and the specific activity in these fractions is low. The total yield of plasminogen is 20 to 25 %.

If lysine is added to the buffers the behaviour of plasminogen is markedly changed. The main part of the caseinolytic and fibrinolytic activity is not adsorbed to the DEAE-cellulose but appears in the front. The remaining part of the activity appears when elution is performed with 0.02 M phosphate buffer pH 6.5. In the frontal

peak about 20 % of the protein of the starting material appears together with about 80 % of the plasminogen. Consequently the starting material has been purified 4 times. This means an increase in purity of 16- to 20-fold as compared to Fraction III and the yield from Fraction III is about 50 %.

The reason for this behaviour of plasminogen is not understood. Attempts to adsorb the plasminogen to CM-cellulose equilibrated with buffers of pH 8.9 containing lysine have not been successful, which indicates that there is no reversal in the electric charge of the plasminogen.

1. Remmert, L. F. and Cohen, P. *J. Biol. Chem.* **181** (1949) 431.
2. Kline, D. L. *J. Biol. Chem.* **204** (1953) 949.
3. Müllertz, S. and Lassen, M. *Proc. Soc. Exptl. Biol. Med.* **82** (1953) 264.
4. Müllertz, S. *Biochem. J.* **61** (1955) 424.
5. Christensen, L. R. *J. Clin. Invest.* **28** (1949) 163.
6. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W. and Gross, P. M. *J. Am. Chem. Soc.* **71** (1949) 541.
7. Sober, H. A. and Peterson, E. A. *J. Am. Chem. Soc.* **78** (1956) 751.
8. Alkjaersig, N., Fletcher, P. and Sherry, S. *J. Biol. Chem.* **234** (1959) 832.
9. Herriott, R. M. *Proc. Soc. Exptl. Biol. Med.* **46** (1941) 642.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.

Received August 10, 1959.

Separation of Glycerol Ethers by Gas-liquid Chromatography

ROLF BLOMSTRAND and JOSEF GURTLER

Department of Clinical Chemistry, University of Lund, Lund, Sweden

In the course of studies [1,2] on the metabolism of the 1-mono-ethers of glycerol with hexadecyl, octadecyl and 9-octadecenyl alcohol, *i.e.* chimyl, batyl and selachyl alcohol, a method for the separation of the individual glycerol ethers was needed. This separation has also earlier been made with adsorption chromatography on alumina [3], but any separation of batyl from selachyl alcohol was not obtained.

The separation of methyl esters of high molecular weight fatty acids by gas-liquid chromatography (GLC) has been reported [4-6]. Since GLC permits microgram mixtures of methyl esters of fatty acids to be resolved, analysis of mixtures of glycerol ethers has been investigated.

The present paper describes the complete separation of chimyl, batyl and selachyl alcohol by GLC after acylation of the two hydroxyl groups of the glycerol ethers.

Apparatus and procedure. The GLC experiments were carried out with an Argon Pye Chromatograph. The temperature of operation was 218°C and the inlet pressure of the gas 72 cm Hg. The rate of flow of the carrier gas (Argon) was 20 cm³/min. The detector voltage was set on 1 250 V. Each 4-foot column was packed with 5 g of acid-washed, alkali-treated Celite (mesh 100—140). As stationary phase a polar polyester LHC-1R-296 (obtained from Cambridge Inc., Mass. U.S.A.) was used in the ratio Celite: stationary phase 4:1. Acylation of the two hydroxyl groups of the glycerol ethers was carried out with acetic anhydride in the presence of pyridine after boiling on a water bath for 12 h. The esterified mixture of glycerol ethers was isolated and dissolved in a few microlitres of ethyl acetate, and the solution taken up in a micropipette and applied to the column.

The retention volumes of the different glycerol ethers were determined using a mixture of known compounds (Table 1). The retention volumes are expressed relative to synthetic batyl alcohol, which was used as internal standard. When determining the retention volumes of the pure compounds, special care was taken not to overcharge the column in order to obtain symmetrical peaks on the chromatogram. The polyester column had a retention time of batyl alcohol diacetin of 49 min.

Table 1. GLC characteristics on LHC-1R-296 as stationary phase of three glycerol ethers after acylation. Temperature 218°C. Argon flow rate 20 cm³/min.

	Apparent retention volume relative to batyl alcohol-diacetin = 1.00
Chimyl alcohol	0.61
Batyl alcohol	1.00
Selachyl alcohol	1.11

peak about 20 % of the protein of the starting material appears together with about 80 % of the plasminogen. Consequently the starting material has been purified 4 times. This means an increase in purity of 16- to 20-fold as compared to Fraction III and the yield from Fraction III is about 50 %.

The reason for this behaviour of plasminogen is not understood. Attempts to adsorb the plasminogen to CM-cellulose equilibrated with buffers of pH 8.9 containing lysine have not been successful, which indicates that there is no reversal in the electric charge of the plasminogen.

1. Remmert, L. F. and Cohen, P. *J. Biol. Chem.* **181** (1949) 431.
2. Kline, D. L. *J. Biol. Chem.* **204** (1953) 949.
3. Müllertz, S. and Lassen, M. *Proc. Soc. Exptl. Biol. Med.* **82** (1953) 264.
4. Müllertz, S. *Biochem. J.* **61** (1955) 424.
5. Christensen, L. R. *J. Clin. Invest.* **28** (1949) 163.
6. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W. and Gross, P. M. *J. Am. Chem. Soc.* **71** (1949) 541.
7. Sober, H. A. and Peterson, E. A. *J. Am. Chem. Soc.* **78** (1956) 751.
8. Alkjaersig, N., Fletcher, P. and Sherry, S. *J. Biol. Chem.* **234** (1959) 832.
9. Herriott, R. M. *Proc. Soc. Exptl. Biol. Med.* **46** (1941) 642.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.

Received August 10, 1959.

Separation of Glycerol Ethers by Gas-liquid Chromatography

ROLF BLOMSTRAND and JOSEF GURTLER

Department of Clinical Chemistry, University of Lund, Lund, Sweden

In the course of studies [1,2] on the metabolism of the 1-mono-ethers of glycerol with hexadecyl, octadecyl and 9-octadecenyl alcohol, *i.e.* chimyl, batyl and selachyl alcohol, a method for the separation of the individual glycerol ethers was needed. This separation has also earlier been made with adsorption chromatography on alumina [3], but any separation of batyl from selachyl alcohol was not obtained.

The separation of methyl esters of high molecular weight fatty acids by gas-liquid chromatography (GLC) has been reported [4-6]. Since GLC permits microgram mixtures of methyl esters of fatty acids to be resolved, analysis of mixtures of glycerol ethers has been investigated.

The present paper describes the complete separation of chimyl, batyl and selachyl alcohol by GLC after acylation of the two hydroxyl groups of the glycerol ethers.

Apparatus and procedure. The GLC experiments were carried out with an Argon Pye Chromatograph. The temperature of operation was 218°C and the inlet pressure of the gas 72 cm Hg. The rate of flow of the carrier gas (Argon) was 20 cm³/min. The detector voltage was set on 1 250 V. Each 4-foot column was packed with 5 g of acid-washed, alkali-treated Celite (mesh 100—140). As stationary phase a polar polyester LHC-1R-296 (obtained from Cambridge Inc., Mass. U.S.A.) was used in the ratio Celite: stationary phase 4:1. Acylation of the two hydroxyl groups of the glycerol ethers was carried out with acetic anhydride in the presence of pyridine after boiling on a water bath for 12 h. The esterified mixture of glycerol ethers was isolated and dissolved in a few microlitres of ethyl acetate, and the solution taken up in a micropipette and applied to the column.

The retention volumes of the different glycerol ethers were determined using a mixture of known compounds (Table 1). The retention volumes are expressed relative to synthetic batyl alcohol, which was used as internal standard. When determining the retention volumes of the pure compounds, special care was taken not to overcharge the column in order to obtain symmetrical peaks on the chromatogram. The polyester column had a retention time of batyl alcohol diacetin of 49 min.

Table 1. GLC characteristics on LHC-1R-296 as stationary phase of three glycerol ethers after acylation. Temperature 218°C. Argon flow rate 20 cm³/min.

	Apparent retention volume relative to batyl alcohol-diacetin = 1.00
Chimyl alcohol	0.61
Batyl alcohol	1.00
Selachyl alcohol	1.11

Before having a gas-liquid chromatography technique available a similar separation was made by reversed phase partition chromatography using 50 % aqueous *iso*propanol as moving phase and 20 % chloroform in heptane as stationary phase. The band containing chimyl and selachyl alcohol was then subjected to renewed chromatography after hydrogenation, when the selachyl alcohol originally present is obtained as batyl alcohol which is easily separated from the chimyl alcohol.

Material. Hexadecyl-α-glyceryl ether (chimyl alcohol) and octadecyl-α-glyceryl ether (batyl alcohol) was prepared according to Holmes *et al.* [7]

Specimens of purified glycerol ethers mainly containing selachyl alcohol were prepared from tiger shark *(Galeocerdo curvier)* liver fat [1,2,6]. The samples obtained were run on the gas-chromatograph before and after hydrogenation.

This method has proved to be useful for biological work with glycerol ethers and it may also be used for purification of such substances.

Using the column and conditions described acylated monoglycerides of different saturated and unsaturated fatty acids can also be separated.

This work is part of investigations supported by the *Swedish Medical Research Council.*

1. Bergström, S. and Blomstrand, R. *Acta Physiol. Scand.* **38** (1956) 166.
2. Blomstrand, R. and Ahrens, E. H., Jr. *Proc. Soc. Exptl. Biol. Med.* **100** (1959) 802.
3. Prelog, V., Ruzicka, L. and Steinman, F. *Helv. Chim. Acta* **27** (1944) 674.
4. James, A. T. and Martin, A. J. P. *Biochem. J.* **63** (1956) 144.
5. Stoffel, W., Insull, W. and Ahrens, E. J., Jr. *Proc. Soc. Exptl. Biol. Med.* **99** (1958) 238.
6. Orr, C. H. and Callen, J. E. *J. Am. Chem. Soc.* **80** (1958) 249.
7. Holmes, N. N., Corbet, R. E., Geiger, W. B., Kornblum, N. and Alexander, W. *J. Am. Chem. Soc.* **63** (1941) 2607.
8. Karnovsky, M. L. and Brumm, A. F. *J. Biol. Chem.* **216** (1955) 689.

Received August 12, 1959.

Chromatography on ECTEOLA * of Sulphate Containing Mucopolysaccharides and Nucleotides

NILS R. RINGERTZ and PETER REICHARD

Department of Cell Research and Genetics and Department of Chemistry I, Karolinska Institutet, Stockholm 60, Sweden

During a study on the enzymatic synthesis of heparin [1] it was found difficult to remove traces of ^{35}S-containing nucleotides from mucopolysaccharides prepared by the cetylpyridinium method [2,3]. We therefore tried chromatography on ECTEOLA * cellulose for the separation of these substances. ECTEOLA has previously been found especially valuable for the chromatography of nucleotides and polynucleotides [4].

Fig. 1 shows the chromatographic behaviour of five different polysaccharides during step-wise elution from ECTEOLA. For good separations it was found necessary to work at a quite acid pH. In the experiments of Fig. 1 the increase in chloride concentration was obtained by adding equimolar amounts of NaCl and HCl. Similar chromatograms were obtained when a constant concentration of 0.05 M HCl was used in all steps, while the chloride concentration was regulated by increasing the amounts of NaCl in the eluent. $CaCl_2$ could be used instead of NaCl. $CaCl_2$ can be removed from the polysaccharide fractions by extraction with alcohol-ether. It interferes, however, strongly with the carbazol reaction [5] and it was therefore not used extensively. The recovery of polysaccharides was found to be close to 100 %. All operations were performed at 0–5°C.

Hyaluronic acid, chondroitinsulfuric acid and heparin had very different affinities for ECTEOLA which allowed their separation.

* ECTEOLA cellulose (E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.* **78** (1956) 751) was obtained from Brown Company, Berlin, New Hampshire, USA.

Before having a gas-liquid chromatography technique available a similar separation was made by reversed phase partition chromatography using 50 % aqueous *iso*propanol as moving phase and 20 % chloroform in heptane as stationary phase. The band containing chimyl and selachyl alcohol was then subjected to renewed chromatography after hydrogenation, when the selachyl alcohol originally present is obtained as batyl alcohol which is easily separated from the chimyl alcohol.

Material. Hexadecyl-α-glyceryl ether (chimyl alcohol) and octadecyl-α-glyceryl ether (batyl alcohol) was prepared according to Holmes *et al.* [7]

Specimens of purified glycerol ethers mainly containing selachyl alcohol were prepared from tiger shark *(Galeocerdo curvier)* liver fat [1,2,6]. The samples obtained were run on the gas-chromatograph before and after hydrogenation.

This method has proved to be useful for biological work with glycerol ethers and it may also be used for purification of such substances.

Using the column and conditions described acylated monoglycerides of different saturated and unsaturated fatty acids can also be separated.

This work is part of investigations supported by the *Swedish Medical Research Council.*

1. Bergström, S. and Blomstrand, R. *Acta Physiol. Scand.* **38** (1956) 166.
2. Blomstrand, R. and Ahrens, E. H., Jr. *Proc. Soc. Exptl. Biol. Med.* **100** (1959) 802.
3. Prelog, V., Ruzicka, L. and Steinman, F. *Helv. Chim. Acta* **27** (1944) 674.
4. James, A. T. and Martin, A. J. P. *Biochem. J.* **63** (1956) 144.
5. Stoffel, W., Insull, W. and Ahrens, E. J., Jr. *Proc. Soc. Exptl. Biol. Med.* **99** (1958) 238.
6. Orr, C. H. and Callen, J. E. *J. Am. Chem. Soc.* **80** (1958) 249.
7. Holmes, N. N., Corbet, R. E., Geiger, W. B., Kornblum, N. and Alexander, W. *J. Am. Chem. Soc.* **63** (1941) 2607.
8. Karnovsky, M. L. and Brumm, A. F. *J. Biol. Chem.* **216** (1955) 689.

Received August 12, 1959.

Chromatography on ECTEOLA * of Sulphate Containing Mucopolysaccharides and Nucleotides

NILS R. RINGERTZ and PETER REICHARD

Department of Cell Research and Genetics and Department of Chemistry I, Karolinska Institutet, Stockholm 60, Sweden

During a study on the enzymatic synthesis of heparin [1] it was found difficult to remove traces of ^{35}S-containing nucleotides from mucopolysaccharides prepared by the cetylpyridinium method [2,3]. We therefore tried chromatography on ECTEOLA * cellulose for the separation of these substances. ECTEOLA has previously been found especially valuable for the chromatography of nucleotides and polynucleotides [4].

Fig. 1 shows the chromatographic behaviour of five different polysaccharides during step-wise elution from ECTEOLA. For good separations it was found necessary to work at a quite acid pH. In the experiments of Fig. 1 the increase in chloride concentration was obtained by adding equimolar amounts of NaCl and HCl. Similar chromatograms were obtained when a constant concentration of 0.05 M HCl was used in all steps, while the chloride concentration was regulated by increasing the amounts of NaCl in the eluent. $CaCl_2$ could be used instead of NaCl. $CaCl_2$ can be removed from the polysaccharide fractions by extraction with alcohol-ether. It interferes, however, strongly with the carbazol reaction [5] and it was therefore not used extensively. The recovery of polysaccharides was found to be close to 100 %. All operations were performed at 0–5°C.

Hyaluronic acid, chondroitinsulfuric acid and heparin had very different affinities for ECTEOLA which allowed their separation.

* ECTEOLA cellulose (E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.* **78** (1956) 751) was obtained from Brown Company, Berlin, New Hampshire, USA.

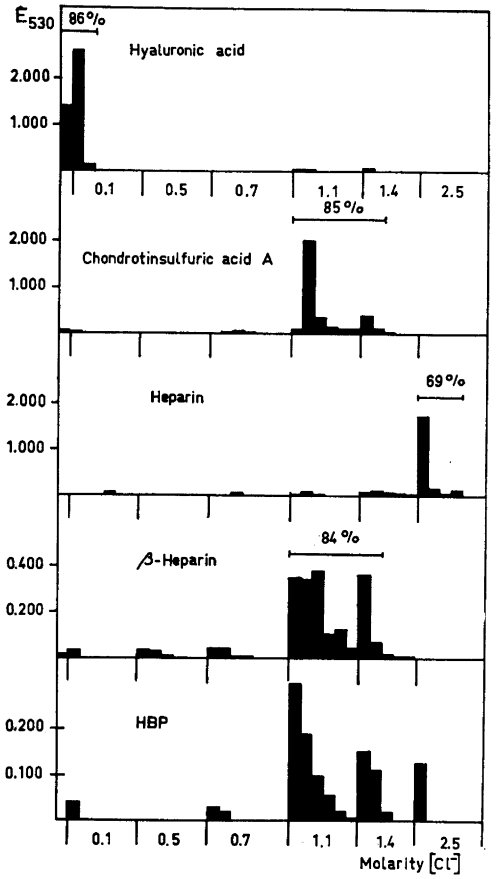


Fig. 1. Chromatography of mucopolysaccharides on ECTEOLA. The resin was washed with 5 liters of 0.5 M NaOH, 5 liters of 3 M NaCl and 3 liters of 0.1 M NaCl-HCl (1:1). Each polysaccharide (10—15 mg dissolved in 10—15 ml of 0.05 M NaCl-HCl) was adsorbed to a separate column (10 × 1 cm). The columns were eluted step-wise with buffers of increasing chloride concentrations (6 × 12 ml step). The fractions were analyzed by the carbazol method [5]. The figures over the main peaks in the chromatograms indicate the recovery of polysaccharides.

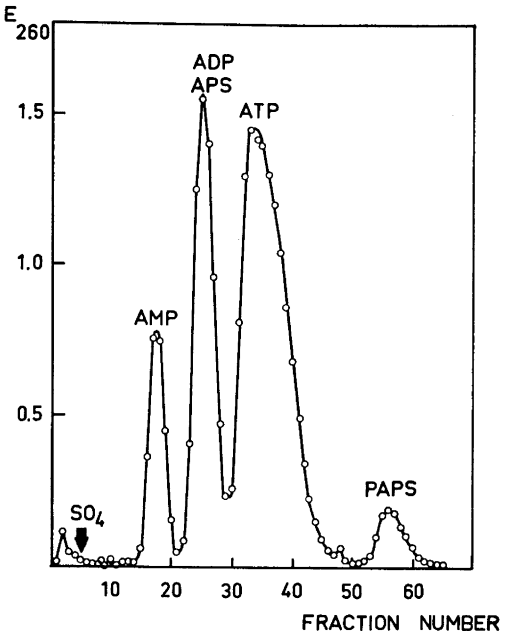


Fig. 2. Chromatography of nucleotides (20 μmoles, pH 7) on an ECTEOLA column (10 × 1 cm). Elution: Linear gradient; vessel 1 = 400 ml 0.01 M NaCl, 0.01 M tris-HCl, (tris-(hydroxymethyl)-aminomethane) pH 7; vessel 2 = 400 ml 0.4 M NaCl, 0.01 M tris-HCl, pH 7. Fractions 7 ml/30 min.

Such separations were actually performed with mixtures of these polysaccharides. It was also evident that the polysaccharides used were inhomogeneous. This was particularly true for a by-product obtained during heparin preparation. This polysaccharide (HBP) separated into four distinct fractions. Upon rechromatography the materials from each of the major fractions were recovered in their original positions. Table 1 shows some analytical data for these HBP-fractions. Evidently they differed largely in composition, *e.g.* as regards the relative proportions of glucosamine and galactosamine.

The anticoagulant activity [6] of heparin after chromatography using the most acid method (equimolar amounts of NaCl and HCl) was 94 Units/mg compared to 105 Units/mg before chromatography. Heparin recovered after elution according to the less acid method (0.05 M HCl) had an activity of 100 Units/mg. Because of the methodological errors involved, these three values are not considered to be significantly different. Analysis of heparin before and after chromatography showed that no loss of sulfate had occurred. This is further support for the absence of degradation.

Table 1. Analytical data for HBP polysaccharide and subfractions.

	SO_4[7]/aminosugar[8] molar ratio	glucosamine/ galactosamine [9] molar ratio	anticoagulant activity [6] units/mg
HBP polysaccharide	1.69	1.17	6
0.7 M subfraction	0.92	1.12	< 4
1.1 M »	2.20	0.60	< 2
1.4 M »	0.96	0.48	8
2.5 M »	1.39	4.83	12—20

Nucleotides were eluted at considerably lower salt concentrations. Adenosine 5'-phosphosulfate (APS) * and adenosine 3'-phosphate-5'-phosphosulfate (PAPS) * could be separated from adenosine 5'-phosphate and adenosine 5'-triphosphate at neutral pH. (Fig. 2). The recovery of nucleotides from the columns was close to 100 %. Chromatography at acid pH caused some degradation of labile nucleotides but served the purpose of removing nucleotides from mucopolysaccharides.

Acknowledgements. We thank Dr. S. Gardell, Dr. B. Högberg and Vitrum Company for generous gifts of mucopolysaccharides. The α-heparin was a gift from Dr. R. W. Jeanloz to Dr. S. Gardell. We also wish to thank prof. E. Jorpes for advice and help in the determination of anticoagulant activity.

1. Ringertz, N. R., Reichard, P. and Roden, L. *Unpublished.*
2. Scott, J. E. *Biochem. J.* **62** (1956) 31 P.
3. Scott, J. E. in Glick, D., Ed., *Methods of Biochemical Analysis*, Interscience Publishers Inc. New York. *To be published.*
4. Rosenkranz, H. S., Bendich, A., Pahl, H. B., Korngold, G. C. and Fresco, J. R. *J. Am. Chem. Soc.* **80** (1958) 3949.
5. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
6. Studer, A. and Winterstein, A. *Helv. Physiol. et Pharmacol. Acta* **9** (1951) 6.
7. Dodgson, K. S. and Spencer, B. *Biochem. J.* **55** (1953) 436.
8. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
9. Gardell, S. *Acta Chem. Scand.* **7** (1953) 207.

Received August 21, 1959.

* APS was synthesized by the carbodiimide method, Reichard, P. and Ringertz, N. R. *J. Am. Chem. Soc* **81** (1959) 878. PAPS was synthesized enzymatically from APS + ATP with a crude rat liver enzyme. Brunngraber, E. G. *J. Biol. Chem.* **233** (1958) 472.

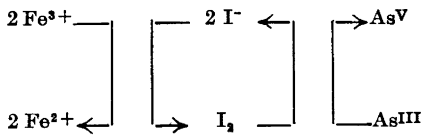
A Sensitive Colour Reaction for the Paper Chromatographic Detection of Iodide, Iodinated Tyrosines and Thyronines

ROLF GMELIN and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The most sensitive chemical reaction for the detection of iodide, iodinated tyrosines and thyronines uses the catalytic action of traces of iodide upon the reduction of ceric sulphate by arsenic acid (Bowden *et al.*[1]). It allows the detection of 0.1 μg thyroxine, for instance. Modifications of the method have been proposed by Fletcher and Stanley [2], Dragunova and Langer [3], Gawienowski [4], and Mandl and Block [5].

We have observed that in the presence of catalytical amounts of iodide also the systems $Fe^{3+} \rightarrow Fe^{2+}$ and $Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$ can be brought in reaction with the system $As^{V} \rightarrow As^{III}$ (Formulas 1 and 2). The reaction becomes especially sensitive when ferri- and ferricyanide-ions are reacting simultaneously, catalyzed by iodide, upon arsenic acid, since the formed ferro- and ferrocyanide-ions, respectively are fixed immediately, forming the insoluble pigment, prussian blue (Formula 3).



(1)

Table 1. Analytical data for HBP polysaccharide and subfractions.

	SO_4[7]/aminosugar[8] molar ratio	glucosamine/ galactosamine [9] molar ratio	anticoagulant activity [6] units/mg
HBP polysaccharide	1.69	1.17	6
0.7 M subfraction	0.92	1.12	< 4
1.1 M »	2.20	0.60	< 2
1.4 M »	0.96	0.48	8
2.5 M »	1.39	4.83	12—20

Nucleotides were eluted at considerably lower salt concentrations. Adenosine 5'-phosphosulfate (APS) * and adenosine 3'-phosphate-5'-phosphosulfate (PAPS) * could be separated from adenosine 5'-phosphate and adenosine 5'-triphosphate at neutral pH. (Fig. 2). The recovery of nucleotides from the columns was close to 100 %. Chromatography at acid pH caused some degradation of labile nucleotides but served the purpose of removing nucleotides from mucopolysaccharides.

Acknowledgements. We thank Dr. S. Gardell, Dr. B. Högberg and Vitrum Company for generous gifts of mucopolysaccharides. The α-heparin was a gift from Dr. R. W. Jeanloz to Dr. S. Gardell. We also wish to thank prof. E. Jorpes for advice and help in the determination of anticoagulant activity.

1. Ringertz, N. R., Reichard, P. and Roden, L. *Unpublished.*
2. Scott, J. E. *Biochem. J.* **62** (1956) 31 P.
3. Scott, J. E. in Glick, D., Ed., *Methods of Biochemical Analysis*, Interscience Publishers Inc. New York. *To be published.*
4. Rosenkranz, H. S., Bendich, A., Pahl, H. B., Korngold, G. C. and Fresco, J. R. *J. Am. Chem. Soc.* **80** (1958) 3949.
5. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
6. Studer, A. and Winterstein, A. *Helv. Physiol. et Pharmacol. Acta* **9** (1951) 6.
7. Dodgson, K. S. and Spencer, B. *Biochem. J.* **55** (1953) 436.
8. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
9. Gardell, S. *Acta Chem. Scand.* **7** (1953) 207.

Received August 21, 1959.

* APS was synthesized by the carbodiimide method, Reichard, P. and Ringertz, N. R. *J. Am. Chem. Soc* **81** (1959) 878. PAPS was synthesized enzymatically from APS + ATP with a crude rat liver enzyme. Brunngraber, E. G. *J. Biol. Chem.* **233** (1958) 472.

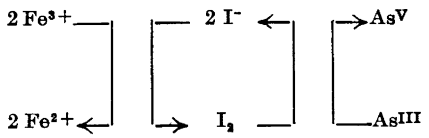
A Sensitive Colour Reaction for the Paper Chromatographic Detection of Iodide, Iodinated Tyrosines and Thyronines

ROLF GMELIN and ARTTURI I. VIRTANEN

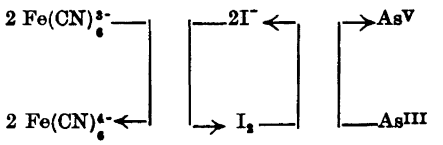
Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The most sensitive chemical reaction for the detection of iodide, iodinated tyrosines and thyronines uses the catalytic action of traces of iodide upon the reduction of ceric sulphate by arsenic acid (Bowden *et al.*[1]). It allows the detection of 0.1 μg thyroxine, for instance. Modifications of the method have been proposed by Fletcher and Stanley [2], Dragunova and Langer [3], Gawienowski [4], and Mandl and Block [5].

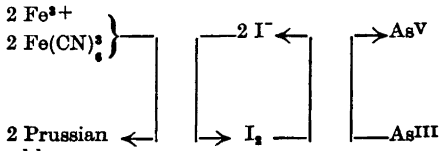
We have observed that in the presence of catalytical amounts of iodide also the systems $Fe^{3+} \rightarrow Fe^{2+}$ and $Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$ can be brought in reaction with the system $As^{V} \rightarrow As^{III}$ (Formulas 1 and 2). The reaction becomes especially sensitive when ferri- and ferricyanide-ions are reacting simultaneously, catalyzed by iodide, upon arsenic acid, since the formed ferro- and ferrocyanide-ions, respectively are fixed immediately, forming the insoluble pigment, prussian blue (Formula 3).



(1)



(2)



(3)

Iodide, iodinated tyrosines and thyronines appear in the ferrichloride-ferricyanide-arsenic acid (FFCA)-reaction as intense blue spots on a light blue background. The limit for the detection is 0.001 μg for iodide or 0.002 μg for thyroxine, triiodothyronine, diiodotyrosine, respectively. In practice it is advisable, however, to work with quantities between 0.01 and 0.1 μg per spot.

This means that the FFCA-reagent is about ten times more sensitive than the ceric sulphate-arsenic acid-reagent. Another advantage of the FFCA-reagent is that the paper chromatograms can be washed after the development of the spots and can be kept after drying indefinitely, while the paper chromatograms treated with the ceric sulphate-arsenic acid reagent will soon be destroyed under the corrosive influence of sulphuric acid.

Disadvantageous for the FFCA-reaction is the fact that also certain reducing substances (phenols, compounds with thiol groups, ascorbic acid, *etc.*) will respond to the reagent by formation of blue spots. Since these compounds are reacting, however, only in amounts above 1 μg per spot, and can be distinguished by their R_F-values, the usefulness of this new method is scarcely influenced by them.

Performance of the colour reaction. The carefully dried paper chromatograms are sprayed evenly from both sides with freshly prepared reagent and are put cautiously and without pressure between two clean glass plates. After a developing time of 15 min the paper is thoroughly washed with distilled water, pressed between filter paper and dried in a current of warm air. The spraying and development of the paper chromatograms has to be carried out in dim light or better in a dark room.

The blue spots which have been formed by iodide, iodinated tyrosines and thyronines can be measured photometrically and can be used for quantitative determinations. Good and reproducible values could be obtained in the same range of concentrations between 0.01 and 0.07 μg per spot with the Zeiss-Extinktionsschreiber II (Filter FE 54).

Preparation of the FFCA-reagent. (All reagents used are analytical grade, Merck.)

Solution A. 2.7 g $FeCl_3 \cdot 6H_2O$ are dissolved in 100 ml 2 N HCl-solution.

Solution B. 3.5 g $K_3Fe(CN)_6$ are dissolved in 100 ml distilled water.

Solution C. 5 g $NaAsO_2$ are dissolved under cooling in 30 ml N NaOH-solution. 65 ml 2 N HCl-solution are added under intense stirring.

Immediately before every use 5 parts of solution A, 5 parts of solution B, and 1 part of solution C are mixed. (Protection against light is important).

This investigation belongs to a research project under U. S. Public Law No. 480, 83rd Congress.

1. Bowden, C. H., Maclagan, N. F. and Wilkinson, I. M. *Biochem. J.* **59** (1955) 93.
2. Fletcher, K. and Stanley, P. G. *Nature* **175** (1955) 730.
3. Dragunova, I. and Langer, P. *Nature* **178** (1956) 537.
4. Gawienowski, A. M. *Analyst* **82** (1957) 452.
5. Mandl, R. H. and Block, R. J. *Arch. Biochem. Biophys.* **81** (1959) 25.

Received September 1, 1959.

Thermal Conductivity of Solid Solutions and the Measuring of Molecular Weights

OLE LAMM

Royal Institute of Technology, Division of Physical Chemistry, Stockholm 70, Sweden

Investigations on the exact measuring of thermal conductivity of solids and certain liquids has resulted in a Report [1] and two publications [2-3]. The "hot wire resistance thermometer" method was chosen by Lars Rombén, and developed by him and H.-E. Nissen. Later, D. G. Gillam brought this method to a present accuracy of ± 0.3 %. One of the goals of this investigation was to find out whether thermal resistivity could be used as a method for determining molecular weights. This seemed especially hopeful in the case of a solid solvent, owing to the high conductivity and the consequently greater and more well-defined effect of the introduction of new centres of scattering of the elastic waves through the solute molecules.

Accurate values have recently been reported by Gillam on the conductivity at 20°C of naphthalene and of four substances dissolved in naphthalene; diphenylmethane, triphenylmethane, diphenyl-*m*-tolylmethane, and diphenyl-*p*-tolylmethane [4]. The results clearly show that in all four solutions the conductivity decreases along one and the same function of the mole fraction. This function is a straight line up to a mole fraction of about 0.03. This is all what is needed for a *method of molecular weight determination* according to a relationship rather analogous to the ones valid for osmotic pressure and light scattering. The validity range of such a simple course will have to be settled by further experimental work. As in the other methods, a more detailed theory will be needed in less ideal cases; a lack of ideality will express itself quite differently when going from one method to another. An essential factor is, in the case considered, the temperature dependency. As a whole, the use of rather low temperatures and solid solvents with high conductivity will give the simplest information. The field of liquid solutions also deserves increased attention.

The determination of molecular weights only represents one aspect of the many possible on the thermal conductivity of mixtures in general. With the exception of the well-known work by Eucken [5] on solid solutions of KCl, KBr, the effects on solids seem to have been mainly referred to as a high sensitivity of the conductance to "impurities". However, the views expressed above, originating from the general ideas of Debye and Eucken, are related to recent, mainly low-temperature investigations on the scattering cross sections of isolated lattice points and on isotope effects, and by the recognition of resistivity to heat conduction as a very sensitive means of studying crystal imperfections, see Berman [6], Klemens [7], Slack [8], and Cohen [9].

1. Rombén, L. and Nissen, H.-E. *Manuscript (unpublished)* p. 1—82, Stockholm 1951 (in English).
2. Gillam, D. G., Rombén, L., Nissen, H.-E. and Lamm, O. *Acta Chem. Scand.* **9** (1955) 641.
3. Gillam, D. G. and Lamm, O. *Acta Chem. Scand.* **9** (1955) 657.
4. Gillam, D. G. *To be published elsewhere.*
5. Eucken, A. and Kuhn, G. Z. *physik. Chem.* **134** (1928) 193.
6. Berman, P. *Proc. Roy. Soc. (London) A* **208** (1951) 90.
7. Klemens, P. G. *Proc. Phys. Soc. (London) A* **68** (1955) 1113.
8. Slack, G. A. *Phys. Rev.* **105** (1957) 829, 832.
9. Cohen, A. F. *J. Appl. Phys.* **29** (1958) 591.

Received September 5, 1959.

NMR Evidence for Hydrogen Bonding in Ethyl Mercaptan

STURE FORSÉN

Research Group for NMR, Division of Physical Chemistry, Royal Institute of Technology, Stockholm, Sweden

Hydrogen bonding of the S—H... S type has for a long time been considered unlikely for mercaptans. Molecular weights determined by the method of freezing point depression have not revealed any tendency towards association [1]. Several investigators have failed to detect any dilution shifts in I.R. spectra of thiophenols and mercaptans [2,3]. Recently, however, Spurr and Byers [4] have studied the fundamental stretching region of the S—H group in some mercaptans and found two bands at about 2 580 cm^{-1} and 2 560 cm^{-1}, which they attribute to the monomer and dimer forms, respectively, of the mercaptans. They showed that the concentration dependent variations in the integrated adsorption coefficients for the two bands could be accounted for on the basis of a monomer-dimer equilibrium. As shown by a number of workers [5] the proton magnetic resonance (PMR) signal of the OH-proton in alcohols and phenols undergoes a shift towards higher fields as the compound is diluted in some inert solvent as CCl_4 or *cyclo*hexane. We have therefore looked for a corresponding dilution shift in the PMR-signal of the SH-group in ethyl mercaptan.

The measurements were carried out on a Varian V-4300 NMR-spectrometer operating at 40 Mc/s. All shifts were measured against an external water standard using coaxial glass cells [6] and correcting for the macroscopic susceptibility effects.

The shifts are discussed in terms of the dimensionless unit δ defined by the expression

$$\delta = 10^6 \cdot \frac{H_{sample} - H_{ext.water}}{H_{ext.water}}$$

The PMR spectrum of pure ethyl mercaptan is shown in Fig. 1 a. The SH-group gives rise to a triple-peak signal which partly covers the CH_3-group signal.

The ethyl mercaptan (Purum. FLUKA, freshly distilled) was diluted by CCl_4 (*pro analysi*, Merck, dried over P_2O_5) in steps down to $x = 0.01$ (mole fraction) and it was found that the SH-signal shifted totally $\Delta\delta_{SH} = 0.38 \pm 0.015$ towards higher fields. Within the accuracy of the measurements no shift of the CH_2- or CH_3-signals could be detected. We take the observed SH-dilution shift as evidence for a small but significant effect of hydrogen bonding. In Fig. 1 b the CH_3-region is shown at $x = 0.2$. The results are shown graphically in Fig. 2.

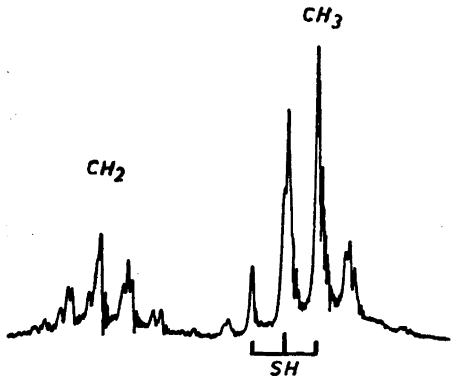


Fig. 1 a. The PMR spectrum of pure ethyl mercaptan. The position of the SH-group is indicated.

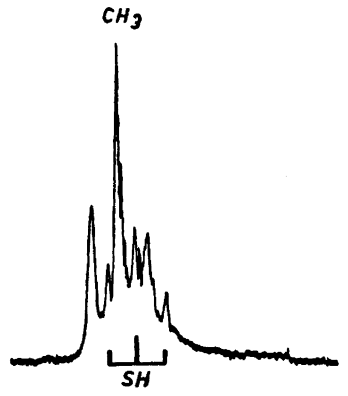


Fig. 1 b. The PMR spectrum of the CH_3-region in ethyl mercaptan diluted with carbon tetrachloride (mole fraction mercaptan = 0.2). The SH-signal has now moved to the right side of the CH_3-signal.

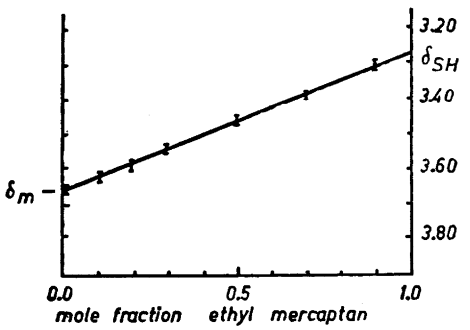


Fig. 2. The dilution shift in CCl_4-solution of the SH-proton resonance in terms of the dimensionless unit δ.

The SH-shift shows an almost linear dependence on the mole fraction mercaptan and this behaviour differs markedly from the one observed for alcohols [7]. The linear dependence and the smallness of the shift imply that the association constant in terms of mole fractions (K_x) should be small and that monomers and dimers dominate in the solution. Spurr and Byers give $K_c = 47$ mole/lit., which corresponds to $K_x = 0.22$. A rather crude approximation based on the formulas in Ref.[5a] is to set the slope of the δ_{SH} *vs.* x plot, $\left(\frac{d\delta}{dx}\right)$, equal to $2K_x\Delta_d$, and, somewhat arbitrarily, $\Delta_d = \delta_d - \delta_m$ to $\frac{1}{2} \cdot 0.38$ *. (See Ref.[5a] p. 1313). This gives $K_x = 0.38$ in reasonable agreement with the results of Spurr and Byers.

Acknowledgements. The author would like to thank the head of the NMR group Dr. Erik Forslind for his kind interest in this work. *Statens Råd för Atomforskning, Statens Naturvetenskapliga Forskningsråd* and *Statens Tekniska Forskningsråd* have provided financial support. The cost of the NMR apparatus has been defrayed by a generous grant from *Knut och Alice Wallenbergs Stiftelse.*

1. Lassettre, E. N. *Chem. Rev.*, **20** (1939) 259.
2. Gordy, W. and Stanford, S. C. *J. Am. Chem. Soc.* **62** (1940) 497.
3. Copley, M. J., Marvel, C. S. and Ginsberg, E. *J. Am. Chem. Soc.* **61** (1939) 3161.
4. Spurr, R. A. and Byers, H. F. *J. Phys. Chem.* **62** (1958) 425.
5a. Huggins, C. M., Pimentel, G. C. and Shoolery, J. N. *J. Phys. Chem.* **60** (1956) 1311.
b. Gutowsky, H. S. and Saika, A. *J. Chem. Phys.* **21** (1953) 1688.
6. Wilmad Glass Co., Landisville, New Jersey.
7. See *e. g.* Becker, E. D., Liddel, U. and Shoolery, J. N. *J. Mol. Spectr.* **2** (1958) 1.

Received September 4, 1959.

* δ_m is the shift of the monomer in relation to the reference ($H_2O_{ext.}$) and is given by δ_{SH} at $x = 0$. δ_d is the shift of the dimer and is not known exactly. It can not be taken as the value of δ_{SH} at $x = 1$.

Composition and Stability Constant of the Complex between Lead(II) and 4-(2-Pyridylazo)-resorcinol (PAR)

H. KRISTIANSEN and F. J. LANGMYHR

University of Oslo, Chemical Institute A, Blindern, Norway

For the EDTA-titration of lead(II) Weber [1] suggested PAR as metal indicator. Pollard, Hanson and Geary [2] recommended PAR as colorimetric reagent for cobalt, lead and uranium. The authors introduced [3] PAR as indicator by indirect EDTA-titration of macro and micro amounts of aluminium, the excess of EDTA being back-titrated with standard lead(II) solution.

In connection with the use of PAR as metal indicator, it is of interest to have information about the complex formed between the indicator and lead(II) ions. In this investigation the composition of the complex and its stability constant were determined spectrophotometrically.

Experimental. The sodium salt of PAR was synthesized according to a procedure given by Chichibabin [4]. The salt was purified by extractions with ether and transformed to the phenol by the passing of carbon dioxide. The phenol was extracted with ether, and again transformed to the sodium salt, which was recrystallized from alcohol. The product was finally dried *in vacuo* over phosphorus pentoxide. The

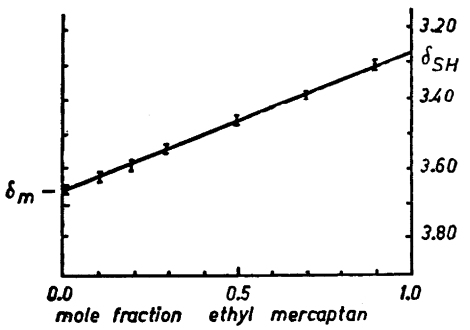


Fig. 2. The dilution shift in CCl_4-solution of the SH-proton resonance in terms of the dimensionless unit δ.

The SH-shift shows an almost linear dependence on the mole fraction mercaptan and this behaviour differs markedly from the one observed for alcohols [7]. The linear dependence and the smallness of the shift imply that the association constant in terms of mole fractions (K_x) should be small and that monomers and dimers dominate in the solution. Spurr and Byers give $K_c = 47$ mole/lit., which corresponds to $K_x = 0.22$. A rather crude approximation based on the formulas in Ref.[5a] is to set the slope of the δ_{SH} *vs.* x plot, $\left(\frac{d\delta}{dx}\right)$, equal to $2K_x\Delta_d$, and, somewhat arbitrarily, $\Delta_d = \delta_d - \delta_m$ to $\frac{1}{2} \cdot 0.38$ *. (See Ref.[5a] p. 1313). This gives $K_x = 0.38$ in reasonable agreement with the results of Spurr and Byers.

Acknowledgements. The author would like to thank the head of the NMR group Dr. Erik Forslind for his kind interest in this work. *Statens Råd för Atomforskning, Statens Naturvetenskapliga Forskningsråd* and *Statens Tekniska Forskningsråd* have provided financial support. The cost of the NMR apparatus has been defrayed by a generous grant from *Knut och Alice Wallenbergs Stiftelse.*

1. Lassettre, E. N. *Chem. Rev.*, **20** (1939) 259.
2. Gordy, W. and Stanford, S. C. *J. Am. Chem. Soc.* **62** (1940) 497.
3. Copley, M. J., Marvel, C. S. and Ginsberg, E. *J. Am. Chem. Soc.* **61** (1939) 3161.
4. Spurr, R. A. and Byers, H. F. *J. Phys. Chem.* **62** (1958) 425.
5a. Huggins, C. M., Pimentel, G. C. and Shoolery, J. N. *J. Phys. Chem.* **60** (1956) 1311.
b. Gutowsky, H. S. and Saika, A. *J. Chem. Phys.* **21** (1953) 1688.
6. Wilmad Glass Co., Landisville, New Jersey.
7. See *e. g.* Becker, E. D., Liddel, U. and Shoolery, J. N. *J. Mol. Spectr.* **2** (1958) 1.

Received September 4, 1959.

* δ_m is the shift of the monomer in relation to the reference ($H_2O_{ext.}$) and is given by δ_{SH} at $x = 0$. δ_d is the shift of the dimer and is not known exactly. It can not be taken as the value of δ_{SH} at $x = 1$.

Composition and Stability Constant of the Complex between Lead(II) and 4-(2-Pyridylazo)-resorcinol (PAR)

H. KRISTIANSEN and F. J. LANGMYHR

University of Oslo, Chemical Institute A, Blindern, Norway

For the EDTA-titration of lead(II) Weber [1] suggested PAR as metal indicator. Pollard, Hanson and Geary [2] recommended PAR as colorimetric reagent for cobalt, lead and uranium. The authors introduced [3] PAR as indicator by indirect EDTA-titration of macro and micro amounts of aluminium, the excess of EDTA being back-titrated with standard lead(II) solution.

In connection with the use of PAR as metal indicator, it is of interest to have information about the complex formed between the indicator and lead(II) ions. In this investigation the composition of the complex and its stability constant were determined spectrophotometrically.

Experimental. The sodium salt of PAR was synthesized according to a procedure given by Chichibabin [4]. The salt was purified by extractions with ether and transformed to the phenol by the passing of carbon dioxide. The phenol was extracted with ether, and again transformed to the sodium salt, which was recrystallized from alcohol. The product was finally dried *in vacuo* over phosphorus pentoxide. The

disodium salt was not analyzed, but an infrared absorption spectrum confirmed the expected structure.

A solution of PAR in water is orange and showed an absorption maximum at 410 mμ. In the pH range 6—10 PAR and lead(II) form a red complex with an absorption maximum at 515 mμ. Curves of continuous variations were plotted at pH 10 (ammonia-ammonium chloride buffer) for solutions of PAR and lead(II), and a distinct maximum was obtained at the mole ratio 1:1. The curves of continuous variations were then utilized for the calculation of the stability constant, which was found to be $K = 3.0 \times 10^6$. Ionic strength of the solutions was about 0.01.

1. Wehber, P. *Z. anal. Chem.* **158** (1957) 10.
2. Pollard, F. H., Hanson, P. and Geary, W. J. *Anal. Chim. Acta* **20** (1959) 26.
3. Langmyhr, F. J. and Kristiansen, H. *Anal. Chim. Acta* **20** (1959) 542.
4. Chichibabin, A. E. *Zhur. Russ. Fiz.-Khim. Obshchestva* **50** (1920) 512.

Received September 7, 1959.

A New Type of Enzymatic Cleavage of Mustard Oil Glucosides. Formation of Allylthiocyanate in *Thlaspi arvense* L. and Benzylthiocyanate in *Lepidium ruderale* L. and *Lepidium sativum* L.

ROLF GMELIN and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The cleavage of mustard oil glucosides to *iso*thiocyanate, sulphate, and glucose, which is effected by myrosinase, is considered to be a specific enzymatic process responsible for the characteristic pungent odour or taste of many representatives of the *Cruciferae, Tropaeolaceae, Capparidaceae, Resedaceae,* and some other plant families. After having worked out a revised structural formula for the mustard oil glucosides, Ettlinger and Lundeen [1] proposed that the enzymatic formation of *iso*thiocyanates takes place through Lossen-rearrangement (reaction 1). In some other representatives of the *Cruciferae* family an unpleasant garlic-like odour is characteristic. In the literature there is no real information on the chemical nature and the way of formation of these compounds. Some plant species even derive their names from the garliclike odour (*Thlaspi alliaceum* L., *Alliaria officinalis* Andrz., *Peltaria alliacea* Jacg.).

We have now investigated the chemical nature and formation of the substances that are responsible for this strong garlic-like odour, which has led to the use of the term "garlic oils" ("Lauchöle") [2] in the literature.

With fresh plants and seeds of *Thlaspi arvense* L., *Lepidium ruderale* L., and to a somewhat smaller extent of *Lepidium sativum* L., there appears an unpleasant odour and taste when the plant material is crushed with some water. Our investigations especially with seeds of *Thlaspi arvense, Lepidium ruderale,* and *Lepidium sativum* led to the following results:

1. An enzymatic process is responsible for the formation of the unpleasant smelling substances.

2. Sinigrin in *Thlaspi arvense* and glucotropaeolin in *Lepidium ruderale* and *L. sativum* are the precursors of the smelling substances.

3. In addition to glucose and sulphate allylthiocyanate in *Thlaspi arvense*, benzylthiocyanate in *Lepidium ruderale*, and a mixture of benzyl*iso*thiocyanate and benzylthiocyanate in *Lepidium sativum* are formed as fission products. Synthetic allyl- and benzylthiocyanates have the same odour and taste as is formed in *Thlaspi arvense* and *Lepidium ruderale*. The substances with similar, unpleasant odours, characteristic of many other *Cruciferae* plants when chewed, are accordingly alkylthiocyanates — a new class of natural S-containing compounds. Reactions 1 and 2 illustrate the formation of *iso*thiocyanates and normal thiocyanates.

Experimental evidence for the conclusions:

These smelling substances are not formed when the seedpowder is treated with boiling water or boiling alcohol. In methanolic extracts from seeds of *Thlaspi arvense*

disodium salt was not analyzed, but an infrared absorption spectrum confirmed the expected structure.

A solution of PAR in water is orange and showed an absorption maximum at 410 mμ. In the pH range 6—10 PAR and lead(II) form a red complex with an absorption maximum at 515 mμ. Curves of continuous variations were plotted at pH 10 (ammonia-ammonium chloride buffer) for solutions of PAR and lead(II), and a distinct maximum was obtained at the mole ratio 1:1. The curves of continuous variations were then utilized for the calculation of the stability constant, which was found to be $K = 3.0 \times 10^6$. Ionic strength of the solutions was about 0.01.

1. Wehber, P. *Z. anal. Chem.* **158** (1957) 10.
2. Pollard, F. H., Hanson, P. and Geary, W. J. *Anal. Chim. Acta* **20** (1959) 26.
3. Langmyhr, F. J. and Kristiansen, H. *Anal. Chim. Acta* **20** (1959) 542.
4. Chichibabin, A. E. *Zhur. Russ. Fiz.-Khim. Obshchestva* **50** (1920) 512.

Received September 7, 1959.

A New Type of Enzymatic Cleavage of Mustard Oil Glucosides. Formation of Allylthiocyanate in *Thlaspi arvense* L. and Benzylthiocyanate in *Lepidium ruderale* L. and *Lepidium sativum* L.

ROLF GMELIN and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The cleavage of mustard oil glucosides to *iso*thiocyanate, sulphate, and glucose, which is effected by myrosinase, is considered to be a specific enzymatic process responsible for the characteristic pungent odour or taste of many representatives of the *Cruciferae, Tropaeolaceae, Capparidaceae, Resedaceae,* and some other plant families. After having worked out a revised structural formula for the mustard oil glucosides, Ettlinger and Lundeen [1] proposed that the enzymatic formation of *iso*thiocyanates takes place through Lossen-rearrangement (reaction 1). In some other representatives of the *Cruciferae* family an unpleasant garlic-like odour is characteristic. In the literature there is no real information on the chemical nature and the way of formation of these compounds. Some plant species even derive their names from the garliclike odour (*Thlaspi alliaceum* L., *Alliaria officinalis* Andrz., *Peltaria alliacea* Jacg.).

We have now investigated the chemical nature and formation of the substances that are responsible for this strong garlic-like odour, which has led to the use of the term "garlic oils" ("Lauchöle") [2] in the literature.

With fresh plants and seeds of *Thlaspi arvense* L., *Lepidium ruderale* L., and to a somewhat smaller extent of *Lepidium sativum* L., there appears an unpleasant odour and taste when the plant material is crushed with some water. Our investigations especially with seeds of *Thlaspi arvense, Lepidium ruderale,* and *Lepidium sativum* led to the following results:

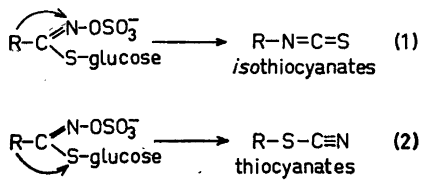
1. An enzymatic process is responsible for the formation of the unpleasant smelling substances.

2. Sinigrin in *Thlaspi arvense* and glucotropaeolin in *Lepidium ruderale* and *L. sativum* are the precursors of the smelling substances.

3. In addition to glucose and sulphate allylthiocyanate in *Thlaspi arvense*, benzylthiocyanate in *Lepidium ruderale*, and a mixture of benzyl*iso*thiocyanate and benzylthiocyanate in *Lepidium sativum* are formed as fission products. Synthetic allyl- and benzylthiocyanates have the same odour and taste as is formed in *Thlaspi arvense* and *Lepidium ruderale*. The substances with similar, unpleasant odours, characteristic of many other *Cruciferae* plants when chewed, are accordingly alkylthiocyanates — a new class of natural S-containing compounds. Reactions 1 and 2 illustrate the formation of *iso*thiocyanates and normal thiocyanates.

Experimental evidence for the conclusions:

These smelling substances are not formed when the seedpowder is treated with boiling water or boiling alcohol. In methanolic extracts from seeds of *Thlaspi arvense*



sinigrin can be detected by paper chromatography, while from seeds of *Lepidium ruderale* and *Lepidium sativum* the glucoside detected is glucotropaeolin. Both glucosides could be isolated (sinigrin in 1.4 % yield from *Thlaspi arvense* seeds, and glucotropaeolin in 1.5 % yield from *Lepidium ruderale*). They proved to be identical with authentic preparations from *Brassica nigra* Koch and *Tropaeolum majus* L. No other glucosides could be found by paper chromatography in *Thlaspi arvense* and in the two *Lepidium* species.

When the finely ground seeds are treated with cold water, after half an hour sinigrin and glucotropaeolin are no longer detectable. On the other hand the content of free glucose and sulphate increases and corresponds to the amount of cleaved glucosides. The formation of allyl*iso*thiocyanate (in *Thl. arvense*) and benzyl*iso*thiocyanate (in *Lep. ruderale*) which normally would be expected is lacking, however. In regard to the typical odour the S-containing compounds which are formed besides glucose and sulphate, are identical with synthetic allyl- and benzylthiocyanate. The identity of the two thiocyanates with allylthiocyanate and benzylthiocyanate was confirmed by the following reactions:

1. Cleavage by nascent hydrogen, by H_2S or by cysteine led to allylmercaptan and benzylmercaptan, respectively, which were isolated and analyzed as Hg-mercaptides.

2. By cleavage with NaSH thiocyanate ions were formed together with allylmercaptan and benzylmercaptan. The colorimetrically determined thiocyanate ions were in good agreement with the amounts of sinigrin or glucotropaeolin originally present.

3. In model experiments the synthetic preparations of allyl- and benzylthiocyanates behaved qualitatively and quantitatively analogously in the formation of mercaptans, mercaptides, and thiocyanate ions.

4. A small amount of benzylthiocyanate could be isolated in crystalline form from moistened, crushed seeds of *Lepidium ruderale* by steam distillation. Melting points and IR-spectra of the natural and synthetic products were identical.

The factors which are responsible for the formation of *iso*thiocyanates in most cases and of thiocyanates in some cases, are not known. Attempts to separate a thiocyanate forming enzyme led in every case to enzyme preparations with myrosinase activity which in the normal way split mustard oil glucosides to *iso*thiocyanate, glucose, and sulphate. If a certain factor which regulates or directs the migration of the alkyl residue to the S-atom instead of to the N-atom exists in the plants where thiocyanates are formed, could not yet be decided. There are, however, indications for this conclusion. It is possible that in the seeds of *Lepidium sativum*, in which benzylthiocyanate and benzyl*iso*thiocyanate are formed simultaneously by the enzymatic process, the factor is present in a smaller quantity than in *Lepidium ruderale*.

We have evidence that this new type of cleavage of the mustard oil glucosides into thiocyanates is rather common in the *Cruciferae* family. The new findings will be reported in a later communication.

Because of their reactivity with thiol-groups and because of their ability to form thiocyanate ions, normal alkylthiocyanates are by no means physiologically harmless substances. Since many important vegetable and fodder plants belong to the *Cruciferae* family, the question of the formation and physiological activity of the thiocyanates seems to be an important problem.

This investigation belongs to a research project under U. S. Public Law No. 480, 83rd Congress.

1. Ettlinger, M. G. and Lundeen, A. J. *J. Am. Chem. Soc.* **78** (1956) 4172.
2. Czapek, Fr. *Biochemie der Pflanzen*, 2. Aufl., G. Fischer, Jena 1921, Vol. 3, p. 183.

Received September 12, 1959.

On the Biological Methylation of Histamine. 4

KERSTIN M. LINDAHL

Institute of Zoophysiology, University of Uppsala, Sweden

In the past few years Schayer and his coworkers have shown that methylation of histamine to 1,4-methylhistamine * and its further oxidation to 1-methylimidazole-4-acetic acid is the principal route of histamine inactivation in man, mouse, cat, and dog. The literature concerning the catabolism of histamine *in vivo* has recently been reviewed by Schayer [1].

In vitro studies concerning the histamine methylating enzyme system have been discussed in earlier communications under the above heading [2-4]. It was reported that the formation of 1,4-methylhistamine requires methionine plus an ATP generating system, this leading on to the finding that S-adenosylmethionine is a most effective methyl donor in this system. These results have later been confirmed in other laboratories [5].

The present communication deals with the influence of some commonly used SH-inhibitors on the histamine methylating enzyme in liver extract.

Enzyme preparation. Pig liver was homogenized in 0.1 M NaAc buffer pH 5.6, centrifuged at 10 000 *g*, heated to 47° for 7 min, and centrifuged at 20 000 *g*. The supernatant liquid was fractionated with $(NH_4)_2SO_4$ at pH 6. The precipitate obtained between 50 and 70 % saturation was dissolved in 0.05 M NaAc pH 5.6, dialyzed overnight, and the resulting solution used for the experiments after centrifugation.

Determination of enzyme activity. The method makes use of the fact that 1,4-methylhistamine is extracted by chloroform from an alkaline solution, while histamine is not [6].

25 μl of the enzyme preparation, containing 25 mg protein per ml, were incubated for 30 min at 37°C with 0.050 μC ^{14}C-histamine and 0.3 μmoles S-adenosylmethionine in 0.5 M Tris buffer of pH 8.0 to make 200 μl. The reaction was interrupted by dipping the test tubes in boiling water. After addition of 50 μl 10 N NaOH saturated with Na_2SO_4 at 100°C and cooling, the samples were extracted with 1 000 μl of chloroform by vigorous shaking for 10 min.

The phases were separated by rapid centrifugation. Samples of 200 μl were withdrawn from the chloroform layer, and evaporated on planchets. The radioactivity was counted under an end window Geiger-Müller tube. From these figures the percentage methylhistamine formed was obtained with the aid of a calibration curve. Details of the method will be published later.

Effect of inhibitors.

o-Iodosobenzoic acid in concentrations 1 to 25 × 10^{-4} M had no influence on the enzymic activity, when the inhibitor was brought in contact with the enzyme 10 min prior to the substrate.

p-Chloromercuribenzoic acid markedly inhibited the histamine methylation (Table 1). The concentration necessary for com-

* Correct name: 1-methyl-4-(β-aminoethyl) imidazole.

Table 1. The effect of *p*-chloromercuribenzoate on the histamine methylating enzyme in pig liver.

Conc. of *p*-chloromercuribenzoate M	No glutathione added		Glutathione added in conc. 5 × 10^{-4} M	
	Histamine methylated %	Inhibition %	Histamine methylated %	Inhibition %
—	70	—	70	0
5 × 10^{-6}	70	0		
5 × 10^{-5}	67	4.3		
1.25 × 10^{-4}	59	15.7		
2.5 × 10^{-4}	31	55.7	70	0
5 × 10^{-4}	0	100	62	11.4
12.5 × 10^{-4}	0	100		

plete inhibition is rather high, *viz.* 5×10^{-4} M, probably on account of the presence of comparatively large amounts of inactive protein in the enzyme preparation. The inhibition was not reversed by dialysis. However, glutathione in slightly higher than equimolar concentrations abolished the inhibition completely.

Chloroacetophenone in high concentrations inhibited the enzyme, to a certain extent, *viz.* 90 % at 2.5×10^{-3} M. *Iodoacetamide* had a similar effect.

Garlic extract contains allicin which is known as an inhibitor of SH-groups [7]. It may be noted that garlic extract, prepared in the proportion 1:200 w/v, inhibits the enzyme completely. Glutathione counteracts this inhibition.

The inhibition of the histamine methylation by *p*-chloromercuribenzoate and the counteraction of glutathione are taken as evidence that SH-groups might be essential for the activity of the methylating enzyme. The inefficiency of *o*-iodosobenzoate may be explained by a protecting effect of other proteins present.

This work is part of investigations made possible by grants from the *Swedish Medical Research Council.*

1. Schayer, R. W. *Physiol. Revs.* **39** (1959) 116.
2. Lindahl, K. M. *Arkiv Kemi* **13** (1958) 149.
3. Lindahl, K. M. *Acta Chem. Scand.* **12** (1958) 1690.
4. Lindahl, K. M. *Acta Chem. Scand.* **12** (1958) 2050.
5. Brown, D. D., Axelrod, J. and Tomchick, R. *Nature* **183** (1959) 680.
6. Rothschild, Z. and Schayer, R. W. *Biochim. et Biophys. Acta* **30** (1958) 23.
7. Wills, E. D. *Biochem. J.* **63** (1956) 514.

Received September 12, 1959.

Long Range Coupling of Nuclear Spins in Some Olefinic and Acetylenic Compounds

RAGNAR A. HOFFMAN and SALO GRONOWITZ

Institutes of Physics and Chemistry, University of Uppsala, Uppsala, Sweden

In view of the growing interest in the theory of the indirect coupling of nuclear spins [1-6] we wish to report some recent findings in connection with our study of long range couplings in proton magnetic resonance spectra.

In an earlier communication [7] one of us suggested that the coupling of methyl group protons over four bond distances with protons bonded to unsaturated carbon atoms should be understood in terms of hyperconjugation.

The need for new evidence for the existence of hyperconjugation in the ground state of unsaturated organic molecules has become obvious as Dewar and Schmeising have recently pointed out [8] that the earlier evidence is inconclusive. It is hoped that a continued investigation of the above mentioned spin couplings may provide such evidence.

In order to verify the interpretation given in Ref.[7], we have tested two predictions that follow from the discussion presented there.

1. The interaction of the π-electron, on an unsaturated carbon atom, with the protons of an attached methyl group is approximately equal to its interaction with a proton directly bound to the unsaturated carbon.

2. The coupling of methyl group protons (to other protons) over triple bonds should be considerably larger (about twice) than those over double bonds.

These predictions were tested in a number of substances. Thus we find, that the coupling between the two non-equivalent methyl groups in tiglaldehyde and in β-bromoangelic acid methylester are 1.0 c/s and 1.5 c/s, respectively *, or of the same order of magnitude as J^{I}_{ab} in *trans*-crotonaldehyde (I), which equals 1.6 c/s or the analogous coupling in *trans*-propenylbenzene, which is reported [9] to equal 1.8 c/s.

Since methyl acetylene is a gas at room temperature, we have instead studied propargyl alcohol and propargyl chloride. Here the coupling of the methylenic protons with the acetylenic proton equals 2.4 c/s and 2.6 c/s, resp., in agreement with the values 2.6—2.8 c/s for the propargyl halides reported by Whipple *et al.*[10]

When the acetylenic hydrogens are substituted by methyl groups, the long range coupling constants (here between the methyl and methylene protons) remain vir-

* All coupling constants reported here are given to $\pm$ 0.1 c/s. We use the symbol J^{I}_{ab} to denote the coupling constant J between groups a and b in compound I.

plete inhibition is rather high, *viz.* 5×10^{-4} M, probably on account of the presence of comparatively large amounts of inactive protein in the enzyme preparation. The inhibition was not reversed by dialysis. However, glutathione in slightly higher than equimolar concentrations abolished the inhibition completely.

Chloroacetophenone in high concentrations inhibited the enzyme, to a certain extent, *viz.* 90 % at 2.5×10^{-3} M. *Iodoacetamide* had a similar effect.

Garlic extract contains allicin which is known as an inhibitor of SH-groups [7]. It may be noted that garlic extract, prepared in the proportion 1:200 w/v, inhibits the enzyme completely. Glutathione counteracts this inhibition.

The inhibition of the histamine methylation by *p*-chloromercuribenzoate and the counteraction of glutathione are taken as evidence that SH-groups might be essential for the activity of the methylating enzyme. The inefficiency of *o*-iodosobenzoate may be explained by a protecting effect of other proteins present.

This work is part of investigations made possible by grants from the *Swedish Medical Research Council.*

1. Schayer, R. W. *Physiol. Revs.* **39** (1959) 116.
2. Lindahl, K. M. *Arkiv Kemi* **13** (1958) 149.
3. Lindahl, K. M. *Acta Chem. Scand.* **12** (1958) 1690.
4. Lindahl, K. M. *Acta Chem. Scand.* **12** (1958) 2050.
5. Brown, D. D., Axelrod, J. and Tomchick, R. *Nature* **183** (1959) 680.
6. Rothschild, Z. and Schayer, R. W. *Biochim. et Biophys. Acta* **30** (1958) 23.
7. Wills, E. D. *Biochem. J.* **63** (1956) 514.

Received September 12, 1959.

Long Range Coupling of Nuclear Spins in Some Olefinic and Acetylenic Compounds

RAGNAR A. HOFFMAN and SALO GRONOWITZ

Institutes of Physics and Chemistry, University of Uppsala, Uppsala, Sweden

In view of the growing interest in the theory of the indirect coupling of nuclear spins [1-6] we wish to report some recent findings in connection with our study of long range couplings in proton magnetic resonance spectra.

In an earlier communication [7] one of us suggested that the coupling of methyl group protons over four bond distances with protons bonded to unsaturated carbon atoms should be understood in terms of hyperconjugation.

The need for new evidence for the existence of hyperconjugation in the ground state of unsaturated organic molecules has become obvious as Dewar and Schmeising have recently pointed out [8] that the earlier evidence is inconclusive. It is hoped that a continued investigation of the above mentioned spin couplings may provide such evidence.

In order to verify the interpretation given in Ref.[7], we have tested two predictions that follow from the discussion presented there.

1. The interaction of the π-electron, on an unsaturated carbon atom, with the protons of an attached methyl group is approximately equal to its interaction with a proton directly bound to the unsaturated carbon.

2. The coupling of methyl group protons (to other protons) over triple bonds should be considerably larger (about twice) than those over double bonds.

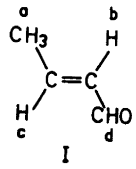
These predictions were tested in a number of substances. Thus we find, that the coupling between the two non-equivalent methyl groups in tiglaldehyde and in β-bromoangelic acid methylester are 1.0 c/s and 1.5 c/s, respectively *, or of the same order of magnitude as J^{I}_{ab} in *trans*-crotonaldehyde (I), which equals 1.6 c/s or the analogous coupling in *trans*-propenylbenzene, which is reported [9] to equal 1.8 c/s.

Since methyl acetylene is a gas at room temperature, we have instead studied propargyl alcohol and propargyl chloride. Here the coupling of the methylenic protons with the acetylenic proton equals 2.4 c/s and 2.6 c/s, resp., in agreement with the values 2.6—2.8 c/s for the propargyl halides reported by Whipple *et al.*[10]

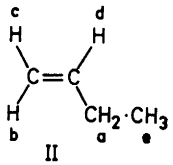
When the acetylenic hydrogens are substituted by methyl groups, the long range coupling constants (here between the methyl and methylene protons) remain vir-

* All coupling constants reported here are given to $\pm$ 0.1 c/s. We use the symbol J^{I}_{ab} to denote the coupling constant J between groups a and b in compound I.

tually unchanged and are found to be 2.4 c/s and 2.5 c/s in the 2-butyne-1-ol and 1-chloro-2-butyne, resp., in perfect agreement with our first prediction. Prediction 2) is reasonably well fulfilled.



I



II

In two compounds studied by us, crotonaldehyde (I) and tiglaldehyde, relative signs of the methyl group coupling constants could be determined. In tiglaldehyde the 40 Mc/s spectrum agrees with that calculated with second order perturbation theory [11] if all coupling constants are assumed to have equal sign. An Y_3 ABX-treatment [12] of the crotonaldehyde (I) spectrum at 40 Mc/s gave two possible assignments: one assuming J^{I}_{ab} and J^{I}_{ac} having equal sign, the other assuming unlike signs of these coupling constants. A well resolved (10^8) spectrum obtained at a radio frequency of 25 Mc/s * favours the latter assignment.

This is in agreement with the results obtained for the similar compound *trans*-propenylbenzene and for but-1-ene (II) by other workers [9,13]. Alexander concluded [13] that J^{II}_{ad} in this latter compound is of opposite sign to J^{II}_{ab} and J^{II}_{ac}. He ascribes the negative value to J^{II}_{ad}. Because the methylene protons have a large shift to the vinyl protons and the influence of the coupling constant J^{II}_{ac} seems to have been excluded from the calculation, we assume that the reverse assignment of signs would give the same calculated spectrum. If this assumption is valid we prefer to ascribe the negative coupling in crotonaldehyde (I) to J^{I}_{ab}. The cases of tiglaldehyde and crotonaldehyde would then form a neat parallel to the VB-calculations of π-electron couplings in even-alternant hydrocarbons by McConnell [5] (*cf.* also a recent note by McLachlan [14]), and also with the relative signs of coupling constants of a conjugated diene as reported by Elvidge and Jackmann [15].

A more comprehensive discussion of the above mentioned results with reference to related observations by other workers in this field will be submitted for publication at a later date.

The authors wish to express their gratitude to professors Kai Siegbahn and Arne Fredga for their interest in this work. A grant to one of us (RAH) from the *State Council of Technical Research* is gratefully acknowledged.

* We are greatly indebted to Dr. H. Primas and Dr. P. Bommer (Zürich) for providing this spectrum.

1. Mc Connell, H. M. *J. Chem. Phys.* **24** (1956) 460.
2. Pople, J. A. *Molecular Phys.* **1** (1958) 216.
3. Karplus, M. and Anderson, D. H. *J. Chem. Phys.* **30** (1959) 6.
4. Karplus, M. *J. Chem. Phys.* **30** (1959) 11.
5. McConnell, H. M. *J. Chem. Phys.* **30** (1959) 126.
6. Williams, G. A. and Gutowsky, H. S. *J. Chem. Phys.* **30** (1959) 717.
7. Hoffman, R. A. *Molecular Phys.* **1** (1958) 326.
8. Dewar, M. J. S. and Schmeising, N. K. *Tetrahedron* **5** (1959) 166.
9. Fessenden, R. W. and Waugh, J. S. *J. Chem. Phys.* **30** (1959) 944.
10. Whipple, E. B., Goldstein, J. H., Mandell, L., Reddy, G. S., and Mc Clure, G. R. *J. Am. Chem. Soc.* **81** (1959) 1321.
11. Anderson, W. A. *Phys. Rev.* **102** (1956) 151.
12. Bernstein, H. J., Pople, J. A. and Schneider, W. G. *Can. J. Chem.* **35** (1957) 65.
13. Alexander, S. *J. Chem. Phys.* **28** (1958) 358.
14. McLachlan, A. D. *Molecular. Phys.* **2** (1959) 223.
15. Elvidge, J. A. and Jackmann, L. M. *Proc. Chem. Soc.* **1959** 89.

Received September 22, 1959.

Preparation and Determination of Sodiumhydrogen S-(2-aminoethyl) Phosphorothioate (Sodiumhydrogen Cysteamine-S-phosphate)

STIG ÅKERFELDT

Eastern Pennsylvania Psychiatric Institute, Philadelphia, Pennsylvania, U.S.A.

In a study of S-phosphorylation in biological systems it became of interest to have access to S-(2-aminoethyl) phosphorothioic acid (cysteamine-S-phosphoric acid *). This substance could possibly be formed by enzymic S-phosphorylation of 2-aminoethanethiol (cysteamine).

The sodiumhydrogen salt of cysteamine-S-phosphoric acid has now been prepared from trisodium phosphorothioate and 2-bromoethylammonium bromide with N,N-dimethylformamide as catalyst according to the following scheme:

$$BrNH_3CH_2CH_2Br + Na_3SPO_3 \longrightarrow NH_2CH_2CH_2SPO_3HNa + 2\ NaBr$$

A slight excess of 2-bromoethylammonium bromide was used to ascertain complete reaction of the phosphorothioate. This reaction gave directly an analytically pure product in good yield (95 %).

From the above scheme it follows that the 2-aminoethyl residue could possibly be bound either to an oxygen or to the sulfur atom of the phosphorothioic acid residue (or to both in a mixture). Therefore, the actual position of this group in the molecule had to be established. From the following experiments it could be concluded that the 2-aminoethyl group in the synthesized substance is exclusively bound to the sulfur atom of the phosphorothioic acid residue: a) unlike phosphorothioate [1] the substance does not reduce iodine in strongly acid solution; b) hydrolysis in 1 M perchloric acid at 100° gave *ortho*-phosphate and a nitroprussiate positive substance. Hydrogen sulfide or phosphorothioate could not be detected. The hydrolysate consumed 98.2 atom-% iodine per mole of compound hydrolyzed *; c) paper chromatographic analysis of untreated and iodine oxidized hydrolysate ** (*n*-butanol: acetic acid: water, 4:1:1, and *n*-propanol: 29 % ammonia: water, 6:3:1) gave the same result as was obtained with authentical cysteamine treated the same way as the hydrolysate. No trace of 2-aminoethanol could be detected.

Mercury(II)ions (and 6-chloromercuri-2-nitrophenol [2]) were found to catalyze the hydrolysis of cysteamine-S-phosphate in acid solution. This fact has been utilized in the colorimetric determination of the substance. The optimum mercury(II)ion concentration was found to be about 45 μM (higher concentrations develop cloudiness in the samples). The same color yield was obtained with this method as is obtained with *ortho*-phosphate alone.

Methods and results. Trisodium phosphorothioate was prepared according to Yasuda and Lambert[3]. Phosphate determinations were based on the method by Gomori[4]. 2-Bromoethylammonium bromide was obtained from Eastman Kodak Company.

Synthesis of sodiumhydrogen cysteamine-S-phosphate. 9.0 g (50 mmole) of trisodium phosphorothioate were dissolved in 50 ml of water. 10.9 g (53 mmole) of 2-bromoethylammonium bromide and 25 ml of N,N-dimethylformamide were added. The solution was vigorously stirred for 40 min during which time a large amount of white crystals separated. (The reaction is conveniently followed by the disappearence of phosphorothioate, which is tested for by the addition of silver ions, whith which a black precipitate is obtained). 300 ml of ethanol were then added and the precipitated crystals (Found: P 12.3; H_2O 28.7***. Calc. for $NH_2CH_2CH_2SPO_3HNa, 4H_2O$: P 12.3; H_2O 28.7) were collected by filtration and washed thoroughly with ethanol. The crystals were dehydrated by stirring them in

* This is in line with the terminology adopted by *Chemical Abstracts* for certain biologically important phosphate esters, *e.g.*, creatine phosphate *etc.* However, to prevent confusion with the isomer cysteamine-N-phosphate the letter S is used to denote the position of the phosphate residue.

* This is about the same yield as was obtained with pure cysteamine after similar treatment.

** To avoid multiple spot formation, which was observed in the presence of perchlorate, hydrochloric acid was used for the hydrolysis in this experiment.

*** Water of crystallization was determined by drying to constant weight at 105°.

200 ml of dry methanol for 1 h. 8.5 g (95 %) of substance were thus obtained after drying *in vacuo*. (Found: C 13.5; H 3.9; P 17.3. Calc. for $NH_4CH_2CH_2SPO_3HNa$ (179.13):C 13.4; H 3.9; P 17.3). The dehydrated product was found to be more stable upon storage than the originally precipitated crystals, which easily lose water of crystallization.

Hydrolysis of cysteamine-S-phosphate. 2.668 mmole of cysteamine-S-phosphate were dissolved in 10 ml of 1 M perchloric acid and heated on a boiling water bath for 30 min in a stream of nitrogen. After hydrolysis this solution consumed 2.62 matom of iodine (98.2 atom-% per mole of cysteamine-S-phosphate) as titrated with a standardized iodine solution.

A similar procedure of hydrolysis was used in the early experiments to determine *ortho*-phosphate.

Determination of cysteamine-S-phosphate by mercury(II)ion catalyzed hydrolysis. To 0.50 ml of sample (containing 0—1 μmole cysteamine-S-phosphate) were added: 0.10 ml mercury(II) acetate (70 mg mercury(II)acetate dissolved in 100 ml of 5 % acetic acid), 2.90 ml of water, 0.50 ml of "elon"[4] and 1.00 ml of molybdate[4]. The solution was set aside for 1 h at room temperature and read against a similarily treated blank (water instead of sample) at 660 mμ in a 1 cm cuvette. One μmole of cysteamine-S-phosphate in the sample was found to give an absorbancy of 0.712. Some quantitative data are presented in Table 1.

Table 1. Recovery experiment for determination of cysteamine-S-phosphate.

Cysteamine-S-phosphate, mmole		% error
Added	Found	
0.213	0.213	0
0.425	0.423	—0.5
0.638	0.643	+0.8
0.851	0.847	—0.5
1.06	1.05	—0.9

The author is indebted to Mrs. M. L. Kip for valuable technical assistance.

1. Thilo, E. and Schöne, E. *Z.anorg.Chem.* **259** (1949) 225.
2. Åkerfeldt, S. *Acta Chem. Scand.* **13** (1959) 627.
3. Yasuda, S. K. and Lambert, J. L. *J. Am. Chem. Soc.* **76** (1954) 5356.
4. Gomori, G. *J. Lab. Clin. Med.* **27** (1942) 955.

Received August 24, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1481–1501

Studies on Nitrosylruthenium Complexes in Nitric Acid Using Repeated Extractions

G. RUDSTAM*

The Gustaf Werner Institute for Nuclear Chemistry, University of Uppsala, Sweden

Repeated extractions using the system tri-*n*-butyl phosphate (TBP) — nitric acid have been applied to the study of nitrosylruthenium nitrates. A detailed investigation of the partition coefficients has been carried out. The equilibrium composition of a solution of nitrosylruthenium nitrates in aqueous nitric acid has been determined, and the kinetics of the interchange between the complexes have been studied.

In the presence of oxides of nitrogen, ruthenium easily forms strong nitroso complexes. These complexes have recently attracted much attention because some of them are effectively extracted by tributyl phosphate. The extraction with tributyl phosphate from nitric acid solutions is a method often used for the separation of uranium and plutonium from fission products. There are two ruthenium isotopes with long half-lives and high fission yields among the fission products [1], namely ^{103}Ru and ^{106}Ru, and ruthenium therefore causes difficulties in the separation process.

Several investigations of the nitrato complexes of nitrosylruthenium have been published [2–9]. In the present investigation an extraction method has been worked out which makes accurate determinations of concentrations and partition coefficients possible. This technique has been used for a study of (a) the composition of a solution of nitrosylruthenium nitrates in aqueous nitric acid as a function of the acidity, (b) the kinetics of the interchange between the complexes, and (c) the dependence of the partition coefficients on various factors such as the tributyl phosphate concentration, the nitric acid concentration, and the temperature.

* Now at CERN, Geneva, Switzerland.

THE METHOD OF REPEATED EXTRACTIONS

Independent constituents

A solution (water phase) containing i independent constituents of the concentrations c_i is extracted repeated times with the same volume of an immiscible liquid (organic phase). The coefficients for the partition of the constituents between the organic phase and the water phase are denoted by q_i. For the sake of simplicity, the symbols p_i are introduced by the relation

$$p_i = \frac{q_i}{1 + q_i} \tag{1}$$

The n:th organic phase will contain the compounds in the total concentration $(C_o)_n$:

$$(C_o)_n = \sum_i c_i q_i (1-p_i)^n \tag{2}$$

For large values of n the constituent with the smallest partition coefficient ($= p_1$) will dominate. If log $(C_o)_n$ is plotted *versus* n a straight line is then obtained:

$$\log(C_o)_n \rightarrow \log c_1 q_1 + n \log(1-p_1) \tag{3}$$

This line has the slope $\log(1-p_1)$, and its intercept with the log C_o-axis is equal to log $c_1 q_1$. Thus q_1 and c_1 can be determined. By the subtraction of $c_1 q_1 (1-p_1)^n$ from eqn. (2) a new function $(C_{o,d})_n$ is obtained. This function can be plotted *versus* n on a semilogarithmic scale and be used for a determination of the partition coefficient and the concentration of the constituent with the next smallest partition coefficient. The same procedure can be repeated to yield the partition coefficients and the concentrations of the remaining constituents of the solution provided that the partition coefficients are sufficiently different *.

If p_1 is very small, eqn. (3) corresponds to a line with a small slope, and it is difficult to determine the partition coefficient. For large values of n, however, one obtains

$$\frac{(C_o)_n}{(C_w)_n} \rightarrow q_1 \tag{4}$$

where $(C_w)_n$ is the total concentration in the water phase after the n:th extraction. The smallest partition coefficient q_1 is determined by plotting the concentration ratio *versus* n.

By repeated extractions of an organic phase with water phases (equal volumes) similar results are obtained:

$$(C'_w)_n = \sum_i \frac{c_i}{q_i} p_i^n \tag{5}$$

(Primed symbols refer in this work to repeated extractions of an organic phase).

* This method of analysis is analogous to the resolution of a complex decay curve of a number of radioactive isotopes into its components.

On a semilogarithmic scale, $(C'_w)_n$ can be resolved into a number of straight lines with their slopes equal to log p_i and their intercepts with the log C'_w-axis equal to log c_i/q_i. The concentration ratio (eqn. (4)) gives in this case the largest partition coefficient.

Repeated extractions of a water phase containing dependent constituents (nitrosylruthenium nitrates)

If the constituents of a solution are dependent of each other, the method of analysis just outlined must be changed in order to take this into account. The nitrosylruthenium nitrates interchange rapidly, and they cannot be considered as independent.

In a solution, which is not too strongly acidified with nitric acid, mainly four different nitrosylruthenium complexes are found [6], namely the trinitrate (RuT), the dinitrate (RuD), the mononitrate (RuM), and a nitrate-free complex (RuN). (Obviously, the complex RuN is not a "nitrato" complex. It is closely related to the others, however, and will therefore be considered in this work). The following formulas have been proposed for these compounds:

$$\begin{aligned} \mathrm{RuT} &= \mathrm{RuNO(NO_3)_3(H_2O)_2}; \\ \mathrm{RuD} &= \mathrm{RuNO(NO_3)_2(OH)(H_2O)_2}; \\ \mathrm{RuM} &= \mathrm{RuNO(NO_3)(OH)_2(H_2O)_2}; \\ \mathrm{RuN} &= \mathrm{RuNO(OH)_3(H_2O)_2}. \end{aligned}$$

These complexes interchange in the following way:

$$\mathrm{RuT} \underset{k_{\mathrm{DT}}}{\overset{k_{\mathrm{TD}}}{\rightleftharpoons}} \mathrm{RuD} \underset{k_{\mathrm{MD}}}{\overset{k_{\mathrm{DM}}}{\rightleftharpoons}} \mathrm{RuM} \underset{k_{\mathrm{NM}}}{\overset{k_{\mathrm{MN}}}{\rightleftharpoons}} \mathrm{RuN}$$

The symbols k_{XY} denote rate constants. The rates of these reactions are all of the first order with respect to ruthenium [6].

In the present investigation the time for each complete extraction step (1 min) was kept short in comparison to the half-time of the reactions. Thus, it is appropriate to assume that the amount of the complex X which is converted into the complex Y during one extraction step is given by the product of its concentration and the "effective" rate constant k_{XY} (in min^{-1}). The actual complex will be found partly in the organic phase and partly in the water phase during the extraction. This is taken into account by using an effective rate constant computed from the partition coefficient and the rate constants in both phases.

Immediately after the n:th extraction the total concentration of nitrosylruthenium nitrates in the water phase and in the organic phase can be written as follows:

$$\left.\begin{aligned} (C_w)_n &= B_{\mathrm{T}n}(1-p_{\mathrm{T}}) + B_{\mathrm{D}n}(1-p_{\mathrm{D}}) + B_{\mathrm{M}n}(1-p_{\mathrm{M}}) + B_{\mathrm{N}n}(1-p_{\mathrm{N}}) \\ (C_o)_n &= B_{\mathrm{T}n}p_{\mathrm{T}} + B_{\mathrm{D}n}p_{\mathrm{D}} + B_{\mathrm{M}n}p_{\mathrm{M}} + B_{\mathrm{N}n}p_{\mathrm{N}} \end{aligned}\right\} \quad (6)$$

Since the actual rate constants are fairly small (<0.1 min^{-1}), all terms which are small of the third or higher orders, *i.e.* which contain more than two rate constants as factors, are now neglected. The following expressions for the B_{Xn}'s can then be deduced:

$$\left.\begin{aligned}
B_{Tn} &= c_T(1-k_T)Q_T^{n-1} + c_D k_{DT} S_{DTn} + c_T P_{TDT} U_{DTn} + c_M P_{MDT} V_{MDTn} \\
B_{Dn} &= c_D(1-k_D)Q_D^{n-1} + c_M k_{MD} S_{MDn} + c_T k_{TD} S_{TDn} + c_D(P_{DMD} U_{MDn} + \\
&\qquad + P_{DTD} U_{TDn}) + c_N P_{NMD} V_{NMDn} \\
B_{Mn} &= c_M(1-k_M)Q_M^{n-1} + c_N k_{NM} S_{NMn} + c_D k_{DM} S_{DMn} + c_M(P_{MNM} U_{NMn} + \\
&\qquad + P_{MDM} U_{DMn}) + c_T P_{TDM} V_{TDMn} \\
B_{Nn} &= c_N(1-k_N)Q_N^{n-1} + c_M k_{MN} S_{MNn} + c_N P_{NMN} U_{MNn} + c_D P_{MDN} V_{DMNn}
\end{aligned}\right\} \quad (7)$$

The concentration symbols c_T, c_D, c_M, and c_N appearing in eqns. (7) denote the concentrations of RuT, RuD, RuM, and RuN, respectively, at the beginning of the experiment. The other symbols have the following meaning:

$$k_T = k_{TD};\ k_D = k_{DM} + k_{DT};\ k_M = k_{MN} + k_{MD};\ k_N = k_{NM}$$

$$Q_X = (1-k_X)(1-p_X)$$

$$P_{XYZ} = k_{XY}(1-p_Y)k_{YZ}$$

$$S_{XYn} = \frac{Q_X^n - Q_Y^n}{Q_X - Q_Y}$$

$$U_{XYn} = \frac{1}{(Q_X-Q_Y)^2}\left[(n-1)Q_Y^n - nQ_Y^{n-1}Q_X + Q_X^n\right]$$

$$V_{XYZn} = \frac{1}{Q_X-Q_Y}\left[Q_X\,\frac{Q_X^{n-1}-Q_Z^{n-1}}{Q_X-Q_Z} - Q_Y\,\frac{Q_Y^{n-1}-Q_Z^{n-1}}{Q_Y-Q_Z}\right]$$

The partition coefficients q_M and q_N are very small (<0.01) for extractions with tributyl phosphate. They will therefore be neglected in this work.

For large values of n both Q_T^n and Q_D^n become negligible. (At the extraction of 1 M HNO_3 with 30 % TBP at 20°, Q_T is about 0.02 and Q_D about 0.75.) In this case the following approximation holds:

$$(C_o)_n \to Q_M^n\left[\frac{c_M k_{MD} p_D}{Q_M-Q_D} + \frac{c_M P_{MDT} p_T}{(Q_M-Q_D)(Q_M-Q_T)} + \frac{c_D P_{DMD} p_D}{(Q_M-Q_D)^2} - \frac{c_N P_{NMD} p_D}{(Q_N-Q_M)(Q_M-Q_D)}\right] + \\ + Q_N^n\,\frac{c_N P_{NMD} p_D}{(Q_N-Q_M)(Q_N-Q_D)} \quad (8)$$

The last term in eqn. (8) is usually small. For this reason $\log(C_o)_n$ will tend to a straight line when plotted *versus* n. From the slope of this line, Q_M is determined. A more accurate value of Q_M can be obtained as a second approximation by taking the last term of eqn. (8) into account.

For somewhat smaller values of n, Q_T^n is still negligible but not Q_D^n. In this case the following difference is obtained by subtracting eqn. (8) (right part) from $(C_o)_n$:

$$\left.\begin{aligned}(C_{o,d})_n \to Q_D^n\Bigg[& c_D q_D - \frac{c_M k_{MD} p_D}{Q_M - Q_D} + \frac{c_T k_{TD} p_D}{Q_D - Q_T} + \frac{c_D k_{DT} p_T}{Q_D - Q_T} + \\ & + \frac{c_N P_{NMD} p_D}{(Q_M - Q_D)(Q_N - Q_D)} - \frac{c_M P_{MDT} p_T}{(Q_M - Q_D)(Q_D - Q_T)} + \frac{c_T P_{TDT} p_T}{(Q_D - Q_T)^2} + \\ & + \frac{c_D P_{DMD} p_D}{(Q_M - Q_D)^2}\left(n - 1 - n\frac{Q_M}{Q_D}\right) + \frac{c_D P_{DTD} p_D}{(Q_D - Q_T)^2}\left(n - 1 - n\frac{Q_T}{Q_D}\right)\Bigg]\end{aligned}\right\} \quad (9)$$

The last two terms within the square brackets are small. Therefore, log $(C_{o,d})_n$ gives a straight line when plotted *versus* n, and Q_D can be determined from the slope of this line.

By subtraction of the right part of eqn. (9) from $(C_{o,d})_n$ a new difference is obtained:

$$(C_{o,d,d})_n = Q_T^n\left[c_T q_T - \frac{c_D k_{DT} p_T + c_T k_{TD} p_D}{Q_D - Q_T} + \right.$$
$$\left. + \text{ smaller terms}\right]. \quad (10)$$

If $\log(C_{o,d,d})_n$ is plotted *versus* n a straight line is obtained with the slope equal to log Q_T. The line is very steep, however, and it is better to determine Q_T by other experiments (repeated extractions of an organic phase).

As an illustration C_o, $C_{o,d}$ and $C_{o,d,d}$ have been plotted *versus* n on a semi-logarithmic scale in Fig. 1.

If the partition coefficients and the rate constants are known, eqn. (10) can be used for a determination of c_T (the term containing c_D is very small and can be neglected).

$$c_T = \frac{(C_{o,d,d})_{n=1}}{Q_T\left[q_T - \dfrac{k_{TD} p_D}{Q_D - Q_T}\right]} \quad (11)$$

For the determination of the relative concentrations c_D, c_M and c_N the following relationships are chosen: (a) the value A_o of the right part of eqn. (9) for n equal to zero as determined by extrapolation of the straight line, and (b) the ratio $(C_o)_n/(C_w)_n = q_n$ for a large value of n (the value $n = 25$ was used). The former relationship depends essentially on the concentration of the dinitrate and the latter on the ratio between the concentrations of the mononitrate and the nitrate-free complex. Besides, all concentrations add up to unity so that a system of equations of the following type is obtained:

$$\left.\begin{aligned} & c_N + c_M + c_D = 1 - c_T \\ \text{(a)} \quad & \alpha_1 c_N + \alpha_2 c_M + \alpha_3 c_D = A_o - \alpha_4 c_T \\ \text{(b)} \quad & (\beta_1 - q_n\gamma_1)c_N + (\beta_2 - q_n\gamma_2)c_M + (\beta_3 - q_n\gamma_3)c_D = (q_n\gamma_4 - \beta_4)c_T\end{aligned}\right\} \quad (12)$$

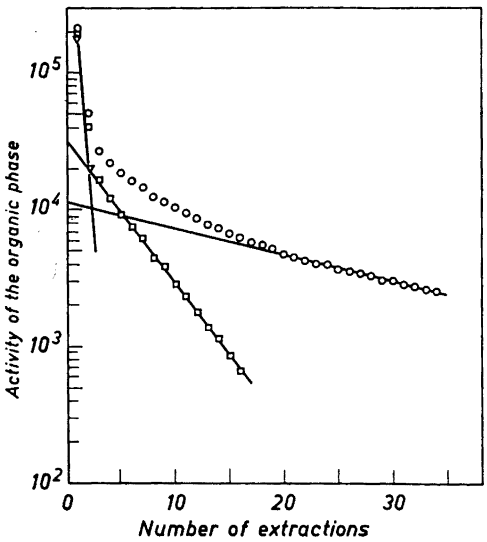


Fig. 1. The activity (proportional to the total concentration of nitrosylruthenium nitrates) of the organic phase *versus* the number of extractions for repeated extractions of a water phase. Circles: $(C_o)_n$; Squares: $(C_{o,d})_n$; Triangles: $(C_{o,d,d})_n$.

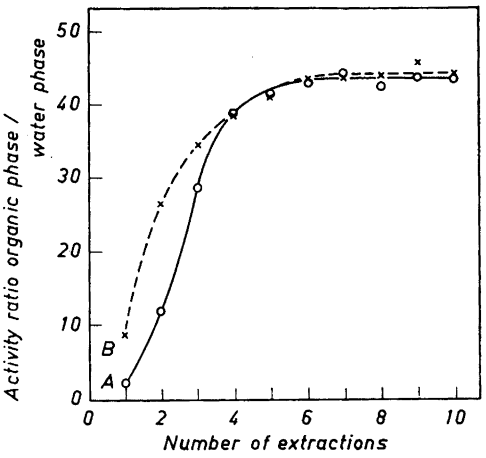


Fig. 2. The ratio between the activity of the organic phase and that of the water phase *versus* the number of extractions for repeated extractions of an organic phase. Curve A: The activity was originally in the water phase. Curve B: The activity was originally in the organic phase.

The α's, β's and γ's depend only on the partition coefficients and the rate constants. They are easily calculated from eqns. (6), (7), and (9). The expressions are somewhat lengthy, however, and they are therefore not written out here.

Repeated extractions of an organic phase containing dependent constituents (nitrosylruthenium nitrates)

For repeated extractions of an organic phase eqns. (6) and (7) are valid if the factor $(1-p_X)$ is changed into p_X in the expressions for Q_X and P_{XYZ}. Thus

$$Q'_X = p_X\ (1-k'_X)$$
$$P'_{XYZ} = k'_{XY} p_Y k'_{YZ}$$

The rate constants, which appear here, differ from the corresponding ones in the preceeding section. Since they turn out to be considerably smaller, terms which are small of the second order, *i. e.* terms containing P'_{XYZ} as a factor, can be neglected. Besides, Q'_N and Q'_M are negligible.

For large values of n, $Q_D'^n$ can be neglected, and the following relation is obtained:

$$\frac{(C_o')_n}{(C_w')_n} \rightarrow q_T\,(1-\delta) \tag{13}$$

with

$$\delta \sim \frac{k_{TD}'}{Q_T'-Q_D'}\left[q_T\,(1-p_D) - p_D\right]$$

From eqn. (13) q_T is determined by successive approximations.

The ratio $(C_o')_n/(C_w')_n$ has been plotted *versus* n in Fig. 2. It tends to become a constant after 4—5 extractions.

For large values of n, $(C_o')_n$ can be written:

$$(C_o')_n \rightarrow c_T Q_T'^n\,(1+\varepsilon) \tag{14}$$

with

$$\varepsilon \sim \frac{p_T\left[k_{TD}'\,\frac{p_D}{p_T} + k_{DT}'\,\frac{c_D}{c_T}\right]}{Q_T'-Q_D'}$$

The right part of eqn. (14) corresponds to a straight line when plotted *versus* n on a semilogarithmic scale. The intercept for n equal to zero can be used for a determination of c_T.

If log $(C_w')_n$ is plotted *versus* n, a straight line is obtained for large values of n. By a subtraction procedure similar to the one leading to $(C_{o,d})_n$ (eqn. (9)), the following difference results:

$$(C_{w,d})_n = Q_D'^n\left[\frac{c_D}{q_D} + \frac{c_M k_{MD}'}{q_D(1-k_D')} - \frac{c_T k_{TD}'(1-p_D) + c_D k_{DT}'(1-p_T)}{Q_T'-Q_D'}\right] \tag{15}$$

A straight line results when log $(C_{w,d}')_n$ is plotted as a function of n. The line has the slope log Q_D' and can be used for a determination of Q_D'. An approximate value of c_D is also obtained.

The accuracy of the determinations. The reproducibility of the determinations is high, especially with regard to RuT. Thus it is possible to determine c_T with an accuracy of better than 1 %, and the q_T-values are in many cases accurate to ± 2 %. The determinations of q_D are reliable to ± 5—10 %.

The accuracy of the determinations of c_D, c_M, and c_N depends essentially upon how well the rate constants are determined. It is probably not better than ± 10—20 %.

EXPERIMENTAL

Solutions. The nitrosylruthenium nitrate contained tracer of ^{106}Ru. A stock solution in 8 M nitric acid was prepared as described in Ref.[6]. The stock solution was carrier-free. An analysis was performed using the dithio-oxamide method [10] after the distilla-

tion of ruthenium as the tetroxide. The analysis showed that the ruthenium concentration of the samples was less than 10^{-7} M.

The tri-*n*-butyl phosphate was first washed with a 5 % solution of sodium carbonate and then distilled at vacuum. After the distillation it was diluted with an inert mixture of hydrocarbons.* The concentration of tributyl phosphate was determined analytically by the method of Allen and DeSesa [11].

The experimental procedure. Repeated extractions of a water phase were carried out using an extraction apparatus (Fig. 3) where the lighter organic phase could be easily removed after each extraction. The extractions were performed by stirring for 30 sec. After this, the phases were separated and a new portion of tributyl phosphate was added. The time allowed for this was also 30 sec. so that the time interval between two successive extractions was exactly 1 min. The experiment was usually carried out at 20° with 0.95 M nitric acid as the water phase and 30 % tributyl phosphate as the organic phase. The volume of each phase was 5 ml, and the number of extractions was 25—35. The activities of all organic phases and the water phase were measured with a scintillation counter. The activity was used as a measure of the concentration.

At the experiments with repeated extractions of an organic phase, a separation funnel was used. The number of extractions was 10.

The acidity of the water phases was determined by titration after the extractions.

RESULTS

Dependence on the extraction time

In a series of experiments with repeated extractions of an organic phase (30 % TBP) with 1 M nitric acid at 20°, the extraction time was varied. The partition coefficients obtained are tabulated in Table 1.

* Nysolvin 160/200, a mainly paraffinic kerosene fraction from Nynäs-Petroleum AB.

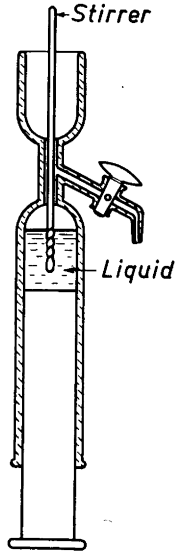


Fig. 3. Extraction apparatus for repeated extractions of the heavier phase.

Table 1. The dependence of the partition coefficients on the extraction time.

Extraction time	q_T	q_D
15 sec.	46.9	0.28
30 »	47.9	0.28
40 »	47.4	0.32

The partition coefficients are independent of the extraction time in the interval studied. Thus stirring for 30 sec is adequate for the equilibrium between the phases to be reached. Similar results regarding RuT have been obtained by Brown *et al.*[6]

Studies on the kinetics of the interchange between the nitrosylruthenium nitrates

(a) Rate constants in 0.95 M nitric acid. In order to make a determination of partition coefficients and concentrations from the extraction data possible, the rate constants must be known. Kinetic experiments are therefore essential. Since the analyses by the method of repeated extractions were usually carried out at 20° and with the system 0.95 M HNO_3—30 % TBP, it was of primary interest to determine the rate constants corresponding to these conditions.

In a series of experiments a pure solution of RuT in 30 % tributyl phosphate was first prepared by removing all other complexes by repeated extractions with 1 M nitric acid. The trinitrate was then reextracted into 8 M nitric acid, and the solution was diluted to 0.95 M with water. It was kept at 20° in a thermostate. Samples were taken at different times for repeated extractions, and the quantities $(C_{o,d,d})_{n=1}$, A_o, and q_{25} (eqns. (11) and (12)) were measured.

Starting from the approximations $k_{TD} = k_{NM} = 0$ and $Q_M = 1-k_{MD}$, the constants of eqns. (12) were calculated and the concentrations determined. Next, new rate constants were determined from the concentrations just obtained. The kinetic equations are of the following form:

$$\left.\begin{aligned} \frac{dc_T}{dt} &= -k_{TD}c_T + k_{DT}c_D \\ \frac{dc_N}{dt} &= -k_{NM}c_N + k_{MN}c_M \end{aligned}\right\} \qquad (16)$$

Now, k_{DT} can be expressed in k_{TD} and k_{MN} in k_{NM} using the concentrations at equilibrium. The derivatives in eqns. (16) are then replaced by the differences $\frac{\Delta c_T}{\Delta t}$ and $\frac{\Delta c_N}{\Delta t}$, and the rate constants k_{TD} and k_{NM} are determined graphically. They are combined with the relation $Q_M = 1-k_M$ and all equilibrium concentrations to yield the remaining rate constants, and the "effective" rate con-

stants are calculated. These are used for new determinations of the concentrations c_X. By successive approximations, values of c_X and k_{XY} are finally found which are not changed by further approximations. The final values of the rate constants in 0.95 M nitric acid and at the temperature 20° are tabulated in Table 2.

Table 2. Rate constants in 0.95 M HNO_3.

k_M	0.051	min^{-1}*
k_{TD}	0.025	»
k_{DT}	0.0024	»
k_{DM}	0.084	»
k_{MD}	0.047	»
k_{MN}	0.0041	»
k_{NM}	0.0025	»

* This value was obtained from $Q_M = 0.949 \pm 0.002$, average of 34 determinations.

The following value of Q_D (average of 34 determinations) has been obtained:

$$Q_D = (1-k_D)\ (1-p_D) = 0.742 \pm 0.007$$

The effective value of k_D is found to be 0.078 min^{-1} from the appropriate rate constants in 0.95 M nitric acid and tributyl phosphate. With this k_D-value one obtains

$$p_D = 0.195;\ q_D = 0.242$$

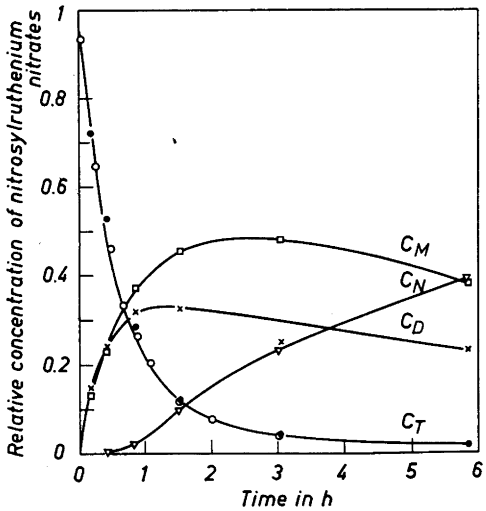


Fig. 4. Relative concentrations of nitrosylruthenium nitrates *versus* time starting from a solution of pure RuT in 0.95 M nitric acid. Temperature: 20°.

Filled circles: c_T
Crosses: c_D
Squares: c_M
Triangles: c_N
} From repeated extractions of a water phase.

Open circles: c_T From repeated extractions of an organic phase.

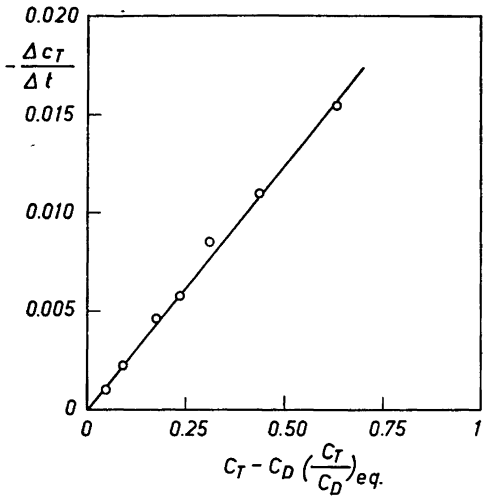


Fig. 5. $\frac{\Delta c_T}{\Delta t}$ *versus* $c_T - c_D \left(\frac{c_T}{c_D}\right)_{eq.}$ for RuT in 0.95 M nitric acid. Temperature: 20°.

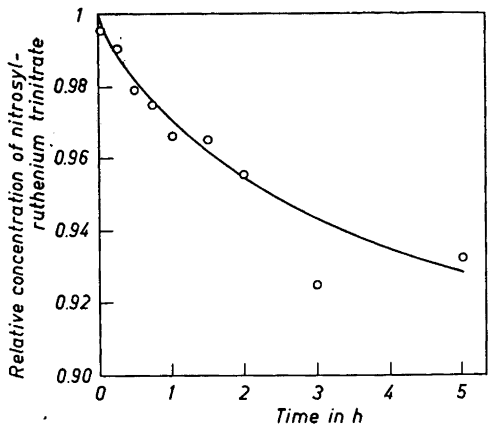


Fig. 6. Relative concentration of RuT *versus* time starting from a solution of pure RuT in 30 % tributyl phosphate. Temperature: 20°.

The concentrations of the nitrosylruthenium nitrates in 0.95 M nitric acid are plotted *versus* time in Fig. 4.

As an example of the rate constant analyses, $\frac{\Delta c_T}{\Delta t}$ has been plotted *versus* $c_T - c_D\left(\frac{c_T}{c_D}\right)_{eq.}$ in Fig. 5. The slope of the straight line corresponds to k_{TD}. The value 0.025 min^{-1} is found.

(b) Rate constants in 30 % tributyl phosphate saturated with 0.95 M nitric acid. The decrease of c_T in a solution of RuT in 30 % tributyl phosphate (saturated with 0.95 M nitric acid) has also been studied. The temperature was 20°. In this case only two components have to be considered, namely RuT and RuD. The following rate constants were obtained:

$$k_{TD,o} = 0.00067 \text{ min}^{-1}$$
$$k_{DT,o} = 0.0059 \text{ min}^{-1}$$

Fig. 6 shows c_T as a function of time.

A conversion of the aquo-complexed nitrosylruthenium trinitrate into the corresponding TBP-complexed compound has been proposed to occur in tributyl phosphate solutions [6]. The latter compound is thought to have a much higher partition coefficient ($> 10^3$) than the former. The half-time of the reaction is estimated to be about 3 min and that of the reverse reaction to be about 6 min. In this work a great number of experiments with repeated extractions of organic phases have been performed in which the ruthenium was originally in the water phase. The experiments cover the temperature range

0°—50° and the acidity range 0.165 M—3.25 M (water phase). In all these experiments results similar to those in Fig. 2 were found. The activity ratio reaches a constant value after 4—5 extractions, *i. e.* after 4—5 min. The same results were obtained also in experiments where the ruthenium was originally in the TBP-phase, in some cases for as long as 18 days. This is not in accord with the hypothesis mentioned above. The conversion proposed would cause the activity ratio to approach a constant value much more slowly. On the other hand, it is quite possible that a more rapid interchange takes place. If this is the case, a measured q_T-value will be a function of the true partition coefficient of the aquo-complex and the rate constants of the interchange.

(c) The temperature dependence of the rate constants in 0.95 M nitric acid. Kinetic experiments have been carried out at three different temperatures: 10°, 20°, and 30°. The nitric acid concentration of the solutions was 0.95 M. The results are given in Table 3 and shown in Fig. 7.

Table 3. The temperature dependence of the rate constants.

Temperature	k_{TD}, min^{-1}	k_{DT}, min^{-1}	k_{MN}, min^{-1}	k_{NM}, min^{-1}
10°	0.0065	0.00096		
20°	0.025	0.0024	0.0041	0.0025
30°	0.068	0.0058	0.012	0.0085

An increase of the temperature by 10° corresponds to a threefold increase of the rate constants. Jenkins and Wain [5] have carried out a series of rate constant determinations. Their results for the rate constant k_{TD} in 0.7—0.8 M nitric acid obtained at 0°, 25°, and 35° have also been plotted in Fig. 7.

(d) The acidity dependence of the rate constants. The dependence of the rate constants on the acidity of the water phase has been studied at the temperature 20°. The results are given in Table 4 and shown in Fig. 8.

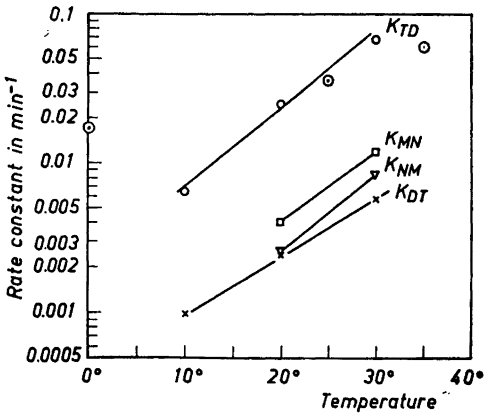


Fig. 7. The temperature dependence of the rate constants in 0.95 M nitric acid. Circles: k_{TD}; Crosses: k_{DT}; Squares: k_{MN}; Triangles: k_{NM}; Dotted circles: k_{TD} from Ref.[5].

Table 4. The acidity dependence of the rate constants.

Acidity	k_{TD}, min^{-1}	k_{DT}, min^{-1}	k_{MN}, min^{-1}	k_{NM}, min^{-1}
0.95 M	0.025	0.0024	0.0041	0.0025
1.92 M	0.019	0.0058	0.0046	0.014
3.80 M	0.018	0.013		
7.11 M	0.020	0.053		

The rate constant k_{TD} is almost independent of the acidity. This is in agreement with the results by Jenkins and Wain [5].

In Fig. 9 c_T has been plotted *versus* time for different acidities.

Relative concentrations of the nitrosylruthenium complexes at equilibrium in aqueous nitric acid solutions

A series of nitrosylruthenium solutions of varying nitric acid concentration were prepared and analysed after about one week by repeated extractions of an organic phase (determination of c_T and c_D) or repeated extractions of a water phase (determination of c_T, c_D, c_M, and c_N). The results are tabulated in Tables 5 and 6, respectively, and plotted *versus* the acidity in Fig. 10.

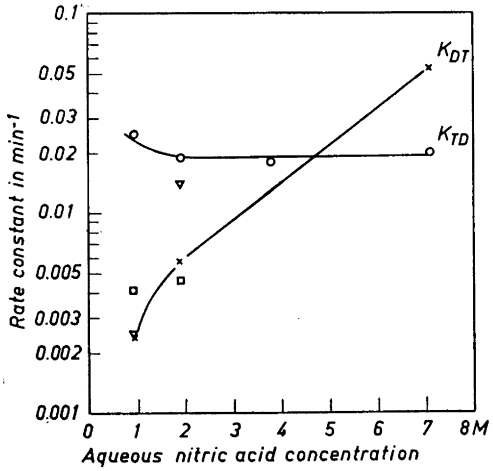


Fig. 8. The acidity dependence of the rate constants. Temperature: 20°. Circles: k_{TD}; Crosses: k_{DT}; Squares: k_{MN}; Triangles: k_{NM}.

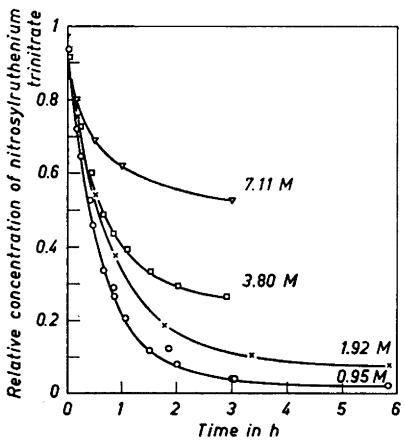


Fig. 9. Relative concentration of RuT *versus* time starting from a solution of pure RuT in aqueous nitric acid. Temperature: 20°. Circles: 0.95 M HNO_3; Crosses: 1.92 M HNO_3; Squares: 3.80 M HNO_3; Triangles: 7.11 M HNO_3.

Table 5. Relative concentrations of nitrosylruthenium nitrates.

Acidity, M	c_D	c_T
0.316	0.00305	0.16
0.517	0.00655	0.21
0.747	0.0115	0.18
1.37	0.0288	0.22
2.03	0.0611	0.27
2.73	0.109	0.26
3.31	0.154	
3.64	0.182	
4.13	0.232	
5.54	0.383	
6.61	0.481	
8.15	0.640	
10.2	0.785	
12.4	0.882	
13.5	0.923	

Table 6. Relative concentrations of nitrosylruthenium nitrates.

Acidity, M	c_T	c_D	c_M	c_N
0.316	0.0028	0.076	0.20	0.72
0.506	0.0069	0.13	0.29	0.58
1.01	0.025	0.19	0.31	0.48
1.94	0.063	0.24	0.36	0.33
2.84	0.124	0.27	0.41	0.20
4.63	0.269	0.28	0.37	0.077
5.96	0.417	0.24	0.30	0.037
8.15	0.582	0.16	0.24	0.014
10.3	0.709	0.11	0.18	0
12.4	0.757	0.084	0.16	0

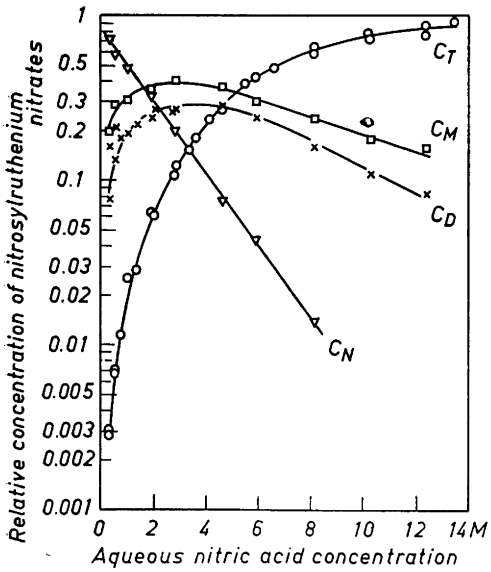


Fig. 10. The concentrations at equilibrium of nitrosylruthenium nitrates in aqueous nitric acid *versus* the acidity. Circles: c_T; Crosses: c_D; Squares: c_M; Triangles: c_N.

Studies on the partition coefficients of the nitrosylruthenium nitrates

(a) Dependence on the tributyl phosphate concentration. Tributyl phosphate solutions with the concentration varying from 10 % to 50 % were extracted at 20° with 1 M nitric acid. The measured partition coefficients are tabulated in Table 7 and plotted *versus* the tributyl phosphate concentration in Fig. 11.

Table 7. Partition coefficients for different concentrations of tributyl phosphate.

Concentration of TBP, %	q_T	q_D
10.0	2.4	
15.6	8.5	0.13
21.0	19.5	0.18
25.9	33.2	0.23
31.2	45.1	0.39
36.1	67.5	0.56
40.9	88	0.54
45.4	113	0.52
50.0	150	0.63

As found by other investigators [6] a plot of log q_T *versus* log C_{TBP} gives a straight line with the slope about 2 (the value 2.3 is obtained from Fig. 11).

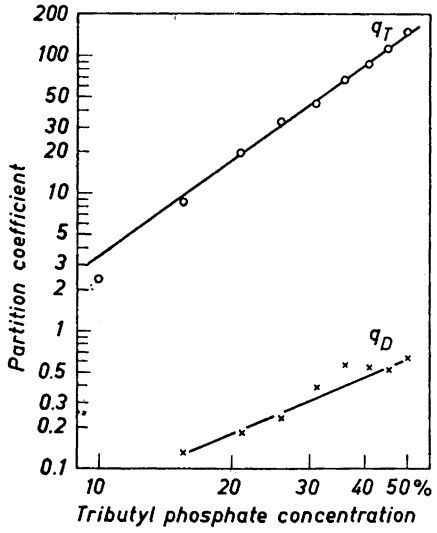


Fig. 11. The dependence of the partition coefficients on the tributyl phosphate concentration for extractions with 1 M nitric acid. Temperature: 20°. Circles: q_T; Crosses: q_D.

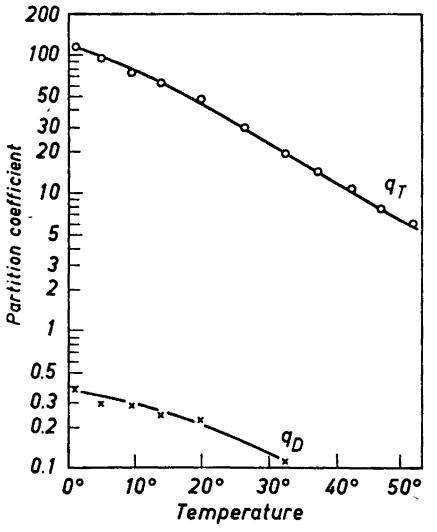


Fig. 12. The temperature dependence of the partition coefficients for extractions of 1 M nitric acid with 30 % tributyl phosphate. Circles: q_T; Crosses: q_D.

(b) Dependence on the temperature. The partition coefficients q_T and q_D were determined in experiments with repeated extractions of organic phases (30 % TBP) with 1 M nitric acid at different temperatures. Eqn. (13) was used for the evaluation of q_T. The effective rate constant k'_{TD}, which forms part of the correction factor δ, contains $k_{TD,w}$ and $k_{TD,o}$. The temperature dependence of $k_{TD,w}$ is obtained from Fig. 7. Only one $k_{TD,o}$-value has been determined, namely that for 20°. The temperature dependence is assumed to be the same as for $k_{TD,w}$, *i. e.* a temperature increase of 10° corresponds to a threefold increase of $k_{TD,o}$.

For the determination of q_D from the experimental values of $Q'_D = p_D(1-k'_D)$, the effective rate constant k'_D must be known. This contains $k_{DT,w}$, $k_{DT,o}$, and $k_{DM,w}$. The first of these three rate constants is obtained from Fig. 7. The others are assumed to vary with temperature in the same way as $k_{TD,w}$. Their values at 20° are known.

The resulting determinations of q_T and q_D are tabulated in Table 8 and plotted *versus* temperature in Fig. 12.

Table 8. The temperature dependence of the partition coefficients.

Temperature, °C	q_T	q_D
1.0	116	0.37
5.0	94	0.29
9.6	75	0.28
14.0	63	0.24
20.0	48.1	0.22
26.5	29.6	0.10
32.6	19.2	0.11
37.6	14.4	
42.8	10.9	
47.2	7.6	
52.0	6	

Table 9. The acidity dependence of the partition coefficients.

Acidity, M	q_T	q_D
0.165	120	0.27
0.326	102	0.24
0.506	94	0.35
0.817	62	0.30
0.937	45	0.19
1.00	39	
1.01	38	
1.22	27.5	
1.62	16.7	
2.06	7.3	
2.37	3.0	
2.79	2.7	
3.25	2.0	

(c) Dependence on the acidity of the water phase. The acidity dependence of the partition coefficients was studied using two different methods for both q_T and q_D. First repeated extractions of an organic phase (30 % TBP) were carried out at the temperature 20° with water phases of the desired acidity. The results are tabulated in Table 9.

In the second method for the determination of q_T, a pure solution of the trinitrate in 30 % tributyl phosphate was prepared by 8—10 extractions with 1 M nitric acid. The organic solution was then extracted at 20° with nitric acid of the desired strength (the acidity was measured after the experiment), and the partition coefficient was determined from the ratio between the activities in the phases after a correction for the formation of dinitrate during the experiment. The results are given in Table 10.

Table 10. The acidity dependence of q_T.

Acidity, M.	q_T
0.0003	24.5
0.0040	63
0.456	92
0.691	79
0.815	66
1.01	44.7
1.34	25.6
1.66	15.6
1.93	7.10
2.28	5.24
2.63	3.02
3.43	0.84
5.30	0.117
7.22	0.073
10.3	0.073
13.5	0.022

The partition coefficient of RuD was also determined by a method similar to the second one for RuT. A solution of nitrosylruthenium nitrates in 1 M nitric acid was extracted four times with 30 % tributyl phosphate. Since q_T is high, all RuT is effectively removed. The solution was then extracted once more, and the organic phase, containing essentially pure RuD, was removed and extracted at 20° with nitric acid of the desired strength. The partition coefficient q_D was obtained from the ratio between the activities in the phases after due correction for the formation of RuT and RuM during the experiment. The results are tabulated in Table 11.

All the data from these experiments have been plotted *versus* the acidity of the water phase in Fig. 13. The agreement with other investigations is reasonable with respect to q_T. The values of q_D are in agreement with measurements using repeated extractions of a water phase (page 1490).

(d) The stability of nitrosylruthenium dinitrate in 30 % tributyl phosphate. A solution of RuD in 30 % tributyl phosphate (saturated with 0.95 M nitric acid) was prepared in the way described in the preceding section. The solu-

Table 11. The acidity dependence of q_D.

Acidity, M	q_D
0.182	0.302
0.396	0.341
0.602	0.304
0.794	0.258
1.42	0.141
1.86	0.079
2.70	0.023
3.50	0.013
5.46	0
7.48	0

tion was kept at 20°. Samples were taken for extraction with 0.95 M nitric acid, and the ratio between the activity of the organic phase and that of the water phase was determined. This ratio is shown in Fig. 14 as a function of the time between the preparation of the solution and the extraction.

If it is assumed that the solution contains only RuT and RuD, their concentrations can be calculated from the activity ratios. From the time-dependence of the concentrations, the rate constant $k_{TD,o} = 0.0067$ min^{-1} is obtained. This agrees well with the value 0.0059 min^{-1} determined earlier.

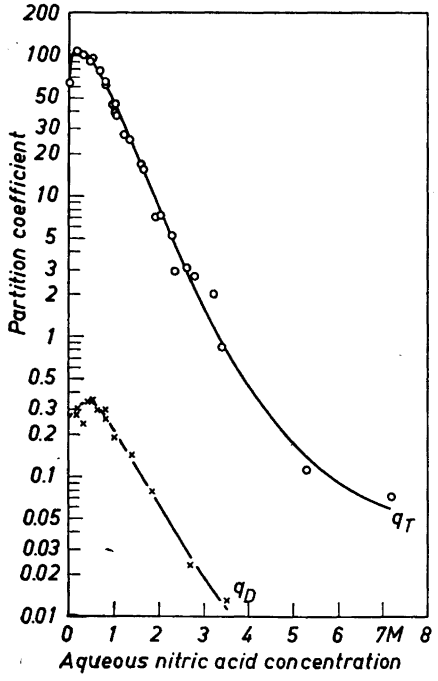


Fig. 13. The acidity dependence of the partition coefficients for extractions with 30 % tributyl phosphate at 20°. Circles: q_T; Crosses: q_D.

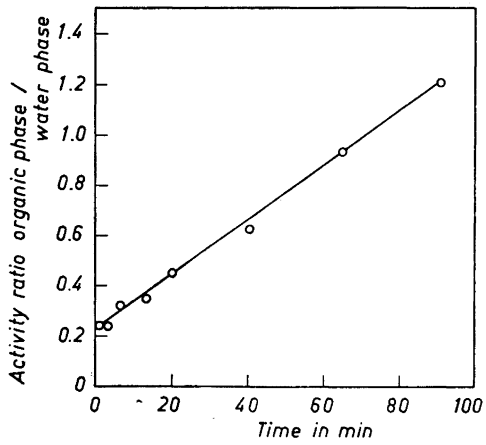


Fig. 14. The ratio between the activity of the organic phase and that of the water phase *versus* time for extractions of a solution of originally pure RuD in 30 % tributyl phosphate with 0.95 M nitric acid.

(e) Upper limits for q_M and q_N. A large number of experiments concerning the partition coefficients q_M and q_N have been performed. The experiments were carried out at 20° with the system 30 % tributyl phosphate — 0.95 M nitric acid. Upper limits of the partition coefficients are immediately obtained from repeated extractions of water phases. For large values of the extraction number n, the ratio between the activity of the organic phase and that of the water phase varies from 0.03 for solutions containing much RuM and little RuN to less than 0.005 for solutions containing mainly RuN. Thus

$$q_M < 0.03 \text{ and } q_N < 0.005$$

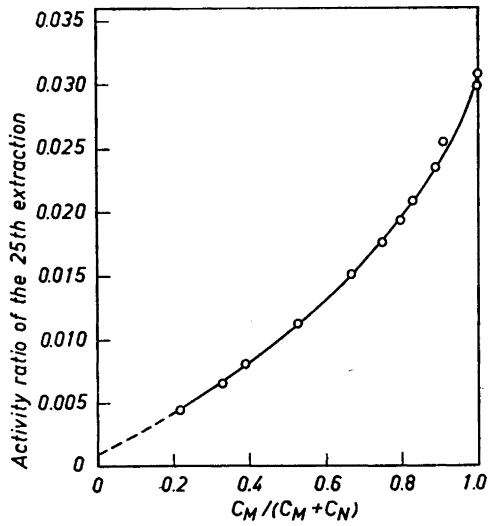


Fig. 15. The ratio between the activity of the organic phase and that of the water phase at the 25th extraction of a water phase with 30 % tributyl phosphate *versus* $c_M/(c_M+c_N)$. Temperature: 20°.

The activity ratio for n equal to 25 has been plotted *versus* the ratio $c_M/(c_M+c_N)$ in Fig. 15. By extrapolation the upper limit 0.001 is obtained for q_N.

A better estimate of the upper limit of q_M can be made. A solution of nitrosylruthenium nitrates in 0.95 M nitric acid was extracted four times with 30 % tributyl phosphate. The fourth organic phase was extracted with 0.95 M nitric acid at 20°, and the ratio between the activities of the phases was measured. Four determinations gave the value 0.260 $\pm$ 0.006 as an average. This corresponds to a q_D-value of 0.245, in excellent agreement with earlier results. In another series of experiments carried out at the same conditions, a water phase was extracted 25 times with 30 % tributyl phosphate, and the 25th organic phase was extracted with 0.95 M nitric acid. The activity ratio 0.237 $\pm$ 0.013 was obtained as an average of seven determinations. This is close to the q_D-value at the given conditions, and the conclusion can be drawn that almost all extracted ruthenium is in the form of RuD. The observed difference between 0.260 and 0.237 corresponds to a q_M-value of 0.003. It should be safe to estimate the upper limit of q_M to 0.01.

Since both q_M and q_N are small, their neglection in this work is justified.

DISCUSSION

The method of repeated extractions has proved to be very useful for the study of the nitrato complexes of nitrosylruthenium. Owing to the possibility of taking the interchange between the complexes during the experiment into account, reliable determinations of concentrations and partition coefficients can be made.

The values determined for rate constants, equilibrium concentrations, and partition coefficients make possible predictions of the behaviour of the nitrosylruthenium nitrates at different conditions with respect to the tributyl phosphate concentration, the nitric acid concentration, the temperature, and the time schedule followed during the experiment. Such predictions are of value in connection with those separation procedures for plutonium and uranium which are based on extractions with tributyl phosphate.

It must be pointed out that the present work has not been concerned with any confirmation of the formulas of the nitrosylruthenium complexes. The trinitrate has been identified merely by its partition coefficient and by comparison with other experiments [6,8]. The identities of the other complexes found have been inferred from their relationships (for instance: a solution of RuD in tributyl phosphate is converted into RuT; a solution of RuM in dilute nitric acid is converted into RuD and not directly into RuT). Furthermore, RuT is probably the most nitrate-rich complex since it dominates at high nitric acid concentrations. Thus, if the identification of RuT as the trinitrate is correct, the identification of the other complexes as the dinitrate, the mononitrate and a nitrate-free complex seems plausible.

The technique of repeated extractions should be useful also for other investigations where a complex mixture of extractable compounds is to be analysed.

Acknowledgements. The author wishes to express his gratitude to Professor The Svedberg for his interest in this work.

Thanks are due to Mrs. Carina Kjellson for her valuable help in carrying out the experiments.

The investigation has been financially supported by AB Atomenergi, which is gratefully acknowledged.

REFERENCES

1. Katcoff, S. *Nucleonics* **16** No. 4 (1958) 78.
2. Bruce, F. R. *Intern. Conf. Peaceful Uses of Atomic Energy* Paper 719 (1955).
3. Fletcher, J. M. and Martin, F. S. *Ibid.* Paper 437 (1955).
4. Fletcher, J. M., Jenkins, I. L., Lever, F. M., Martin, F. S., Powell, A. R. and Todd, R. *J. Inorg. & Nuclear Chem.* **1** (1955) 378.
5. Jenkins, I. L. and Wain, A. G. *Ibid.* **3** (1956) 28.
6. Brown, P. G. M., Fletcher, J. M. and Wain, A. G. *UKAEA Report AERE C/R* 2260 (1957).
7. Brown, P. G. M., Fletcher, J. M., Hardy, C. J., Kennedy, J., Scargill, D., Wain, A. G. and Woodhead, J. L. *Second United Nations Intern. Conf. Peaceful Uses of Atomic Energy* Paper 31 (1958).
8. Zvyagintsev, O. E., Nikolsky, V. D., Starostin, S. M., Kurbanov, A. and Schmidt, V. S. *Ibid.* Paper 2143 (1958).
9. Fletcher, J. M. *J. Inorg. & Nuclear Chem.* **8** (1958) 277.
10. Ayres, G. H. and Young, F. *Anal. Chem.* **22** (1950) 1281.
11. Allen, R. J. and DeSesa, M. A. *Nucleonics.* **15** No. 10 (1957) 88.

Received May 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1502–1510

On the Structure of the Reversibly Oxidized and Reduced Group of Sperm Antagglutins

P. E. LINDAHL and S. ROSS

Institute of Zoophysiology, University of Uppsala, Sweden

When sperm antagglutins are inactivated by oxidation a carbonyl group is formed. This has been detected by blocking with sodium bisulphite of the reactivation of the antagglutin by reducing agents. The oxidized biologically inactive sperm antagglutins are reduced by DPNH. This reaction seems to be non-enzymatic, and to be blocked by diacetyl and lactate. The location of the easily reducible carbonyl group in the oxidized antagglutin molecule is discussed.

In semen from most mammals a factor occurs which prevents head-to-head agglutination of the spermatozoa. This anti-agglutinating factor, named male sperm antagglutin, is formed in the prostatic gland in cattle [1] and man [2]. Male sperm antagglutin has been isolated and purified by precipitation with ammonium sulphate and chromatography on calcium carbonate [3]. It is a proteid with an active group containing carbohydrate [1,3], sulphuric acid residues [4,5], and a probably carbocyclic compound [5,6,7].

Also in the female genital tract of several mammals a sperm anti-agglutinating factor, named female sperm antagglutin, has been detected and isolated by chromatography on silica [8]. It contains no protein, but seems to be very similar to the active group of the male sperm antagglutin. However, no sulphuric acid residues could be detected [8].

Both these sperm antagglutins are inactivated by being kept in aqueous solution at room temperature in contact with air. However, this process is very much accelerated by bubbling oxygen through the solution after addition of a minute amount of platinum black. When a reducing agent as sodium thioglycolate, sodium ascorbate, or hydrogen and platinum black is added to these biological inactive solutions the activity is recovered [1,3]. The inactivation of sperm antagglutins is thus brought about by oxidation of some group in the antagglutin molecules.

As the oxidation and the inactivation of the sperm antagglutins occur simultaneously, it was of interest to try to acquire some knowledge about the chemical structure of the group of the antagglutin molecule which is so

easily reversibly oxidized and reduced. To obtain such information we have to study the biological activity of pretreated samples of sperm antagglutin. By adding different reagents with known qualities to oxidized, biologically inactive sperm antagglutin some substances with the power of inhibiting the reactivation of the oxidized antagglutin might possibly be found. In case the same reagent, added to reduced antagglutin, should not influence the biological activity of the antagglutin, it could be concluded that the reagent reacts in some way with the oxidized form of the group of the antagglutin molecule which is reversibly oxidized and reduced, by preventing its reduction. In its oxidized form this group might be quinonelike or it might be a disulphide, carbonylic, carboxylic, epoxy, or ethylenic group. Since female sperm antagglutin has been shown to contain no sulphur [8], disulphide groups can be excluded from discussion. Quinones as well as carbonyls should react with carbonylic reagents, although in somewhat different ways. We have therefore used for this purpose sodium bisulphite, and also tried some other carbonyl reagents.

All substances so far studied with the ability of reducing the oxidized sperm antagglutins have an oxidation-reduction potential (E_o') equal to 0.06 or lower [9]. Among these sodium thioglycolate as well as reduced diphosphopyridine nucleotide (DPNH) were found to induce very high biological activity. Combined with one of several dehydrogenases DPNH has the ability of reducing different carbonyl compounds. On the other hand the possibility is not excluded that DPNH might by itself reduce oxidized antagglutin, although it is known to be rather inert. We have therefore tried to decide whether DPNH, when reducing oxidized sperm antagglutin is acting by itself or combined with an enzyme.

MATERIALS

The male sperm antagglutin was prepared according to Lindahl and Kihlström [3]. The relatively pure preparations were used in aqueous solution of about 50 μg antagglutin per ml, estimated according to Lindahl and Kihlström [3]. The female sperm antagglutin which was prepared according to Lindahl and Nilsson [8] has been used in an aqueous solution equivalent in its biological activity to the above mentioned male sperm antagglutin solution. The sperm antagglutin solutions were oxidized by aeration during one hour in presence of platinum black. Sodium thioglycolate ($4.4-5.7 \times 10^{-4}$ M) was regularly used as reducing agent in experiments in which reactions with reduced antagglutins were studied. When investigating the reduction of oxidized antagglutin by DPNH we used this compound, enzymatically reduced (Sigma), in a concentration of 5.7×10^{-4} M.

Bull spermatozoa were used for tests on biological activity. The spermatozoa in 0.5 ml semen were washed three times by suspending them in 8 ml of the diluter of Phillips and Spitzer [10], slightly modified, and by centrifugation. This diluter was prepared by mixing equal amounts of two solutions, one containing 0.6 % glucose, and 0.8 % galactose, and the other 0.2 % KH_2PO_4 and 0.2 % $Na_2HPO_4 12H_2O$, both made up with water twice distilled in glass, and autoclaved. This is here named diluter I. Another diluter with the double osmotic pressure must also be used to compensate for the very low osmotic pressure of the dilute antagglutin solutions and of the reagent solutions used. This diluter here named diluter II, contains twice as much solids as diluter I. — All reagents with exception of diacetyl were of *pro analysi* quality. All solutions have been freshly prepared every day.

EXPERIMENTS AND RESULTS

The experiments are arranged in such a way that conclusions may be drawn from comparisons between values of anti-agglutination index, AAI, found. Thus the AAI of any sample of antagglutin is determined with a control containing all the reagents with the exception of the antagglutin, and in the same final concentrations as in the relevant sample. In cases in which one or more reagents are brought to react with the sperm antagglutin the sample is prepared in the following way: To 0.03 ml of a solution of sperm antagglutin in a test tube an equal volume of solution of the reagent to be studied is added, the tube shaken, and left for 10 min at room temperature (time of reaction, *cf.* tables). The same volume of a second reagent solution may be added, the mixture shaken, and left for another 10 min, and in a few cases a third reagent may be added in the same way. A volume of diluter II equal to that of the mixture already in the tube is added, and the final volume (*cf.* tables) minus 0.03 ml made up with diluter I. Then 0.03 ml of the sperm suspension is added. Each addition is followed by thorough mixing. As the reduction of oxidized sperm antagglutin is a comparatively fast process a "reaction time" of less than 10 min is generally allowed to pass after the addition of his reagent, in case it being the last one, the time elapsing until the beginning of the counting being long enough.

The percentage of spermatozoa agglutinated head-to-head (P_2) in the experimental sample and the corresponding percentage (P_1) in the control are determined in a haemocytometer as described by Lindahl and Nilsson [8]. The anti-agglutination index (AAI) is expressed according to the formula:

$$AAI = \frac{P_2 - P_1}{P_1}$$

Differences in AAI determined with this method, when greater than 0.1 are generally significant. Negative values of AAI of oxidized antagglutin preparations are the result of agglutinating impurities, whereas positive values depend upon incomplete oxidation. Real quantitative comparisons of values of AAI's are possible only, when the same antagglutin preparation and the same spermatozoa have been used. However, considering that the errors in the many measurings of small amounts of liquids in some samples may accumulate, still greater differences may appear between samples which might be expected to give identical AAI values.

Reaction between oxidized sperm antagglutin and sodium bisulphite. When a dilute aqueous solution of sodium bisulphite is added to oxidized male or female sperm antagglutin, the biological activity remains zero also after addition of sodium thioglycolate (Table 1:III). Reduced sperm antagglutins on the other hand maintain their biological activity after addition of the sodium bisulphite solution (Table 1:V). The oxidized, but not the reduced form of the sperm antagglutins obviously reacts with sodium bisulphite, the complex formed preventing reduction of the antagglutins. The reaction between oxidized sperm antagglutins and sodium bisulphite is, however, reversible as is shown by the following: Addition only of sodium pyruvate which has a large affinity to bisulphite, and thus also to that in the complex formed by the

Table 1. Blocking of male (a—b) and female (c) sperm antagglutin with sodium bisulphite. I—VII.

A. Composition of test samples (1 volume = 0.03 ml). Total volume 0.27 ml.

Test samples	Ox. antagglutin ~1 μg/vol.	$NaHSO_3$ 1.6×10^{-2} M	Na thioglycolate 4×10^{-3} M	Na pyruvate 3×10^{-2} M	Diluter I	Diluter II	Sperm susp.
I	1				6	1	1
II	1	1			4	2	1
III	1	1	1		2	3	1
IV	1		1		4	2	1
V	1	1	1		2	3	1
VI	1	1		1	2	3	1
VII	1	1	1	1		4	1

B. Order of adding reagents, and AAI:s found.

		I	II	III	IV	V	VI	VII
1.		Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin
2.		Dilut.	$NaHSO_3$	$NaHSO_3$	Na thioglycol.	Na thioglycol.	$NaHSO_3$	$NaHSO_3$
3.	Time of reaction		10 min	10 min		10 min	10 min	10 min
4.			Dilut.	Na thioglycol.	Dilut.	$NaHSO_3$	Na pyruvate	Na pyruvate
5.	Time of reaction					10 min	10 min	10 min
6.				Dilut.		Dilut.	Dilut.	Na thioglycol. Dilut.
7.	Expts a	0.01		+0.13	+0.56		+0.03	+0.40
	Expts b	−0.20	0.00			+0.31		
	Expts c	−0.12	+0.08		+0.52	+0.53		

oxidized antagglutin and sodium bisulphite, does not change the biological activity from zero (Table 1:VI). When, however, the reducing agent sodium thioglycolate is then added, the activity is immediately recovered (Table 1:VII). The reaction studied may thus be written:

oxidized antagglutin + $NaHSO_3$ ⇌ oxidized antagglutin $H \cdot SO_3Na$

Sodium bisulphite is a wellknown carbonyl reagent which reacts reversibly. It is thus probable that the oxidized sperm antagglutins contain at least one carbonyl group which is reduced to a hydroxygroup by the reducing agent when the antagglutins are reactivated. It is further obvious that the velocity with which bisulphite combines with the carbonyl must be much higher than that of the reduction effected on the same group. The latter is readily reduced by sulphite [9].

Sperm antagglutins reacting with phenylhydrazine hydrochloride and hydroxylamine hydrochloride, respectively. Both oxidized (Table 2:II) and reduced

Table 2. Blocking of oxidized (II) and reduced (IV) sperm antagglutin with phenylhydrazine hydrochloride and hydroxylamine hydrochloride (2.3×10^{-3} M).

A. Composition of test samples (1 vol. = 0.03 ml). Total volume 0.21 ml.

Test samples	Ox. antagglutin ~1 μg/ml	Studied compound 1.6×10^{-2} M	Diluter I	Diluter II	Sperm susp.
I	1		4	1	1
II	1	1	2	2	1
III	1		4	1	1
IV	1	1	2	2	1

B. Order of adding reagents, and AAI:s found.

	I	II	III	IV
1.	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin
2.	Diluters	Studied compound		Na thioglycolate 10 min
3. Time of reaction				Studied compound
4.				
5. Dialysis (4×10^{-3} M Nathioglycolate solution)		12 h	12 h	12 h
6.		Diluters	Diluters	
Phenylhydrazin · HCl		+0.01		+0.10
Hydroxylamin · HCl	+0.08 *		+0.37	
		+0.05		+0.08

* Tested against other spermatozoa than samples II—IV.

(Table 2:IV) male sperm antagglutin are inactivated by these two carbonyl reagents. Since these reagents react with sugars, we had to replace the sugars of the diluters by sodium chloride when determining biological activities. In order to prevent that the effects observed are due to influences of the reagents on the spermatozoa the experiments were performed with male sperm antagglutin, and the reaction mixtures II and IV of Table 2 dialyzed against 4×10^{-3} M sodium thioglycolate solution over night. Thus the surplus of the reagents was removed before the addition of the diluters and the spermatozoa, no thioglycolate solution being added. The activity of the oxidized antagglutin was estimated immediately (Table 2:I), and another volume of this solution, diluted with 2 volumes of distilled water, was dialyzed together with samples II and IV to give the AAI of untreated reduced antagglutin. The inactivation even of the reduced male sperm antagglutin by these compounds probably depends on a reaction with the sugar residues in the antagglutin molecule. Male sperm antagglutin is known to give a positive reaction with benzidine [3] which indicates the presence of reducing sugar. Experiments with these two carbonyl reagents therefore do not give any information as to the nature of the reversibly oxidized and reduced group. — In special control experiments the treatment of spermatozoa with the two relevant

reagents was found to be without any effect upon the estimations of the biological activity of female antagglutin. An experiment with phenylhydrazine on the same lines, however, with exclusion of the dialysis was performed with reduced female antagglutin with the same result. Here a reaction with sugar residues is less probable, as no positive reaction on reducing sugars with benzidine has been obtained [8]. The inactivation of reduced female sperm antagglutin by phenylhydrazine may possibly indicate the existence of a second carbonyl, much more resistant against reducing agents and not localized in the saccharide constituent. However, a possible coupling of this carbonyl with bisulphite should not result in inactivation.

The non-enzymatic reduction of oxidized sperm antagglutins with DPNH. In order to be able to decide, whether or not an enzyme is involved in the oxidation of DPNH by oxidized antagglutin, experiments were performed with female sperm antagglutin as this does not contain any protein, and thus is free from contaminating enzymes. The spermatozoa used in the tests for biological activity represent another possible source of enzymes. In order to exclude the participation of enzymes of the test spermatozoa in the studied reaction the experiments were arranged in such a way that the reaction between DPNH and the oxidized antagglutin takes place before the addition of the sperm suspension. This was secured by adding bisulphite to the mixture of the two compounds, blocking in this way any oxidized antagglutin before the addition of the spermatozoa. Thus a sample (Table 3:II) was prepared in the following way: Sodium bisulphite was added to female sperm antagglutin. Ten minutes later DPNH, and after another 10 min the diluters and the sperm suspension were introduced into the mixture. In another sample (Table 3:III) DPNH

Table 3. Inhibition of the power of DPNH ($\sim 6 \times 10^{-4}$ M) to reduce oxidized female sperm antagglutin in presence of sodium bisulphite (2.3×10^{-3} M).

A. Composition of test samples (1 vol. 0.03 ml). Total volume 0.21 ml.

Test samples	Ox. antagglutin ~1 µg/vol.	$NaHSO_3$ 1.6×10^{-2} M	DPNH 4×10^{-3} M	Diluter I	Diluter II	Sperm susp.
I	1			4	1	1
II	1	1	1		3	1
III	1	1	1		3	1
IV	1		1	2	2	1

B. Order of adding reagents, and AAI:s found.

	I	II	III	IV
1.	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin	Ox. antagglutin
2.	Diluters	$NaHSO_3$	DPNH	DPNH
3. Time of reaction		10 min	10 min	10 min
4.		DPNH	$NaHSO_3$	Diluters
5. Time of reaction		10 min	10 min	
6.		Diluters	Diluters	
	−0.12	−0.12	+0.33	+0.45

was mixed with the antagglutin solution, and sodium bisulphite added only 10 min later. From Table 3 it is obvious that in the first case the sodium bisulphite blocks the reduction of the antagglutin, whereas in the second case the antagglutin is reduced by DPNH. The reduction of oxidized female sperm antagglutin by DPNH thus seems to be non-enzymatic, and this probably also applies to the male sperm antagglutin.

Spectrophotometric recording of the oxidation of DPNH by oxidized male sperm antagglutin. A few experiments were performed in order to study the decrease in the absorption maximum of DPNH at 340 mμ, expected to occur, when oxidized sperm antagglutin is reduced by DPNH. The result of such an experiment is shown in Table 4. To 0.7 mg of oxidized male sperm antag-

Table 4. Decrease in optical density at 340 mμ during incubation of DPNH (0.3 μmole) with oxidized male sperm antagglutin (0.7 mg) in bidistilled water (2.9 ml). Temperature 22°C.

Time in min	0	1	2	3	5
$-\Delta D_{340\mu}$	0.000	0.020	0.028	0.032	0.040

glutin in 2.8 ml of distilled water 0.1 ml of 3×10^{-3} M DPNH was added in a quarz cell. Immediately after mixing the absorption at 340 mμ was read on a Beckmann DU spectrophotometer against a blanc containing 0.1 ml of 3×10^{-3} M DPNH and 2.8 ml of distilled water. The absorption of the sample was then read after 1, 2, 3, and 5 min, and was found to decrease during this time in relation to that of the blank, DPNH being oxidized to DPN by the sperm antagglutin (Table 4).

Inhibition of the reduction of oxidized sperm antagglutins with DPNH by diacetyl and sodium lactate. Starting from the idea that the reaction between DPNH and oxidized sperm antagglutin could be involved in the regulation of sperm metabolism we investigated the influence of a substance, reacting as metabolite with the diphosphopyridine nucleotide, *viz.* lactate, and diacetyl.

If diacetyl or sodium lactate in concentrations of 4.5×10^{-3} M are added to a solution of oxidized sperm antagglutin before the addition of DPNH, the biological activity is not restored (Table 5:III, expts b and c). If other reducing agents, like sodium thioglycolate or sodium ascorbate, are used neither diacetyl nor sodium lactate inhibit the reduction (Table 5:IV). Diacetyl obviously is the more potent of the two compounds in this respect. In another experiment (Table 5:III, expt d) increased quantities of diacetyl were mixed with a constant quantity of antagglutin prior to the addition of DPNH. In this case the inhibition of the reduction of the oxidized antagglutin was complete, when the added quantity of diacetyl was about equivalent to the amount of DPNH present (Table 5:III, expt e). It is probable that the inhibition of the reduction of antagglutin by DPNH is dependent on some interaction between DPNH and diacetyl or sodium lactate, respectively, which brings about a blocking in the reducing power of DPNH. In the oxidized state diphosphopyridine nucleotide forms additive compounds with dihydroxyacetone and similar α-hydroxy carbonyl compounds [11]. Additive compounds between DPNH and carbonyl compounds seem not to be known.

Table 5. Blocking with diacetyl or sodium lactate of the oxidation of DPNH by oxidized male sperm antagglutin.

A. Composition of test samples (1 vol. 0.03 ml). Total volume 0.21 ml.

Test samples	Ox. antagglutin ~1 μg/vol.	Studied compound 3×10^{-3} – 3×10^{-2} M	DPNH 4×10^{-3} M	Na thio-glycolate 4×10^{-3} M	Diluter I	Diluter II	Sperm susp.
I	1				4	1	1
II	1		1		1	2	1
III	1	1	1			3	1
IV	1	1		1		3	1

B. Order of adding reagents, and AAI:s found.

			I Ox. antag-glutin	II Ox. antag-glutin	III Ox. antag-glutin	IV Ox. antag-glutin
1.						
2.			Diluters	DPNH	Compound studied	Compound studied
3. Time of reaction				10 min	10 min	10 min
4.				Diluter	DPNH	Na thio-glycolate
5. Time of reaction					10 min	10 min
6.					Diluter	Diluter
Experi-ments	Studied compound	Final conc.				
a	Na lactate	4.5×10^{-4} M	0.00	+0.51	+0.24	
b	Na lactate	4.5×10^{-3} M	+0.01	+0.46	+0.16	+0.40
c	Diacetyl	4.5×10^{-3} M	+0.05	+0.46	+0.06	+0.45
d	Diacetyl	1.5×10^{-4} M	+0.08	+0.44	+0.39	
		3×10^{-4} M			+0.29	
		12×10^{-4} M			−0.12	
e	Diacetyl	4.5×10^{-4} M	+0.08	+0.64	+0.02	

How the ability of inhibiting the reaction between DPNH and oxidized sperm antagglutin is connected with the structure of the compounds studied is so far poorly understood.

DISCUSSION

The easiness with which the sperm antagglutins are oxidized into a biological inactive compound, and are reactivated by reducing agents is notable. The inhibition of the reduction of oxidized antagglutins by sodium bisulphite points to a carbonyl or possibly a quinone-like structure in the oxidized antagglutin. It is thus probable that at least one hydroxyle in the reduced form is oxidized into a carbonyl, when the antagglutins are reversibly inactivated at oxidation. Depending on stereochemical configurations quinones may react with sodium bisulphite either irreversibly producing hydroquinone sulphonic acids or reversibly leading to α-hydroxysulphonic acids like those of aliphatic carbonyl compounds. A quinone should have a specific absorption

in the ultra-violet part of the spectrum. The oxidized male sperm antagglutin, however, has the same absorption curve as the reduced antagglutin [3]. This speaks against a quinone-like structure in the oxidized male sperm antagglutin.

On previous occasions a compound has sometimes been extracted in this laboratory with ethyl ether from male sperm antagglutin, and spectrophotometrically compared with tocopherols [5]. This aromatic compound seems to be of diphenolic character as it gives after oxidation a substance with a quinone-like spectrum in ultra-violet light. It appears most probable that this aromatic compound is an artefact, originating from an aliphatic precursor bound in the antagglutin molecule. When male sperm antagglutin is heated in molten potassium bisulphate, a product is formed which has some properties in common with corresponding products obtained in the same way from α- and δ-tocopherols, and also with p-benzoquinone disulphonic acid [12].

It is a notable fact that the reduction of oxidized antagglutins with DPNH is a non-enzymatic reaction. Carbonyl compounds are inert to DPNH, whereas a compound as p-benzoquinone is reduced by DPNH [13]. From this it is obvious that the carbonyl group of the oxidized antagglutin is more reactive than those, *e.g.*, in sugars, having other neighbouring groups. In this connection it must, however, be stressed that we have not been able to detect ascorbic acid as a constituent of the sperm antagglutin.

Acknowledgements. This study was aided by grants from the *Population Council*, New York, the *Swedish Natural Science Research Council*, and the *Swedish Medical Research Council*. We are very much indebted to Drs. B. Ström and T. Svensson for semen material. We thank Mr. O. Heidenberger for valuable technical assistance.

REFERENCES

1. Lindahl, P. E. and Kihlström, J. E. *Proc. II Intern. Congr. Animal Repr., Copenhagen* 1952, p. 70.
2. Lindahl, P. E. *Proc. III Intern. Congr. Animal Repr., Cambridge* 1956.
3. Lindahl, P. E. and Kihlström, J. E. *Fertility and Sterility* **5** (1954) 241.
4. Kihlström, J. E. *Arkiv Kemi* **7** (1954) 399.
5. Lindahl, P. E., Kihlström, J. E. and Ross, S. *Acta Chem. Scand.* **10** (1956) 1597.
6. Lindahl, P. E. and Kihlström, J. E. *Nature* **174** (1954) 600.
7. Lindahl, P. E., Kihlström, J. E. and Ross, S. *Acta Chem. Scand.* **11** (1957) 1.
8. Lindahl, P. E. and Nilsson, A. *Biochim. et Biophys. Acta* **25** (1957) 22.
9. Lindahl, P. E. *J. Repr. and Fertility* **1** (1960). *In press.*
10. Phillips, P. H. and Spitzer, R. R. *J. Dairy Sci.* **29** (1946) 407.
11. Burton, R. M. and Kaplan, N. O. *J. Biol. Chem.* **206** (1954) 283.
12. Ross, S. *Acta Chem. Scand.* **13** (1959) 409.
13. Wosilait, W. D. and Nason, A. J. *J. Biol. Chem.* **206** (1954) 255.

Received June 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1511–1541

A Moving-Bomb Method for Combustion Calorimetry of Organic Bromine Compounds

LARS BJELLERUP

Department of Organic Chemistry, University of Lund, Lund, Sweden

For the purpose of developing a method for obtaining accurate heat of formation data for organic bromine compounds, a moving-bomb calorimeter for measuring heats of combustion at 25°C of solid and liquid samples has been constructed. The calorimetric system is calibrated by combustion of standard benzoic acid under certified bomb-calibrating conditions. However, in order to obtain well-defined, equilibrium final states in the bomb in combustion experiments with bromine compounds the bomb liquid must differ both in nature and amount from that specified for calibration experiments with benzoic acid. This may give rise to non-cancelling systematic errors. As a first step in defining errors, the reproducibility in calibration as well as combustion experiments has been studied. The paper describes in detail the apparatus, the procedure and the calculation of results, and reports data obtained in calibration experiments and in combustion experiments with paraffin oil and *p*-bromobenzoic acid.

Results from calibration experiments with benzoic acid and combustion experiments with paraffin oil demonstrated that the moving-bomb apparatus, the experimental procedure and the method of calculation of results, presented in this paper, were capable of giving results having a precision slightly better than 0.01 % which is comparable to that obtained with best available modern bomb-calorimetric techniques for compounds containing carbon, hydrogen and oxygen. Furthermore, the experiments with *p*-bromobenzoic acid showed that the various additional complications introduced into the procedure by the presence of a reasonable percentage of bromine in the sample did not affect the precision obtainable with the given apparatus and procedure.

Although the precision of the present method was found to be satisfactorily high, no conclusions could be drawn as to the accuracy of heat of combustion data obtained with this method. Experimental work designed to give information on this point has been done and will be reported in another paper.

The available heat of formation data for organic bromine compounds are few, and only a small number have an uncertainty interval * comparable

* The terms *uncertainty interval, reproducibility, precision, accuracy* and related quantities used in this work have the same meaning as given in Ref.[1] The terms *reproducibility* and *precision* are used synonymously.

to that of modern heat of formation data for other groups * of organic compounds **. Experimental data, from which heat of formation values with small uncertainty intervals can be calculated, have been reported on: (i) heats of bromination of certain alkenes [4,5] and *cyclo*-alkenes [5], (ii) heats of hydrobromination of *cyclo*-propane [6,7], propene [6,7] and the normal butenes [8], (iii) the heat of hydrogenation of vinyl bromide [9], and (iv) equilibrium constants for the system ethylene — hydrogen bromide — ethyl bromide [10]. Therefore, heat of formation values of low uncertainty are at present available for the following sixteen organic bromine compounds: bromoethane, bromoethene, 1-bromopropane, 2-bromopropane, 2-bromobutane, 1,2-dibromoethane, 1,2-dibromopropane, 1,2-dibromobutane, *meso*-2,3-dibromobutane, *dl*-2,3-dibromobutane, 2,3-dibromo-2-methylbutane, 1,2-dibromoheptane, 1,2-dibromo*cyclo*pentane, 1,2-dibromo*cyclo*hexane, 1,2-dibromo*cyclo*heptane, and 1,2-dibromo*cyclo*octane. It is striking, however, that data are lacking for a number of organic bromine compounds of considerable interest to pure as well as to applied chemistry, including most of the lower monobromoalkanes, several of the lower dibromoalkanes, bromobenzene, the dibromobenzenes, benzyl bromide *etc.* Evidently, further work is required, aiming at the determination of experimental thermochemical data from which accurate heats of formation of organic bromine compounds can be calculated.

One of the heat of formation values referred to above was derived from experimental data obtained in a study of chemical equilibria, and the others could be calculated from data obtained by application of the methods of reaction calorimetry *** to various chemical reactions each involving one of the mentioned organic bromine compounds with other compounds of accurately known heats of formation. However, the experience of earlier experimental work on the thermochemistry of organic compounds suggests that neither equilibrium studies nor reaction calorimetry could compete in versatility and width of applicability with combustion calorimetry for obtaining experimental data leading to high-accuracy heat of formation values for organic bromine compounds. Therefore an accurate combustion-bomb calorimetric method for organic bromine compounds would certainly be capable of providing the majority of heat of formation data for such compounds. Heat of combustion work has been applied to organic bromine compounds in eight previous investigations [11–18]. None of the methods used in these can, however, be considered fully satisfactory for present-day requirements on accuracy.

---

* "Group" should here be understood as "containing a certain combination of elements" such as C,H,O or C,H,O,N or C,H,O,Br or C,H,O,S.

** The uncertainties attached to the best available heat of formation data for groups of organic compounds vary according to the nature of the groups. As a rough generalization, however, it can be stated that modern thermochemical apparatus and technique [2,3] is capable of giving experimental results leading to standard heat of formation values having uncertainty intervals for the lower members of various homologous series in the range ± 0.1 to ± 0.6 kcal/mole.

*** Although no general agreement exists it has become practice among workers in thermochemistry to use the name *reaction calorimetry* only for the measurement of heats of reactions other than combustion in oxygen.

From, (i), the results obtained using the various combustion techniques for bromine compounds mentioned above, (ii), experience from recent applications of moving-bomb methods to organic sulfur compounds [23,24], and (iii), results from recent systematic studies of certain details of various moving-bomb techniques [25,26], it was evident that further development of the moving-bomb methods described in Refs.[15,17,18] should provide a combustion-bomb calorimetric method for organic bromine compounds capable of yielding data of comparable accuracy to those given by the best present-day methods for other groups of organic compounds [3].

Work on such a development has been in progress in this laboratory for some time. This paper describes the present status of development of apparatus and experimental technique, and reports results sufficient to indicate the precision of the method.

METHOD

The conventional substitution method was used, comprising two types of experiments: calibration experiments with benzoic acid and combustion experiments with bromine compound. In all experiments the temperature rise of the calorimeter was virtually the same.

The calorimetric system was calibrated by combustion of a standard sample of benzoic acid under certified bomb-calibrating conditions.

The bromine compound was burned in the platinum-lined bomb with paraffin oil of known heat of combustion as a burning aid. The amounts of sample and paraffin oil were so adjusted that virtually the same quantities of heat were released in the combustion experiments as in the calibration experiments. From 95 to 98 % of the bromine content of the sample was transformed to elemental bromine through the combustion process and the rest to hydrogen bromide. In these experiments the bomb contained an appropriate amount of arsenious oxide solution for reduction of the elemental bromine to bromide ion. Bomb and calorimeter vessel were so designed that during part of the reaction period of the calorimetric measurement a rotating motion could be imparted to the bomb to bring about proper agitation of the bomb contents. Hereby the elemental bromine was quantitatively reduced to bromide ion, the whole interior of the bomb was well rinsed by the final bomb solution and equilibrium between the liquid and the gaseous phase was attained. This established a homogeneous, stable and thermodynamically definable final liquid-gas system in the bomb. After completion of the calorimetric measurement the bomb liquid was quantitatively analyzed for the various constituents, in particular, arsenious oxide and nitric acid.

APPARATUS

The calorimetric system was of the stirred-liquid-calorimeter with constant-temperature-jacket type. The construction, and the influence of various constructional details on the performance of such calorimetric systems, have been discussed in considerable detail by several authors [27-33]. The adaptation of this type of calorimetric system for use with moving-bomb techniques has also been described in detail previously [23,24]. Furthermore, certain experimental details of various moving-bomb techniques have also been treated in some detail previously [25,26]. For these reasons and because several of the characteristics of the apparatus used in the present investigation are similar to those described in Refs.[23,24], the description of the apparatus and the discussion of its functioning will be more thorough on certain selected points than on others.

Calorimetric assembly. The calorimetric assembly, shown diagrammatically in Fig. 1, consisted of the following main parts: combustion bomb B, calorimeter vessel C_1-C_{10} with moving mechanism M_1-M_{13} and other accessories, thermometer T, and jacket J_1-J_{25}.

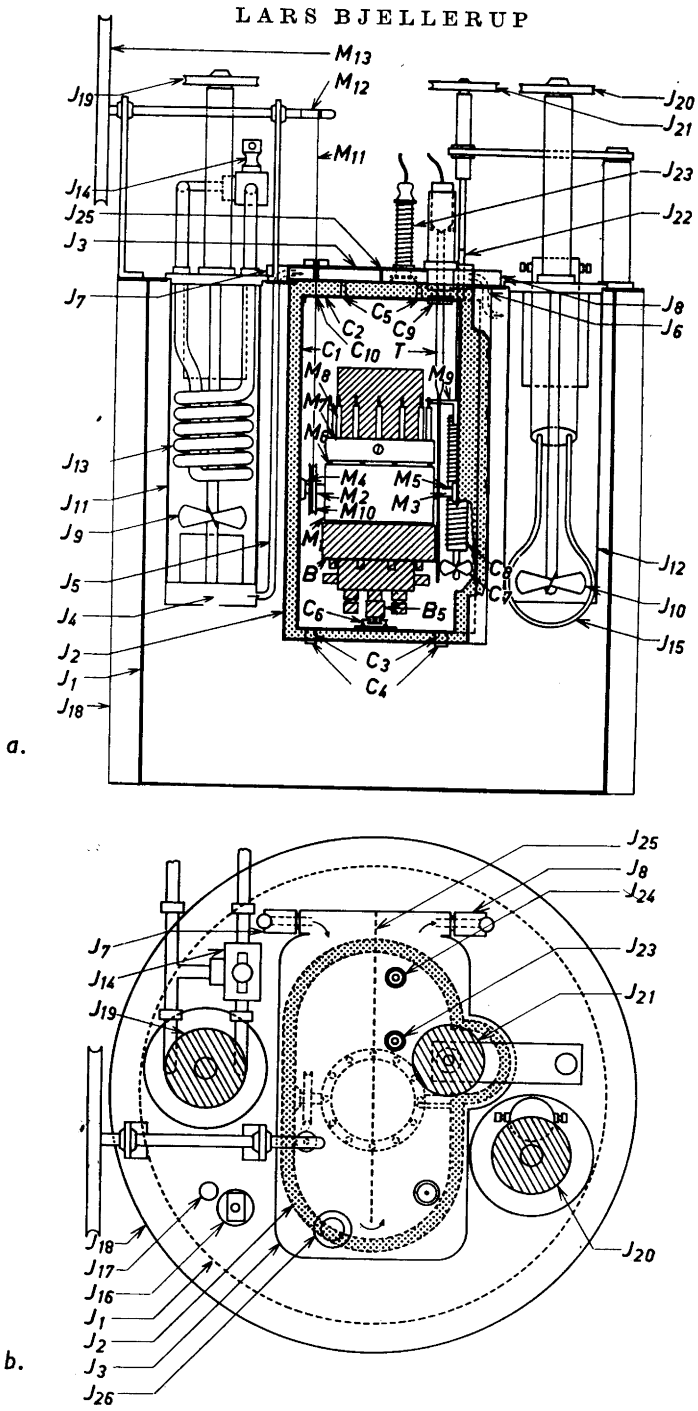


Fig. 1. Assembled calorimetric system: a. Sectional view. b. Top view.

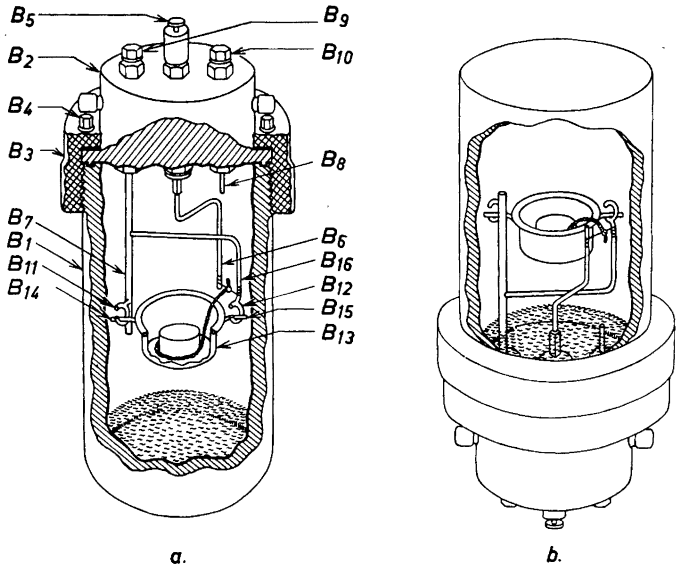


Fig. 2. Charged combustion bomb: a. In upright position. b. In inverted position.

Combustion bomb. The combustion bomb, shown in Fig. 2, was of a conventional three-part type consisting of body B_1, head B_2, and sleeve B_3. All three parts were made of stainless steel. The inner surface of the body, the bottom of the head and all internal fittings were lined with platinum of 0.20 mm thickness. A gas-tight seal between body and head was obtained through a gold gasket situated in a groove in the bottom of the flange of the head. The head was pressed against the body by the aid of eight set-screws B_4 in the sleeve and a chromium-plated, hard steel ring lying on top of the flange of the head below the set-screws. The design was very similar to that described in Ref.[34]

The head was equipped with a terminal B_5 for ignition, electrically insulated from the rest of the head, mainly through teflon washers, and ending with the platinum rod B_6. The head was also equipped with two platinum tubes, one B_7 for gas-inlet into the bomb and one B_8 for gas-outlet. The openings to the tubes could be closed by the two valves B_9 and B_{10} with gold packings.

To the gas-inlet tube were welded two open platinum rings B_{11} and B_{12} between which the platinum combustion crucible B_{13} could be suspended by the aid of a platinum ring having two short axles B_{14} and B_{15} as seen in Fig. 2. The design was similar to that described in Ref.[24] This device made it possible to charge the bomb in a conventional way, as described later, while it was in an ordinary upright position, place it in its suspension ring in the calorimeter vessel and then rotate the ring through 180 degrees about the axis M_2—M_3 so that the bomb could be temporarily fixed in an inverted position, as shown in Figs. 1 a and 2 b, while the crucible and its contents retained their original upright position. The charge was then ignited with the bomb in this position which directed the hot gases from the combustion blast towards the body of the bomb instead of towards the more sensitive head. When rotation of the bomb was started after completion of the combustion process the axles of the crucible suspension ring slipped out through the openings in the platinum rings B_{11} and B_{12} so that the crucible and suspension ring fell down into the bomb liquid and were thoroughly rinsed.

For ignition of the sample the two platinum rods B_6 and B_{16} were connected through a 0.05 mm platinum wire of approximately 10 mm length. One end of a fuse, consisting of a weighed piece of cotton string about 90 mm long, was attached to the mid-point of the thin platinum wire and the other end of the fuse was put in contact with the sample.

Application of a potential of 12 V to the ignition circuit caused such a rapid heating of the thin platinum wire to its melting point that only a small amount of energy (estimated to be less than 0.5 cal) was introduced into the calorimeter before the circuit was broken. During this process, however, the fuse was ignited and the fuse in turn ignited the sample. The variation in the electrical ignition energy from experiment to experiment was considered small enough to permit incorporation of this energy into the energy equivalent of the calorimeter. The internal volume of the bomb was 0.2670 liter.

Calorimeter vessel. The calorimeter vessel, consisting of can C_1 and lid C_2, had the shape shown in Fig. 1. The dotted space in Fig. 1 represents the air-gap between the calorimeter vessel and the constant-temperature-jacket. The distance between the outer surface of the calorimeter and the inner surface of the jacket was 10 mm at all points[32,p.168]. The can was supported on four cylindrical Bakelite pegs C_3 snugly fitting in four corresponding pits in the jacket well C_4. The three Lucite pegs C_5 in the calorimeter lid C_2 were just long enough to be slightly pressed against the under side of the jacket lid J_3 when this was placed in its closed position as shown in Fig. 1. Spring clip C_6, attached to the bottom of the can, could be lifted a few millimeters from the bottom and slid on to a knob on the electrical ignition terminal B_5. In this way, the bomb could be kept in the inverted position, shown in Fig. 1, during the fore period and the first part of the reaction period of the calorimetric experiments. Also, the spring clip served as an electrical lead to the ignition terminal.

The calorimeter lid was equipped with a calorimeter stirrer C_7, a 10 W calorimeter heater C_8, a rubber washer C_9 through which the thermometer T could be inserted and tightly positioned in the calorimeter, a small orifice C_{10} for the copper wire M_{11}, and two flat electrical contacts (not shown in the figure) leading to the heater C_8 and to the spring clip C_6, respectively.

Moving mechanism. The device used to effect appropriate agitation of the bomb contents during the reaction period consisted of the following parts shown in Fig. 1: the bomb supporting ring M_1 with axles M_2 and M_3 and bearings M_4 and M_5 attached to the wall of the calorimeter can; the flat ball bearing M_6; the bomb fastening ring M_7 with twelve evenly spaced rods M_8; the removable stud M_9 projecting from the wall of the calorimeter can; the pulley M_{10} on axle M_2 of the supporting ring; the 0.3 mm copper wire M_{11} wound on pulley M_{10}; the pulley M_{12} driven by a motor operating on pulley M_{13}.

Withdrawal of drive wire M_{11} from pulley M_{10} by rotation of pulley M_{12} gave the bomb a rotational motion about the horizontal axis M_2-M_3 perpendicular to the bomb axis. During this end-over-end rotation of the bomb, each passage through the inverted position shown in Fig. 1, brought one of the rods M_8 into contact with stud M_9, which pushed the rod, causing the bomb to rotate about the bomb axis (axial rotation) through one twelfth of a full revolution. Since the bomb in the inverted position was supported entirely by the ball bearing M_6, and did not fit too tightly in the supporting ring M_1, it was very easily axially rotated whilst in the inverted position. In all other positions during the end-over-end rotation, however, the bomb was more or less pressed directly against the supporting ring so that no axial rotation occurred with the bomb in these positions. Consequently, the moving mechanism, during the withdrawal of the drive wire, effected a compound rotation of the bomb about an axis perpendicular to the bomb axis and about the bomb axis at the same time. The speed of the axial rotation was one twelfth of the speed of the end-over-end rotation *.

In all experiments — calibration as well as combustion experiments — in the present work a length of 600.0 ± 0.5 cm of the 0.3 mm copper wire was wound around pulley M_{10} in 43 turns. The withdrawal of the wire was made at such a rate as to give the bomb an end-over-end rotation rate of 12 rpm and consequently an axial rotation rate of 1 rpm. Unwinding of all 43 turns of drive wire thus required a time of 215 sec. The agitation, due to this rotation, of the 30 ml of bomb solution used in all combustion experiments with bromine compounds, was found to be satisfactory for establishing a homogeneous and stable final liquid-gas system in the bomb.

The amount of frictional energy introduced into the calorimeter through the rotation was estimated, from a crude measurement of the force necessary to effect the rotation,

* The author's thanks are due to Dr. S. Sunner, Thermochemistry Laboratory, University of Lund, for cooperation in the construction of the moving mechanism.

at approximately 2.5 cal per combustion experiment. The variation in this rotation energy from one experiment to another was difficult to assess but since the rotation was performed exactly in the same way in all experiments the rotation energy was considered constant and was incorporated into the energy equivalent of the calorimeter.

Thermometric system. The thermometric system consisted of a flat, 25 ohm platinum resistance thermometer T (Leeds & Northrup Co., Catalog No. 8160) positioned in calorimeter and jacket lid as shown in Fig. 1 and a type G-2 Mueller bridge (L. & N., Catalog No. 8069) in conjunction with a type HS reflecting galvanometer (L. & N., Catalog No. 2284-d) and a telescope equipped with a 1 000 mm scale which had been properly curved *. The distance between scale and galvanometer mirror was 93 cm in all directions. Using a thermometer current of about 4 mA and taking the last figure from galvanometer deflections the resistance could be read to the nearest 0.00001 ohm which was equivalent to 0.0001°C in the corresponding temperature value.

Jacket. The constant-temperature-jacket consisted of a water-filled, 43 l, cylindrical tank J_1 enclosing the calorimeter well J_2, which had a volume of about 10 l. The well was covered by a double-walled lid J_3, through which was circulated water from the jacket tank by the aid of a centrifugal pump J_4 and tubes J_5 and J_6 in hinges J_7 and J_8 as described in Ref.[24] The water in the tank was stirred by two 750 rpm propeller-stirrers J_9 and J_{10} operating in stirrer wells J_{11} and J_{12} (so that water was forced from bottom to top in well J_{11} and in the opposite direction in well J_{12}). Through a coil of copper tubing J_{13}, positioned around the stirrer shaft in well J_{11}, a constant flow of 21°C water was passed at a rate of 50 ml/min. J_{14} was a by-pass switch for the water flow. Around stirrer J_{10} was mounted a 22 W immersion heater J_{15}. The temperature of the jacket water was kept constant within a temperature interval of 0.003°C at about 25.22°C by balancing the constant cooling effect from coil J_{13} against an intermittent energy input from heater J_{15}. The heater was operated by a mercury-in-glass thermoregulator J_{16} (shown only in Fig. 1 b) *via* an electronic relay. The temperature of the jacket water was checked after each experiment by placing the resistance thermometer in hole J_{17} and reading it continuously for some minutes. J_{18} was a plastic sheet surrounding the jacket tank. J_{19}, J_{20} and J_{21} were pulleys for the two jacket stirrers and the calorimeter stirrer, respectively. J_{22} was a Lucite stud for coupling stirrer C_7 with pulley J_{21}. In the jacket lid were mounted two electrical terminals J_{23} and J_{24} which could be pushed down into the calorimeter well to make connection with the two flat contacts (*cf.* above) in the calorimeter lid leading to the heater and to the ignition spring clip [23, p. 21]. J_{25} was an interior wall in the jacket lid and J_{26} a glass window for inspection of the flow of water in the lid.

The whole apparatus was kept in a thermostated room at a temperature of 23.0±0.2°C.

EXPERIMENTAL PROCEDURE

Calibration experiments

The calorimetric system was calibrated by combustion of NBS standard benzoic acid, under the conditions given in the NBS certificate [35]. The recommendations, also given in the certificate, for certain details of the procedure were followed except for the method of igniting the sample. The ignition was effected with the device described above (p. 1515) using the procedure given below for the heat of combustion experiments. In all other details (including for example rotation of the bomb) the procedure of the calibration experiments was, wherever possible, the same as that for the combustion experiments. From what will be said later it will be evident that the most important difference in procedure between calibration and combustion experiments was in the nature and amount of the liquid placed in the bomb before the experi-

* Thermometer and bridge had both been calibrated at the National Bureau of Standards, Washington, D. C.

ments. In the calibration experiments 0.810 ml (corresponding to 3 g per liter of bomb volume) of water was introduced into the bomb but in the combustion experiments with the bromine compound 30.05 ml of an arsenious oxide solution (corresponding to 112 g of water per liter of bomb volume) were placed in the bomb. The corresponding effect on the results obtained will be discussed later.

Heat of combustion experiments

Preparation and assembly of the initial system. A portion of the *p*-bromobenzoic acid sample was made into a pellet having a mass corresponding to an amount of 6.0 mg-atoms of bromine. The pellet was lightly polished with a soft brush to remove any loose fragments of substance from its surface. It was then placed in the pre-weighed platinum combustion crucible and the whole was weighed to the nearest 0.00001 g. Paraffin oil of known composition and heat of combustion was added to the pellet in the crucible in such an amount that the temperature rise obtained in the combustion experiment would be, within 1 %, the same as that obtained in the calibration experiments. The weight of paraffin oil added was obtained by weighing crucible with contents to the nearest 0.00001 g. The cotton string fuse was weighed and attached to the mid-point of the thin platinum ignition wire. The crucible with its contents was placed in the crucible support in the bomb head and the free end of the fuse was immersed in the oil in the crucible. In the bottom of the bomb body was placed 30.05 ml of 0.0627_9 M arsenious oxide (As_2O_3) solution and the bomb was assembled. The gas-inlet opening of the bomb was connected to a cylinder of oxygen containing 0.3 % of nitrogen as an impurity but no combustible impurities. To remove the air from the bomb the latter was flushed with oxygen for 3 min at a rate of flow of 800 ml/min (at 23°C and 1 atm). The gas-outlet valve was closed and the bomb filled with oxygen to a final pressure of 30.0 $\pm$ 0.05 atm at 25°C. The filling required a time of about 12 min. The bomb was tested for leaks by immersion in water for 2 min. The outer surface of the calorimeter can and lid and the inner side of the well were polished with chamois leather and the copper wire of the moving mechanism was wound around pulley M_{10} (Fig. 1). Fastening ring M_7 was placed on the bottom of the calorimeter can under the supporting ring M_1. Ball bearing M_6 was placed in its groove on the fastening ring and the bomb was lowered into the supporting ring, ball bearing and fastening ring. The fastening ring with the ball bearing was pressed against the supporting ring and locked in position by two set-screws in the fastening ring. Stud M_9 was put in place and the bomb was carefully rotated through 180° about axis M_2—M_3 and fastened in the inverted position, shown in Fig. 1, by spring clip C_6. Water, of 24.1°C temperature, was then added to the can until can and contents balanced, within 0.05 g, a counterpoise which remained unchanged throughout the work *. The mass of water added was about 5 000 g. Immedi-

* Thus the mass of water in the calorimeter can varied from experiment to experiment with the total mass of contents introduced into the bomb, all other masses in the calorimetric system being kept constant. The variations thus introduced into the energy equivalent of the calorimetric system were accounted for in the calculations of the experiments.

ately after weighing, the calorimeter can with contents was placed in the jacket, and the calorimeter lid was pressed into place after the drive wire had been fed through orifice C_{10} (Fig. 1). The wire was then fed through the corresponding orifice in the jacket lid and the latter was put in place over the well. The end of the drive wire was fastened to pulley M_{12} (Fig. 1) and the calorimeter stirrer was connected with pulley J_{21}. The resistance thermometer was put in place in the calorimeter and the calorimeter stirrer was started.

Calorimetric observations. The calorimeter was heated to a temperature of 24.057°C and left to reach thermal equilibrium. After about 20 min, when equilibrium had been established, the temperature of the calorimeter had reached the value 24.087°C. At this temperature, which in all experiments was taken as starting point for the calorimetric observations, the resistance-*versus*-time readings of the fore period were begun. The fore period had a duration of 20 min and the thermometer resistance was read every 2 min at the times 0, 2, 4, ..., 18 min. During these readings the bridge current was adjusted to give a bridge sensitivity corresponding to a galvanometer deflection of 1.0 mm of scale length per 0.0001 ohm of change in the bridge setting. After the 18th min reading the bridge sensitivity was reduced to give a galvanometer deflection of 400 mm. of scale length per 0.1000 ohm of change in the bridge setting * and the bridge was set at a preselected reading corresponding to about two thirds of the total temperature rise of the calorimeter during the reaction period **. At time 20 min the charge was ignited and the reaction period began. During the first 3 min of the reaction period, when the temperature rose very rapidly, resistances were read from the galvanometer deflections without changing the bridge setting from the preselected value mentioned above. For convenience the resistance was not observed at certain selected times, but instead the time was recorded on a chronograph when passing through certain selected resistance values. These values were so chosen as to give time-temperature data at small enough intervals to permit calculation of the energy exchange between calorimeter and surroundings with sufficient accuracy, *cf.* Ref.[32] and below. The intervals were in order, in °C, 0.004 (approx.), 0.05, 0.05, 0.1, 0.1, 0.1, 0.1, 0.1, 0.1, 0.05, 0.05, 0.025, 0.025, 0.0125. Immediately before time 23 min, when the temperature rise was becoming slow again, the bridge sensitivity was reset at the value it had during the fore period, and the rest of the calorimeteric observations were done by reading the resistance — operating the bridge in the conventional way — at selected times. From time 23 min to time 25 min the resistance was read every half minute and each minute from 25 min to 36 min. Thermal equilibrium had always reestablished not later than at time 36 min and therefore the reaction period was always taken to be 16 min long. During the after period, which began at time 36 min and had a duration of 20 min, the resistance was read every 2 min at the times 38, 40, ..., 56 min. Finally the jacket temperature was measured with the calorimeter thermometer.

* A change in resistance of 0.1000 ohm corresponded in this interval to a temperature change of 0.9950°C.

** The temperature rise obtained in all experiments in this work was 0.88 ± 0.01°C.

Chemical analysis of the final system. After disassembly of the apparatus the bomb gases were slowly let out and the liquid phase in the bomb was quantitatively transferred to a volumetric flask and diluted to 250 ml. In order to determine the chemical composition at the end of the reaction period of the liquid phase in the bomb, aliquot portions of the diluted bomb solution were withdrawn and used for determination of the concentrations of the following components: (i) nitric acid, (ii) bromoaurous acid, (iii) bromoplatinous acid, (iv) arsenious oxide, and (v) hydrobromic acid.

(i) Nitric acid was determined colorimetrically by a diphenylamine method [36] as follows. A 2 ml sample of the diluted bomb solution was mixed with 10 ml of conc. sulphuric acid in a glass-stoppered flask and the mixture was allowed to cool to room temperature. 1 ml of a diphenylamine solution was then added, consisting of 200 mg of diphenylamine dissolved in a mixture of 26 ml of water and conc. sulphuric acid to a volume of 250 ml. After a selected standard time-interval (usually 15 min) the absorbance of the solution was measured with a photoelectric colorimeter using a red filter. The nitric acid concentration was then read from a reference curve obtained with nitrate standards containing the same amounts of arsenious oxide, arsenic oxide and hydrobromic acid as the samples. The amount of nitric acid formed in each combustion experiment was about 0.1 mmole.

(ii) Bromoaurous acid was determined colorimetrically on a 2 ml sample by a rhodanine method [37,38]. The amount of bromoaurous acid formed in each combustion experiment was found to be about 0.005 mmole.

(iii) Bromoplatinous acid was determined on a 2 ml sample by oxidation with bromine to bromoplatinic acid which latter was determined colorimetrically by an iodide method [38,39]. The amount of bromoplatinous acid formed in each combustion experiment was found to be about 0.0015 mmole.

(iv) Arsenious oxide was determined by titration with potassium permanganate [40] using the following procedure. To a 50 ml sample of the diluted bomb solution was added 10 ml of 3 M sodium hydroxide, 15 ml of 6 M hydrochloric acid and one drop of 0.002 M potassium iodide. The mixture was titrated with 0.1 N potassium permanganate solution using a platinum electrode for indication of the end point, at which a change in potential of about 250 mV was obtained for an addition of 0.005 ml of the permanganate solution. The amount of hydrobromic acid present in the sample was found not to interfere with the determination of the arsenious oxide.

(v) Hydrobromic acid was determined by potentiometric titration of a 20 ml sample (to which had been added 30 ml of water and 6 ml of 5 M nitric acid) with 0.04 M silver nitrate solution. The amounts of arsenious oxide and arsenic oxide present in the sample were found not to interfere with the determination of hydrobromic acid.

CALCULATION OF RESULTS

The corrected temperature rise

The difference between the temperature of the calorimeter at the end of the reaction period (*i.e.* at time 36 min) t_f and the temperature of the calori-

meter at the beginning of the reaction period (*i.e.* at time 20 min) t_i constitutes "the observed temperature rise" of the calorimeter $t_f - t_i$. This quantity is conveniently considered as the sum of two terms Δt and Δt_{ex}, where Δt is "the corrected temperature rise", *i.e.* the temperature rise of the calorimeter during the reaction period due to the heat of the actual bomb process, and Δt_{ex} is "the heat exchange correction", *i.e.* the temperature rise of the calorimeter during the reaction period due to energy exchange between calorimeter and environment. The value of Δt, needed in the further calculations, was obtained from eqn. 1.

$$\Delta t = t_f - t_i - \Delta t_{ex} \tag{1}$$

Values of all three quantities on the right in this equation were calculated from the temperature-time ($t-\tau$) observations of the calorimetric experiment in the following way *.

The mean temperatures t_I and t_{II} (in ohms **) and the rates of temperature rise $R_I = \left(\frac{dt}{d\tau}\right)_I$ and $R_{II} = \left(\frac{dt}{d\tau}\right)_{II}$ (in ohm/min) of the calorimeter during the fore and after periods, respectively, were computed from the ten resistance readings of each period using eqns. 2—5.

$$t_I = \frac{1}{10}\,(t_o + t_2 + \ldots + t_{18}) \tag{2}$$

$$t_{II} = \frac{1}{10}\,(t_{38} + t_{40} + \ldots + t_{56}) \tag{3}$$

$$R_I = \frac{1}{330}\,[9(t_{18} - t_0) + 7(t_{16} - t_2) + \ldots + (t_{10} - t_8)] \tag{4}$$

$$R_{II} = \frac{1}{330}\,[9(t_{56} - t_{38}) + 7(t_{54} - t_{40}) + \ldots + (t_{48} - t_{46})] \tag{5}$$

The obtained values for t_I, t_{II}, R_I and R_{II} were inserted in eqns. 6 and 7 to give values for t_i and t_f.

$$t_i = t_I + 11\ R_I \tag{6}$$

$$t_f = t_{II} - 11\ R_{II} \tag{7}$$

The value of Δt_{ex} was calculated from eqn. 8 ***

$$\Delta t_{ex} = [R_{II} + K(t_{II} - \frac{1}{\tau_f - \tau_i}\int_{\tau_i}^{\tau_f} t\,d\tau)](\tau_f - \tau_i) \tag{8}$$

where K is the leakage modulus of the calorimeter system (in ohm/min ohm), τ is the time at which the temperature of the calorimeter is t, τ_i is the time

* The computation of "the heat exchange correction" has been discussed by several authors, see for example Refs. 2 (p. 743), 27 (p. 228), 28 (p. 37), 32, 33, 41—44, 45 (p. 94), 46 (p.136).

** Throughout the calculations for obtaining the value of Δt all temperature values were expressed in resistance (in ohms) of the given resistance thermometer, as measured with the given bridge.

*** This equation is identical with "the improved form of the Regnault-Pfaundler formula" given in Ref. 28 (p. 41, eqn. 13) and Ref. 2 (p. 746, eqn. 18).

at which the reaction period begins and τ_f the time where it ends. The term containing the integral should be physically interpreted as the mean temperature of the calorimeter during the reaction period. In all experiments τ_i and τ_f were 20 min and 36 min, respectively, the value of K was calculated from eqn. 9

$$K = -\frac{R_{II}-R_I}{t_{II}-t_I} \tag{9}$$

and the value of the integral was obtained from the temperature-time data of the reaction period by numerical integration using the trapezium method.

It should be mentioned that a necessary condition for using eqn. 8 in the computation of Δt_{ex} is that, within the temperature interval of the calorimetric observations, the rate of temperature rise of the calorimeter, due to energy exchange with the environment, should be a linear function of the calorimeter temperature, which could be represented for example by the following relation

$$R = \frac{dt}{d\tau} = K(t_j-t) + w \tag{10}$$

where t_j is the constant temperature of the jacket and w is the rate of temperature rise of the calorimeter, due to constant energy leakage from the environment to the calorimeter mainly through the stirring of the calorimeter water. The present apparatus was checked in respect of this requirement by measuring, as given in Refs.[32,47], R for various calorimeter temperatures t while the temperature of the jacket t_j was kept constant. The results are given in Table 1.

The energy equivalent of the calorimetric system

The energy equivalent of the calorimetric system varied with nature and amount of the contents introduced into the bomb. In addition, the experimental procedure used in this work required the amount of water in the calorimeter vessel to vary, as described on p. 1518, with the total mass of contents in the bomb, so that the total mass of bomb-contents plus water remained constant. For convenience in calculation of the results from calibration as well as heat of combustion experiments, the energy equivalent of the calorimetric system was divided into three parts: (i) the energy equivalent of a "standard calorimetric system" consisting of the whole system as assembled for a fictive experiment with no contents in the bomb ε°(Calor.)*, (ii) the energy equivalent of an amount of water equal in mass to the contents of the bomb $C_P(H_2O)m^i$(Cont.), where $C_P(H_2O)$ is the heat capacity of liquid water at temperature t_i and m^i(Cont.) is the total mass of the contents of the bomb, and (iii) the energy equivalent of the contents of the bomb ε(Cont.) **. The last quantity was different in the initial and final states of the experiment, *i.e.* the states in the bomb at the beginning and at the end of the reaction

* The notation used in this section is the same as that in Ref.[48]

** With "contents of the bomb" was understood all removable matter in the interior of the bomb, including the platinum combustion crucible and its suspension ring.

period. These two energy equivalents are denoted by ε^{i}(Cont.) and ε^{f}(Cont.), respectively. Let $-\Delta E_{\text{I.B.P.}}$ be "the heat of the isothermal bomb process", *i.e.* the amount of heat released in the actual bomb process if it occurred isothermally at a selected temperature t_h, let $\Delta E_{\text{ign.}}$ be the amount of electrical energy introduced into the calorimetric system to ignite the sample, and let $\Delta E_{\text{rot.}}$ be the amount of energy introduced into the calorimetric system by the rotation of the bomb in the reaction period: then the following relation holds

$$-\Delta E_{\text{I.B.P.}} + \Delta E_{\text{ign.}} + \Delta E_{\text{rot.}} =$$

$$\begin{aligned}&\ulcorner\varepsilon^{\circ}(\text{Calor.}) - C_P(H_2O)m^{i}(\text{Cont.}) + \varepsilon^{i}(\text{Cont.})](t_h - t_i) + [\varepsilon^{\circ}(\text{Calor.}) - \\ &C_P(H_2O)m_i(\text{Cont.}) + \varepsilon^{f}(\text{Cont.})](t_f - \Delta t_{ex} - t_h) = \\ &[\varepsilon^{\circ}(\text{Calor.}) - C_P(H_2O)m^{i}(\text{Cont.})](t_f - \Delta t_{ex} - t_i) + \varepsilon^{i}(\text{Cont.})(t_h - t_i) + \\ &+ \varepsilon^{f}(\text{Cont.})(t_f - \Delta t_{ex} - t_h) \qquad (11)\end{aligned}$$

In the present work t_h was taken as 25°C and all experiments were so arranged as to give $t_f - \Delta t_{ex}$ a value which was virtually 25.0°C. Without significant loss in accuracy the relation

$$t_h = t_f - \Delta t_{ex}$$

could therefore be inserted in eqn. 11 which together with eqn. 1 gave the following relation

$$\begin{aligned}&-\Delta E_{\text{I.B.P.}} + \Delta E_{\text{ign.}} + \Delta E_{\text{rot.}} = \\ &[\varepsilon^{\circ}(\text{Calor.}) - C_P(H_2O)m^{i}(\text{Cont.}) + \varepsilon^{i}(\text{Cont.})]\,\Delta t \qquad (12)\end{aligned}$$

which could be rearranged to give eqn. 13

$$\begin{aligned}&\varepsilon^{\circ}(\text{Calor.}) - (\Delta E_{\text{ign.}}/\Delta t) - (\Delta E_{\text{rot.}}/\Delta t) = \\ &-\Delta E_{\text{I.B.P.}}/\Delta t + C_P(H_2O)m^{i}(\text{Cont.}) - \varepsilon^{i}(\text{Cont.}) \qquad (13)\end{aligned}$$

In all calibration and heat of combustion experiments the quantities $\Delta E_{\text{ign.}}$, $\Delta E_{\text{rot.}}$ and Δt differred from certain fixed values by amounts small enough to permit considering the quantities $\Delta E_{\text{ign.}}/\Delta t$ and $\Delta E_{\text{rot.}}/\Delta t$ in eqn. 13 as being constant throughout the work and incorporating them into the constant value of ε°(Calor.). For simplicity this was done by removing the two terms from eqn. 13 which thereby changed to:

$$\varepsilon^{\circ}(\text{Calor.}) = -\Delta E_{\text{I.B.P.}}/\Delta t + C_P(H_2O)m^{i}(\text{Cont.}) - \varepsilon^{i}(\text{Cont.}) \qquad (14)$$

The values of m^{i}(Cont.) and ε^{i}(Cont.) in eqn. 14 could be computed, as given in detail in Ref.[48], from data obtained in each calibration experiment. The value of the term $-\Delta E_{\text{I.B.P.}}$ could also be computed from the data obtained in a calibration experiment by use of the relation

$$-\Delta E_{\text{I.B.P.}} = -m'\Delta Ec/M(\text{Benzoic acid}) - \Delta E(HNO_3) - m'''\Delta Ec/M(\text{Fuse}) \qquad (15)$$

where m' is the mass of benzoic acid burned, $-\Delta Ec/M$(Benzoic acid) is the quantity of heat evolved by combustion at t_h°C of one unit of mass of the standard benzoic acid under the actual bomb conditions of the calibration experiment, $-\Delta E(HNO_3)$ is the small quantity of heat evolved during the

combustion process by the formation from water, oxygen and nitrogen (present as impurity in the oxygen) of a certain amount of nitric acid, m''' is the mass of fuse burned to ignite the benzoic acid sample, and $-\Delta E_c/M$(Fuse) is the heat of combustion of the fuse under the actual bomb conditions of the calibration experiment.

From the data obtained in each calibration experiment a value of $\varepsilon°$(Calor.) could thus be computed by the aid of eqn. 14. A set of calibration experiments was made in all of which the initial contents of the bomb were within practical limits the same. Therefore the values of m^i(Cont.) and ε^i(Cont.) were within required limits the same for all experiments in the set. Therefore the values of $-\Delta E_{I.B.P.}/\Delta t$ computed from the individual experiments were averaged before eqn. 14 was used for calculation of the $\varepsilon°$(Calor.) value of the calorimetric system, *cf.* Table 2.

The heat of combustion of the sample

The most useful thermodynamic quantity that can be calculated from the experimental data obtained in a combustion experiment with an organic bromine compound is the standard heat of combustion of the compound, $-\Delta E_c°$(Compound), at the reference temperature, t_h, of the combustion experiment. This is the change in energy associated with the "idealized combustion reaction" represented by eqn. 16, in which all reactants and products are in their thermodynamic standard reference states [49]:

$$C_aH_bO_cBr_d(c \text{ or liq}) + (a + b/4 - c/2)\ O_2(g) \rightarrow \rightarrow a\ CO_2(g) + b/2\ H_2O(liq) + d/2\ Br_2(liq) \qquad (16)$$

The various steps in which the value of $-\Delta E_c°$(Compound) is computed from the experimental data will be treated in this section.

The heat of the isothermal bomb process, $-\Delta E_{I.B.P.}$, was calculated, using eqn. 14, directly from the experimental data obtained in a heat of combustion experiment and the $\varepsilon°$(Calor.) value determined in the calibration experiments. To assign the measured heat-quantity to the corresponding amount of chemical reaction, it was only necessary to define the initial and final states of the bomb contents with an accuracy equivalent to the precision in the calorimetric data. This required a chemical analysis of the final system as described previously (p. 1520). The description of the initial and final states is treated in full detail in Ref.[48] The chemical change of the contents of the bomb from the initial to the final state in the bomb process, which is usually called "the chemistry of the bomb process", is conveniently described through a set of arbitrary reactions consistent with the actual initial and final states. The various reactions are selected so that the associated energy changes shall be known with appropriate accuracy.

The chemistry of the bomb process. Through the present and previous work the chemistry of the bomb process in combustion experiments with organic bromine compounds is well established. The combustion of an organic bromine compound in a bomb in oxygen at 30 atm pressure results in the following transformations of the elements in the sample: the carbon is quantitatively transformed to carbon dioxide, part of the hydrogen is transformed to hydrogen

bromide and the rest to water, and part of the bromine is transformed to hydrogen bromide and the rest to elemental bromine. This part of the bomb process is conveniently represented by eqn. 17. Of the products formed in reaction 17 the elemental bromine is quantitatively reduced to hydrobromic acid by the arsenious oxide solution, as represented in eqn. 18, while the hydrogen bromide and the water are absorbed by the bomb solution forming hydrobromic acid as given in eqn. 19:

$$C_aH_bO_cBr_d(c \text{ or } liq) + \{a + [b-(1-x)d]/4-c/2\}\ O_2(g) \rightarrow$$
$$\rightarrow a\ CO_2(g) + xd/2\ Br_2(g) + (1-x)d\ HBr(g) + [b-(1-x)d]/2\ H_2O(liq) \quad (17)$$

$$Br_2(g) + 1/2\ As_2O_3(aq) + (2n + 1)\ H_2O(liq) \rightarrow$$
$$\rightarrow 2\ (HBr \cdot nH_2O)(liq) + 1/2\ As_2O_5(aq) \quad (18)$$

$$HBr(g) + n\ H_2O(liq) \rightarrow (HBr \cdot nH_2O)(liq) \quad (19)$$

The actual bomb process can thus be described as the sum of eqn. 17, xd/2 times eqn. 18 and (1—x)d times eqn. 19. For calculation purposes it is, however, useful to represent the actual bomb process not by the more realistic eqns. 17—19 but instead by the hypothetical eqns. 20 and 21:

$$C_aH_bO_cBr_d(c \text{ or } liq) + [a + (b-d)/4-c/2]\ O_2(g) +$$
$$+ [nd-(b-d)/2]\ H_2O(liq) \rightarrow a\ CO_2(g) + d\ (HBr \cdot nH_2O)(liq) \quad (20)$$

$$As_2O_3(aq) + O_2(g) \rightarrow As_2O_5(aq) \quad (21)$$

It is seen that the sum of eqn. 20 and xd/4 times eqn. 21 is identical with the aforementioned sum of eqn. 17, xd/2 times eqn. 18 and (1—x)d times eqn. 19. The bomb process was therefore considered to comprise one reaction in which all of the bromine compound reacted according to eqn. 20 and another reaction in which the amount of arsenious oxide consumed during the process reacted according to eqn. 21. Further reactions during the bomb process were the combustion of the auxiliary oil and of the fuse, respectively, according to the following equations:

$$C_aH_b(liq) + (a + b/4)\ O_2(g) \rightarrow a\ CO_2(g) + b/2\ H_2O(liq) \quad (22)$$

$$C_aH_bO_c(c) + (a + b/4-c/2)\ O_2(g) \rightarrow a\ CO_2(g) + b/2\ H_2O(liq) \quad (23)$$

Since a significant part of the gaseous carbon dioxide formed in reactions 20, 22 and 23 was dissolved in the bomb liquid the reaction given in eqn. 24 also contributed to the bomb process.

$$CO_2(g) \rightarrow CO_2(aq) \quad (24)$$

Finally, to account for the small amounts of nitric acid, bromoaurous acid and bromoplatinous acid present in the final bomb solution, side reactions according to eqns. 25—27 were also assumed to occur in the bomb process.

$$1/2\ N_2(g) + 5/4\ O_2(g) + 1/2\ H_2O(liq) \rightarrow HNO_3(aq) \quad (25)$$

$$Au(c) + 1/4\ O_2(g) + 2\ HBr(aq) \rightarrow HAuBr_2(aq) + 1/2\ H_2O(liq) \quad (26)$$

$$Pt(c) + 1/2\ O_2(g) + 4\ HBr(aq) \rightarrow H_2PtBr_4(aq) + H_2O(liq) \quad (27)$$

From this is evident that the isothermal bomb process could conveniently be described as the sum of the main combustion reaction 20 and the auxiliary combustion reactions 21—27.

The heat effect, $-\Delta E$(Compound), associated with the main combustion reaction 20 can be called the "heat of the combustion of the sample in the bomb process". This quantity was obtained from the observed "heat of the isothermal bomb process", $-\Delta E_{\text{I.B.P.}}$, by subtraction of the heat effects $-\Delta E$(As), $-\Delta E$(Oil), $-\Delta E$(Fuse), $-\Delta E(CO_2)$, $-\Delta E(HNO_3)$, $-\Delta E$(Au), and $-\Delta E$(Pt), these being the heats associated with the actual amounts of reaction according to eqns. 21—27, respectively. For computation of the latter quantities it was necessary to know (i) the amounts of reaction and (ii) the molar heats of reaction for the various auxiliary combustion reactions.

The amounts of reaction in the bomb process. The amounts of reactions 20, 22, and 23 were computed from composition and mass of the bromine compound, the paraffin oil, and the fuse, respectively, that were placed in the bomb. The amount of reaction 21 was determined from the amount of arsenious oxide consumed in the bomb process, obtained from the analytical determinations of arsenious oxide in the initial and final bomb solutions. The amount of reaction 24 was determined as given in Ref.[48] The amounts of reactions 25—27, finally, were obtained from the experimentally determined amounts of nitric acid, bromoaurous acid and bromoplatinous acid in the final bomb solution.

Heats of reaction for the auxiliary combustion reactions. The values used for the molar heats of reaction for the various auxiliary combustion reactions are summarized below. The data were either obtained directly as results from experimental thermochemical work or computed from data given in Ref.[49]

$-\Delta$E (reaction 21) = $(77.4 \pm 0.1) \times 10^3$ cal/mole at 25°C, Ref.[50]

$-\Delta$E°(reaction 22) = $-\Delta Ec°/M$(Oil) = 10 980.5 ± 1.2 cal/g at 25°C, this work

$-\Delta E°$(reaction 23) = $-\Delta Ec°/M$(Fuse) = 3 971 ± 4 cal/g at 25°C, this work

$-\Delta E$ (reaction 24) = $(3.89 \pm 0.14) \times 10^3$ cal/mole at 25°C, Ref.[48]

$-\Delta E°$(reaction 25) = 14.176×10^3 cal/mole at 25°C, Ref.[49]

$-\Delta E°$(reaction 26) = 8×10^3 cal/mole at 25°C, Ref.[49] *

$-\Delta E°$(reaction 27) = 43.4×10^3 cal/mole at 25°C, Ref.[49] **

---

* Since no value has been found for the standard heat of formation of $HAuBr_2$(aq) a guess, from related data given in Ref. [49] of −32 kcal/mole was used for this quantity in calculating $-\Delta E°$(reaction 26). As will be seen in the following the error resulting from use of this uncertain value for $-\Delta E°$(reaction 26) will be small.

** In calculating this heat of reaction the value −91.0 kcal/mole was used for the standard heat of formation of H_2PtBr_4(aq) at 25°C.

For rigorous calculation of the "heat of the combustion of the sample in the bomb process", $-\Delta E$(Compound), according to the scheme outlined above, the given auxiliary heat of reaction data should all be applicable to actual conditions of the bomb process instead of being standard heats of reaction as some of them are. This apparent inconsistency is explained and eliminated through the mode of calculation used for the reduction of the obtained heat of combustion in the bomb process to the standard heat of combustion [48].

Reduction to standard heat of combustion. Through the various steps of the calculation described above a value was obtained for the heat of the combustion of the sample in the bomb process, $-\Delta E$(Compound), at the temperature $t_h = 25°C$. This value referred to reaction according to eqn. 20, with reactants and products in their actual states in the bomb process. The thermodynamically more useful standard heat of combustion, $-\Delta Ec°$(Compound), according to eqn. 16 was finally computed from the obtained $-\Delta E$(Compound) value by application of the Washburn reduction as given in Ref.[48] The various terms of this reduction were conveniently expressed as the sum of two quantities: (i) $-\Delta E$(HBr), the amount of heat associated with oxidation of the hydrobromic acid, formed in reaction 20, to elemental bromine according to eqn. 28

$$(HBr \cdot nH_2O)(liq) + 1/4\ O_2(g) \rightarrow (n + 1/2)\ H_2O(liq) + 1/2\ Br_2(liq) \quad (28)$$

and, (ii) $-\Delta E_\Sigma$, the amount of heat corresponding to "the remaining Washburn corrections" (see Ref.[48]). The amount of reaction for reaction 28 was computed from the mass and composition of the combustion sample, and the molar heats of reaction for various values of n were calculated from data in Ref.[49] (*cf.* Ref.[48]).

All the various heat quantities discussed above were combined in the over-all eqn. 29 to give the resulting value for $-\Delta Ec°/M$(Compound), the standard heat of combustion per gram of compound according to eqn. 16.

$$-\Delta Ec°/M(\text{Compound}) = \frac{1}{m'}\{-\Delta E_{\text{I.B.P.}} - [-\Delta E(\text{As}) - E\Delta(\text{Oil}) - \Delta E(\text{Fuse}) - \\ -\Delta E(CO_2) - \Delta E(HNO_3) - \Delta E(\text{Au}) - \Delta E(\text{Pt})] - \Delta E(\text{HBr}) - \Delta E_\Sigma) \quad (29)$$

In eqn. 29, m' denotes the mass of the bromine compound placed in the bomb and all other symbols have been defined previously.

Final over-all precision. The estimation of the final over-all precision in the $-\Delta Ec°/M$(Compound) values obtained, will be discussed in a later paper [51].

RESULTS

The energy exchange between calorimeter and environment

The calorimetric system was checked for linearity in the relation between calorimeter temperature and rate of energy exchange between calorimeter and environment by the method given on p. 1522. The results given in Table 1 were obtained from a series of measurements of the rate of temperature rise of the calorimeter at various calorimeter temperatures, while the temperature

of the jacket was kept constant at 25.495 ± 0.001°C *. In the various columns of the table are listed: (i) t, the average temperature of the calorimeter during the time interval in which the corresponding rate of temperature rise was measured; (ii) $(dt/d\tau)_{obs.}$, the observed rate of temperature rise; (iii) $(dt/d\tau)_{calc.}$, the rate of temperature rise calculated from the equation

$$dt/d\tau = [13.67_8\ (25.4950 - t) + 1.00_6] \cdot 10^{-4}$$

which is a "least squares" fit of eqn. 10 to the measured values of t and $(dt/d\tau)_{obs.}$; and (iv) $(dt/d\tau)_{obs.} - (dt/d\tau)_{calc.}$, the difference between the observed and the calculated rate of temperature rise.

Table 1. Results from the checking of the calorimetric system for linearity in the relation between calorimeter temperature and rate of energy exchange between calorimeter and environment.

t°	$(dt/d\tau)_{obs.}$ 10^{-4}deg/min	$(dt/d\tau)_{calc.}$ 10^{-4}deg/min	$(dt/d\tau)_{obs.} - (dt/d\tau)_{calc.}$ 10^{-4}deg/min
24.2218	18.37	18.42	−0.05
24.2476	18.13	18.07	+0.06
24.6224	13.02	12.94	+0.08
24.8056	10.36	10.44	−0.08
24.8175	10.18	10.27	−0.09
24.9856	8.03	7.97	+0.06
25.2201	4.79	4.77	+0.02

Since the uncertainty in the measured $(dt/d\tau)_{obs.}$-values in the first column of Table 1 was about ± 0.04 × 10^{-4} deg/min it was evident from the values listed in the last column that the agreement between observed and calculated $dt/d\tau$-values was satisfactory and that therefore the calorimetric system was unctioning so as to permit calculation of the energy exchange between calorimeter and environment by eqn. 8.

Calibration experiments

The experimental procedure used in the calibration experiments and the method of calculation of results have already been described, p. 1517 and 1522.

The benzoic acid was National Bureau of Standards standard sample 39 g [35] and the fuse was a cotton string having a percentage composition corresponding to the empirical formula $CH_{1.85}O_{0.91}$.

For reduction of weight in air to mass and calculation of the energy equivalent of the initial contents of the bomb, the following values at 25°C of density, ϱ, and specific heat, c_p, were used: for benzoic acid, $\varrho = 1.320$ g/ml [35,52], $c_p = 0.287$ cal/g deg [53]; for fuse, $\varrho = 1.5$ g/ml [45 (p. 81)], $c_p = 0.4$ cal/g deg [45 (p. 93)].

* It should be observed that all other calorimetric experiments in the present work were performed with the jacket having a temperature of 25.22°C, *cf.* p. 1517.

The results obtained are presented in Table 2. The heading of the table gives the experimental conditions which were common to all the calibration experiments, and in the various columns are listed the data obtained for the individual experiments. The symbols denote:

t_h	the reference temperature to which the calorimetric experiments are ascribed
t_i	the initial temperature of the actual bomb process
P^i(gas.)	the initial pressure, at t_h, of the gaseous phase in the bomb
V(Bomb)	the volume of the bomb
V^i(H_2O tot.)	the volume of water introduced into the bomb
m(Pt)	the mass of removable platinum parts in the bomb (crucible and suspension ring)
m^i(Cont.)	the mass of the initial contents of the bomb (see p. 1522)
$-\Delta Ec/M$(Benzoic	acid NBS 39 g) heat of combustion (per gram mass) of standard benzoic acid NBS 39 g under the actual bomb conditions
$-\Delta Ec/M$(Fuse)	heat of combustion (per gram) of the fuse under the actual bomb conditions
$\varepsilon°$(Calor.)	the energy equivalent of the standard calorimetric system (see p. 1522)
ε^i(Cont.)	the energy equivalent of the initial contents of the bomb (see p. 1523)
m'	the mass of benzoic acid burned
m'''	the mass of fuse burned
Δt	the corrected temperature rise (see p. 1520)
$-\Delta E(HNO_3)$	the heat evolved through the formation of the nitric acid
$-\Delta E_{I.B.P.}$	the heat of the isothermal bomb process (see p. 1523)

The value given in Table 2 for the heat of combustion of the standard benzoic acid under the actual bomb conditions of the calibration experiments, $-\Delta Ec/M$(Benzoic acid NBS 39g), was computed from the certified value [35] (*cf.* Refs.[54,55,52]) of 26.4338 ± 0.0026 abs. kj/g mass (6317.83 ± 0.62 cal/g mass) under certificate conditions by multiplication with a factor obtained as given in the certificate [35], *cf.* Refs.[43 (p. 37), 54 (p. 261)]. The actual conditions adhered to certificate conditions so closely that the required factor did not differ from unity to any significant extent. The value given in Table 2 for the heat of combustion of the fuse under the actual bomb conditions, $-\Delta Ec/M$(Fuse), is in fact the standard heat of combustion of the fuse, $-\Delta Ec°/M$(Fuse), obtained from heat of combustion experiments with the fuse. The error introduced into the results of the calibration experiments by this approximation was not significant.

Heat of combustion experiments

Two sets of combustion experiments were performed, one with the sample of paraffin oil used as auxiliary material in the combustion experiments with the bromine compound, and one with a sample of *p*-bromobenzoic acid. The

Table 2. Results of calibration experiments.

t_h = 25°C	V(Bomb) = 0.2670 liter	m^i(Cont.) = 20.745 g	ε^i(Cont.) = 3.198 cal/deg	
t_i = 24.1°C	$V^i(H_2O$ tot.) = 0.810 ml	$-\Delta Ec/M$(Benzoic acid NBS 39 g) = 6 317.83 ± 0.62 cal/g [a]		
P^i (gas.) = 30.0 atm	m(Pt) = 8.528 g	$-\Delta Ec/M$(Fuse) = 3 971 ± 4 cal/g [a]		

m' g	m''' g	Δt deg	$-\Delta E(HNO_3)$ cal	$-\Delta E_{I.B.P.} / \Delta t$ cal/deg
0.79944	0.00535	0.87412	1.00	5 803.52
0.79986	0.00502	0.87431	1.65	5 804.53
0.80154	0.00583	0.87660	1.10	5 804.52
0.80168	0.00562	0.87687	1.00	5 802.68
0.80117	0.00480	0.87555	0.70	5 803.69
0.80103	0.00561	0.87573	0.70	5 805.16
			Mean	5 804.02
			Standard deviation of mean	± 0.36
				ε°(Calor.) = 5 821.54 ± 0.36 cal/deg

[a] At 25°C under the actual bomb conditions; see text for further comments.

experimental procedure used in the heat of combustion experiments and the method of calculation of results have been described previously, p. 1518 and 1524. It should, however, be noted that since no reducing agent was required in the combustion experiments with the paraffin oil, the initial bomb liquid used in the paraffin oil experiments was the same as that used in the calibration experiments, *i.e.* 0.810 ml of water.

Paraffin oil USBM-P3a. The oil was a sample of redistilled mineral oil obtained from the Thermodynamics Laboratory, Petroleum Experiment Station, Bureau of Mines, Bartlesville, Oklahoma, under the laboratory designation Paraffin oil USBM-P3a. The oil was found to have a percentage composition corresponding to the empirical formula $CH_{1.89}$ and a density, ϱ, of 0.8755_0 g/ml at 20.00°C and 0.8723_5 g/ml at 25.00°C. For use in calculation of the energy equivalent of the initial contents of the bomb and in reduction to standard states the following values at 25°C of specific heat, c_P, and energy of compression, $(\partial E/\partial P)_T$, were used: $c_P = 0.53$ cal/deg [45 (p. 93)], $(\partial E/\partial P)_T = -0.0060$ cal/g atm. The latter value was calculated from the density values given above using the approximate relation $(\partial E/\partial P)_T = -T(\partial V/\partial T)_P$ as discussed in Ref.[56 (p. 542–43)].

The results obtained in the combustion experiments with the paraffin oil are presented in Table 3. The denotation of the symbols is the same as in Table 2. The following additional symbols are used:

$-\Delta E_c^\circ/M$(Fuse)	the standard heat of combustion (per gram) of the fuse
ε(Calor.)	the energy equivalent of the actual calorimetric system minus the contents of the bomb (see p. 1522); this quantity is related to ε°(Calor.) through the equation ε(Calor.) = ε°(Calor.) − $c_P(H_2O)m^i$(Cont.)
$-\Delta E_\Sigma/m''$	the heat to be evolved, per gram of paraffin oil burned, through the reactions constituting the sum of the remaining Washburn corrections (p. 1527)
$-\Delta E(CO_2)/m''$	the heat evolved, per gram of paraffin oil burned, from the dissolution of carbon dioxide in the final bomb solution
m''	the mass of paraffin oil burned
$-\Delta E_B/M$	the heat of combustion per gram of the compound in the isothermal bomb process at the temperature t_h (see Ref.[48])
$-\Delta E_c^\circ/M$	the standard heat of combustion per gram of compound at the temperature t_h; as discussed in Ref.[48] the following relation holds: $-\Delta E_c^\circ/M = -\Delta E_B/M - \Delta E_\Sigma/m''$

p-*Bromobenzoic acid.* The *p*-bromobenzoic acid used in the combustion experiments was a highly purified sample *.

* The principal aim with the combustion experiments in the present work was to investigate the precision obtainable with the described bomb-calorimetric method and not to determine an accurate value for the heat of combustion of the sample used in the combustion experiments. For this reason it was necessary to have access to a suitable organic bromine compound sample of fairly high purity, but the detailed description of the purification process and of the determination of the purity of the sample was not of primary interest. It is intended to report on these details in a later paper dealing with the heats of combustion and formation of *p*-bromobenzoic acid and with the possible use of this compound as a test substance for bomb combustions of organic bromine compounds.

Table 3. Results of combustion experiments on paraffin oil USBM-P3a.

t_h = 25.0°C	V(Bomb) = 0.2670 liter	$\varepsilon°$(Calor.) = 5 821.54 ± 0.36 cal/deg	ε(Calor.) = 5 801.16 cal/deg	
t_i = 24.1°C	V^i(H_2O tot.) = 0.810 ml		ε^i(Cont.) = 3.21 cal/deg	
P^i(gas.) = 30.0 atm	m(Pt) = 8.528 g	$-\Delta Ec°/M$(Fuse) = 3 971 ± 4 cal/g	$-\Delta E_\Sigma/m'' - \Delta E(CO_2)/m''$ = −3.4 cal/g	
m″ g	m‴ g	Δt deg	$-\Delta$E(HNO_3) cal	$-\Delta E_B/M$ cal/g
0.46138	0.00508	0.87687	0.70	10 986.2
0.46100	0.00525	0.87590	0.80	10 981.3
0.46186	0.00567	0.87788	0.65	10 982.5
0.46122	0.00534	0.87694	2.05	10 985.7
			Mean	10 983.9
			Standard deviation of mean	± 1.2
				$-\Delta Ec°/M$ = 10 980.5 ± 1.2 cal/g

For use in reducing weight in air to mass in the calculation of the energy equivalent of the initial contents of the bomb and in the reduction to standard states, the following values at 25°C of density, specific heat and energy of compression were used: $\varrho = 1.889$ g/ml, $c_p = 0.18$ cal/deg g, $(\partial E/\partial P)_T = -0.0029$ cal/g atm. These values were obtained as follows: the density value was calculated from the value $\varrho^{20} = 1.894$, given in Ref.[57], using a value for the temperature coefficient of the density of 0.00054 g/ml deg, which is the average of the two values given for benzoic acid in Ref.[52]; the specific heat value was obtained from the value given earlier in this paper for benzoic acid, by assuming the molar heat capacities for benzoic acid and *p*-bromobenzoic acid to be approximately the same; and the energy of compression value given above is that obtained for benzoic acid from data given in Ref.[52]

The results obtained in the combustion experiments with the *p*-bromobenzoic acid sample are presented in Table 4. The denotation of symbols is the same as in Tables 2 and 3 except for m', which see below. The following additional symbols denote:

V^i(soln.)	the volume of arsenious oxide solution introduced into the bomb
C^i(soln.)	the concentration of As_2O_3 in the initial bomb solution
$-\Delta Ec^\circ/M$(Oil)	the standard heat of combustion per gram of the auxiliary paraffin oil at the temperature t_h
m'	the mass of *p*-bromobenzoic acid burned
$n^f(As_2O_5)$	the number of moles of As_2O_5 in the final solution
$-\Delta E(CO_2)$	the heat evolved from the dissolution of carbon dioxide in the final bomb solution
$-\Delta E$(Au)	the heat evolved through the formation of the bromoaurous acid
$-\Delta E$(Pt)	the heat evolved through the formation of the bromoplatinous acid
$-\Delta E$(HBr)	the heat to be evolved through oxidation to elemental bromine of the hydrobromic acid in the final solution
$-\Delta E_\Sigma$	the heat to be evolved through the reactions constituting the sum of the remaining Washburn corrections (see p. 1527)

DISCUSSION OF METHOD AND RESULTS

The precision of the method

The over-all precision [1 (p. 430)] of heat of combustion data obtained with the present method will be discussed in a later paper [51]. The immediate precision [1 (p. 430)] of the results from the calibration and from the combustion experiments presented above can, however, be used for comparison of the precision of the present technique with that of other bomb-calorimetric techniques.

As is seen from Table 2 the precision obtained in the calibration experiments with benzoic acid was 0.006 % in terms of standard deviation of the mean of the six experimental values. This figure is comparable to the best

Table 4. Results of combustion experiments on *p*-bromobenzoic acid.

t_h = 25.0° C V(Bomb) = 0.2670 liter $\varepsilon°$(Calor.) = 5 821.54 ± 0.36 cal/g m(Pt) = 8.529 g

t_i = 24.1°C V^i(soln.) = 0.03005 liter $-\Delta Ec°/M$(Oil) = 10 980.5 ± 1.2 cal/g ε(Calor.) = 5 772.09 cal/deg

P^i(gas.) = 30.0 atm C^i(soln.) = 0.0627$_9$ mole As_2O_3/liter $-\Delta Ec°/M$(Fuse) = 3 971 ± 4 cal/g ε^i(Cont.) = 32.09 cal/deg

m′ g	m″ g	m‴ g	Δt deg	$n^f(As_2O_5)$ mole	$-\Delta E(CO_2)$ cal	$-\Delta E(HNO_3)$ cal	$-\Delta E$(Au) cal	$-\Delta E$(Pt) cal	$-\Delta E$(HBr) cal	$-\Delta E_\Sigma$ cal	$-\Delta Ec°/M$ cal/g
1.19492	0.05444	0.00568	0.88022	0.001444	14.71	2.00	0.04	0.08	31.34	−1.99	3 673.37
1.20108	0.04585	0.00568	0.86800	0.001473	14.56	0.70	0.04	0.06	31.50	−2.03	3 673.45
1.20034	0.04690	0.00518	0.86899	0.001451	14.59	1.35	0.05	0.10	31.48	−2.03	3 673.35
1.19825	0.05258	0.00553	0.87899	0.001462	14.70	1.00	0.04	0.06	31.43	−2.01	3 674.50
1.20048	0.05637	0.00573	0.88758	0.001468	14.82	1.35	0.04	0.05	31.49	−2.00	3 673.16
1.20161	0.04606	0.00549	0.86873	0.001470	14.59	0.65	0.04	0.04	31.52	−2.03	3 674.31
1.20115	0.05425	0.00535	0.88377	0.001465	14.77	0.75	0.04	0.06	31.50	−2.01	3 674.06
										Mean	3 673.74
										Standard deviation of mean	± 0.20

precision obtained in calibration experiments with other present-day combustion calorimetric systems of static-bomb as well as moving-bomb type. The combustion experiments with paraffin oil, presented in Table 3, were performed under the same experimental conditions as the calibration experiments. The precision obtained was 0.011 %, from a set of only four individual experiments (see table). In a later series of combustion experiments on paraffin oil [51] with the same apparatus the standard deviation of the mean of six experimental values was found to be 0.008 %. Although slightly high these figures are also satisfactory in comparison with results from other modern bomb-calorimetric work. Thus the results obtained in the experiments with benzoic acid and paraffin oil demonstrated that the reproducibility obtainable with the apparatus and technique was satisfactory. More specifically, the assumption made earlier in this paper was confirmed that for certain energy quantities, such as the ignition energy $\Delta E_{ign.}$ and the bomb-rotation energy $\Delta E_{rot.}$, the variation in value from one calorimetric experiment to another was small enough to accept them as constant in all the calorimetric experiments. Tables 2 and 3 also prove that the apparatus and technique gave satisfactory precision in the temperature—time measurements and in the calculation of heat exchange corrections Δt_{ex}.

The precision obtained in the heat of combustion experiments with *p*-bromobenzoic acid, presented in Table 4, was 0.005 % in terms of standard deviation of the mean of seven experimental values. This high precision was virtually the same as that obtained in the calibration experiments with benzoic acid, which showed that neither did the presence of bromine in the compound affect the reproducibility of the combustion process of a pelleted sample, nor was the reproducibility affected by the presence of a large amount of bomb liquid or by the additional uncertainty in the determination of the amount of heat associated with the oxidation of arsenious oxide to arsenic oxide.

The various experiments of the present work thus have shown that the precision of heat of combustion results obtained with the described moving-bomb method for organic bromine compounds is comparable to the precision obtained with the best modern combustion calorimetric systems for other groups of organic compounds.

The accuracy of the method

Although the precision obtainable with the present method has been found to be high, the presence of undetected systematic errors might render the accuracy of obtained heat of combustion data much lower than actually would be attainable with this high precision. Therefore the accuracy of the method has to be investigated, and there are several possible ways of doing this. Since the principal use for heat of combustion data is to derive corresponding heats of formation, a suitable approach would be to compare, for certain selected bromine compounds, heats of formation calculated both from heat of combustion data and from calorimetric data on heats of reactions other than combustion. Such a comparison has been made and the results will be reported in a later paper [51]. It will, however, be appropriate to include in the

present discussion of method and results an examination of certain details of the technique which could possibly cause systematic errors.

The calorimetric part of the measurement

The energy exchange between calorimeter and environment. It was experimentally demonstrated earlier that the functioning of the present calorimetric system allowed calculation of the energy exchange between calorimeter and environment during the reaction period of an experiment by eqn. 8 given on p. 1521. A necessary prerequisite for this is, however, that equilibrium has been reestablished in the bomb before the after period starts. The question about attainment of equilibrium in the bomb will be discussed later in this paper.

The energies of ignition and of rotation of the bomb. It was evident from the discussion on p. 1523 that, as long as both calibration and combustion experiments were so arranged that the obtained Δt-values were virtually the same in all experiments, it was only the *variation* in the energies of ignition and of rotation from one set of experiments to another that might give rise to systematic errors. As has already been pointed out, such variations would also affect the precision in the various sets of measurements. Since this precision, however, was found to be as high as could be expected, the variations in the two mentioned quantities could not be significant.

The heats of reaction for the auxiliary combustion reactions. Any uncertainties in the molar heat of reaction values for the auxiliary combustion reactions 21—27, given on p. 1525, will give rise to corresponding systematic errors in obtained heat of combustion values. As is seen from Table 4 the quantities $-\Delta E(HNO_3)$, $-\Delta E$(Au) and $-\Delta E$(Pt) associated with the actual amounts of reactions 25—27 are small enough to permit large uncertainties in the corresponding molar heats of reaction *. Therefore, although the molar heat of reaction 26 has a high degree of uncertainty (p. 1526), the systematic errors from reactions 25—27 are probably small enough to be neglected. The variation in value of the quantity $-\Delta E$(Fuse) in calibration as well as combustion experiments is small enough to give with the uncertainty in the $-\Delta E^\circ$ (reaction 23)-value satisfactory cancellation of errors from this quantity and therefore this error in the obtained heat of combustion values is also small enough to be neglected. The systematic errors associated with the uncertainties in the molar heats of reaction 21, 22 and 24 are, however, significant. These errors will be treated more fully in a later paper [51] dealing with the evalaution of the final over-all precision in the obtained heats of combustion. It may be pointed out that all these three molar heats of reaction were obtained, as specified on p. 1526, from recent experimental work directly in connection with the work presented in this paper.

Chemical part of the measurement

Homogeneity and equilibrium in the final state in the bomb. It has already been stressed that, as the method requires a large volume of bomb liquid, it is

* Since the quantity $-\Delta E(HNO_3)$ appears in calibration as well as combustion experiments the error from this quantity is partly cancelled through the procedure of the given method.

imperative that homogeneity in the final liquid phase and equilibrium between the gaseous and the liquid phase in the bomb should be achieved before the after period of a measurement has started. The presence of a large volume of bomb liquid and the mode and duration of the rotation of the bomb in the present work brought about a thorough rinsing of the whole interior surface of the bomb, except for certain parts of the platinum rod B_6 (Fig. 2), and the gas-inlet tube B_7. Homogeneity of the liquid phase was therefore rapidly obtained.

Considering equilibration of the bomb gases with the bomb solution, it was found (see below) that, at the end of the reaction period, no hydrogen bromide or elemental bromine could be detected in the gaseous phase and therefore these components must have been quantitatively transferred to the bomb solution during the reaction period. The carbon dioxide was a third component, the equilibration of which between the gaseous and the liquid phase was of primary importance for the present method. Recently, the influence of various experimental factors on the rate of absorption of carbon dioxide in the final bomb liquid and the time required for reaching equilibrium between the gaseous and the liquid phase has been experimentally studied for different moving-bomb techniques [26]. Since the results of this investigation were utilized in designing the moving mechanism used in the present work, it can be confidently assumed that in all experiments the distribution of carbon dioxide between the gaseous and the liquid phase in the bomb was complete within the reaction period.

Chemistry of the bomb process. The accuracy of the heat of combustion data can only compare with the over-all precision of the experimental results if the nature and amount of the various chemical reactions 21—27, constituting the bomb process, are known to a corresponding degree of accuracy.

The amounts of reactions 20, 22, and 23 were obtained, as described earlier, from the composition and mass of the bromine compound, the paraffin oil, and the fuse, respectively. A possible check on the completeness of the sum of these three reactions would be by an experimental determination of the total amount of carbon dioxide present in the bomb after the combustion. Due to the large amount of bomb liquid such a determination would be experimentally very difficult, and no such measurement has been attempted.

A check on the completeness of the transformation of the bromine in the combustion sample to hydrogen bromide according to eqn. 20 would be the analytical determination of the amount of bromide ion in the final bomb liquid *. This was done in the experiments presented in Table 4 by potentiometric titration of aliquot 20 ml samples of the diluted bomb solutions with silver nitrate solution and calculation of the total amount of hydrogen bromide present in the final bomb liquid. It was necessary to correct for the small amounts of hydrogen bromide consumed in reactions 26 and 27 and therefore present in the final bomb liquid as $HAuBr_2$ and H_2PtBr_4, respectively. From

* That no detectable amounts of bromine, in the form of hydrogen bromide or elemental bromine, were present in the final gas phase was demonstrated in separate combustion experiments by discharging the bomb gases, at the end of the reaction period, through a sodium hydroxide solution and analyzing the solution for bromide ion.

available stability constants [58] for these compounds it was estimated * that by the silver nitrate titration approximately one of the bromines in $HAuBr_2$ and none of the bromines in H_2PtBr_4 were titrated as bromide ion. Taking account of this in the calculation of the total amount of bromine present in the final bomb liquid the following figures were obtained for the recovery of the amount of bromine introduced into the bomb with the combustion sample: 99.3, 99.4, 99.6, 99.0, 99.5, 99.7, 99.8, and 99.8 %, respectively. Because of the uncertainties in the analytical method the deviation of these figures from the theoretical value 100.0 could not be considered significant and therefore the figures could be taken as an indication of the absence of gross "chemical" errors in this part of the method.

The actual oxidation state of the dissolved gold and platinum in the bomb liquid was difficult to assess. Combination of available data on oxidation potentials [59] with the concentrations of the various constituents in the final bomb solution implied that the gold should be present as Au(0) and the platinum as Pt(II). It was, however, experimentally found that the precipitation of metallic gold from the bomb solution was very slow and therefore it could be assumed that the oxidation state of the entire amount of gold, which had been dissolved during the combustion process was — at the end of the reaction period — Au(I) and that the precipitation of gold during the after period was completely negligible.

If arsenious oxide solution is not affected by gaseous oxygen under bomb conditions the amount of reaction 21 should be equivalent with the amount of elemental bromine formed in the actual combustion reaction 17. It is a principal requirement that no reaction between the arsenious oxide solution and the oxygen in the bomb should take place in either the initial or the final period of the calorimetric experiment. This question will be discussed next.

Stability of the arsenious oxide solution. For possible use as a reductant in combustion experiments with organic compounds containing chlorine the former I.U.C. Standing Commission for Thermochemistry discussed [60] three different reducing agents: arsenious oxide, hydrazine monohydrochloride and formic acid. Of these compounds only arsenious oxide has been further studied [61]. Instead of the monohydrochloride, hydrazine dihydrochloride has also been studied for such purposes [62]. A comparison has recently been made [63] of arsenious oxide and hydrazine dihydrochloride for use in static-bomb combustion calorimetry of organic chlorine compounds. Arsenious oxide was used as reductant in the introductory moving-bomb work on organic bromine compounds in this laboratory [17,18] and since a closer study (see below) showed that an arsenious oxide solution of the composition used in the present work was completely stable under the experimental bomb conditions of the present technique, this substance was chosen as a reductant for the further work.

The stability of the arsenious oxide solution and the accuracy of the analytical procedure were tested by treating 30.01 ml samples of the arsenious oxide solution in the following ways: (1) The sample was diluted in a volumetric flask to 250.0 ml, (2) The sample was placed in the bomb body and the bomb

* The author's thanks are due to Mr. Ragnar Larsson, Department of Inorganic and Physical Chemistry, University of Lund, for making the calculations required in this estimation.

was closed. It was shaken for a minute and then opened. The contents were carefully transferred to a volumetric flask by repeated rinsing with water and diluted to 250.0 ml, (3) The sample was placed in the bomb which was then closed and filled with oxygen to a pressure of 30 atm. After 24 h the oxygen was discharged and the liquid contents were transferred to a volumetric flask and diluted as above, (4) The sample was placed in the bomb and used for a combustion experiment with benzoic acid or paraffin oil. After the experiment the bomb liquid was transferred as above to a volumetric flask and diluted to 250.0 ml. Numerous experiments of this type were performed. From all the solutions obtained under items 1 to 4 aliquot samples of 50 ml were withdrawn and titrated with potassium permanganate solution according to the method described above (p. 1520). The volume of permanganate solution consumed was in all the experiments 15.05 ml within $\pm$ 0.1 % *. These results indicated that, within the precision of the analytical method, no oxidation of the arsenious oxide solution occurred during combustion experiments with compounds containing only carbon, hydrogen and oxygen. Therefore it could be confidently assumed that the stability of the reductant during the initial and final period of an experiment was satisfactory.

Amount of reaction. From what has been said above on the analytical determinations of the amounts of carbon dioxide and hydrogen bromide present in the final state in the bomb it was evident that the only feasible way of determining the amount of reaction for the main combustion reaction 20 was from the mass and composition of the organic bromine compound placed in the bomb. This makes the question of the purity of the combustion sample most important.

Purity of the sample. The use of the above method for determination of the amount of reaction 20 requires that the impurities in the combustion sample are known in nature and amount or, preferably, that the purity of the sample is high enough to give no significant contribution to the uncertainty of the over-all method. In the latter case the absence of inert impurities, such as water, is especially important.

Application of the principle of comparative measurements to the present method

The application of the method of comparative measurements to combustion-bomb calorimetric studies of organic sulfur, chlorine and bromine compounds, respectively, has been discussed recently by various authors [64–66]. Three different methods, which should be adaptable for use in studies of bromine compounds, were mentioned in Ref.[66] The technique described above constitutes a fourth possible method. On account of the nature of this work the discussion of the present technique from the method-of-comparative-measurements point of view will, however, be deferred to a later paper.

Acknowledgement. The author's thanks are due to the *Swedish Natural Science Research Council* for financial support of this investigation.

* For two solutions from combustion experiments with benzoic acid a slightly lower result was obtained.

REFERENCES

1. Rossini, F. D. and Deming, W. E. *J. Wash. Acad. Sci.* **29** (1939) 416.
2. Sturtevant, J. M. *Calorimetry* Chapter XIV of Weissberger, A. (Ed.) *Physical Methods of Organic Chemistry*, 2nd Ed., Part I, Interscience, New York 1949.
3. Rossini, F. D. (Ed.) *Experimental Thermochemistry*, Interscience, New York 1956.
4. Conn, J. B., Kistiakowsky, G. B. and Smith, E. A. *J. Am. Chem. Soc.* **60** (1938) 2764.
5. Lister, M. W. *J. Am. Chem. Soc.* **63** (1941) 143.
6. Lacher, J. R., Walden, C. H., Lea, K. R. and Park, J. D. *J. Am. Chem. Soc.* **72** (1950) 331.
7. Lacher, J. R., Kianpour, A. and Park, J. D. *J. Phys. Chem.* **61** (1957) 1124.
8. Lacher, J. R., Billings, T. J., Campion, D. E., Lea, K. R. and Park, J. D. *J. Am. Chem. Soc.* **74** (1952) 5291.
9. Lacher, J. R., Kianpour, A., Montgomery, P., Knedler, H. and Park, J. D. *J. Phys. Chem.* **61** (1957) 1125.
10. Lane, M. R., Linnett, J. W. and Oswin, H. G. *Proc. Roy. Soc. London* **216** (1953) 361.
11. Berthelot, M. *Ann. chim. et phys.* [5] **23** (1881) 214.
12. Thomsen, J. *Thermochemische Untersuchungen*, Barth, Leipzig 1882–1886, Bd. 4, p. 112.
13. Kablukov, I. and Perelmann, F. *Compt. rend. acad. sci. U.R.S.S.* Ser. A **1930** 519.
14. Pongratz, A., Griengl, F. and Cecelsky, J. *Sitz. ber. Akad. Wiss. Wien, Math. naturw. Kl. Abt. II b* **141** (1932) 833.
15. Popoff, M. M. and Schirokich, P. K. *Z. physik. Chem. A.* **167** (1933) 183.
16. Brüll, L. *Gazz. chim. ital.* **65** (1935) 19.
17. Smith, L. and Sunner, S. *The Svedberg*, Almqvist & Wiksell AB, Uppsala 1944, p. 352.
18. Smith, L. and Bjellerup, L. *Acta Chem. Scand.* **1** (1947) 566.
19. Berthelot, M. *Ann. chim. et phys.* [5] **23** (1881) 160.
20. Ref.[12], Bd. 2, p. 349.
21. Roth, W. A., Ginsberg, H. and Lassé, R. *Z. Elektrochem.* **30** (1924) 417.
22. Sunner, S. *Svensk Kem. Tidskr.* **58** (1946) 71.
23. Sunner, S. *Studies in Combustion Calorimetry Applied to Organo-Sulphur Compounds* (Diss.) University, Lund 1949.
24. Hubbard, W. N., Katz, C. and Waddington, G. *J. Phys. Chem.* **58** (1954) 142.
25. Sunner, S. and Bjellerup, L. *Acta Chem. Scand.* **5** (1951) 261.
26. Bjellerup, L. and Sunner, S. *Rec. trav. chim.* **73** (1954) 862.
27. Dickinson, H. C. *Bull. Bur. Standards* **11** (1915) 189.
28. White, W. P. *The Modern Calorimeter*, Chemical Catalog Company, New York 1928.
29. Coops, J., van Nes, K., Kentie, A. and Dienske, J. W. *Rec. trav. chim.* **66** (1947) 113.
30. Coops, J. and van Nes, K. *Rec. trav. chim.* **66** (1947) 131.
31. Coops, J. and van Nes, K. *Rec. trav. chim.* **66** (1947) 142.
32. Coops, J. and van Nes, K. *Rec. trav. chim.* **66** (1947) 161.
33. Challoner, A. R., Gundry, H. A. and Meetham, A. R. *Trans. Roy. Soc. (London) Ser. A.* **247** (1955) 553.
34. Prosen, E. J. and Rossini, F. D. *J. Research Natl. Bur. Standards* **27** (1941) 289.
35. *Provisional Certificate of Analysis of Standard Sample 39 g, Benzoic Acid*, National Bureau of Standards, Washington, D.C., 1949.
36. Smith, L. *Svensk Kem. Tidskr.* **45** (1933) 110.
37. Sandell, E. B. *Anal. Chem.* **20** (1948) 253.
38. Bjellerup, L. *To be published.*
39. Sandell, E. B. *Colorimetric Determination of Traces of Metals*, Interscience, New York 1944, p. 361.
40. Kolthoff, I. M., Belcher, R., Stenger, V. A. and Matsuyama, G. *Volumetric Analyses Vol. III*, Interscience, New York 1957, p. 41–45.
41. King, A. and Grover, H. *J. Appl. Phys.* **12** (1941) 557.
42. Jessup, R. S. *J. Appl. Phys.* **13** (1942) 128.
43. Coops, J., Jessup, R. S. and van Nes, K. *Calibration of Calorimeters for Reactions in a Bomb at Constant Volume* Chapter 3 of Ref.[3]

44. Rossini, F. D. *Calibrations of Calorimeters for Reactions in a Flame at Constant Pressure* Chapter 4 of Ref.[3]
45. Hubbard, W. N., Scott, D. W. and Waddington, G. *Standard States and Corrections for Combustions in a Bomb at Constant Volume* Chapter 5 of Ref.[3]
46. Prosen, E. J. *Combustion in a Bomb of Compounds Containing Carbon, Hydrogen, Oxygen, and Nitrogen* Chapter 6 of Ref.[3]
47. Jessup, R. S. and Green, C. B. *J. Research Natl. Bur. Standards* **13** (1934) 469.
48. Bjellerup, L. *To be published.*
49. Rossini, F. D., Wagman, D. D., Evans, W. H., Levine, S. and Jaffe, I. *Selected Values of Chemical Thermodynamic Properties*, Circular of the National Bureau of Standards 500, U.S. Government Printing Office, Washington, D. C., 1952.
50. Bjellerup, L., Sunner, S. and Wadsö, I. *Acta Chem. Scand.* **11** (1957) 1761.
51. Bjellerup, L. *To be published.*
52. Jessup, R. S. *J. Research Natl. Bur. Standards* **36** (1946) 421.
53. Furukawa, G. T., McCoskey, R. E., and King, G. J. *J. Research Natl. Bur. Standards* **47** (1951) 256.
54. Jessup, R. S. *J. Research Natl. Bur. Standards* **29** (1942) 247.
55. Prosen, E. J. and Rossini, F. D. *J. Research Natl. Bur. Standards* **33** (1944) 439.
56. Washburn, E. W. *Bur. Standards J. Research* **10** (1933) 525.
57. Steinmetz, H. *Z. Kryst. Mineral.* **53** (1913) 475.
58. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability Constants. Part II: Inorganic Ligands*, The Chemical Society, London 1958.
59. Latimer, W. M. *The Oxidation States of the Elements and Their Potentials in Aqueous Solutions*, 2nd Ed. Prentice-Hall, New York 1952.
60. Union Internationale de Chimie, Commission Permanente de Thermochimie *Revue Analytique & Critique de Thermochimie Organique*, Paris 1936, p. 23.
61. Smith, L., Bjellerup, L., Krook, S. and Westermark, H. *Acta Chem. Scand.* **7** (1953) 65, p. 76.
62. Hubbard, W. N., Knowlton, J. W. and Huffman, H. M. *J. Phys. Chem.* **58** (1954) 396.
63. Smith, L. and Hubbard, W. *Combustion in a Bomb of Organic Chlorine Compounds* Chapter 8 of Ref.[3], p. 198.
64. Waddington, G., Sunner, S. and Hubbard, W. *Combustion in a Bomb of Organic Sulfur Compounds* Chapter 7 of Ref.[3], p. 166.
65. Ref.[63], p. 195.
66. Smith, L. and Bjellerup, L. *Combustion in a Bomb of Organic Bromine Compounds* Chapter 9 of Ref.[3], p. 214.

Received May 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1542–1546

Isolation of Five Myoglobins from Seal (*Phoca vitulina*)

NEVENKA M. RUMEN

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

1. Crystalline seal myoglobin contains 5 components. They have been separated by the aid of a cellulose ion-exchanger.
2. All five myoglobins have been crystallized from ammonium sulphate and their crystal shapes were found to be identical under the microscope.
3. The iron content was the same for all five.
4. Mb I is a basic protein, the others have IP values in the pH range 7.6—6.2.
5. Glycine is the terminal group in all five Mb's. Mb IV may contain serine as a second terminal residue.
6. The only differences found amongst the components is the isoelectric point and slight differences in the extinction coefficient of certain bands of the spectrum.

Already in 1934 Theorell [1] found an exceptionally high concentration of myoglobin in the seal, where the muscle press juice contains from 5 to 10 % of myoglobin. This is something like ten times the concentration normally found in mammals. Kendrew, Parrish and others [2] have also found that aquatic mammals, like whales, seals and penguins are particularly rich sources for myoglobin, so that their skeletal muscles are almost black in colour.

In this paper the isolation and some of the properties of seal myoglobin will be described.

ISOLATION

Seal myoglobin was prepared from the meat of the common seal, *Phoca vitulina*, by the method described for sperm-whale myoglobin by Kendrew and Parrish [3], and kindly given by Dr. Kendrew for this work. The crude preparation, which was sent as a thick paste in phosphate buffer, pH 6.8, was dissolved in a very small amount of water and dialyzed three times against distilled water in the cold, and four or five times against 0.01 M tris-buffer, pH 8.50 ± 0.02.

Carboxymethyl cellulose was prepared according to Peterson and Sober [4], and a column was packed with it and charged with tris buffer pH 8.5. The column dimensions were 1.9×23 cm. The load of the protein was 119 mg in a volume of 2.5 ml. The temperature was 4°C; no difference was noticed if the chromatograms were run at room temperature.

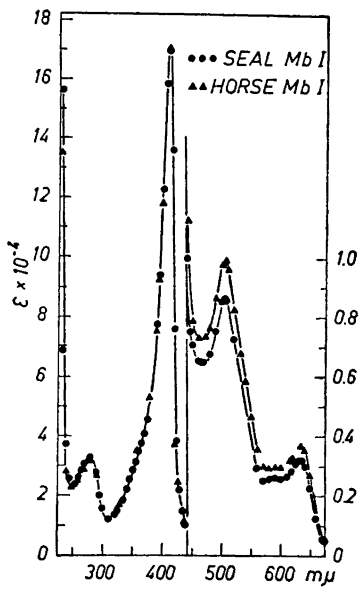


Fig. 1. Light absorption curves of seal ferri-Mb I and horse ferri-Mb I.

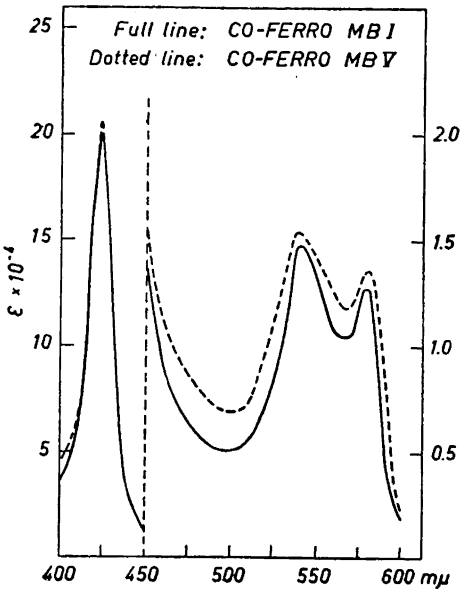


Fig. 2. Light absorption curves of seal CO-myoglobins I and V.

The chromatogram was eluated with the same buffer. On the column five distinct bands separated, moving with different speeds. The fastest component was named Mb V, and the slowest, which contained about 90 % of the total amount, was named Mb I. The distances between the bands were: Mb I 3 cm from the top, the distance between Mb I and II was 3.7 cm, between II and III 4.5 cm, between III and IV 2.5 cm, and Mb V came rather close to Mb IV. The yield of Mb I was about 90 % of the total; Mb II 2.5; Mb III 1.7; Mb IV 1.7 and Mb V 3.4 %. The high content of Mb I may be explained by the repeated crystallization from phosphate and ammonium sulphate, which followed after the extraction from the meat.

The five components were dialyzed over night in the cold against distilled water, and each component was put separately on a column, and eluated with tris buffer. In this way it was possible to concentrate the solutions, and at the same time to complete purification.

PROPERTIES

The *homogeneity* of the five myoglobins was demonstrated by moving boundary electrophoresis in phosphate buffer $\mu = 0.1$, at various pH in the range 6.1—9.0. The isoelectric point, determined by boundary electrophoresis, was found to be near pH 8.05 for myoglobin I and for myoglobin II at pH 7.6. The isoelectric points for Mb III, IV and V were not determined, because of lack of protein.

The *sedimentation constant* of Mb I determined in the Spinco centrifuge, was found to be $S_{20} = 1.95\ S$, a value which is similar to those obtained for horse myoglobin I by Theorell and Åkeson [5].

The *minimum molecular weight* deduced from the iron content (0.30 % for Mb I, 0.29 % for Mb II, 0.30 % for Mb III, 0.29 % for Mb IV, and 0.30 % for Mb V) is 18 600 for all five myoglobins. These values are comparable with those found by Theorell [5] for pure and homogeneous horse myoglobin.

The *spectrophotometric analysis* of the seal myoglobins showed that the five myoglobins have light adsorption maxima and minima exactly at the same wave lenghts as pure horse myoglobin I, but they differ in extinction coefficients. Seal myoglobin I and horse Mb I have the same molar light absorption at 280, 315 and 409 mμ, but they differ at the wave lenghts 465, 505, and 635 mμ, which can be seen from Fig. 1. The results for all five myoglobins are given in the Tables 1 and 2.

The *prosthetic group* of the seal myoglobin I is identical with that of horse Mb I; this was demostrated by resynthesis from seal apo-protein and protohematin. Resynthesized Mb I was crystallized and the crystals were identical with those of the native seal myoglobin. The yield of Mb I after resynthesis from apo-protein and protohematin was 93.5 %, determined from the optical density of the solution at 409 mμ. Myoglobin I was crystallized from ammonium sulfate (80 %) at pH 8. For Mb II the pH was adjusted to pH 7.5, and for the other components to 7. All five myoglobins crystallized in the same form.

Free amino group of the five myoglobins. For the determination of the N-terminal group of the seal myoglobins Sanger's method [6], slightly modified, was used.

Table 1. Ferrimyolglobins, $\varepsilon \times 10^{-4}$.

mμ	250	280	315	409	465	505	590	635
Seal Mb I	2.31	3.31	1.22	17.1	0.657	0.868	0.260	0.323
» » II	1.93	2.96	0.99	17.2	0.591	1.02	0.284	0.366
» » III	2.58	3.66	1.44	17.2	0.755	1.01	0.310	0.374
» » IV	2.45	3.35	1.39	17.1	0.721	0.95	0.280	0.355
» » V	2.76	3.64	1.39	17.2	0.781	1.05	0.329	0.383

Table 2. CO-ferromyoglobins, $\varepsilon \times 10^{-4}$.

mμ	580	560	540	500	424
Mb I	1.28	1.05	1.45	0.502	20.55
Mb II	1.24	1.00	1.43	0.505	20.50
Mb III	1.32	1.06	1.52	0.565	20.60
Mb IV	1.30	1.06	1.48	0.522	19.40
Mb V	1.36	1.19	1.54	0.694	20.45

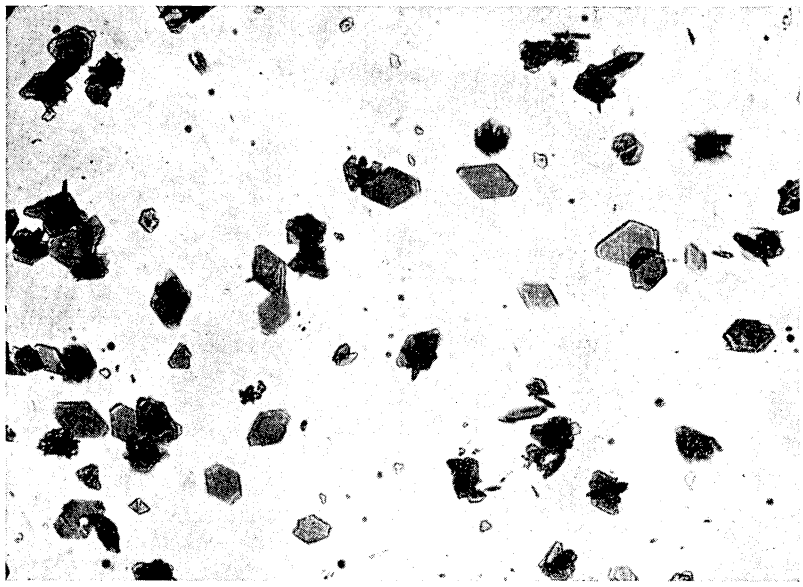


Fig. 3. Crystals of native seal Mb I from ammonium sulfate (× 83).

In each experiment 3 mg (0.25 ml) of pure salt-free myoglobin, denatured with 0.75 ml ethanol was used, and 0.5 ml of a 10 % solution of $NaHCO_3$ and 0.005 ml liquid 1,2,4-fluorodinitrobenzene were added. The mixture was then vigorously stirred for 2 h at room temperature. The precipitate formed was centrifuged off, washed with water, ethanol and finally with ether. The precipitate was hydrolyzed with 6 N HCl at 110°C for 4.6 and 8 h. After hydrolysis, the solution was cooled and evaporated to dryness, water added and dried. After dissolving in a small volume of water, the solution was extracted twice with ethyl acetate. The extracts were combined, evaporated under vacuum to a small volume, applied to a corner of a Whatman No. 1 paper and run by the ascending technique with butanol-ammonia [7] (*n*-butanol was equilibrated with the same volume of 5 % ammonia and the top phase used). After 24 h the paper was dried at 38°C for 4 h. The paper was then run in the second dimension using the descending technique and 1.5 M aqueous phosphate buffer, pH 6. The paper was dried and the R_F values were compared with those of known DNP-amino acids. In myoglobins I, II, III, and V only one spot was observed, which was found to be DNP-glycine. In Mb IV two spots were observed, with R_F values corresponding to ε-DNP-lysine or DNP-serine, and DNP-glycine.

To identify them, the DNP-amino acids were eluated from the paper after chromatographic separation and heated with concentrated ammonia [8] in a sealed capillary for 2 h at 108°C. The DNP-derivatives had been split, and the free amino acids could be run by one-dimensional paper chromatography, using phenol as solvent [9]. On the paper lysine could be identified, but whether serine

was present or not was difficult to see, because of the tailing of the spots. The experiment could not be repeated because of the shortage of fraction IV. The N-terminal group of unpurified seal myoglobin has been determined by Orláns [2], who found glycine as the terminal residue.

Acknowledgements. Grateful acknowledgement is made to Professor H. Theorell for his interest and advice during the course of this work. The author wishes to thank Dr. John C. Kendrew for kindly supplying the myoglobin mixture.

REFERENCES

1. Theorell, H. *Biochem. Z.* **268** (1934) 73.
2. Kendrew, J. C., Parrish, R. G., Marrack, J. R. and Orláns, E. S. *Nature* **174** (1954) 946.
3. Kendrew, J. C. and Parrish, R. G. *Proc. Roy. Soc. London* **A 238** (1956) 305.
4. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
5. Theorell, H. and Åkeson, Å. *Ann. Acad. Sci. Fennicae.* A. II. 60 (1955) 303.
6. Sanger, F. *Biochem. J.* **39** (1945) 507.
7. Koch, G. and Weidel, W. *Z. physiol. Chem. Hoppe-Seyler* **303** (1956) 3.
8. Lowther, A. G. *Nature* **167** (1951) 767.
9. Maehly, A. C. and Paléus, S. *Acta Chem. Scand.* **4** (1950) 508.

Received May 5, 1959

ACTA CHEMICA SCANDINAVICA 13 (1959) 1547–1560

Hydrolysis of Carboxylic Acid Esters of Thiocholine and its Analogues

3. Hydrolysis Catalysed by Acetylcholine Esterase and Butyrylcholine Esterase

EDITH HEILBRONN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

The hydrolysis of thiocholine esters, α-methylthiocholine esters and β-methylthiocholine esters, catalysed by the enzymes acetylcholine esterase and butyrylcholine esterase, was studied with an automatic recording titrator at pH 8.00 and 25°C. Michaelis-Menten constants, maximum velocities and reaction rates are given. It was shown that acetylthiocholine is easier split by butyrylcholine esterase than acetylcholine. Both are split at the same rate by acetylcholine esterase.

The influence of some reversible and irreversible inhibitors on the hydrolysis of acetylcholine and acetylthiocholine, catalysed by acetylcholine esterase and butyrylcholine esterase, was studied at pH 8.00 and 25°C. It was shown that the substrates are about equally good protectors for acetylcholine esterase, but that the thioester is the better protector for butyrylcholine esterase.

The hydrolytic effect of imidazole and cysteine on the hydrolysis of the thioesters was shown. Hydrolysis was enhanced about 4 times with imidazole and about 50 times with cysteine.

The bonds between the enzymes and the substrates are discussed on the basis of the results obtained.

Choline esters are known to be of interest *in vivo* for their potent pharmacodynamic effects and *in vitro* as substrates for choline esterases. Some of their thiocholine analogues have been prepared during the 1930's and were investigated for their pharmacodynamic actions [1]. Acetylthiocholine was used by Koelle [2] and others in histochemical methods for the determination of choline esterase activity, and by Zajiceck [3] and others in a diver technique method for determination of choline esterase activity in single cells. Thiocholine esters were also used in a few studies on the structure of choline esterases by, *e.g.*, Wilson [4] and Bergmann [5].

In two preceding papers the acid [6] and alkaline [7] hydrolysis of thiocholine esters and analogues has been described. This paper reports studies on the hydrolysis, catalysed by acetylcholine esterase (AChE) and butyrylcholine esterase (BuChE), of the thiocholine esters and their methylsubstituted analogues, acetylhomothiocholine and acetylcholine. The work was done in order to gain more knowledge about the reaction of this type of ester with choline esterases. Earlier studies [7] had demonstrated the catalytic effect of some buffer anions, e.g. phosphate, on the hydrolysis of the thioesters. To avoid this effect an automatic recording titrator [8], allowing fairly exact studies of hydrolysis, was used for the study of the enzymic hydrolysis. Besides the measurements on the enzymic hydrolysis a few experiments were carried out in order to show that the thiocholine esters and analogues interact with the same groups in the enzymes as the choline esters do. The protective effect of the thioesters against some reversible and irreversible inhibitors was studied, and the catalytic effect of some organic compounds, proposed in literature [9,10] to be related to the active centre in choline esterases, on the hydrolysis of acetyl-β-methylthiocholine and acetylthiocholine, was confirmed.

EXPERIMENTAL

Materials

Substrates. The same esters as described earlier [6,7] were used. Acetylhomothiocholine was prepared by Hansen according to the same method as was used for the other thiocholine esters [11,12].

Enzymes. The source for *AChE* was freeze-dried electric organs from *Torpedo oscellata*. *BuChE* was obtained from fraction IV-6-3, prepared from retroplacentar serum (AB Kabi, Stockholm).

Catalysts. Imidazole was from Schuchardt GMBH, L-cysteine hydrochloride and DL-serine were from Merck & Co. 2-Methyloxazoline was synthetized according to Wenker [13].

Inhibitors. Sarin was prepared by Fagerlind [14], eserine salicylate was from Smith Ltd, London, choline iodide and thiocholine iodide were prepared by Hansen.

Methods

pS-Activity curves. Reaction velocities were determined with an automatic recording titrator [8] at 25°C and pH 8.00. A 0.100 M solution of sodium hydroxide was added. For reasons explained later reaction velocities are expressed as recorder units, proportional to the amount of sodium hydroxide consumed by the hydrolysed ester, per unit of time (relative activities). The values used for calculations of reaction rates refer to hydrolysis from 1 to 10 min after addition of the substrate.

The activities of the enzyme solutions were checked each day with acetylthiocholine at 10^{-2} M substrate concentration. All activity values were corrected to the activity obtained the first day. The activity of BuChE solutions was constant during about 14 days when the solutions were kept in a refrigerator. The solutions of AChE were less stable.

Enzyme solutions. AChE: 500 mg of freeze-dried electric organs were homogenised in 100 ml of 0.10 M potassium chloride with a Potter-Elvehjem homogenizer. The solution was filtered through filter paper Munktell No. 3 and diluted 1:4 with 0.10 M potassium chloride. BuChE: 25 mg of serum fraction IV-6-3 were dissolved in 100 ml of 0.10 M potassium chloride.

Substrate solutions. The final concentration of the substrate was in all pS-activity experiments 0.100 M, 0.050 M, 0.010 M, 0.005 M, 0.001 M and 0.0005 M. The substrates were dissolved in 0.10 M potassium chloride.

Procedure and reaction mixture. The reaction mixture without enzyme was titrated to pH 8.00 with 0.100 M sodium hydroxide. The enzyme solution was added and the pH immediately again corrected to pH 8.00. The following reaction mixtures were used: enzyme solution 1 ml (AChE) or 3 ml (BuChE), substrate solution 30 ml, 0.10 M potassium chloride to make a total volume of 40 ml.

Rate of hydrolysis. In these experiments the automatic recording titrator [9] was used to keep pH constant at 8.00, and the amount of unhydrolysed ester was determined as iron-hydroxamate complex as described earlier [15]. The concentration of the esters was 0.001 M, enzyme concentration as described for pS-activity determinations. The temperature was 25°C.

Spontaneous hydrolysis and catalytic effects of some organic compounds. The spontaneous hydrolysis of each thioester was determined for 0.050 M and 0.010 M solutions. In these experiments the enzyme solution was replaced by 0.10 M potassium chloride. From the values obtained the spontaneous hydrolysis for the other substrate concentrations was calculated. Enzymic hydrolysis was calculated according to eqn (2) below. The spontaneous hydrolysis of acetylcholine was calculated from values found in this laboratory and published earlier [7]. The following equations were used for the corrections of the observed hydrolysis of acetylcholine:

$$k1_{\text{spont.}} \times C_S = k0_{\text{spont.}} \quad (1)$$

$$k0_{\text{obs.}} - k0_{\text{spont.}} = k0_{\text{enz.}} \quad (2)$$

All enzyme activity values were corrected for spontaneous hydrolysis.

In experiments with imidazole, 2-methyloxazoline, cysteine and serine the final concentration of the catalyst was the same as the concentration of the substrate, 0.001 M. The substance was dissolved in 0.10 M potassium chloride and pH adjusted to 8.00. The solution was then added to the reaction mixture. The total volume was 40 ml and the temperature 25°C.

Inhibition studies. An electrometric apparatus [16] was used for all experiments, except those where the substrate was used to protect the enzymes against inhibition by sarin. In these experiments the automatic recording titrator [8] was used.

Electrometric method. Enzyme solutions and procedure. AChE: 50 mg of freeze-dried electric organs were homogenised in 20 ml of Michels' buffer, run through filter-paper Munktell No. 3 and diluted 1:20 with Michels' buffer. BuChE: 50 mg of serum fraction IV-6-3 were dissolved in 200 ml of Michels' buffer. The reaction mixtures were incubated at 25°C. The final concentration of inhibitor was the same in all experiments, 10^{-7} M eserine (experiments with BuChE) or 10^{-2} M butyrylcholine (experiments with AChE). The concentration of the substrate was varied between 10^{-1} M and 10^{-3} M.

In experiments with sarin, thiocholine or choline the inhibitor concentration causing 50 % inhibition of enzyme activity was determined for the different pairs of substrates and inhibitors. The concentration of the substrates was 8.0×10^{-3} M. Incubation was allowed to proceed for 30 min before the addition of the substrate.

Automatic recording titrator. Procedure. Reaction velocities for inhibition with sarin after protection of the enzymes by substrate were determined with 3.9×10^{-7} M (BuChE) or 1.2×10^{-6} M (AChE) sarin and the substrates acetylcholine or acetylthiocholine, 4×10^{-3} (BuChE) or 5×10^{-3} M (AChE). The method and enzyme solutions were the same as described for pS-activity curves, but 2 ml of sarin were added after about 4 min. The concentrations refer to the reaction mixture. Calculations were done analogously to Guggenheim's [17] method in all cases where the spontaneous hydrolysis of substrates and inhibitors could be neglected, but otherwise as described by Tammelin [18].

Ionic Strength. Procedure and reaction mixture. Enzyme solutions were as described for pS-activity curves. By addition of the necessary amounts of potassium chloride the ionic strength was varied between 0.10 – 0.25 M. The following reaction mixture was used: 38 ml of 0.10 M potassium chloride + 1 ml of 0.40 M acetylthiocholine bromide + 1 ml of enzyme solution + varying amounts of solid potassium chloride.

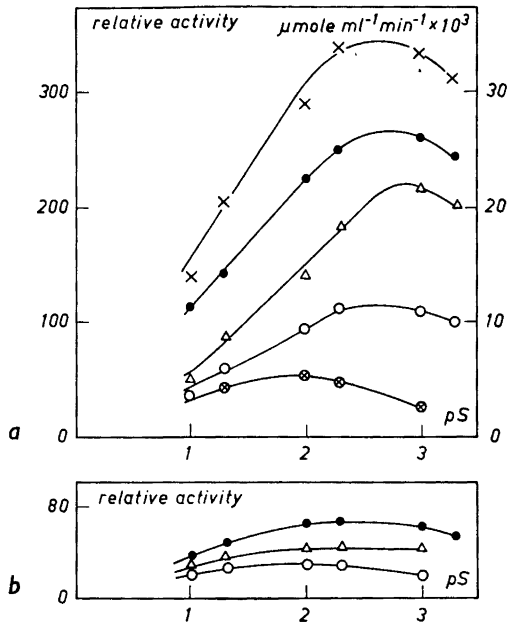


Fig. 1. Acetylcholine esterase: a. pS-Activity curves for the acetyl esters of thiocholine (●), α-methylthiocholine (○), β-methylthiocholine (△), homothiocholine (⊗) and choline (×) at pH 8.00 and 25°C. Only acetylcholine refers to the y-axis on the right hand side. b. pS-Activity curves for the propionyl esters of thiocholine (●), α-methylthiocholine (○) and β-methylthiocholine (△).

RESULTS

pS-Activity curves

Relative activities and values of maximum velocity given here are only comparable if the alcohol group in the esters is the same.

AChE. Fig. 1 shows the pS-activity curves for acetylcholine esterase and the acetyl and propionyl esters of thiocholine, α-methylthiocholine and β-methylthiocholine, and acetylcholine. Michaelis-Menten constants were not calculated, as the enzyme is inhibited by excess of substrate as seen by the decreasing activity at higher substrate concentrations. Only the right limb of each curve would have allowed calculations of the constants, and this was not obtained over a large enough substrate range. The activity values are expressed as relative activities, except for acetylcholine, as the pK_a-values for some of the products of hydrolysis, α-methylthiocholine and β-methylthiocholine, could not be determined, because these compounds were not available. Studies on the spontaneous hydrolysis of the thioesters [7] also show that secondary reactions occur with some of the products of hydrolysis. Therefore it was impossible to calculate the amount of sodium hydroxide equivalent to 1 mole of hydrolysed ester.

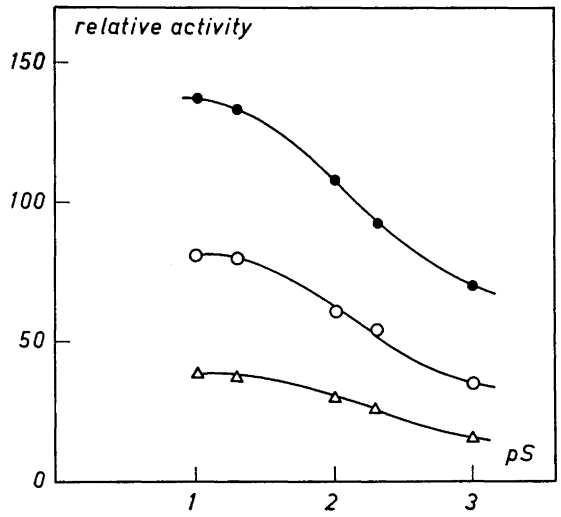


Fig. 2. Butyrylcholine esterase. pS-Activity curves for the acetyl esters of thiocholine (●), α-methylthiocholine (△) and β-methylthiocholine (○) at pH 8.00 and 25°C.

The experiments show that the catalytic effect of AChE resembles the one demonstrated for hydroxide ions [7], but decreases more rapidly with elongation of the acyl group in the ester. All acetyl esters are split easily but propionyl esters are split at a lower rate. The hydrolysis of *n*-butyryl, *iso*-butyryl, valeryl and benzoyl esters is extremely slow and not measurable with the method used. As in hydroxide-ion-catalysed hydrolysis the decreasing rate of hydrolysis with the length of the acyl group in the ester may be explained by combined steric and inductive effects of this group. The more rapid decrease in AChE-catalysed hydrolysis compared with hydroxide-ion-catalysed hydrolysis is probably caused by the importance of steric hindrance in enzyme-catalysed hydrolysis. The effect of the acyl group in the esters on the rate of AChE-catalysed hydrolysis is well known from choline esters [19]. The substrate maximum with thioesters was obtained at about the same concentration of ester as with acetylcholine, as seen in Fig. 1. The effect of substitution in the alcohol group in the esters upon the AChE-catalysed hydrolysis was also the same as observed with hydroxide ions as catalysts. All three types of esters are readily split and show the same type of curve. Homothiocholine shows a very slow hydrolysis and a somewhat different substrate curve.

BuChE: Fig. 2 shows curves obtained with butyrylcholine esterase and thioesters. When the enzyme velocity (v) is plotted against $v/[S]$ according to the Michaelis-Menten equation, expressed as

$$v = -\frac{v}{[S]} \cdot K_M + V_{max} \qquad (3)$$

the lines are not straight, just as has earlier been shown with the substrate butyrylcholine [20]. This deviation from straight lines in the Michaelis-Menten plot was explained as a sign of a possible existance of two enzymes able to split thiocholine esters as well as choline esters. One of the enzymes seems to have a fairly low substrate maximum [20] and to have reached its maximum velocity (V_{max_2}) below the substrate concentration of 10^{-3} M. Michaelis-Menten constants (K_M) and maximum velocities (V_{max}) for the BuChE-catalysed hydrolysis of the thioesters were therefore calculated from the determinations at the four highest substrate concentrations and are seen in Table 1. If there are two enzymes, then the values of the Michaelis-Menten constant are still correct for the enzyme with the highest V_{max} (V_{max_1}), because V_{max_1} is reached at the substrate concentrations used for the calculations. A subtraction of V_{max} with V_{max_2} would only cause a parallel shift of the curve obtained for the enzyme with V_{max_1}. The values of maximum velocity given in Table 1 would then be too high. It is seen from Table 1 that K_M

Table 1. Michaelis-Menten constants and maximum velocities for the reaction of BuChE with some carboxylic acid esters of thiocholine, α-methylthiocholine and β-methylthiocholine at pH 8.00 and 25°C.

R_2	$-CH_2-CH_2-\overset{+}{N}(CH_3)_3$		$-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-\overset{+}{N}(CH_3)_3$		$-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-\overset{+}{N}(CH_3)_3$	
R_1	V_{max} relative activity	K_M mole $\times l^{-1} \times 10^3$	V_{max} relative activity	K_M mole $\times l^{-1} \times 10^3$	V_{max} relative activity	K_M mole $\times l^{-1} \times 10^3$
CH_3-	140	2.6	—	—	82	2.6
CH_3CH_2-	287	2.3	—	—	146	2.3
$CH_3CH_2CH_2-$	308	0.9	31	1.2	144	1.1
$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH_2-$	54	3.1	—	—	—	—
$CH_3CH_2CH_2CH_2-$	271	0.8	29	0.8	144	0.4

becomes smaller with elongation of the acyl group of the thioesters. Branching of the acyl group gives a larger constant. The benzoyl ester was not split to any measurable extent. V_{max} has its highest value for the butyryl esters and falls again before K_M becomes larger. The dependence of V_{max} from the length and shape of the carbon chain of the acyl group in the esters confirms the result obtained by, e.g., Whittaker[19] with choline esters. Elongation of the acyl group has thus the reverse effect in BuChE-catalysed hydrolysis from that in hydroxide-ion and AChE-catalysed hydrolysis, but the steric effect of the acyl group seems to be the same.

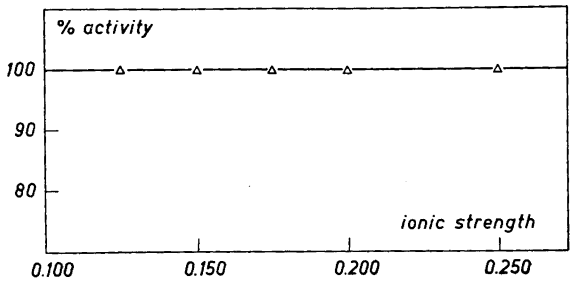


Fig. 3. Acetylcholine esterase. Dependence of enzyme activity at pH 8.00, 25°C and substrate concentration 10^{-3} M (acetylcholine) on ionic strength.

It has been observed [21] that the catalytic effect of BuChE on acetyl-β-methylthiocholine is different from its effect upon the analogous oxygen ester. It is shown here that this difference exists for all the esters examined. The K_M-values for thiocholine esters and β-methylthiocholine esters, seen in Table 1, are the same in contrast to those for the corresponding choline esters, where the β-methyl compound is known to be split at a nonmeasurable rate by BuChE. Catalysis by hydroxide ions was shown [7] to have the same effect. Only a few values for α-methylthiocholine esters are given because in the other cases it was difficult to reproduce the values. Acetylhomothiocholine was split at a nonmeasurable rate. The Michaelis-Menten constant for reaction with acetylcholine was measured for comparison and found to be $3{,}7 \times 10^{-3}$ moles $\times$ l^{-1}. The thiocholine ester is thus more easily split.

Influence of ionic strength. Varying the ionic strength between 0.10 and 0.25 M was shown to have no influence on AChE-catalysed reactions, see Fig. 3. The effect on the BuChE-catalysed hydrolysis has been demonstrated earlier [20].

Rate of hydrolysis

In a few experiments the rate of enzymic hydrolysis of the acetyl esters was measured at constant pH by determination of the amount of unhydro-

le 2. First order rate constants for the enzymic hydrolysis of acetylcholine, acetylthiocholine, acety hylthiocholine and acetyl-β-methylthiocholine at pH 8.00 and 25°C. Enzymes are AChE and BuC

Ester / yme	$-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$	$-S-CH_2-CH_2-\overset{+}{N}(CH_3)_3$	$-S-CH_2-\underset{CH_3}{CH}-\overset{+}{N}(CH_3)_3$	$-S-\underset{CH_3}{CH}-CH_2-\overset{+}{N}(CH_3)_3$
hE min^{-1}	1.7×10^{-2}	1.6×10^{-2}	1.2×10^{-2}	1.4×10^{-2}
ChE min^{-1}	1.1×10^{-2}	1.8×10^{-2}	6.6×10^{-3}	9.9×10^{-3}

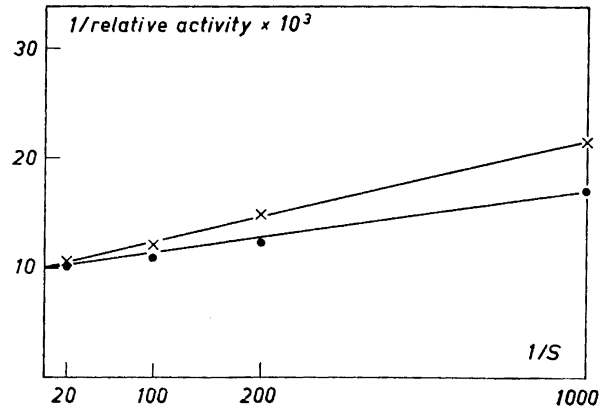


Fig. 4. Butyrylcholine esterase. Enzyme activity in presence (×) or absence (●) of eserine. pH 8 and 25°C. Substrate acetyl-β-methylthiocholine.

lysed ester. The curves obtained show a first order reaction with respect to ester concentration. The pseudo-first order rate constants are seen in Table 2. It is seen that AChE catalyses the hydrolysis of acetylcholine and acetylthiocholine at practically the same rate at the substrate concentration used, while BuChE is a better catalyst for acetylthiocholine.

Effect of inhibitors

Eserine and butyrylcholine. Fig. 4 shows the curve obtained with the system BuChE — acetyl-β-methylthiocholine in the presence of, or without, the inhibitor eserine, which is competitive with choline esters. These curves are representative for a competitive reaction. As expected the thioesters react with at least some of the same active groups in the enzyme as the inhibitor does. An experiment to demonstrate this with acetyl-β-methylcholine as inhibitor did not work. The affinity of this compound for BuChE was too low to show any inhibitory effect even at a high concentration (5×10^{-2} M). For AChE, butyrylcholine was used as inhibitor to demonstrate the competitive effect.

Choline and thiocholine. Table 3 shows the negative logarithm of the inhibitor concentration causing 50 % inhibition after incubation of the enzymes with choline for 30 min at 25°C and pH 8. Substrates were acetylcholine and acetylthiocholine, respectively. The higher concentration of choline necessary to inhibit the enzymes to 50 % when acetylthiocholine is used as substrate shows that thioesters have a greater affinity for BuChE than the oxygen analogues. In the case of AChE the difference in pI_{50} is very small. From the equations according to Briggs-Haldane [22] for the rate of enzyme reaction in the presence of, (eqn 4), or without, (eqn 5), a competitive reversible inhibitor

Table 3. pI_{50}- values for the inhibition of AChE and BuChE by choline or thiocholine in the presence of acetylcholine or acetylthiocholine and equilibrium constants for the inhibition of BuChE. Concentration of substrates 8×10^{-3} M. 25°C and pH 8. Incubation time 30 min.

	Enzyme					
	AChE			BuChE		
Inhibitor	choline		thiocholine	choline		thiocholine
Substrate	acetyl-choline	acetyl-thiocholine	acetyl-choline	acetyl-choline	acetyl-thiocholine	acetyl-choline
pI_{50}	1.46	1.38	1.92	1.36	0.80	2.45
K_I, moles $\times l^{-1} \times 10^2$	–	–	–	1.4	3.9	0.11

$$v = \frac{V}{1 + \frac{K_M}{[S]}\left(1 + \frac{[I]}{K_I}\right)} \quad (4)$$

$$v = \frac{V}{1 + \frac{K_M}{[S]}} \quad (5)$$

the values of the dissociation constants for the enzyme-inhibitor complex K_I could be calculated for the systems BuChE-acetylcholine-choline, BuChE-acetylthiocholine-choline and BuChE-acetylcholine-thiocholine. They are seen in Table 3.

Sarin. It was shown that after inhibition by an irreversible phosphorylating inhibitor, such as sarin, the active groups of the enzyme are blocked in such a way that no difference in the enzymic hydrolysis of oxygen esters and thioesters after inhibition can be demonstrated. This is seen from the values of pI_{50} in Table 4. When, however, the esters were used to protect the enzymes against inhibition, it could be demonstrated that the thioester was the better protector for BuChE. Acetylcholine and acetylthiocholine were found to be about equally good protectors for AChE. The results are seen in Table 5.

Effect of some organic compounds

The effect of imidazole, serine, cysteine and 2-methyloxazoline upon the hydrolysis of acetylcholine, acetyl-β-methylcholine, acetyl-β-methylthiocholine and acetylthiocholine was studied with the automatic recording titrator at pH 8.00 and 25°C. None of the compounds had any

Table 4. Concentration of sarin, expressed as the negative logarithm, causing a 50 % inhibition of the hydrolysis of the acetyl esters of choline, thiocholine, α-methylthiocholine, β-methylthiocholine and homothiocholine by the enzymes AChE and BuChE at pH 8, $t = 25°C$. Incubation time 30 min.

Substrate \ Enzyme	AChE	BuChE IV-6-3
Acetylcholine	8.80 ± 0.01	8.29 ± 0.01
Acetylthiocholine	8.93 ± 0.01	8.36 ± 0.01
Acetyl-α-methylthiocholine	8.92 ± 0.01	8.24 ± 0.005
Acetyl-β-methylthiocholine	8.92 ± 0.05	8.29 ± 0.01
Acetylhomothiocholine	8.85 ± 0.05	—

observable effect upon the hydrolysis of the choline esters, nor could any effect be noted on the hydrolysis of the thioesters by serine and methyloxazoline. Imidazole enhanced the hydrolysis of the thiocompounds about 4 times and cysteine about 50 times. The rate constants obtained with cysteine were found to be 0.144 min^{-1} for acetylthiocholine and 0.139 min^{-1} for acetyl-β-methylthiocholine. The effect of the above mentioned compounds seems to be related to the pK_a of the substances, the compound with the higher pK_a having the better hydrolytic effect. It has been pointed out by Larsson [23], that the basicity of a reagent seems to be a significant factor for its reactivity.

Table 5. Second order velocity constants for reaction of sarin with AChE and BuChE in the presence of acetylcholine iodide or acetylthiocholine iodide at pH 8.00 and 25°C.

	Enzyme			
	AChE		BuChE	
Type and concentration of inhibitor, moles × l^{-1}	sarin 1.2×10^{-8}		sarin 3.9×10^{-7}	
Type and concentration of substrate, moles × l^{-1}	acetylcholine iodide 5.0×10^{-3}	acetylthiocholine iodide 5.0×10^{-3}	acetylcholine iodide 4.0×10^{-3}	acetylthiocholine iodide 4.0×10^{-3}
Inhibition of enzyme, $k_2 \times l \times mole^{-1} \times min^{-1}$	$(2.4 \pm 0.2) \times 10^5$	$(2.9 \pm 0.2) \times 10^5$	$(9.3 \pm 0.1) \times 10^5$	$(5.2 \pm 0.1) \times 10^5$

DISCUSSION

The catalytic activity of esterases [24,25] is generally described as one of bifunctional type, a basic and an acid group taking part in the reaction. There are reasons to believe that the choline esterases have at least two sites in their active center [24,25], called the anionic and the esteratic sites. The anionic site seems to bind and direct substrates or inhibitors with quaternary nitrogen by means of an ionic bond. There is some evidence [24,25] that the anionic site is a carboxyl group. The hydrolytic activity of the enzyme seems to originate from the esteratic site, which seems to consist of a nucleophilic part, maybe a nitrogen atom in an imidazole ring (the basic group) and an electrophilic part, for instance the hydrogen atom in an amino group or a phenolic hydroxyl group (the acid group). The basic group in the enzyme is thought to form a covalent bond with the carbonyl carbon of a substrate, resulting in an addition complex. With the aid of the acid group in the esteratic site, which may form a hydrogen bond to the oxygen in the substrate, the combined acid-base attack upon the substrate results in the formation of an acylated enzyme.

There are several theories about the nature of the nucleophilic group. There are reasons to believe that it is an imidazole ring in histidine [10], but degradation experiments with choline esterases and other esterases show that, after inhibition with organophosphorus compounds, phosphorylated serine is always found, and no imidazole is found near the phosphorylated serine [25,26]. These findings caused a revival of a theory first suggested by M. Bergmann,

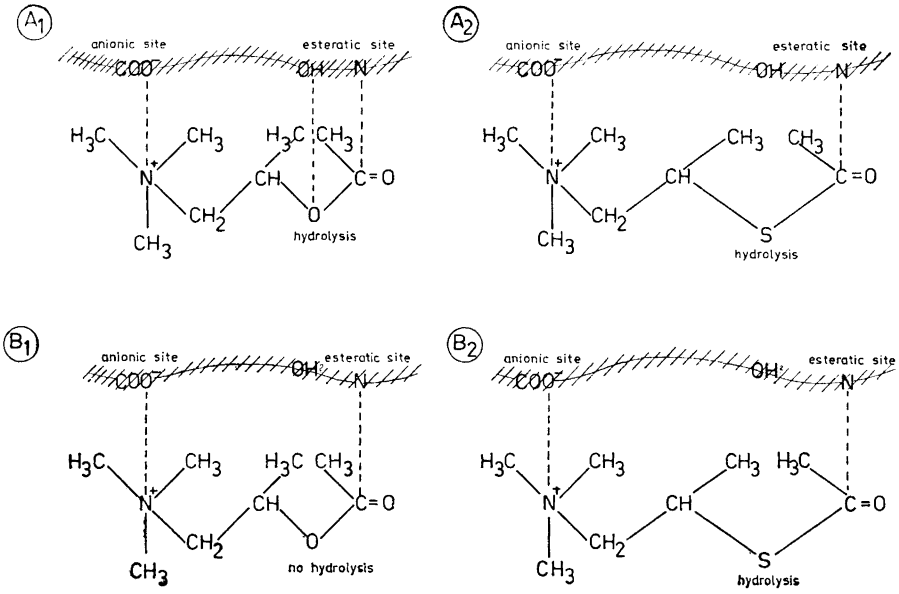


Fig. 5. Models showing attraction of choline esters (1) and thiocholine esters (2) to the surface of acetylcholine esterase (A) and butyrylcholine esterase (B). Dotted lines show points of attraction between enzymes and esters.

about the existence of an oxazoline ring formed from the aminoacid sequence aspargyl-serine [9]. However there is the possibility that the catalytically active imidazole is working near the phosphorylated serine, but situated in another part of the α-helix of the protein and therefore lost in degradation experiments.

The experiments here described confirm for *AChE* the picture of a combined acid-base attack at the enzyme and the picture of the esteratic and anionic sites and their importance and limiting effect in the orientation and binding of the substrate. The difference in radius of oxygen and sulphur and the difference in their influence upon the electrophilic properties of the carbonyl carbon seem not very important as seen from the small differences in the rates of hydrolysis and in the protection effects of the two types of esters.

Bergmann *et al.*[5] have published values for the rate of the AChE-catalysed hydrolysis of acetylcholine and acetylthiocholine at pH 7.5 and 28°C. They found that acetylthiocholine at the substrate concentration used (10^{-3} M) was split 5 times faster than acetylcholine. We were not able to confirm this result but obtained the same rate for the AChE-catalysed hydrolysis of acetylcholine and acetylthiocholine (Table 2). Our measurements were done at pH 8.0 and 25°C. To get the right value for the hydrolysis of acetylthiocholine we had to use the Hestrin method and to keep the pH constant with the automatic recording titrator, otherwise we would have obtained catalysis by buffer ions and difficulties in keeping the pH constant because of the acid properties of thiocholine ($pK_a \sim 7.7$) [7], one of the products of hydrolysis. Thiocholine would also have consumed sodium hydroxide, if the titration method had been used, or evolved CO_2, if the Warburg method had been used. Of course this latter effect would not have increased 5 times the value obtained for the rate of hydrolysis.

It may be worthwhile to discuss the shape of the pS-activity curves for AChE and substrates. The same type of curve was obtained with thioesters as substrate as with acetylcholine. The shape of the acetylcholine curve was found by some authors [5,27] to be a rather narrow bell showing maximum activity at pS around 2.25. These curves were mostly determined at pH 7.4 with the Warburg method. From our laboratory already in 1954 curves were published [28] showing a less narrow bell and an activity which still was near its maximum value at pS 3. Maximum activity was around pS 2.75 and this pS-value increased with the pH. It was observed that this type of curve was obtained as soon as the enzyme activity was measured from near the starting point of the reaction. It was clearly seen during the experiments that the activity in each determination decreased very soon if low substrate concentrations were used. The curves were at that time measured with the electrometric method at pH 8. In the meantime we have used the automatic recording titrator, which allows measurements of enzyme activity from the starting point of the reaction and the use of a second syringe [20,23] containing substrate solution to keep the concentration of the substrate constant. pS-Activity curves were measured at pH 7.5 [18] and now at 8.0 and showed the same shape as published earlier by us.

BuChE is known to be a less "specific" enzyme than AChE. The rate of hydrolysis of oxygen esters and thioesters increases, up to a certain limit,

with the length of the acyl group in the ester in spite of the inductive effects of the acyl group, when BuChE catalyses the reaction. The limit may depend on steric hindrance from the long acyl group in the formation of the enzyme-substrate addition complex. Furthermore the probably somewhat less electrophilic sulphur ester is more easily split than the oxygen analogue and is a better protector aginst inhibitors. Both hydroxide-ion- and BuChE-catalysed hydrolysis of thioesters was shown to be independent of a shielding methyl group near the ester sulphur, the hydrolysis possibly, therefore, taking place without an attack on the sulphur. It may be that BuChE is less ready to attack a substrate with the aid of an acid group than AChE and therefore able to split the thioester but not the oxygen ester. The shape of the pH-activity curve for the enzyme with acetylcholine gives no definite information concerning an acid group, because the decreasing activity at high pH [29] seems to be the result of more general protein denaturation as well as the dissociation of a functional acid group. The catalytic effect of imidazole on thioesters, but not on oxygen esters, demonstrated by Bergmann *et al.*[5] for acetylthiocholine and here for acetyl-β-methylthiocholine, demonstrates the ability of sulphur esters to split on attack of this nucleophilic group alone. That the thioesters are less susceptible to an attack by hydrogen ions than oxygen esters was demonstrated by their slower hydrolysis in acid medium.

The enzymes AChE and BuChE differ in some important way in the reaction with their substrates. Summarizing the results obtained in this and two preceding papers [6,7] the following points are found:

The thiocholine esters and their methylsubstituted analogues hydrolyse slower in acid medium than their oxygen ester analogues.

The hydrolysis of the thioesters is affected by some buffer ions, e.g. phosphate. Secondary reactions with the products of hydrolysis seem to occur in alkaline medium at least with the methylsubstituted thioesters.

The hydrolysis of the thioesters at pH 8.00 is catalysed by imidazole and enhanced by cysteine in contrast to their oxygen analogues.

The activation energies in hydroxide-ion-catalysed hydrolysis are about equal for acetylthiocholine, acetyl-β-methylthiocholine and acetylcholine, but about 33 % higher for acetyl-β-methylcholine.

Acetyl-β-methylthiocholine is split by BuChE in contrast to its oxygen analogues.

The reaction rates between AChE and acetylcholine or acetylthiocholine are about equal, but BuChE reacts faster with acetylthiocholine than with acetylcholine.

Choline and thiocholine are about equally good inhibitors for AChE, but thiocholine is a better inhibitor for BuChE.

Acetylcholine and acetylthiocholine protect AChE about equally well against sarin, but acetylthiocholine is the better protector for BuChE.

On the basis of these results one way to explain the difference between the two enzymes and their reaction with the substrates is shown in Fig. 6. A hydrogen bond is thought to exist between the enzyme and the ether oxygen to explain the shielding effect of the β-methyl group in hydroxide-ion- and enzyme-catalysed hydrolysis [7] of β-methylcholine esters. For perhaps steric reasons, BuChE is not able to create this hydrogen bond between its acid group and

the ether oxygen in β-methylcholine esters. Competition with water may occur and be unfortunate for the enzyme. The thioesters seem to be able to split without this hydrogen bond and are therefore split also by BuChE.

My sincere thanks are due to the Director of the Research Institute of National Defence, Dept. 1, Professor G. Ljunggren, to Professor A. Tiselius, Director of the Institute of Biochemistry, University of Uppsala, for their kind interest in this work, and to Docent B. Malmström, Institute of Biochemistry, University of Uppsala, for helpful discussions.

REFERENCES

1. Renshaw, R. R., Dreisbach, P. F., Ziff, M. and Green, D. *J. Am. Chem. Soc.* **60** (1938) 1765.
2. Koelle, G. B. and Friedenwald, J. S. *Proc. Soc. Exptl. Biol. Med.* **70** (1949) 617.
3 Zajicek, I *Acta Physiol. Scand.* **40** (1957) Suppl 138.
4. Wilson, I. B. *J. Biol. Chem.* **197** (1952) 215.
5. Bergmann, F., Rimon, S. and Segal, R. *Biochem. J.* **68** (1958) 493.
6. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1481.
7. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1492.
8. Larsson, L. and Hansen, B. *Svensk Kem. Tidskr.* **68** (1956) 521.
9. Rydon, H. N. *Nature* **182** (1958) 928.
10. Barnard, E. A. and Stein, W. D. *Advances in Enzymol.* **20** (1958) 51.
11. Hansen, B. *Acta Chem. Scand.* **11** (1957) 537.
12. Hansen, B. *Acta Chem. Scand.* **13** (1959) 159.
13. Wenker, H. *J. Am. Chem. Soc.* **57** (1935) 1079.
14. Tammelin, L.-E. and Fagerlind, L. *Unpublished results.*
15. Heilbronn, E. *Acta Chem. Scand.* **10** (1956) 337.
16. Tammelin, L.-E *Scand. J. Clin. & Lab. Invest.* **5** (1953) 267.
17. Guggenheim, E. A. *Phil. Mag.* **2** (1926) 538.
18. Tammelin, L.-E. *Arkiv Kemi* **12** (1958) 31.
19. Whittaker, V. P. *Physiol. Revs.* **31** (1951) 312.
20. Heilbronn, E. *Acta Chem. Scand.* **12** (1958) 1879.
21. Koelle, G. B. *J. Pharmacol. Exptl. Therap.* **100** (1950) 158.
22. Dixon, M. and Webb, E. *Enzymes.* Longmans, Green & Co., London 1958.
23. Larsson, L. *Svensk Kem. Tidskr.* **70** (1958) 423.
24. Bergmann, F. *Catalysis* **1958** 130.
25. Davies, D. R. and Green, A. L. *Advances in Enzymol.* **20** (1958) 283.
26. Oosterbaan, R. A., Warringa, M. G. P. Y., Yansz, H. S., Berends, F. and Cohen, J. A. *Abstracts*, IV. *Intern. Congr. Biochem.* **1958**, 38.
27. Augustinsson, K. B. and Nachmansohn, D. *J. Biol. Chem.* **179** (1949) 543.
28. Heilbronn, E. *Acta Chem. Scand.* **8** (1954) 1368.
29. Heilbronn, E. *Acta Chem. Scand* **13** (1959) 1255.

Received May 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1561–1564

Acetate Carboxyl Oxygen (^{18}O) as Donor for Phenolic Hydroxy Groups of Orsellinic Acid Produced by Fungi

STEN GATENBECK and KLAUS MOSBACH

Institute of Biochemistry, University of Lund, Lund, Sweden

Oxygen-18 labelled sodium acetate was administered to the culture medium of the orsellinic acid producing fungus *Chaetomium cochliodes* Pall. The ^{18}O content in the carboxyl group and the hydroxy groups of orsellinic acid was determined. A high degree of incorporation was found, where the ^{18}O content of the carboxyl group was half that of each hydroxy group. These results confirm the hypothesis of a β-polyketoacid as an intermediate in the biosynthesis of orsellinic acid.

During the last five years it has been shown by several investigators [1-4] that the biogenesis of some aromatic substances proceeds by head to tail coupling of acetate units. However, from these results nothing can be said with any certainty about the intermediate steps in the course of the biosynthesis of the final aromatic substance from acetate.

Structural analysis of naturally occurring phenolic compounds proposes an intermediate of β-polyketo character [5,6]. The distribution of the oxygen atoms in for example orsellinic acid corresponds exactly to the sequence of the carbonyl groups in an unbranched β-polyketo acid (Fig. 1).

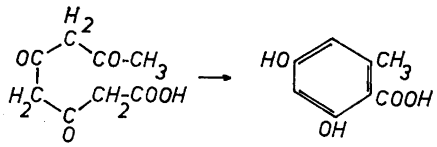


Fig. 1.

If there is a direct condensation of acetate units to the postulated β-polyketo acid, administration of ^{18}O labelled acetate to the orsellinic acid [7] producing fungus *Chaetomium cochliodes* should result in ^{18}O-labelling of the orsellinic acid.

EXPERIMENTAL

Preparation of $CH_3-{}^{14}CO^{18}ONa$. Oxygen-18 labelled water (344 mg) with 90 atom% of ^{18}O was slowly added to a slight excess of the equivalent amount of icecold acetyl chloride. The acetic acid and the hydrochloric acid formed were neutralized with 1 N NaOH by titration. To this solution 1 mC (17 mg) of ^{14}C-carboxyl labelled CH_3COONa was added. A representative sample of the mixture was evaporated to dryness and its isotope content measured.

Culture conditions. Four culture flasks, each containing 500 ml of Czapek-Dox medium, were inocculated with perithecia from *Chaetomium cochliodes* Pall. The culture medium was removed by suction after 20 days incubation at 28°C in the dark. Care was taken not to rupture the mycelium. Fresh Czapek-Dox medium and the labelled acetate were added after rinsing the mycelium twice with distilled water. The incubation was continued for further 8 days.

Isolation of orsellinic acid. The mycelium was filtered off. The culture medium after taking out a small volume for ^{18}O analysis, was acidified with HCl and extracted three times with ether. The residue (300 mg) of the evaporated ether extract was recrystallized from diluted acetic acid yielding orsellinic acid (150 mg), m.p. 176°C.

Determination of ^{14}C. A part of the dry sample of the sodium acetate mixture was combusted to CO_2 according to van Slyke and Folch [8]. The carbon dioxide was trapped in carbonate-free barium hydroxide solution. The barium carbonate formed was isolated and its radioactivity measured with a G.M.-counter. The found radioactivity was 52 600 cpm/mg C. Thus the radioactivity of the carboxyl group will be 2 × 52 600 cpm/mg C.

The same procedure was undertaken with orsellinic acid. The found radioactivity after the total combustion was 2 200 cpm/mg C. As orsellinic acid is built up by four acetate units, which has been shown by one of us (M)[9], the labelled C atoms in the molecule will contain 2 × 2 200 cpm/mg C.

Determination of ^{18}O. The analytical data of all the ^{18}O measurements are listed in Table 1.

The oxygen of the sodium acetate was isolated as carbon dioxide which was obtained after cracking the substance in the presence of heated (1 100°C) carbon and oxidation of the formed carbon monoxide with iodine pentoxide to carbon dioxide according to Unterzaucher [10]. One of the oxygen atoms in the resulting carbon dioxide will thus be derived from the test sample. The liberated iodine was fixed on a slightly heated silber gauze and the carbon dioxide flushed with nitrogen into a clear solution of octadecylamine in petroleum ether (b.p. 40–60°C). The precipitate of octadecyl carbamate was isolated by centrifugation and dried in vacuum over solid paraffine. The dry octadecyl carbamate was used directly for measurement in a masspectrometer whereby the carbon dioxide was released by heating the carbamate to about 100°C. The same procedure of isolating the oxygen as carbon dioxide has been applied throughout this work. The use of octadecylamine as absorbing agent for carbon dioxide has been introduced by Ehrensvärd [18]. This method has turned out to be most convenient for ^{18}O determinations.

The spared test sample of the culture medium was distilled. A few milligrams of the water were taken out, transformed to carbon dioxide in the same way as described above and the ^{18}O content measured.

The average ^{18}O content of orsellinic acid was determined from 5 mg of the substance.

For decarboxylation of the orsellinic acid, 30 mg were heated in a stream of nitrogen in a quartz tube at 180°C for 20 min. The formed carbon dioxide was driven over into a solution of octadecylamine in petroleum ether and its ^{18}O content measured.

The residue consisting of orcinol, shown to be pure by paper chromatography, was analyzed for the ^{18}O amount in the two remaining hydroxy groups.

RESULT AND DISCUSSION

The data in Table 1 show that the ^{18}O from the administered acetate has been incorporated to a high degree in orsellinic acid.

This confirms the hypothesis of direct condensation of activated acetate units to orsellinic acid. Furthermore, a condensation of the acetate *via* acetyl-

Table 1. ^{18}O and ^{14}C analysis.

Samples analyzed	Average ^{14}C- radio-activity	Radio-activity of labelled C-atoms	^{18}O-content	^{18}O-content	$^{18}O/^{14}C \times 10^4$	Dilution ^{18}O *versus* ^{14}C
	cpm/mg C	cpm/mg C	atom %	atom % excess		
Standard			0.20	0.00		
Sodium acetate	52 600	105 200	33.20	33.00	3.13	1
H_2O (culture medium)			0.20 (3)	0.00 (3)		
Orsellinic acid	2 200	4 400	0.72	0.52		
Carboxyl group		4 400	0.59	0.39	0.89	3.6
Orcinol		4 400	0.92	0.72	1.64	1.9

coenzyme A should end up in a hydrolysis of the terminal thiolester. The atom % excess of ^{18}O in the carboxyl group should then be half that of the hydroxy groups in the orsellinic acid. This is also confirmed by our experiment (COOH: 0.39 atom % excess; OH: 0.72 atom % excess ^{18}O).

By comparing the radioactivity in the carboxyl group of the original sodium acetate with that in orcinol a dilution of 24 times is observed whereas the ^{18}O excess of the carboxyl group is diluted 46 times. The ratio $^{14}C/^{18}O$ dilution is almost 1:2 giving place for speculations since the activation of the acetate through the adenosine triphosphate-coenzyme A system would not give rise to dilution of the acetyl oxygen unless there is hydrolysis of the intermediates. Of course there is the possibility of exchange of the oxygen with water before activation of the acetate. Nevertheless, the ratio 1:2 invites one to think of an organized exchange of the acetyl oxygen. It could be explained by a hydratization of one of the intermediates or there could be an hitherto unknown intermediate step in the course of acetate activation.

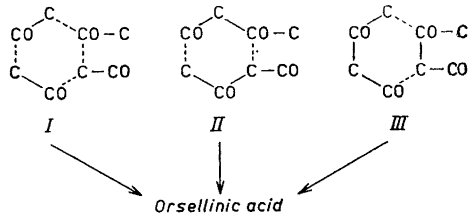


Fig. 2. Alternative pathways of the condensation of acetate to orsellinic acid. I. Successive condensation of acetyl-coenzyme A. II and III. Two different ways of formation of orsellinic acid from acetoacetyl-coenzyme A.

The condensation of the acetate units to orsellinic acid may proceed in one of two possible ways, either by successive condensation of four C-2 units presumably acetyl-coenzyme A or by coupling of two C-4 compounds like acetoacetyl-coenzyme A (Fig. 2). The alternative that a β-polyketo unit underlying the synthesis of orsellinic acid is formed by an oxidative mechanism acting upon a long chain fatty acid could be excluded, since in that case the oxygen atoms entering in successive β-positions should be nonlabelled in view of the very low ^{18}O content of the water in the culture medium (0.003 atom % excess).

It is reasonable to draw a parallel between orsellinic acid and fatty acid synthesis. In the first case the acetate condensation stops at the keto level whereas in the fatty acid synthesis the keto stage is further reduced *via* a DPN-system.

The high degree of incorporation of the ^{18}O from the acetate into the phenolic hydroxy groups of orsellinic acid seems to be promising for a further use of this procedure as a common method of distinguishing between oxidatively and non-oxidatively formed hydroxy groups in compounds derived from acetic acid.

The authors are much indebted to Prof. G. Ehrensvärd for valuable discussions and to Dr. C. Karrman for putting the resources of his analytical department at our disposal.

REFERENCES

1. Birch, A. J. *et al. Austr. J. Chem.* **8** (1955) 539; *J. Chem. Soc.* **1958** 360.
2. Thomas, R. *Proc. Chem. Soc.* **1959** 88.
3. Gatenbeck, S. *Acta Chem. Scand.* **12** (1958) 1211, 1985.
4. Schwenk, E., Alexander, G. J., Gold, A. M. and Stevens, D. F. *J. Biol. Chem.* **233** (1958) 1211.
5. Robinson, R. *Structural Relations of Natural Products*, Clarendon Press 1955.
6. Birch, A. J. and Donovan, F. W. *Austr. J. Chem.* **6** (1953) 360.
7. Mosbach, K. *Z. Naturforsch.* **14 b** (1959) 69.
8. *Cf.* Calvin, Heidelberg, Reid, Tolbert and Yankwich, *Isotopic Carbon* Wiley, New York 1949, p. 92.
9. Mosbach, K. *To be published.*
10. Unterzaucher, J. *Ber.* **73** (1940) 391; *Mikrochemie ver. Mikrochim. Acta* **36/37** (1951) 706.
11. Ehrensvärd, G. *In press.*

Received June 9, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1565–1574

Amino Acid Studies. Part II.* Structure and Synthesis of Albizziine (L-2-Amino-3-ureidopropionic Acid), an Amino Acid from Higher Plants**

ANDERS KJÆR and P. OLESEN LARSEN

Organic Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, Denmark

Albizziine is an amino acid, recently isolated from various species of the botanical family *Mimosaceae*. The suggested structure, L-2-amino-3-ureidopropionic acid (I), has now been unequivocally established by comparison of N-benzoylalbizziine methyl ester with a synthetic specimen of methyl L-2-benzamido-3-ureidopropionate (III).

A simple and satisfactory synthesis of L-albizziine from L-2,3-diaminopropionic acid has been developed, based on temporary masking with cupric ions of the 2-amino-grouping during carbamylation at the desired 3N-position. The same procedure has been applied in the preparation of DL-albizziine. L-2-Ureido-3-aminopropionic acid (II), the positional isomeride of L-albizziine, has been synthesized for comparison purposes.

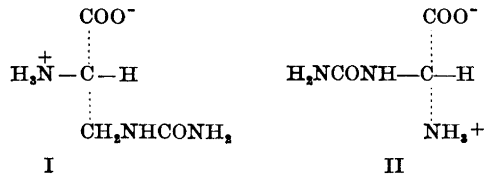
Various observations relating to the acid hydrolysis and biogenesis of albizziine are briefly discussed.

Albizziine constitutes a recent addition to the rapidly increasing class of free amino acids occurring in higher plants (for a summary *cf. e.g.* Ref.[1]). It was discovered in and isolated from seeds of the botanical species *Albizzia julibrissin* Durazz. by Gmelin *et al.*[2]. The same authors demonstrated commercially available seed material of the species *Albizzia lophantha* Benth. to be an alternative and satisfactory source of albizziine and provided paper-chromatographic evidence for the presence of the new amino acid in several other species of the same or related genera, all belonging to the family *Mimosaceae* [2]. Still more recently, Gmelin *et al.*[3] listed additional sources of albizziine and mentioned *Enterolobium cyclocarpum* as a particularly rich source. The elementary composition [2,3], positive reaction with ninhydrin and Ehrlich's reagent [2], and the products formed upon acid hydrolysis [3], suggested that

* The communication which appeared in *Experientia* 15 (1959) 253 should be regarded as Part I of this series.

** Presented in abstract before the 10th Meeting of Scandinavian Chemists in Stockholm, Sweden, August 1959.

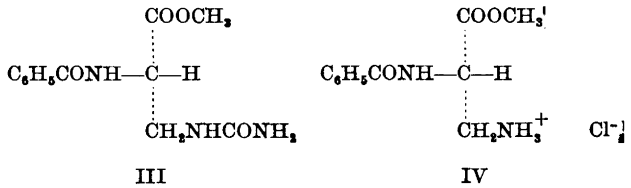
albizziine was L-2-amino-3-ureidopropionic acid (I), although the isomeric structure (II) was not incompatible with the available evidence [3].



I II

In a preliminary communication from this laboratory [4], (I) has been unequivocally established as the correct structure for albizziine. It is the purpose of the present paper to expand on the structure determination, as well as to describe a single-step synthesis of albizziine from L-2,3-diaminopropionic acid. Furthermore, various observations in connexion with the acid hydrolysis of the latter will be discussed.

Upon benzoylation in aqueous carbonate solution, albizziine yielded an oily derivative which, however, could be transformed into a crystalline methyl ester on treatment with diazomethane. The identity of the ester as methyl L-2-benzamido-3-ureidopropionate (III) was established upon critical comparison with an unambiguously synthesized specimen.



III IV

The latter was produced by interaction at pH 7 of potassium cyanate and *levo*rotatory methyl 2-benzamido-3-aminopropionate hydrochloride (IV). Synthesis of this was achieved essentially as described by Schneider [5] through a series of reactions departing from configurationally known L-asparagine and involving the preparation of L-2,3-diaminopropionic acid. Additional and independent corroboration of the L-configuration of (IV) was available through its experimentally established relationship with L-serine [5]. The above transformations unequivocally established the projection formula (I) as the correct expression for albizziine.

The formally simple structure of albizziine rendered the development of a synthesis of this lower homologue of L-citrulline a logic extension of the structure determination. L-2,3-Diaminopropionic acid offered itself as a suitable starting material, and attempts were consequently directed towards selective carbamylation of its terminal amino-grouping. From analogous cases it appeared natural to proceed *via* selective protection of the 2N-grouping, carbamylation at the 3N-position and, eventually, removal of the protecting group under mild conditions. Apart from its circumstantial character this approach, however, suffered from the disadvantage that no 2N-monoacylated derivatives of

diaminopropionic acid had ever been prepared by direct acylation *. From theoretical considerations this should, in principle, be possible by direct acylation under controlled conditions [6]; steric factors may, however, be responsible for the failure in effecting the process as desired.

An alternative approach, based on Hofmann-degradation of a suitable N-acyl-L-asparagine and introduction of the carbamido-grouping into the 3-amino-substituent of the 2N-acylated intermediate, ultimately followed by removal of the acyl-group, constitutes a variant of a method formerly employed in the synthesis of L-2,3-diaminopropionic acid [8,5]. Though this approach seemed promising in principle **, a route involving fewer steps still appeared desirable. Hence, attention was directed towards the possibility of introducing a carbamido-grouping into L-2,3-diaminopropionic acid selectively and in a single reaction step.

First, the diamino-acid was subjected to reaction with potassium cyanate in a series of experiments in which the relative amounts of reactants, as well as the pH-value of the solutions, were varied. As estimated from paper chromatography ***, mixtures of the isomerides, (I) and (II), were consistently obtained, accompanied by varying amounts of the 2,3-diureido-derivative; the latter appeared on the chromatograms as Ehrlich-positive, ninhydrin-negative spots possessing higher R_F-values than the monosubstituted products. Clearly, masking of the 2-amino-grouping was required to secure a homogeneous reaction product.

In the synthesis of ω-acylated derivatives of 2,ω-diaminocarboxylic acids, copper complexes have often proved very useful. Thus, Kurtz [10] prepared DL-citrulline from urea and the copper complex of ornithine, a procedure subsequently extended by the same author [11] to include syntheses of L-citrulline, DL- and L-2-amino-6-ureidohexanoic acid, as well as DL- and L-2-amino-4-ureidobutyric acid. More recently, Smith [12] modified the synthesis of L-2-amino-4-ureidobutyric acid, L-citrulline and L-2-amino-6-hexanoic acid from urea or cyanate and the appropriate 2,ω-diamino acids. In the 2,4-diaminobutyric acids, the two amino groupings are sufficiently close to form an alternative site of complex formation, as evident from the strong complexing action between cupric ions and 1,3-diaminopropane. Still, however, the 1,2-complex must be present, in view of the successful preparation of the 4-ureido-

* Several years ago, one of us (A.K.) demonstrated monocarbobenzoxylation of DL-diaminopropionic acid to take place exclusively at the 3N-position [6]. However, recent reinvestigation of the mono-derivative has revealed its content of a slight amount of the 2N-isomeride. The alternative course, leading predominantly to the latter and suggested as prevalent in a less alkaline environment, has not since been experimentally verified [6]. Lately, Poduška *et al.* [7] demonstrated that DL-diaminopropionic acid upon treatment with tosylglycyl chloride afforded the 3N-tosyl-glycyl-derivative.

** After the conclusion of the present work, we were privately informed that Dr. J. Rudinger, Institute of Chemistry, Czechoslovakian Academy of Science, Prague, has, in actuality, synthesized L-2-amino-3-ureidopropionic acid (I) along similar lines, utilizing the tosyl-derivative of L-asparagine. Specimens of his synthetic material, natural albizziine, and a sample synthesized in this laboratory as described below, were all found to possess coinciding infra-red spectra.

*** For the present purpose a general paperchromatographic method was devised [9], according to which 2-monoaminocarboxylic acids can be distinguished from other ninhydrin-positive compounds. Thus, the isomeric aminoureidopropionic acids, (I) and (II), can be individually recognized by this procedure.

derivatives. The copper complexes of 2,3-diaminopropionic acid have been studied by Ley and Hegge [13]. Visually these authors noticed a red-violet colour of the copper complex of this acid, deviating from that given by ordinary 2-amino-acids and hence attributed to participation of the 3-amino-grouping. On acidification, the colour changed to a bluish-purple, characteristic for the ordinary 2-amino-acids. From the comprehensive studies of Albert [14,15] on copper complexes of amino acids it can be inferred that the deviating properties exhibited by 2,3-diaminopropionic and 2,4-diaminobutyric acid must be ascribed to their contents of terminal amino-groups, in sufficient proximity to the other active site to permit additional complex formation of a different type. Calculations, based on the quantitative treatment of Albert (*l.c.*), suggested that in the pH-range 3—5, ionic species containing a positively charged amino group in 3-position should be predominant and, hence, selective carbamylation feasible. Explorative experiments indicated that this was, in fact, the case. The individual runs were controlled by paper chromatography in acid solvent systems, in which the copper complexes are sufficiently dissociated to permit the free amino acids to migrate (*cf.* Ref.[16]). Development of the chromatograms were performed with Ehrlich's reagent as well as with ninhydrin according to the modification devised in this laboratory [9]. Best results were obtained when a solution of L-2,3-diaminopropionic acid hydrochloride and half a molecular proportion of cupric sulphate was maintained at pH 5 and 58° during the slow addition of a large excess of potassium cyanate. The soluble copper complex of the reaction product was then decomposed by hydrogen sulphide, the resulting amino acids absorbed on an acid exchange resin, and finally isolated by elution with ammonia. On addition of ethanol, albizziine, uncontaminated with the isomeride (II), separated in about 50 % yield. The usual procedure for the preparation of copper complexes of 2-amino acids consists in boiling the latter with excess cupric oxide or carbonate. In the present case, however, it was observed that considerable quantities of the desired complex was lost by adherence to the excess of solid reagents, and it was found advantageous and convenient to form the complex simply by adding a soluble copper salt to the amino acid solution. Most likely, this modification could be employed also in other, analogous cases. On critical comparison, the pure, synthetic compound proved indistinguishable from natural albizziine. The infra-red spectrum is presented in Fig. 1.

The new plant amino acid possessed a surprisingly high *levo*rotation in water ($[\alpha]_D^{22} - 67°$), yet displaced towards more *dextro*rotatory values on acidification in accordance with the Lutz-Jirgenson rule for L-2-amino acids [17]. Table 1 presents a collection of rotatory values for the next higher homologues, measured under identical conditions. The numerically high rotation of albizziine probably reflects a strong vicinal influence from the carbamido-grouping.* The isomeride (II) is seen to deviate principally from the 2-amino-acid derivatives in its rotatory pattern.

* Through the courtesy of Dr. W. Klyne, Postgraduate Medical School, London, the rotatory dispersion curves of L-albizziine and the higher homologues of the same configurational series have been determined over the wave-length range 290—600 mμ. Whereas in aqueous solution the latter all possess similar, plain and positive curves, L-albizziine exhi-

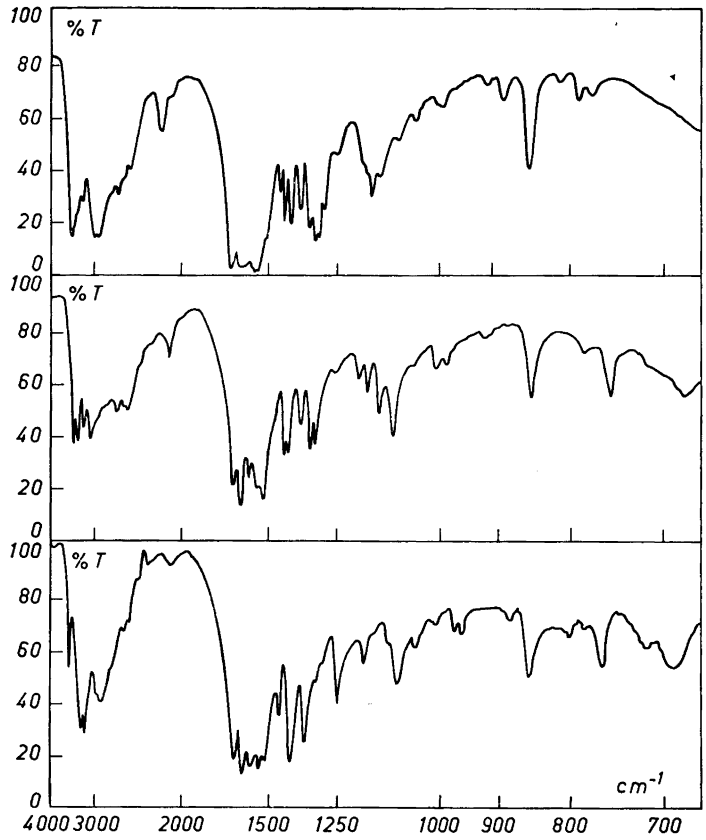


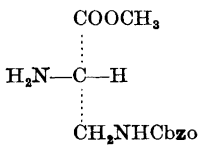
Fig. 1. Infra-red spectra, determined in potassium bromide discs. Upper curve: natural L-albizziine (I); middle curve: racemic albizziine; lower curve: the L-albizziine-isomeride (II) (L-2-ureido-3-aminopropionic acid).

The synthesis of DL-2-amino-3-ureidopropionic acid (DL-albizziine) from DL-2,3-diaminopropionic acid hydrobromide was achieved as described above for the L-series, with the sole exception that the intermediate copper complex was slightly soluble and therefore separated during preparation. Again, racemic albizziine was considerably less soluble in water than the natural isomeride and possessed a distinctly different infra-red spectrum in the solid state (Fig. 1).

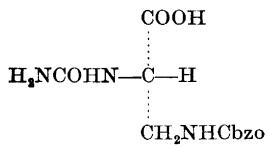
During the present studies it became of interest to synthesize L-2-ureido-3-aminopropionic acid (II), the positional isomeride of albizziine. Carbobenz-

bits an almost mirror image-formed, negative curve. This pattern is a telling illustration of the frequently observed, dramatic influence of chromophores in positions adjacent to an optically active centre and of the caution required in interpreting dispersion curves devoid of Cotton effect. In acid and alkali the deviations are less conspicuous.

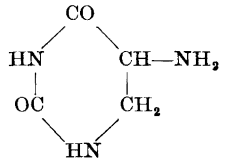
oxylation of the hydrochloride of L-2,3-diaminopropionic acid at pH 7 proceeded to give a homogeneous mono-derivative, transformed by diazomethane into an ester, which was proved to be methyl L-2-amino-3-benzyloxycarbamido-propionate (V, Cbzo = $C_6H_5CH_2OCO$) upon comparison with an authentic specimen, synthesized as described by Schneider [5]. Hence, the monocarbobenzoxylated L-diaminopropionic acid carried the acyl-grouping in 3N-position.



V



VI



VII

Reaction of the latter acid with potassium cyanate afforded crystalline L-2-ureido-3-benzyloxycarbamidopropionic acid (VI, Cbzo = $C_6H_5CH_2OCO$) which, on hydrogenolysis, yielded (II). On paper chromatograms, run in several solvent systems, this albizziine-isomeride migrated at virtually the same rate as albizziine but could be distinguished from the latter by its much weaker and slower ninhydrin-reaction or, still better, by its deviating behaviour when treated with copper salt and ninhydrin according to the method developed in this laboratory [9]. Both albizziine and (II) yielded, as expected, the same positive Ehrlich-reaction, but clearly deviated in their rotations (Table 1) and infra-red spectra (Fig. 1).

Table 1. Molecular rotation of L-albizziine and various homologues, determined in this laboratory.

Compound	Water			2 N HCl			0.11 N NaOH		
	$[M]_D$	g/100 ml	t°C	$[M]_D$	g/100 ml	t°C	$[M]_D$	g/100 ml	t°C
L-2-Amino-6-ureidohexanoic acid	+ 7.6°	2.1	24	+37.4°	3.8	24	+18.9°	1.0	26
L-Citrulline	+ 6.1°[a]	5		+37.1°	3.8	24	+18.9°	1.1	25
L-2-Amino-4-ureidobutyric acid	− 1.0°	2.1	24	+42.3°	3.2	24	+14.5°	1.0	26
L-Albizziine	−98.5°	2.1	23	−32.2°	2.1	22	− 8.2°	1.0	26
L-2-Ureido-3-aminopropionic acid	+ 4.7°	1.0	25	−63.2°[b]	1.0	25	+36.2°	1.0	27

[a] Gornall, A. G. and Hunter, A. *Biochem. J.* **33** (1939) 170. [b] In 0.1 N HCl.

In course of their degradation studies on albizziine, Gmelin *et al.*[3] isolated 2,3-diaminopropionic acid hydrohalides of lower specific rotation than previously recorded, subsequent to hydrolysis with strong mineral acid. The authors suggested acid-induced racemization of L-diaminopropionic acid as a likely explanation, an assumption which has now been confirmed in this laboratory. Upon heating at 125° the specific rotation of a solution of L-2,3-diaminopropionic acid hydrochloride in 48 % hydrobromic acid decreased from an initial value of 19.3° to 1.2° in the course of 6 h. Paper chromatography confirmed that no extensive decomposition was responsible for the loss in rotatory power. On the other hand, paperchromatographic analysis of an acid hydrolysis mixture of albizziine as a function of time suggested the concomitant or intermediate formation of a ninhydrin-positive product with higher R_F-values, possibly one of the unknown 5-aminodihydrouracils (VII). The obviously limited formation of this cyclized product is in marked contrast to the customary, and usually very facile cyclodehydration of 3-ureido-substituted carboxylic acids [18,19]. Possible explanations for this fact, supplemented by a detailed investigation of the albizziine hydrolysis, will be presented in a forthcoming communication.

A possible relationship between albizziine and a hydrouracil derivative merits further consideration in connexion with reflections on the biogenesis of the former. Formally, it recalls the well-established biosynthesis in animals and microorganisms of the uracil ring from ureidosuccinic acid *via* dihydroorotic and orotic acid. In lack of evidence to the contrary, however, albizziine may equally well, if at all, be on the pathway of reductive pyrimidine degradation [20]. A different biochemical perspective in connexion with the occurrence of albizziine is suggested by its formal similarity with citrulline, a key compound in the formation of arginine and urea, or decomposition products of the latter, in mammalian tissues and certain microorganisms. In this connexion, the recent establishment by Gmelin *et al.*[3] of the occurrence of free L-2,3-diaminopropionic acid, a formal analogue to L-ornithine, in seeds of *Mimosa Palmeri* and *M. hemiendyta* of the family *Mimosaceae*, appears particularly interesting. Botanically, these species are close allies of the known albizziine-producing plants and it may well be that the new amino acids within this family are results of a particular and interesting enzymic mechanism.

EXPERIMENTAL

Melting points are uncorrected and determined in an electrically heated block. Rotations are measured in a 1 dm tube at the D-line. Infra-red spectra of all the compounds described have been determined in potassium bromide pellets on a Perkin-Elmer "Infracord"-instrument.

N-Benzoylalbizziine methyl ester. To a cooled and stirred aqueous solution of albizziine* (149 mg) and anhydrous soda (285 mg), a total of 0.175 ml of benzoyl chloride was added in small portions. Extraction with ether, acidification, extraction with pentane, and treatment of the aqueous phase with Norite gave a solution from which the reaction product was removed by extraction with ethyl acetate. The colourless, glassy product was

* The specimen employed was isolated from seeds of *Albizzia lophantha* Benth. according to the procedure of Gmelin *et al.* [2] and had an $[\alpha]_D^{23}$ of —67.0° (*c* 2.1, H_2O).

dissolved in anhydrous methanol and treated with excess diazomethane. Evaporation of the solvent and trituration of the residue with pentane yielded the crystalline ester (78 mg) which was recrystallized twice from water before analysis (15 mg), m. p. 171—174° (decomp.). (Found: C 54.40; H 5.68; N 15.89. Calc. for $C_{12}H_{15}N_3O_4$: C 54.33; H 5.70; N 15.84.) A slightly impure specimen, isolated from the mother liqour, had the rotation value $[\alpha]_D^{21}$ −30° (*c* 1.0, CH_3OH). The infra-red spectrum of the pure product was indistinguishable from that of the synthetic specimen described in the sequel.

Synthetic methyl L-*2-benzamido-3-ureidopropionate* (III). A sample of methyl L-2-amino-3-benzyloxycarbamidopropionate hydrochloride * (488 mg) was benzoylated according to Schneider [5] to give *methyl* L-*2-benzamido-3-benzyloxycarbamidopropionate* (480 mg). An analytical specimen separated from chloroform as colourless needles, m. p. 98.5—99.5°, $[\alpha]_D^{28}$ −17.3° (*c* 1.2, CH_3OH). (Found: C 63.85; H 5.67; N 7.74. Calc. for $C_{19}H_{20}N_3O_5$: C 64.04; H 5.66, N 7.86.) Literature value [5]: m. p. 102°, (no rotation reported). Again, decarbobenzoxylation was performed with hydrogen and palladium black as described [5]. It was found advantageous, however, to run the reaction at 40°. *Methyl* L-*2-benzamido-3-aminopropionate hydrochloride* (IV) was obtained in 80 % yield as colourless crystals, m. p. 171—174° (decomp.), $[\alpha]_D^{30}$ −48.3° (*c* 1.0, CH_3OH). (Found: C 51.10; H 5.87; N 10.67. Calc. for $C_{11}H_{15}N_3O_3$, HCl: C 51.06; H 5.84; N 10.83.) Literature value [5]: m. p. 179° (decomp.), (no rotation reported).

A solution of (IV) (130 mg) and potassium cyanate (113 mg) in water (3 ml) deposited, in the course of 3 h, a crystalline product the amount of which was increased (to 92 mg) upon concentration. A pure specimen of *methyl* L-*2-benzamido-3-ureidopropionate* (III) (69 mg) separated from water as clusters of colourless needles, m. p. 172—173.5° (decomp.), alone or in admixture with the methyl ester of N-benzoylalbizziine described above. $[\alpha]_D^{28}$ −32.5° (*c* 1.2, CH_3OH). The infra-red spectra of the two specimens were identical and possessed all the bands expected.

Synthesis of L-*albizziine* (I). A solution of L-2,3-diaminopropionic acid hydrochloride ** (300 mg) and cupric sulphate (2.2 ml of a 0.5 M solution) in water (28 ml) was brought to pH 5 by addition of 2 N NaOH. The stirred solution was kept at 58° and placed in a pH-stat, preset to maintain a constant pH of 5 by automatic addition of 4 N HCl during the introduction of a solution of potassium cyanate (430 mg in 4.8 ml) in the course of 100 min. At the end of another 100 min, the colour had changed from an initial reddish-violet to a bluish-purple and the HCl-consumption was very slow. The solution was then treated with hydrogen sulphide and filtered. The combined filtrate and washings (200 ml) were percolated through a column containing a cation exchange resin (Zeokarb 215, packing volume: 100 ml) in the acid form. The column was washed with water, and the amino acids eluted by means of 2 % ammonia. Evaporation of the eluate afforded crystalline needles (255 mg), consisting of reasonably pure albizziine. Two recrystallizations from aqueous ethanol afforded pure L-albizziine (150 mg), m. p. 206—211° (decomp.), alone or when mixed with a natural specimen, $[\alpha]_D^{26}$ −66.0° (*c* 2.0, H_2O). The infra-red spectrum was identical with that of a natural sample of albizziine [3] (Fig. 1).

Synthesis of DL-*albizziine.* When a solution of DL-2,3-diaminopropionic acid hydrobromide (555 mg) was processed as described for the corresponding L-isomeride, the light-blue copper complex (273 mg) separated and was filtered off. After decomposition with hydrogen sulphide and evaporation of the resulting solution, a crystalline residue (239 mg) was obtained. It was recrystallized twice from aqueous ethanol to give prisms of pure DL-albizziine (138 mg), m. p. 210—216° (decomp.). (Found: C 32.30; H 6.29; N 28.28. Calc. for $C_4H_9N_3O_3$: C 32.65; H 6.17; N 28.56.) The infrared spectrum (Fig. 1) deviates distinctly from that of the optical isomeride.

L-*2-Amino-3-benzyloxycarbamidopropionic acid.* To a cooled and stirred solution of L-2,3 diaminopropionic acid hydrochloride (1.41 g) in a phosphate buffer of pH 7 (15 ml, 1/15 M) and water (10 ml), a toluene solution (1.9 ml) containing 11 mequiv. of carbo-

* The sample employed was prepared as formerly described [5] and had the rotation $[\alpha]_D^{21}$ —4.2° (*c* 2.0, H_2O); (no rotation was previously recorded).

** The diamino-acid employed was isolated from seeds of *Mimosa Palmeri* as described by Gmelin *et al.*[3] and had the rotation value $[\alpha]_D^{24}$ + 24.8° (*c* 4.7, 1 N HCl).

benzoxy chloride was gradually added in the course of 1 h. Intermittent addition of 1 N NaOH served to maintain the pH-value at 6.5—7. After standing for 2.5 h at room temperature the solution was cooled. The crystalline precipitate was filtered off and washed with ice-cold water and ether. The carbobenzoxy-derivative (1.85 g) separated from water in colourless needles, m. p. 227—229° (decomp.), $[\alpha]_D^{21}$ −18.7° (*c* 1.0, 1 N HCl); $[\alpha]_D^{18}$ −4° (*c* 1.1, 0.1 N NaOH). (Found: C 55.45; H 5.80; N 11.80. Calc. for $C_{11}H_{12}N_2O_4$: C 55.45; H 5.92; N 11.76.) The specimen proved to be uncontaminated with the 2N-acylated isomer when tested by our spot test technique [9].

That the carbobenzoxy-group had entered the L-2,3-diaminopropionic acid in 3N-position appeared from the transformation of the above carbobenzoxyacid into the corresponding methyl ester hydrochloride. The acid (96 mg) was suspended in anhydrous methanol (2 ml) and treated with an excess of ethereal diazomethane. After filtration and evaporation an oil was obtained which upon solution in methanol and addition of methanolic HCl yielded an ester hydrochloride (36 mg) which was recrystallized from methanol and ether; m. p. 161—162° (decomp.), alone or in admixture with a specimen of *methyl* L-*2-amino-3-benzyloxycarbamidopropionate hydrochloride* (V) prepared as described by Schneider [5], who reported the m. p. 164°. Furthermore, the infra-red spectra of the two preparations were completely identical.

L-*2-Ureido-3-benzyloxycarbamidopropionic acid* (VI). A solution of the above described L-2-amino-3-benzyloxycarbamidopropionic acid (875 mg) and potassium cyanate (1 440 mg) in water (100 ml) was boiled under reflux for 20 min. After concentration to 30 ml and ether extraction, acidification with conc. HCl to pH 3 and cooling caused the ureido-acid to separate in crystalline form (721 mg), m. p. 181—182° (decomp.), $[\alpha]_D^{17}$ −1.5° (*c* 1.0, 0.1 N NaOH). A specimen was recrystallized twice from water before analysis, m. p. 188—190° (decomp.). (Found: C 51.10; H 5.58; N 15.01. Calc. for $C_{12}H_{15}N_3O_5$: C 51.24; H 5.38; N 14.94.)

L-*2-Ureido-3-aminopropionic acid* (II). A suspension of palladium black (90 mg) in a solution of the carbobenzoxy-acid (VI) (500 mg) in 50 % methanol (50 ml) was kept at 50° while a stream of hydrogen was passed through, until the production of CO_2 ceased. After filtration and concentration of the filtrate, the latter was treated with Norite, and ethanol was cautiously added, resulting in separation of needles of the colourless ureido-acid (II) (225 mg). A sample for analysis was recrystallized twice from water by addition of ethanol, m. p. 204—210° (decomp.); $[\alpha]_D^{25}$ +3.2° (*c* 1.0, H_2O), $[\alpha]_D^{25}$ −43.0° (*c* 1.0, 0.1 N HCl), $[\alpha]_D^{27}$ +24.6° (*c* 1.0, 0.1 N NaOH). (Found: C 32.75; H 6.24; N 28.70. Calc. for $C_4H_9N_3O_3$: C 32.65; H 6.17; N 28.56.) The infra-red spectrum of this albizziine-isomeride is reproduced in Fig. 1.

Racemization of L-*2,3-diaminopropionic acid.* A solution in constantly boiling hydrobromic acid of L-2,3-diaminopropionic acid hydrochloride, with an initial rotation of $[\alpha]_D^{20}$ +19.3° (*c* 1.6), was heated at 125° in a closed vessel. After 4 h, the rotation had decreased to $[\alpha]_D^{23}$ +4.6° (*c* 1.6) and at the end of 6 h a rotation of $[\alpha]_D^{21}$ +2° (*c* 1.6) indicated almost complete racemization. Upon paper chromatography only one spot was observed, *viz.* that of 2,3-diaminopropionic acid.

The authors are grateful to their former research associate, Dr. R. Gmelin, for the gift of an albizziine specimen as well as various seed samples. Thanks are due also to Professor S. Bergström who provided us with useful reference samples of various citrulline-homologues and to Dr. J. Rudinger for advance information on his synthesis of albizziine. Microanalyses were performed by Mr. P. Hansen at the Chemical Laboratory of the University of Copenhagen.

Grants from *The Research Council of the Technical Sciences, (Det Teknisk-Videnskabelige Forskningsråd)* and *The Carlsberg Foundation Stipendium for the Royal Veterinary and Agricultural College (Carlsbergfondets Legat for Den Kgl. Veterinær- og Landbohøjskole)* for the acquisition of the employed infra-red and potentiometric equipment, respectively, are gratefully acknowledged.

REFERENCES

1. Fowden, L. *Biol. Revs. Cambridge Phil. Soc.* **33** (1958) 393.
2. Gmelin, R., Strauss, G. and Hasenmaier, G. *Z. Naturforsch.* **13b** (1958) 252.
3. Gmelin, R., Strauss, G. and Hasenmaier, G. *Hoppe-Seyler's Z. physiol. Chem.* **314** (1959) 28.
4. Kjær, A., Olesen Larsen, P. and Gmelin, R. *Experientia* **15** (1959) 253.
5. Schneider, F. *Ann.* **529** (1937) 1.
6. Kjær, A. *Acta Chem. Scand.* **3** (1949) 1087.
7. Poduška, K., Rudinger, J. and Šorm, F. *Collection Czechoslov. Chem. Communs.* **20** (1955) 1174.
8. Karrer, P. and Schlosser, A. *Helv. Chim. Acta* **6** (1923) 411.
9. Olesen Larsen, P. and Kjær, A. *Biochim. et Biophys. Acta* **16** (1959) *In press.*
10. Kurtz, A. C. *J. Biol. Chem.* **122** (1937) 487.
11. Kurtz, A. C. *J. Biol. Chem.* **180** (1949) 1253.
12. Smith, L. H. *J. Am. Chem. Soc.* **77** (1955) 6691.
13. Ley, H. and Hegge, H. *Ber.* **48** (1915) 70.
14. Albert, A. *Biochem. J.* **47** (1950) 531.
15. Albert, A. *Biochem. J.* **50** (1952) 690.
16. Honda, K., Miura, K. and Tamaoki, T. *J. Agr. Chem. Soc. Japan* **26** (1952) 453; *cf. Chem. Abstracts* **47** (1953) 6480.
17. Neuberger, A. *Advances in Protein Chem.* **4** (1948) 297, p. 303.
18. Batt, R. D., Martin, J. K., Ploeser, J. McT. and Murray, J. *J. Am. Chem. Soc.* **76** (1954) 3663.
19. Tarlton, E. J. and McKay, A. F. *Can. J. Chem.* **36** (1958) 496.
20. Campbell, Jr., L. L. *J. Biol. Chem.* **233** (1958) 1236.

Received June 26, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1575–1584

*iso*Thiocyanates XXXVI. * (+)-4-Methyl-2-oxazolidinethione, the Enzymic Hydrolysis Product of a Glucoside (Glucosisymbrin) in Seeds of *Sisymbrium austriacum* Jacq.

ANDERS KJÆR and BO W. CHRISTENSEN

Organic Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, Denmark

Previous reports on the presence of *iso*thiocyanates and their parent glucosides in various species of the cruciferous genus *Sisymbrium* are discussed.

Paperchromatographic evidence for the presence of three new *iso*thiocyanate glucosides (A, B and C) in seeds of *Sisymbrium austriacum* Jacq. is presented. Upon enzymic hydrolysis the glucosides A and B afford substituted 2-oxazolidinethiones, probably originating in initially formed and spontaneously cyclizing β-hydroxysubstituted *iso*thiocyanates, different from those formerly recognized. Glucoside C, the structure of which has yet to be established, appears to furnish a non-cyclizing mustard oil.

By synthesis of its antipode, the heterocyclic enzymic hydrolysis product produced from glucoside A is proved to be the hitherto unknown (+)-4-methyl-2-oxazolidinethione (V) of established absolute configuration. This represents the first example of a naturally derived 4-substituted 2-oxazolidinethione. The nature of glucoside B is being investigated at the present.

Certain configurational and biogenetical implications are briefly discussed.

The cruciferous genus *Sisymbrium* is of nearly cosmopolitan distribution and comprises about 80 individual species, a few of which have formerly been quoted as sources of *iso*thiocyanate glucosides. Thus, Lepage [1] in 1846 reported on the presence of a mustard oil-producing glucoside in seeds of *S. officinale* Scop.** A few years ago, Schultz and Gmelin [2] recorded paperchro-

* Presented in abstract before the 10th Meeting of Scandinavian Chemists, August 1959, in Stockholm, Sweden.

Part XXXV of this series: *Acta Chem. Scand.* **13** (1959) 851.

** Another species cited by the same author as a glucoside source, *S. cheiranthoides* Et. et W., is more commonly recognized as *Erysimum cheiranthoides* L.

Like several cruciferous genera that of *Sisymbrium* represents a far from well-defined collection of taxa. Numerous species, at one time regarded as belonging to the genus *Sisymbrium* have subsequently been transferred to other genera and *vice versa*, a fact which calls for attention in chemotaxonomic considerations.

matographic evidence for the presence of sinigrin in fresh plants of *S. officinale* and *S. Loeselii*, a finding which was later supplemented by data indicating the occurrence of the same glucoside also in seeds of the former species [3,4]. Paper chromatography of seed material of *S. strictissimum* revealed a content of two glucosides, tentatively interpreted [5] as glucoputranjivin * and glucocochlearin. In this laboratory the latter assignment was supported by independent studies of volatile mustard oils liberated from seed of *S. strictissimum* L., indicating their identity as *iso*propyl and *sec.*-butyl *iso*thiocyanate [6]. In seed material of the common weed, *Sisymbrium sophia* L.**, the presence of sinigrin was established by formation of paperchromatographically identified N-allylthiourea subsequent to enzymic hydrolysis and ammonia treatment of the liberated mustard oil. Fresh plants of *S. luteum* (Maxim) O. E. Schulz, on the other hand, were found to be devoid of volatile mustard oils [6].

In the course of systematic studies in this laboratory on the distribution of *iso*thiocyanate glucosides in a large number of plant species, the presence of three strong glucoside spots were noticed upon paper chromatography of a methanolic seed extract of *Sisymbrium austriacum* Jacq.*** Paperchromatographic experiments, conducted in various solvent systems and in the presence of several authentic reference glucosides, suggested that the three compounds (A, B and C) were different from all mustard oil glucosides previously encountered in Nature (*cf.* Refs. [7,8]). In butanol:ethanol:water (4:1:4), glucoside A migrated slowly, corresponding to an R_B-value [4] of 0.34, whereas compound B possessed an R_B-value of 0.59. The third glucoside (C) was much more lipophilic (R_B 1.67). Two additional, but weaker spots, located between compounds B and C, were possibly attributable to glucoputranjivin and glucocochlearin, two glucosides affording *iso*propyl and *sec.*-butyl *iso*thiocyanate on enzymic hydrolysis, respectively. Solutions of the individual glucosides, A, B and C, were obtained by elution of the appropriate horizontal zones from paper band chromatograms developed in the same solvent system and subjected to enzymic hydrolysis with a cell-free myrosinase preparation. It appeared that both glucoside A and B afforded Grote-positive hydrolysis products directly, whereas compound C underwent fission to a component which gave positive Grote-reaction only after treatment with ammonia. These observations, supplemented with characteristic UV-absorption patterns of the hydrolysis products derivable from A and B, suggested that the latter belonged to the group of 2-oxazolidinethiones, formerly recognized as cyclized products of initially produced β-hydroxy-substituted *iso*thiocyanates (*cf.* Refs.[7,8]). Glucoside C, in contrast, appeared to yield a reasonably stable mustard oil which was transformed into its thiourea derivative on treatment with ammonia. Upon paper chromatography, with the upper layer of the solvent system benzene:heptane:water (9:2:9) as the mobile phase, the supposable 2-oxazolidine-

* This glucoside was differently interpreted in Ref. [3], *viz.* as sinigrin.

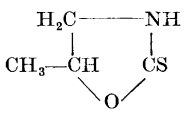
** This plant is now more commonly recognized as a species of the genus *Descurainia*, *viz.* *D. sophia* (L.) Webb; *cf.* foot note on p. 1575.

*** This species is indigeneous to the mountain areas of Middle Europe. The authenticity of the seed material employed in the present studies has been controlled through the courtesy of the *Botanical Garden of the University of Copenhagen* by sowing and subsequent botanical determination of the resulting plants.

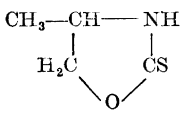
thiones derived from glucoside A and B ('THIOX A' and 'THIOX B') migrated at a rate corresponding to an R_{Ph}-value * of 0.27 and 0.62, respectively. These figures, as well as direct chromatography with various 2-oxazolidinethiones as reference substances, indicated that both compounds, and hence the glucosides A and B, were different from all previously encountered natural compounds. The present communication reports on the structural elucidation of 'THIOX A' and hence glucoside A for which the name *glucosisymbrin* is proposed. The chemical identity of the glucosides B and C will form the subject of forthcoming papers.

In a preliminary isolation experiment a small specimen of pure, crystalline 'THIOX A' was secured after a rather long and tedious series of purification steps (see Exptl. Part). The crystalline product proved very helpful as seeding material for a subsequently obtained portion of 'THIOX A', isolated from a larger sample (220 g) of seeds of *Sisymbrium austriacum* Jacq.** The seed material was finely ground, defatted with carbon tetrachloride and thoroughly extracted with 70 % methanol. After removal of impurities with lead acetate, the resulting solution was enzymically hydrolyzed with myrosinase, and the ether-extractable reaction products purified by means of alumina to give an almost colourless oil (1.6 g). After extraction with water and lyophilization of the aqueous solution an oil remained which on seeding with the previously obtained, crystalline specimen of 'THIOX A' yielded a portion of crystals (133 mg) which was repeatedly recrystallized from various solvents to yield an analytically pure sample of chromatographically homogeneous 'THIOX A', m.p. 64°, which proved to be *dextro*rotatory in ethanol solution.

The analytical composition, C_4H_7ONS, in conjunction with the ultra-violet and infra-red spectra, strongly suggested that 'THIOX A' was a methyl-substituted 2-oxazolidinethione. Of these, both the (±)-5-methyl-derivative (I) [9,10] (m.p. 72-73°[9], 74.5-75.0°[10]) and the racemic 4-methyl-2-oxazolidinethione [11]



I



II

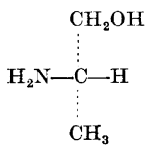
(II) (m.p. 75-76°) had been formerly described. Previous experience within this series has indicated that the racemic compounds often melt higher than the enantiomorphs and deviate considerably from the latter in their infra-red spectra. Careful paper chromatography in benzene:heptane:water (9:2:9), however, showed that 'THIOX A' travelled at a slightly, but consistently, higher rate than a specimen of racemic (I), prepared in this laboratory. Again, it was noticed that in the same solvent system (±)-4-vinyl-2-oxazolidinethione

* *i.e.* the ratio between the distances travelled by the said substance and (±)-5-phenyl-2-oxazolidinethione on the same chromatogram.

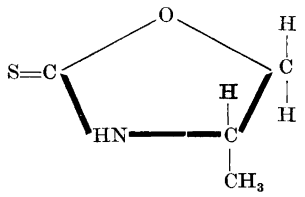
** The seed material employed was produced by cultivation on a larger scale through 1957—58 in the *Botanical Garden of the University of Copenhagen*. The authors are very grateful to the Garden for its valuable assistance.

migrated slightly faster than the (±)-5-isomeride *. This fact, in conjunction with the observation that 'THIOX A' compared with the 4-vinyl-isomeride, produced more blue and stable colours with Grote's reagent than the 5-methyl- and 5-vinyl-substituted ring compounds, supported the belief that 'THIOX A' was, in fact, one of the stereoisomeric 4-methyl-2-oxazolidinethiones (II). Accordingly, the correctness of this assumption was tested by the following synthetic approach.

The 2-amino-alcohol required for the synthesis of one of the optically active 4-methyl-2-oxazolidinethiones (II) is either (+)- or (−)-2-amino-1-propanol (alaninol). Consequently, natural L(+)-alanine was reduced with lithium aluminium hydride in tetrahydrofuran as described by Vogl and Pöhm [12] to give (+)-2-amino-1-propanol (III) in 78 % yield. The amino-alcohol was characterized as its acid oxalate [12,13], whereas a neutral oxalate with the reported m.p. [12–14] could not be obtained despite many attempts, possibly on account of the formation of unresolvable solid solutions of the acid and neutral salts.

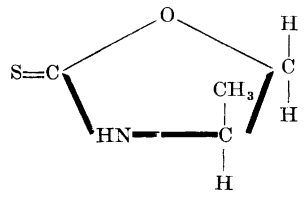


III



(−)

IV



(+)

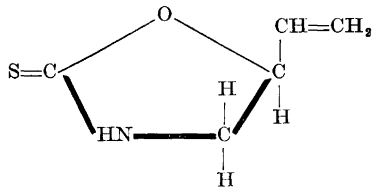
V

L-Alaninol (III) was then treated with thiocarbonyl chloride and triethylamine in chloroform solution to give an oil which was induced to crystallize only after seeding with 'THIOX A'. As shown below the synthetic compound constitutes, in actuality, the antipode of 'THIOX A'. Hence, the successful seeding should probably be attributed to a case of oriented overgrowth on crystals of intermediately formed traces of the racemic compound (*cf. e.g.* Ref.[15]). The synthetic preparation was indistinguishable from 'THIOX A' on paper chromatography and furthermore possessed the same elementary composition and melting point as well as ultra-violet and infra-red spectra as 'THIOX A'. Its optical rotation, however, was similar in magnitude *but opposite in sign* to that of the naturally derived, *dextro*rotatory 2-oxazolidinethione. The enantiomorphous relationship between the two compounds further appeared from the fact that a mixture of equal amounts of the two separated from methanol as the racemic 4-methyl-2-oxazolidinethione (II) possessing the m.p. 75—76°, in agreement with the value previously reported [11]. Not unanticipated, the infra-red spectrum of the latter deviated considerably from that of the enantiomers (Fig. 1).

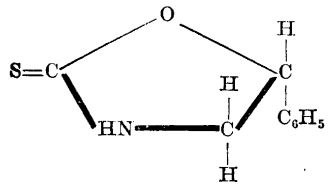
* Synthetic specimens of the vinyl-substituted 2-oxazolidinethiones were kindly supplied by Dr. M. G. Ettlinger of the Chemistry Department, Rice Institute, Houston, Texas, U.S.A.

The above synthetic route, departing from configurationally known (+)-alanine, immediately establishes the absolute configuration (IV) for the *levo*rotatory, synthetic product *. Consequently, (V) depicts the absolute configuration for the naturally derived, *dextro*rotatory 4-methyl-2-oxazolidinethione ** ('THIOX A').

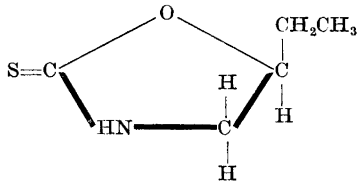
The new 2-oxazolidinethione represents the first example of a 4-substituted derivative of natural provenance. Previously reported compounds of this type include 5,5-dimethyl-[17], (−)-5-vinyl-[18] and (−)-5-phenyl-2-oxazolidinethione [19]. To the last two of these we have assigned the absolute configurations (VI)[20] and (VII)[21], respectively. Furthermore, it has been pointed out [20] that the synthetic, *levo*rotatory 5-ethyl-derivative possesses the absolute configuration (VIII).



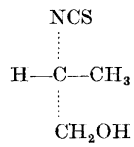
VI



VII



VIII



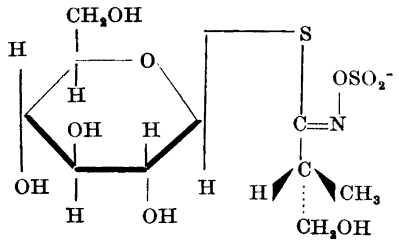
IX

It is of interest to note that exchange of a vinyl- or ethyl-grouping in 5-position with a methyl-substituent in 4-position on the same side of the ring plane, results in a change in sign of rotation, whereas no such alteration accompanies the exchange of the 5-phenyl- with the 4-methyl-substituent. It is expected that further insight into the relationship between configuration and optical rotation in the present class of substances will be obtained from rotatory dispersion studies at present under way.

On the very likely premises that *dextro*rotatory 4-methyl-2-oxazolidinethione (V) results from spontaneous intramolecular cyclization of an initially produced β-hydroxy*iso*propyl *iso*thiocyanate, the above determination of the absolute configuration of (V) immediately establishes the spatial arrangement (IX) around the asymmetric carbon atom of the latter.

* *(S)*-4-Methyl-2-oxazolidinethione in the specification system of Cahn *et al.*[16]

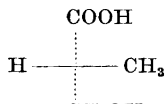
** *(R)*-4-Methyl-2-oxazolidinethione in the same system.



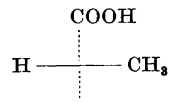
X

Although no experimental proof is available, it appears likely that the configuration depicted in (IX) is that prevailing also in the side-chain of the parent glucoside glucosisymbrin which, therefore, should be formulated as (X), in accordance with the revised general glucoside structure forwarded by Ettlinger and Lundeen [22]. In an analogous case, *viz.* that of glucocochlearin which on enzymic hydrolysis affords (+)-2-butyl *iso*thiocyanate, the same authors [22] suggested that the intramolecular rearrangement accompanying the enzymic reaction would probably proceed with retention of configuration as a consequence of the reaction mechanism involved.* This view has indirectly received some support from the establishment in this laboratory of the same absolute configuration of naturally derived (+)-2-butyl mustard oil as that prevailing around the β-carbon atom of natural *iso*leucine [23] which may be envisaged as biogenetically related with glucocochlearin.

The structural elucidation of glucosisymbrin raises the question as to its biogenetic relationship with glucoputranjivin, the glucoside producing *iso*propyl *iso*thiocyanate and present in *inter alia* several *Sisymbrium* species [24]. It appears likely that one may be derivable from the other by enzymic oxidation or reduction, a possibility which, however, needs further clarification. A reaction sequence analogous to the well-established valine catabolism in animal tissues, proceeding *via iso*butyryl-CoA and β-hydroxy*iso*butyric acid [25], may conceivably be operative also in higher plants. In this connexion attention should be drawn to the establishment by Suchý *et al.*[26] of one of the enantiomorphous β-hydroxy*iso*butyric acids as the acid moiety of the sesquiterpene lactone arctiopicrin.



XI



XII

* *Added in proof.* Provided this assumption proves tenable, acid hydrolysis of glucosisymbrin, analogous to that recorded for other *iso*thiocyanate glucosides by Ettlinger and Lundeen [22], should afford optically active β-hydroxy*iso*butyric acid possessing the absolute configuration (XI).

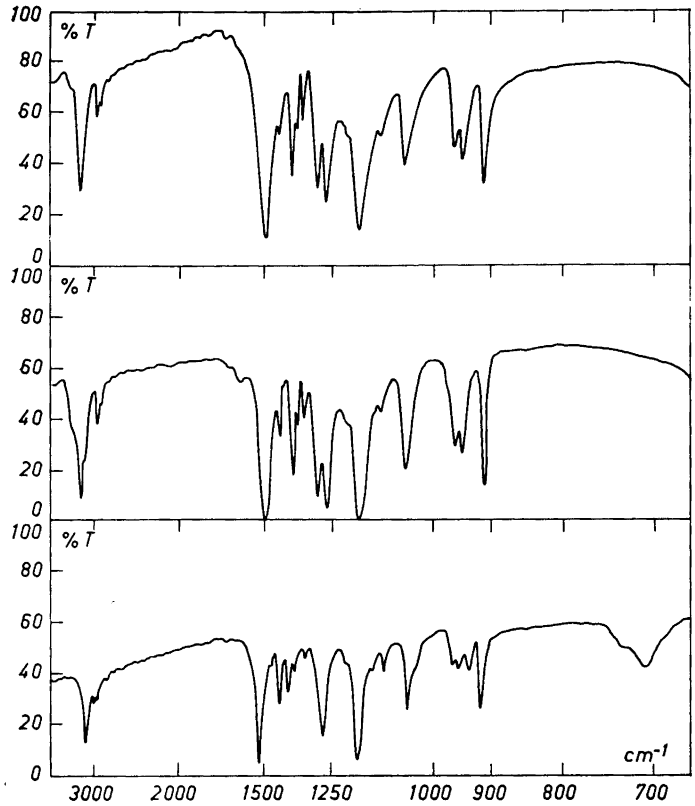


Fig. 1. Infra-red spectra, determined in KBr pellets. Upper curve: naturally derived, *dextro*rotatory 4-methyl-2-oxazolidinethione ('THIOX A'). Middle curve: synthetic, *levo*rotatory enantiomer of the same substance. Lower curve: racemic 4-methyl-2-oxazolidinethione.

Very recently, Asen *et al.*[27] isolated (−)-β-amino*iso*butyric acid from bulbs of *Iris tingitana*. The determination by Balenović and Bregant [28] of the absolute configuration (XII) for *levo*-rotatory β-amino*iso*butyric acid establishes identical configurations around the asymmetric β-carbon atom of (XI) and (XII), both derivable from plant sources. Again, it appears interesting that methylmalonic acid semialdehyde, a product on the catabolic pathway of β-hydroxy*iso*-butyric acid in mammalian tissues, has been shown to undergo a reversible transamination with glutamate to furnish β-amino*iso*butyric acid [25].

EXPERIMENTAL

All melting points reported are uncorrected and, if not otherwise stated, determined in capillary tubes in a slowly heated bath. Rotations are measured in a 1 dm tube. Infra-red spectra are determined in KBr pellets on a Perkin-Elmer "Infracord" instrument.

Exploratory paperchromatographic investigations. A glucoside solution, prepared by boiling a finely ground seed sample of *Sisymbrium austriacum* Jacq. (0.5 g) with 70 % methanol (5 ml), was chromatographed descendingly on Schleicher and Schüll paper

No. 2243b together with a reference sample of glucotropaeolin, with the upper layer of the solvent system *n*-butanol:ethanol:water (4:1:4) as the mobile phase. Three spots were easily distinguishable upon dipping the chromatogram through methanolic silver nitrate, and were attributed to the glucosides A, B and C with R_B-values [4] of 0.34, 0.59 and 1.67, respectively. In *n*-butanol:pyridine:water (6:4:3) the R_B-values were: 0.65, 0.80 and 1.19. That the three spots in this solvent system also represented the glucosides A, B and C in the same sequence was proved by chromatographing the individual compounds, obtained by elution with water of the appropriate zones from chromatograms run as bands in the neutral system, in the pyridine solvent.

To the individual aqueous eluates, each of a volume of 1 ml, were added a phosphate buffer (pH 6.7, 1/15 M, 0.25 ml) and 3 drops of a cellfree myrosinase solution. After 1 h at room temperature, the three solutions were extracted with chloroform. The extracts derivable from the glucosides A and B yielded a strong blue colour with Grote's reagent in contrast to that originating from compound C. The first two solutions, together with a reference solution of (±)-5-phenyl-2-oxazolidinethione [19], were subjected to descending chromatography on Whatman paper No. 1 with the upper layer of the solvent system benzene:heptane:water (9:2:9) as the mobile phase. On spraying with Grote's reagent two blue spots were observed, derivable from glucoside A and B and possessing R_{Ph}-values of 0.27 and 0.62, respectively. Neither of these spots could be assigned to any previously encountered product of natural origin but both exhibited the same characteristic UV-spectrum in ethanol (λ_{max} 243 mμ, λ_{min} 219 mμ), very similar to the pattern previously registered for 2-oxazolidinethiones. After treatment with a chloroform solution of dry ammonia the reaction product of glucoside C yielded a Grote-positive derivative, travelling near the solvent front in water-saturated chloroform.

Preliminary isolation experiment. Dry seeds (220 g) of *Sisymbrium austriacum* Jacq. were covered with carbon tetrachloride (500 ml) and finely ground in a Waring blendor without cooling. The process was repeated with fresh solvent (500 ml) and the suspension was finally refluxed for 1 h. The filtered, defatted seed powder (155 g) was then extracted with three 1 l portions of 70 % methanol. After filtration through Celite, the extract was concentrated *in vacuo* to a sticky brown mass, which was dissolved in the lower phase (200 ml) of the solvent system employed for paper chromatography (*n*-butanol:ethanol: water, 4:1:4). The solution was introduced into No. 1 of a total of 11 separatory funnels, all preloaded with the corresponding upper phase (200 ml in each funnel). The crude mixture was now distributed in a counter-current manner through all vessels. Troublesome emulsion formation was overcome by centrifugation before the next following transfer. Paper chromatography indicated that glucoside C was primarily concentrated in the separatory funnels No. 5—8, supplemented with the upper phase of funnel No. 9, whereas the lower layer of the latter plus the vessels No. 10 and 11 contained essentially a mixture of glucoside A and B, contaminated with considerable quantities of an almost black syrup. To a solution of the crude fraction containing A and B in water (400 ml), a 10 % solution of basic lead acetate was added until the separation of a yellow-brown precipitate ceased (a total of *ca.* 120 ml). The latter was removed by filtration through Celite, and hydrogen sulphide was introduced into the filtrate to precipitate excess lead ions. The filtered solution was concentrated *in vacuo* to an almost colourless syrup. This was redissolved in water (400 ml), equal volumes (20 ml) of 1/15 M solutions of primary and secondary potassium phosphate were added, followed by 25 ml of a myrosinase solution. After having been kept for 3 h at room temperature, the solution was extracted with four 300 ml portions of ether which were pooled and dried. The residual yellow oil (1.3 g) was dissolved in benzene (20 ml) and rapidly washed through a small column of alumina (4 × 0.8 cm) with additional benzene, until the Grote-reaction became negative. This procedure yielded an almost colourless oil (1.1 g) which was thereafter subjected to careful adsorption chromatography on neutral alumina (column diameter: 2.2 cm, height of packing: 25 cm) with 1 % of methanol in benzene as the eluant. Fractions of 5 ml were taken; the Grote-positive constituents were located in fractions No. 147—178. Paper chromatography revealed that the fractions No. 147—155 contained exclusively 'THIOX B', No. 170—181 solely 'THIOX A' and the intermediate fractions a mixture of the two.

The 'THIOX A'-fractions were taken to dryness, redissolved in pure benzene and a small insoluble residue discarded. The oily product (134 mg) was subjected to fractional sublimation at 0.2 mm Hg. At 40° (bath temperature) an oily fraction distilled and was discarded. The bath was slowly raised to 80° when a deposit of crystalline material

(34 mg) on the 'cold finger' was noticed. The somewhat oily preparation was recrystallized from anhydrous ether at low temperature to give thin colourless prisms, $[\alpha]_D^{24}$ +22.1° (*c* 0.85, MeOH). The recovered material from the rotation determination, supplemented by a fraction of crystalline material obtained directly from fraction No. 166–169 on seeding, was purified by repeated recrystallizations from a mixture of benzene and petroleum ether to give a pure specimen (16.8 mg) of 'THIOX A' as colourless prisms with m. p. 64–65°.

Isolation of (+)-4-methyl-2-oxazolidinethione. Another seed portion (220 g) was processed exactly as described above with the sole exception that the cumbersome countercurrent extraction was omitted. The lead-purified glucoside extract was enzymically hydrolyzed as described in the foregoing. Again, yellow contaminants were partially removed from the ethereal extract by means of a small alumina column. The residue was treated with water (80 ml) causing a considerable amount of a gummy product (polymerisation?) to separate. The latter was thoroughly washed and the aqueous solution lyophilized to give a colourless oil. This was taken up in ether (5 ml), the solution was cooled to 0° and seeded with previously obtained crystals of 'THIOX A' when the separation of crystalline, but slightly oily 'THIOX A' (133 mg) started. Two recrystallizations from a mixture of benzene and petroleum ether (1:1), followed by another two from anhydrous ether afforded a pure specimen of 'THIOX A' (17 mg), m. p. 63.5°–64.5°. (Found: C 41.06; H 6.15; N 11.81. Calc. for C_4H_7ONS: C 40.99; H 6.02; N 11.95.)

An additional 9.6 mg of the same purity was secured by repeated recrystallizations of the mother liquor preparation, m. p. 64°; $[\alpha]_D^{24}$ +21.1° (*c* 0.72, EtOH). The infra-red spectrum is reproduced in Fig. 1.

Synthesis of (−)-4-Methyl-2-oxazolidinethione. L-Alanine (8.01 g, $[\alpha]_D^{28}$ +14.7° (*c* 7.0, 1 N HCl)) was reduced by means of a solution of $LiAlH_4$ (10.5 g) in tetrahydrofuran (120 ml), according to the procedure described by Vogl and Pöhm [12]. Distillation *in vacuo* afforded L(+)-alaninol in 78 % yield as a colourless oil, b. p. 67°/8 mm, n_D^{25} 1.4487, $[\alpha]_D^{25}$ +22.0° (*c* 6.45, 96 % EtOH). Literature values: b. p. 78–80°/12 mm, $[\alpha]_D^{17}$ +20.1° (alcohol) [13]; b. p. 85°/10 mm, $[\alpha]_D^{17}$ +20.3° (EtOH) [12].

The amino-alcohol was characterized as its acid oxalate, produced by mixing hot solutions of the former and of oxalic acid dihydrate (20 % excess). The salt separated from ethanol in colourless needles, m. p. 151°, $[\alpha]_D^{26}$ +13.8° (*c* 2.5, H_2O). (Found: C 36.35; H 6.56; N 8.55. Calc. for C_3H_9ON, $C_2H_2O_4$: C 36.35; H 6.71; N 8.48). Literature values: m. p. 140–141°[13]; m. p. 141–142°[12]; (no rotation data reported). Repeated attempts to prepare the formerly described neutral oxalate proved unsuccessful, despite variations in the procedure and amounts of reactants employed. Beautiful, crystalline salts were obtained with the reported rotation value ($[\alpha]_D^{23}$ +18.0° (*c* 2.3, H_2O)) but invariably melting over a wide temperature range (*e. g.* 125–140°).

To a cooled and stirred solution of thiocarbonyl chloride (1.16 g) in chloroform (15 ml), another solution of L(+)-alaninol (0.65 g) and triethylamine (1.76 g) in chloroform (10 ml) was added in the course of 10 min. Shortly after the addition of the amino-alcohol was completed the thiophosgene smell disappeared, and the yellow solution was evaporated to dryness *in vacuo*. Repeated extractions with ether removed the Grote-positive product from triethylamine hydrochloride. The concentrated extract was decolourized with Norite, filtered and again concentrated to a final volume of 7 ml. Only after addition of seed crystals of 'THIOX A' a crystalline precipitate appeared. Next day, the product (346 mg) was recrystallized thrice from ether to give an analytical specimen (180 mg) of *(−)-4-methyl-2-oxazolidinethione*, m. p. 65°, $[\alpha]_D^{26}$ −22.9° (*c* 1.12, 96 % EtOH). (Found: N 11.77; S 27.42. Calc. for C_4H_7ONS: N 11.95; S 27.36.) The infra-red spectrum of the synthetic preparation was indistinguishable from that of 'THIOX A' (Fig. 1). The latter, therefore, is the antipodal (+)-4-methyl-2-oxazolidinethione.

Racemate formation. Synthetic material (2.36 mg) was mixed with 'THIOX A' (2.36 mg) and the solid dissolved in a few drops of methanol. The residual oil crystallized on scratching, and the racemate was recrystallized from two drops of water to give thin, colourless needles, m. p. 75.5–76.0° (microscope). Literature value: m. p. 75–76°[11].

The infra-red spectrum, which is conspicuously different from that of the optically active forms, is presented in Fig. 1.

Microanalyses were performed by Mr. P. Hansen at the Chemical Laboratory of the University of Copenhagen. Thanks are also due to Dr. R. Gmelin for valuable assistance in the preliminary phases of these studies.

The present work is part of investigations supported by *The Danish State Research Foundation (Statens Almindelige Videnskabsfond)* and *Kai Hansen's Fond.* One of us (A.K.) wishes to express his gratitude to *The Research Council of the Technical Sciences (Det Teknisk-Videnskabelige Forskningsråd)* for the appropriation of an infra-red spectrophotometer.

REFERENCES

1. Lepage, J. *J. Chim. Méd.* [3] **2** (1846) 171.
2. Schultz, O.-E. and Gmelin, R. *Z. Naturforsch.* **7b** (1952) 500.
3. Schultz, O.-E. and Gmelin, R. *Z. Naturforsch.* **8b** (1953) 151.
4. Schultz, O.-E. and Wagner, W. *Z. Naturforsch.* **11b** (1956) 73.
5. Gmelin, R. *Präparative und analytische Versuche über Senfölglucoside* (Diss.) University, Tübingen 1954.
6. Kjær, A., Conti, J. and Larsen, I. *Acta Chem. Scand.* **7** (1953) 1276.
7. Kjær, A. *Suomen Kemistilehti* **A32** (1959) 53.
8. Kjær, A. In *Organic Sulfur Compounds*, Pergamon Press. London-New York 1959, vol. I. *In press.*
9. Hopkins, C. Y. *Can. J. Research* B**20** (1942) 268.
10. Rosen, A. A. *J. Am. Chem. Soc.* **74** (1952) 2994.
11. Viscontini, M. and Adank, K. *Helv. Chim. Acta* **33** (1950) 2251.
12. Vogl, O. and Pöhm, M. *Monatsh.* **83** (1952) 541.
13. Karrer, P., Portmann, P. and Suter, M. *Helv. Chim. Acta* **31** (1948) 1617.
14. Stoll, A., Peyer, J., Hofmann, A. *Helv. Chim. Acta* **26** (1943) 929.
15. Bunn, C. W. *Chemical Crystallography*, Oxford University Press 1946, p. 60.
16. Cahn, R. S., Ingold, C. K. and Prelog, V. *Experientia* **12** (1956) 81.
17. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
18. Astwood, E. B., Greer, M. A. and Ettlinger, M. G. *J. Biol. Chem.* **181** (1949) 121.
19. Kjær, A. and Gmelin, R. *Acta Chem. Scand.* **11** (1957) 906.
20. Kjær, A., Christensen, B. W. and Hansen, S. E. *Acta Chem. Scand.* **13** (1959) 144.
21. Kjær, A. and Gmelin, R. *Acta Chem. Scand.* **12** (1958) 1693.
22. Ettlinger, M. G. and Lundeen, A. J. *J. Am. Chem. Soc.* **78** (1956) 4172.
23. Kjær, A. and Hansen, S. E. *Acta Chem. Scand.* **11** (1957) 898.
24. Kjær, A. *Unpublished observation.*
25. Robinson, W.G., Nagle, R., Bachhavat, B.K., Kupiecki, F.P. and Coon, M.J. *J. Biol. Chem.* **224** (1957) 1.
26. Suchý, M., Herout, V. and Šorm, F. *Collection Czechoslov. Chem. Communs.* **22** (1957) 1902.
27. Asen, S., Thompson, J.F., Morris, C.J. and Irreverre, F. *J. Biol. Chem.* **234** (1959) 343.
28. Balenović, K. and Bregant, N. *Tetrahedron* **5** (1959) 44.

Received June 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1585–1606

Studien über Lokalanästhetica

XVII. * Synthese von N-Diäthylaminoalkyl-substituierten 2,6-Dimethylbenzamiden und 2,6-Dimethylbenzylideniminen

NILS LÖFGREN und WILLY STOFFEL

Institut für organische Chemie und Biochemie der Universität, Stockholm, Schweden

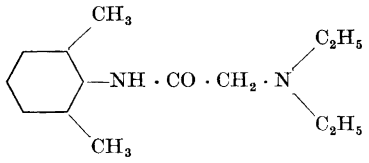
Es werden drei für die Synthese von N-Dialkylaminoalkyl-2,6-dimethylbenzamide geeignete Methoden, sowie die Synthesen von sechs Verbindungen dieser Reihe beschrieben. Die Reaktion von N-Halogenalkyl-2,6-dimethylbenzamiden mit sekundären Aminen führt ausser zur Bildung obiger Amide, durch Ringschluss zu heterocyclischen Verbindungen, von denen das 2-(2,6-Dimethylphenyl)-2-oxazolin und das 2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin isoliert wurden. Die synthetisierten Amide sind äussert stabil gegen hydrolytische Einflüsse, was aus einem vergleichenden Hydrolyseversuch mit dem kernisomeren, sterisch nicht gehinderten N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid hervorgeht, das auch synthetisiert wurde. Für diese Stabilität wird eine elektronentheoretische Deutung gegeben. Ferner wurde das früher nicht beschriebene N,N-Dimethyl-2,6-dimethylbenzamid synthetisiert, und für die Darstellung des N-(2-Diäthylamino-1,2-dimethyläthyl)-2,6-dimethylbenzamids wurde das bisher nicht bekannte 2-Amino-3-(diäthylamino)butan präpariert. Über die Synthese zweier Schiff-Basen des bisher wenig untersuchten 2,6-Dimethylbenzaldehyds, nämlich das N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzylidenimin und das N-(3-Diäthylaminopropyl)-2,6-dimethylbenzylidenimin wird auch berichtet. Die neueren Methoden zur Synthese von Aldehyden von Plieninger und Werst [2], Grundmann [3], Weygand und Mitarbeitern [4], erwiesen sich für die Darstellung des 2,6-Dimethylbenzaldehyds, wegen der sterischen Hinderung, als nicht geeignet.

Es wird weiter über die lokalanästhetische Wirksamkeit und die Toxizität der synthetisierten *N*-Dialkylaminoalkyl-*eso*-dimethylbenzamide berichtet.

Auf Grund experimenteller Befunde sind die bemerkenswerten Eigenschaften des Xylocains® (α-Diäthylamino-2,6-dimethylacetanilid), vor allem im Vergleich mit den kernisomeren Verbindungen — sowohl in physikalisch-chemischer Hinsicht (U.V.-Spektrum [5,6] Dipolmoment [6], Molrefraktion [5], Dis-

* Mitteilung XVI dieser Reihe, siehe Löfgren, Tegnér und Takman [1].

soziationstant [5], Resistenz gegen Säuren und Basen [5]), als auch in pharmakologischer Hinsicht [5,7,8] — auf den durch die vicinale Gruppierung bedingten Ortho-Effekt zurückzufüren. Der Ortho-Effekt äussert sich in einer stark

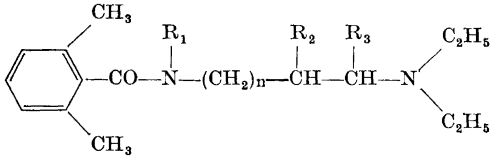


Xylocain ®

verminderten Resonanz zwischen Kern und Amidstickstoff [5].

Invertiert man im Xylocain die Amidgruppierung so kommt man zum N-(Diäthylaminomethyl)-2,6-dimethylbenzamid. Die Darstellung dieser Verbindung, die als Xylocainisomer von besonderem Interesse erschien, wurde über eine Mannich-Reaktion (vgl. S. 1594) versucht. Da aber die Substanz nicht befriedigend rein erhalten werden konnte, wird auf die Beschreibung der Syntheseversuche verzichtet *. Es werden hier sechs andere N-(Diäthylaminoalkyl)-2,6-dimethylbenzamide (I—VI) beschrieben, die zwei oder mehrere Kohlenstoffatome zwischen Amid- und Amin-Stickstoff enthalten (vgl. Tabelle

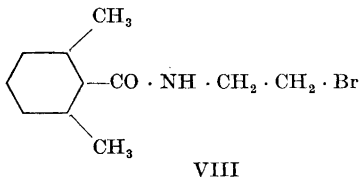
Tabelle 1. Synthetisierte Verbindungen vom Typus



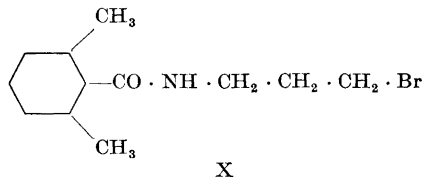
Verbdg.	n	R_1	R_2	R_3
I	0	H	H	H
II	0	CH_3	H	H
III	1	H	H	H
IV	0	H	H	CH_3
V	0	H	CH_3	H
VI	0	H	CH_3	CH_3

1). Sie sind — wie Xylocain — durch die ortho-ständigen Methylgruppen sterisch gehindert (vgl. S. 1593). Alle sechs Verbindungen wurden auf ihre lokalanästhetische Wirksamkeit geprüft.

Das N-(Diäthylaminoäthyl)-2,6-dimethylbenzamid (I) und das N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) wurden über das früher nicht beschriebene N-(2-Bromäthyl)-2,6-dimethylbenzamid (VIII) und das N-(3-



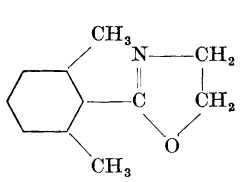
VIII



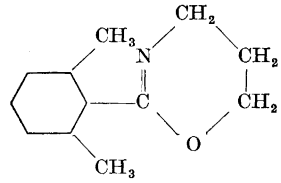
X

* Die Arbeiten mit der Substanz werden fortgesetzt.

Brompropyl)-2,6-dimethylbenzamid (X) dargestellt. Als Nebenprodukte bei der Darstellung von I und III über VIII bzw. X wurden das 2-(2,6-Dimet-



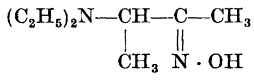
IX



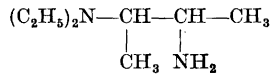
XI

hylphenyl)-2-oxazolin (IX) und das 2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin (XI) isoliert.

Die Synthese von N-(2-Diäthylamino-1,2-dimethyläthyl)-2,6-dimethylbenzamid (VI) wurde über die Stufen 3-Brom-2-butanon, 3-Diäthylamino-2-butanon, 3-Diäthylamino-2-butanon-Oxim (XII) und 2-Amino-3-(diäthylamino)-butan (XIII) durchgeführt. XII und XIII sind früher nicht in der



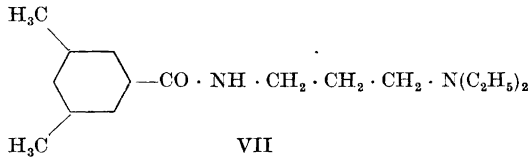
XII



XIII

Literatur beschrieben worden.

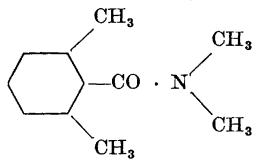
Um den Effekt von den in den 2,6-Stellungen sterisch hindernden Methylgruppen hinsichtlich der lokalanästhetischen Aktivität und hinsichtlich der Stabilität gegenüber hydrolytischen Einflüssen zu studieren, wurde die der Verbindung N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) kernisomere, sterisch ungehinderte Verbindung N-(3-Diäthylaminopropyl)-3,5-dimet-



VII

hylbenzamid (VII) dargestellt. Die Verbindung VII wurde auf ihre lokalanästhetische Wirksamkeit geprüft und in einem Hydrolyseversuch mit III verglichen.

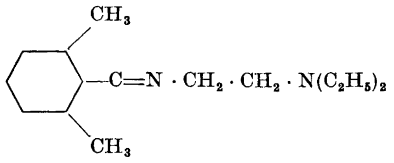
Das bisher noch nicht beschriebene N,N-Dimethyl-2,6-dimethylbenzamid



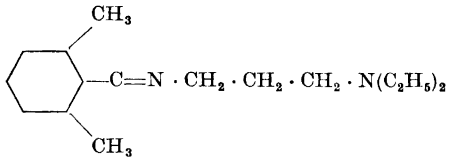
XIV

(XIV) wurde auch synthetisiert.

Von den Kondensationsprodukten des 2,6-Dimethylbenzaldehyds ist bisher nur das von Lock und Schmidt [9] beschriebene Semicarbazon bekannt. Offenbar verhinderte der Ortho-Effekt in den Versuchen letztgenannter Autoren die Bildung des Phenylhydrazons und die Reaktion nach Perkin. Hier gelang es, das N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzylidenimin (XV) und das N-(3-Diäthylaminopropyl)-2,6-dimethylbenzylidenimin (XVI) darzustellen. —



XV



XVI

Ferner wurde versucht, bei der Synthese des sterisch gehinderten 2,6-Dimethylbenzaldehyds neuere Methoden zur Darstellung von Aldehyden (Plieninger und Werst [2], Grundmann [3], Weygand und Mitarbeiter [4]) anzuwenden. Wegen der sterischen Hinderung erwiesen sich hier diese Methoden als nicht geeignet (vgl. S. 1595).

CHEMISCH-THEORETISCHER TEIL

Für die Synthese der N-Diäthylaminoalkyl-2,6-dimethylbenzamide wurden drei Methoden (A, B, C) ausgearbeitet. Die Ausgangssubstanz für die beiden ersten Methoden A und B war das 2,6-Dimethylbenzoylchlorid. Es wurde über die folgenden Stufen synthetisiert:

vic.-m-Xylidin $\xrightarrow[CuCN]{\text{Diazotierung}}$ 2,6-Dimethylbenzonitril $\xrightarrow[H_2SO_4(96\ \%)]{\text{Verseifung}}$

2,6-Dimethylbenzamid $\xrightarrow[H_3PO_4(100\ \%)]{\text{Verseifung}}$ 2,6-Dimethylbenzoesäure $\xrightarrow{SOCl_2}$

2,6-Dimethylbenzoylchlorid.

Analog dem Verfahren von Clarke und Read [10] zur Synthese des *o*-Tolunitrils wurde *vic.-m*-Xylidin in das Diazoniumsalz und weiter durch Sandmeyer-Reaktion mit CuCN in das 2,6-Dimethylbenzonitril mit 60 % Ausbeute überführt. Nach Berger und Oliver [11] wurde durch Verseifung des Nitrils mit konzentrierter Schwefelsäure bei konstant 80° in der ersten Stufe das 2,6-Dimethylbenzamid (50 % Ausbeute) erhalten. Mit 100 % Phophorsäure wurde die Verseifung bis zur Säure weiter geführt. Diese fiel hier in 60 % Ausbeute an. Die Darstellung des Säurechlorids mit Thionylchlorid verläuft fast quantitativ und wurde dem Verfahren von Lock und Schmidt [9], in dem Phosphorpentachlorid angewendet wird, vorgezogen.

Die erschwerte Verseifung des 2,6-Dimethylbenzonitrils beruht auf sterischen Faktoren, die durch die ortho-ständigen Methylgruppen bedingt sind.

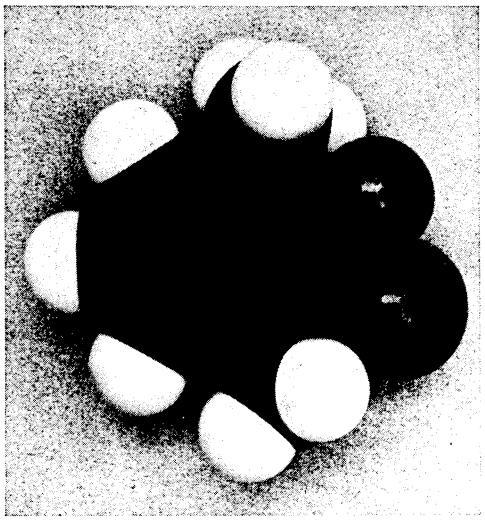
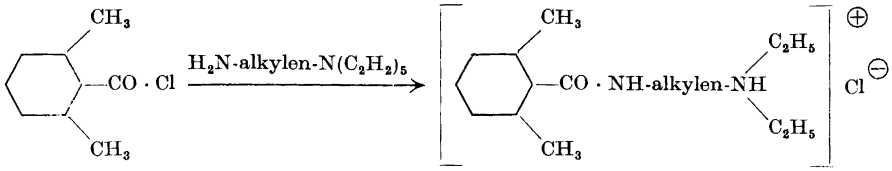


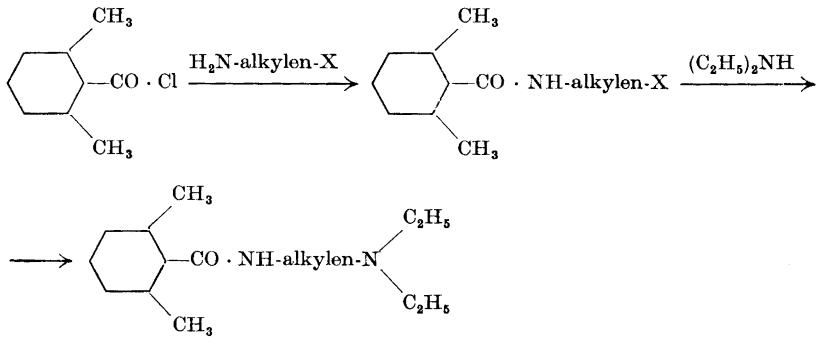
Fig. 1. Stuart-Modell des 2,6-Dimethylbenzoylchlorid-Moleküls.

Aus Versuchen Hoffmanns [12], das Pentamethylbenzonitril zu verseifen, geht hervor, dass auch die kräftigsten Verseifungsmethoden erfolglos blieben und lediglich zum Pentamethylbenzol führten. Das dargestellte 2,6-Dimethylbenzoylchlorid wird ebenso wie das Mesitylencarbonsäurechlorid fast wie ein aliphatisches Säurechlorid durch Wasser stürmisch zersetzt. Das Stuart-Modell zeigt (vgl. Fig. 1), dass von den beiden Atomen Sauerstoff und Chlor das eine oberhalb und das andere unterhalb der Ebene des Benzolkerns liegt. Die Drehbarkeit der $-C\begin{matrix}\diagup\!\!\!\diagup O \\ \diagdown Cl\end{matrix}$ Gruppe um die C_{kern} — C-Achse wird dadurch auf Oszillationen um eine Mittellage beschränkt, die dadurch bestimmt ist, dass die durch $-C\begin{matrix}\diagup\!\!\!\diagup O \\ \diagdown Cl\end{matrix}$ gelegte Ebene senkrecht zur Ebene des Benzolkernes liegt. Dadurch wird eine Interferenz der p-Elektronen der Carbonylgruppe mit dem π-Bindungssystem des Kerns herabgesetzt. Die Ausbildung von mesomeren Grenzstrukturen ist somit stark zurückgedrängt, wodurch das reaktive Verhalten des Säurechlorids eine Erklärung findet.

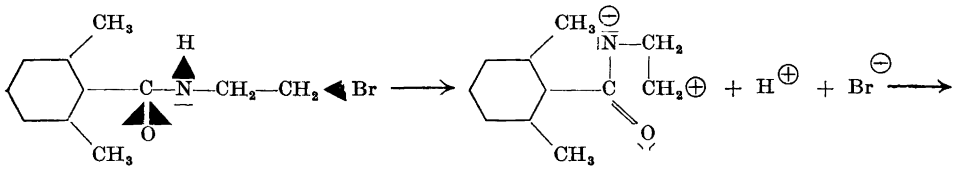
Nach *Methode A* wurde ohne Zusatz eines Protonenakzeptors das 2,6-Dimethylbenzoylchlorid mit dem entsprechenden Diäthylaminoalkylamin zur Reaktion gebracht. Die unter der Reaktion entstehende Base fungiert selbst als Protonenakzeptor:

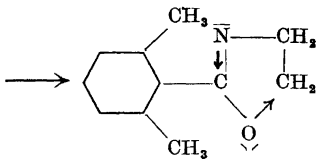


Nach der *Methode B* lassen sich N-Diäthylaminoalkyl-2,6-dimethylbenzamide stufenweise synthetisieren. Man bringt das Säurechlorid mit zwei Mol des betreffenden Halogenalkylamins zur Reaktion. Über das so gewonnene N-Halogenalkyl-2,6-dimethylbenzamid wird dann in der gewöhnlichen Weise die Diäthylaminobase dargestellt:



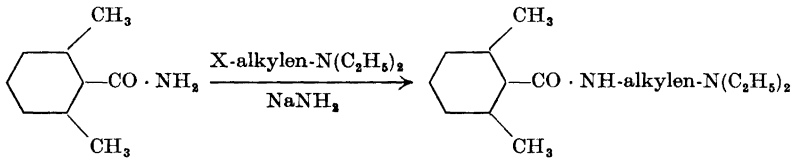
Nach Methode B wurden N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid (I) und N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) über N-(2-Bromäthyl)-2,6-dimethylbenzamid (VIII) resp. N-(3-Brompropyl)-2,6-dimethylbenzamid (X) dargestellt. Nach der Umsetzung mit Diäthylamin fielen bei der fraktionierten Destillation des Reaktionsproduktes jeweils eine niedriger und eine höher siedende Fraktion an. Die niedriger siedende Fraktion konnte im ersten Falle als das 2-(2,6-Dimethylphenyl)-2-oxazolin (IX), im zweiten Falle als das 2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin (XI) identifiziert werden. Gabriel und Heymann [13] sowie Gabriel und Elfeldt [14] stellten Oxazoline durch Behandeln von Halogenalkylbenzamiden mit Natronlauge dar. Der Ringschluss zu dem hier bei der Synthese als Nebenprodukt in 20 bzw. 30 % Ausbeute anfallenden Oxazolin bzw. „Pentoxazolin" erfolgte beim Erhitzen der entsprechenden N-Halogenalkyl-2,6-dimethylbenzamide mit Diäthylamin in benzolischer Lösung:





Durch die Schlüsselatome O und Br wird die Amidwasserstoff-Bindung stark polarisiert. Unter der Einwirkung von Diäthylamin kommt es zur Abspaltung des Wasserstoff- und Bromions und zur Bildung des fünfgliedrigen Oxazolin- bzw. sechsgliedrigen „Pentoxazolinringes". Mit Bromwasserstoffsäure gaben die beiden „Oxazoline" die erwartete Reaktion (vgl. Gabriel und Heymann [13]): Sie wurden unter Ringöffnung in die entsprechenden Ausgangsverbindungen (VIII, X) verwandelt. Die beiden N-Diäthylaminoalkyl-2,6-dimethylbenzamide (I und III) wurden in 50 % Ausbeute erhalten.

Die Ausgangssubstanzen in der *Methode C* sind das 2,6-Dimethylbenzamid und Diäthylaminoalkylhalogenide oder deren Salze. Als Protonenakzeptor dient das Natriumamid:



Es wurden zwei Mol Natriumamid eingesetzt, wenn das Hydrochlorid oder das Hydrobromid des Diäthylaminoalkylhalogenids angewandt wurde.

Durch den Ortho-Effekt wird die Hydrolyse von ortho-substituierten Benzoesäureestern verschwert. So konnten Hinshelwood und Newling [15] zeigen, dass die Verseifung des *o*-Nitrobenzoesäureäthylesters eine um rund 6 000 cal höher liegende Aktivierungsenergie erfordert als die des isomeren *p*-Nitrobenzoesäureäthylesters. Die über die Silbersalze mit Alkyljodid gewonnenen Mesitylencarbonsäureester lassen sich nicht wieder verseifen [16]. Da der Verlauf der Hydrolyse von Amiden in elektronentheoretischer Hinsicht dem der Ester sehr verwandt ist, wurde das Verhalten der ebenfalls dem Ortho-Effekt unterliegenden synthetisierten N-Diäthylaminoalkyl-2,6-dimethylbenzamide gegenüber hydrolytischen Einflüssen untersucht. Als Vergleichssubstanz wurde das N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid (VII) synthetisiert und unter den gleichen Bedingungen wie die entsprechende 2,6-Verbindung (III) der sauren Hydrolyse unterworfen. Der Versuch zeigte (siehe exp. Teil S. 1601), dass die 2,6-Verbindung unter diesen eingreifenden Bedingungen fast überhaupt nicht hydrolysiert wird. Sie konnte in 91 % Ausbeute unverändert zurückgewonnen werden. Dagegen wurde die dem Ortho-Effekt nich unterliegende 3,5-Verbindung (VII) nahezu vollständig hydrolysiert. Die 3,5-Dimethylbenzoesäure wurde in 86 % Ausbeute isoliert.

Die erschwerte Hydrolyse der 2,6-Dimethylbenzamide kann auf folgende Weise gedeutet werden: Die Stuart-Modelle des synthetisierten 2,6-Dimethyl-

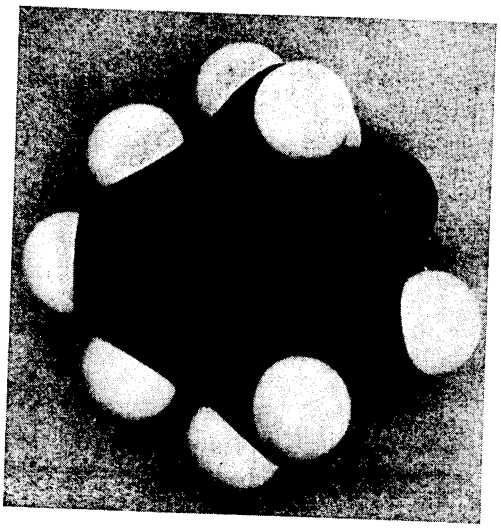


Fig. 2. Stuart-Modell des 2,6-Dimethylbenzamid-Moleküls.

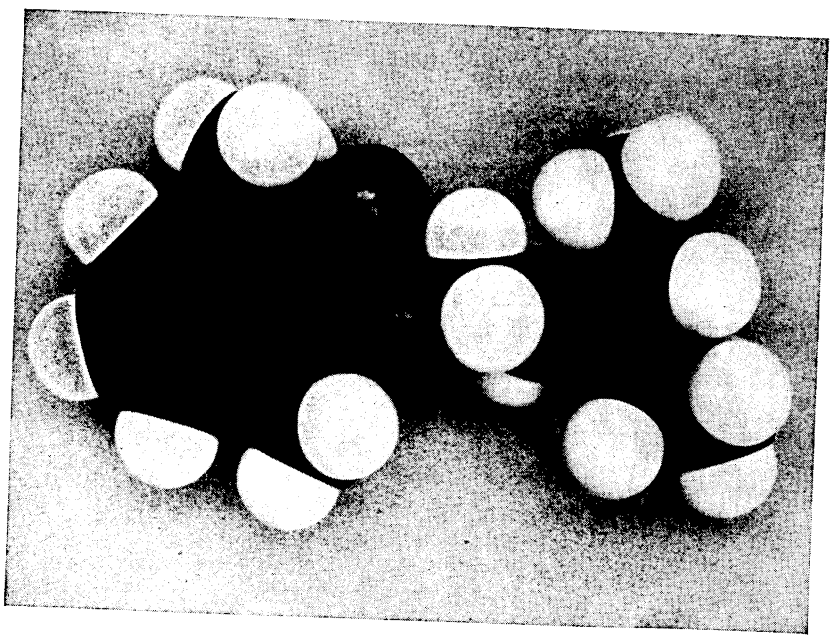


Fig. 3. Stuart-Modell des N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid-Moleküls.

benzamids und der N-Diäthylaminoalkyl-2,6-dimethylbenzamide zeigen deutlich (vgl. Fig. 2 und 3), dass eine sterische Hinderung zwischen den Wasserstoffatomen der ortho-ständigen Methylgruppen und sowohl dem Carbonylsauerstoff als auch dem Amidstickstoff besteht. In Wasserstoffbrücken beansprucht praktisch der Wasserstoff keinen Raum, vielmehr wird der Kernabstand der durch die H-Brücken verbundene Atome verringert. Wie aus einem Studium der sterischen Verhältnisse der hier aktuellen Verbindungen (vgl. Fig. 2 und 3) hervorgeht, so ist die Möglichkeit für die Bildung intramolekularer Wasserstoffbrückenbindungen (Chelatbindungen), einmal zwischen dem Carbonylsauerstoff- und einem Wasserstoffatom der einen *o*-Methylgruppe, zum anderen zwischen dem Amidstickstoff- und einem Wasserstoffatom der anderen *o*-Methylgruppe gegeben, d.h. die Repulsion der einander nahe kommenden Gruppen wäre aufgehoben und statt dessen gegen eine Attraktion durch Chelatbindungen vertauscht worden (vgl. Branch und Calvin [17]). Durch die Bildung dieser Wasserstoffbrücken entstehen — wie aus dem Stuart-Modell ersichtlich ist — zwei zum Benzolring *koplanar* liegende sechsgliedrige Chelatringe. Die durch die erwähnten H-Brücken gebildete koplanare Drei-Sechsring-Struktur, die also den Benzolring mit einbezieht, wird ferner durch Resonanz stabilisiert. Fig. 4 veranschaulicht das Wasserstoffbrücken-System, und unter den Formeln stellen *d* und *e* solche dar, die aus der durch die koplanare Lage bedingten Resonanz mit dem Benzolkern resultieren *.

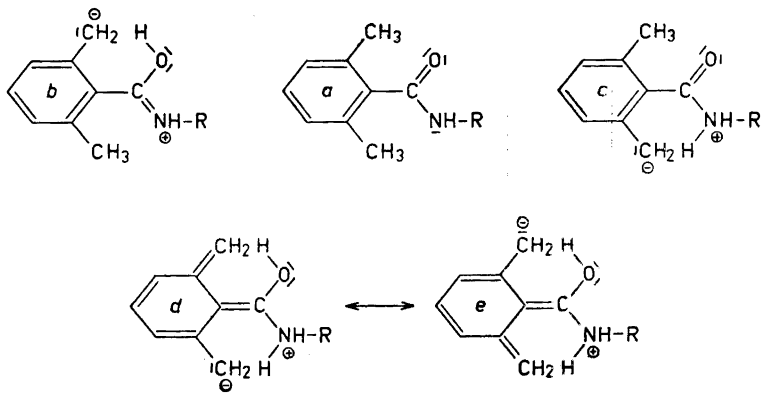


Fig. 4. Wasserstoffbrücken-System in 2,6-Dimethylbenzamiden.

Wasserstoffbrücken zwischen ortho-Methylgruppen und Aminostickstoff werden von Branch und Calvin [17] z.B. im Falle des *o*-Methylanilins beschrieben. Löfgren [5] (vgl. auch Lindström [18]) hat auf Grund experimenteller Befunde das Vorkommen eines solchen den Amid-Stickstoff und den *o*-Methyl-Wasserstoff mit einschliessenden fünfgliedrigen Chelatringes im *a*-Diäthylamino-2,6-

* Die hier angewandte Schreibweise für die Wasserstoffbrückenbindung schliesst sich der von Branch und Calvin [17] (siehe Beispiel *o*-Toluylsäure) an.

dimethylacetanilid (Xylocain) wahrscheinlich gemacht. Nach Watson [19] sowie Branch und Calvin [17] ist die Bildung sechsgliedriger Chelatringe — wie im vorliegenden Fall — am stärksten begünstigt. — Durch den oben beschriebenen Ortho-Effekt in den 2,6-Dimethylbenzamiden sollte die Amid-Gruppe weitgehend gegen Attacken von Reagentien, z.B. von Protonen, geschützt sein. — Somit ist eine Erklärung für die Hydrolyse-Resistenz gegeben.

Über eine Mannich-Reaktion wurde versucht, das N-(Diäthylaminomethyl)-2,6-dimethylbenzamid aus 2,6-Dimethylbenzamid, Formaldehyd und Diäthylammoniumchlorid zu synthetisieren. Da jedoch die Reindarstellung der Base durch Destillation wegen der schnellen Zersetzung unmöglich war, die dargestellten gewöhnlichen Salze unbefriedigende Analysenwerte gaben, und die Freisetzung der Base aus den Salzen zur Zersetzung führte, wurde vorläufig von dieser Synthese Abstand genommen.

Bei der Alkylierung des 2,6-Dimethylbenzamids mit 1-Diäthylamino-2-chlorpropan konnte erwartet werden, dass als hauptsächliches Reaktionsprodukt nicht das N-(2-Diäthylamino-1-methyläthyl)-2,6-dimethylbenzamid (V) entsteht, sondern das N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamid (IV). Schultz, Robb und Sprague [20] sowie andere Autoren [21-23] haben nämlich gefunden, dass bei der Alkylierung mit 1-Dialkylamino-2-chlorpropanen in alkalischem Medium eine solche Wanderung der Methylgruppe stattfindet. Der Reaktionsverlauf der Alkylierung des im alkalischen Milieu als

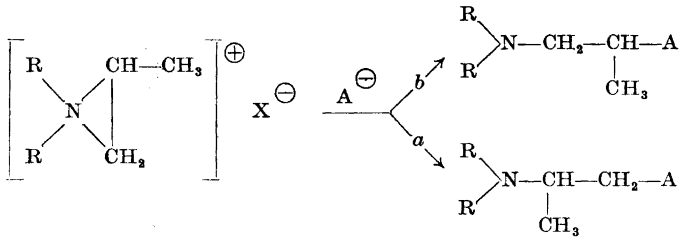


Fig. 5.

Anion vorliegenden Kupplungspartners (A^-) kann so gedeutet werden, dass aus dem 1-Dialkylamino-2-chlorpropan ein Äthylenimmoniumion als Zwischenprodukt ensteht; vgl. Fig. 5. In diesem Äthylenimmoniumion ist die Bindung zwischen Stickstoffatom und Methylengruppe leichter angreifbar, so dass der im Fig. 5 angegebene Reaktionsverlauf *a* begünstigt ist. — Bei der Alkylierung des 2,6-Dimethylbenzamids mit 1-Diäthylamino-2-chlorpropan nach Methode C trat diese oben beschriebene Umlagerung tatsächlich ein. Die sorgfältige Destillation des Reaktionsproduktes gab nur eine im Brechungsindex einheitliche Fraktion. Entsprechend obiger Hypothese sollte diese Fraktion das Umlagerungsprodukt N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamid (IV) sein. Zur Klärung dieser Frage wurde 2-Amino-1-(diäthylamino)-propan nach Breslow und Mitarbeitern [24] durch reduktive Aminierung von Diäthylaminoaceton synthetisiert. Das 1-Amino-2-(diäthylamino)propan wurde durch Reduktion des nach Luten [25] synthetisierten 2-Diäthylaminopropionitrils

erhalten. Die Diamine wurden mit dem 2,6-Dimethylbenzoylchlorid nach Methode A zur Reaktion gebracht. Wie im experimentellen Teil beschrieben, sind die physikalischen Eigenschaften (Kp., Fp., n_D^{20}) des Umlagerungsproduktes mit denen nach Methode A synthetisierten N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamids (IV) identisch. Die betreffenden Eigenschaften des Umlagerungsproduktes und des N-(2-Diäthylamino-1-methyläthyl)-2,6-dimethylbenzamids (V) unterscheiden sich hingegen eindeutig. Weiterhin zeigt die Mischschmelzpunktprobe des Umlagerungsproduktes mit dem N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamid (IV) keine Depression. — Die Wanderung der Methylgruppe hat im vorliegenden Fall offenbar quantitativ stattgefunden.

N-(2-Diäthylamino-1,2-dimethyläthyl)-2,6-dimethylbenzamid (VI) wurde durch Kupplung des 2,6-Dimethylbenzoylchlorids mit dem 2-Amino-3-(diäthylamino)butan (XIII) synthetisiert. Das Diamin erhielt man über die Stufen 3-Brom-2-butanon, 3-Diäthylamino-2-butanon und 3-Diäthylamino-2-butanon-Oxim (XII). Das 3-Diäthylamino-2-butanon ist früher von Rose und Weedon [26] durch Hydratisierung von 3-Diäthylamino-1-butin synthetisiert worden. Das dem Keton entsprechende Oxim (XII) sowie das durch die Reduktion des Oxims erhaltene 2-Amino-3-(diäthylamino)butan (XIII) sind bisher nicht in der Literatur beschrieben. Bei der Bromierung von Methyläthylketon wurde die von Catch und Mitarbeitern [27] angegebene Vorschrift befolgt. Bei der Reduktion des Ketoxims mit $LiAlH_4$ wurde eine Ausbeute von 50 %, mit Natrium und Äthanol von 60 % d.Th. erzielt. Auf eine Trennung des bei der Synthese entstehenden Gemisches von zwei Racematen musste wegen Mangel an Substanz verzichtet werden.

Analog dieser Derivate der 2,6-Dimethylbenzoesäure, waren die des 2,6-Dimethylbenzaldehyds, die Diäthylaminoalkyl-2,6-dimethylbenzylidenimine (Schiff-Basen) von Interesse. Der 2,6-Dimethylbenzaldehyd wurde nach Lock und Schmidt [9] durch Rosenmund-Reduktion [28] in rund 60 % Ausbeute erhalten. Bei der Darstellung dieses Aldehyds wurden weiterhin moderne Verfahren für die Synthese von Aldehyden geprüft. Es war nämlich von Interesse zu ermitteln, ob diese Methoden sich auch mit sterisch gehinderten Molekülen als erfolgreich erweisen würden. Nach der Aldehyd-Methode von Plieninger und Werst [2] hydriert man das betreffende Nitril katalytisch in Gegenwart von Semicarbazid und fängt den anfallanden Aldehyd als Semicarbazon ab. Diese Methode erwies sich hier als erfolglos: unter Berücksichtigung der angegebenen Versuchsbedingungen wurde kein Wasserstoff aufgenommen. — Nach Grundmann [3] gelangt man zum Benzaldehyd über die Stufen Benzoylchlorid, Diazoacetophenon, ω-Acetoxy-acetophenon und Phenylglykol (durch Reduktion nach Meerwein-Ponndorf), das durch Bleitetraacetat gespalten wird. Schon die Bildung des 2,6-Dimethyldiazoacetophenons war in wiederholten Versuchen nicht zu erreichen. Die Möglichkeit einer Hydrolyse des empfindlichen Säurechlorids, durch Spuren von Feuchtigkeit in der ätherischen Diazomethanlösung, wurde durch Einleiten von gasförmigen Diazomethan nach der Vorschrift von Arndt und Amende [29] ausgeschlossen. — Das von Weygand und Mitarbeitern [4] ausgearbeitete Verfahren, den Aldehyd aus dem entsprechenden N-Methylanilid durch Reduktion mit $LiAlH_4$ darzustellen, scheiterte ebenfalls an der Präparation des Ausgangsproduktes N-Methyl-2,6-dimethyl-

benzanilids. — Der durch Rosenmund-Reduktion des 2,6-Dimethylbenzoylchlorids in befriedigender Ausbeute dargestellte 2,6-Dimethylbenzaldehyd ist äusserst luftempfindlich und die Oxydation zur Säure ist nur eine Frage von Stunden. Daher wurden die beschriebenen Versuche unmittelbar im Anschluss an die Vacuumdestillation des Aldehyds durchgeführt. — Die Kondensation des 2,6-Dimethylbenzaldehyds mit dem 1-Amino-2-(diäthylamino)äthan und dem 1-Amino-3-(diäthylamino)propan zu den entsprechenden N-Diäthylaminoalkyl-2,6-dimethylbenzylideniminen XV und XVI war ohne Schwierigkeiten zu erzielen und verlief mit guter Ausbeute. Diese Verbindungen werden leicht durch verdünnte Säuren hydrolysiert.

CHEMISCH-EXPERIMENTELLER TEIL *

Die N-Diäthylaminoalkyl-2,6-dimethylbenzamide wurden, wie schon im theoretischen Teil (S. 1588) berichtet, nach den drei für die Synthese letztgenannter Amide allgemein anwendbaren Methoden A, B und C synthetisiert.

Methode A. In einen 250 ml Dreihalskolben, der mit einem durch Quecksilberverschluss abgedichteten Hershbergrührer, Rückflusskühler und Tropftrichter (beide mit $CaCl_2$-Rohren) versehen war, wurde eine Lösung von 0,030 Mol Diäthylaminoalkylamin in 50 ml trockenem Äther eingetragen. Unter Umrühren wurden 0,030 Mol 2,6-Dimethylbenzoylchlorid, in 25 ml Äther gelöst und unter Eiskühlung langsam eingetropft. Der Äther wurde vom Kristallbrei dekantiert und das Salz mit Äther gewaschen. Es wurde in 50 ml Wasser gelöst, die Base mit 30 % Natronlauge in Freiheit gesetzt und in Äther aufgenommen. Nach dem Trocknen über K_2CO_3 und Abdampfen des Lösungsmittels, wurde die Base durch fraktionierte Destillation als Öl erhalten. Einige Verbindungen gingen in den kristallinen Zustand über und konnten durch Umkristallisieren gereinigt werden.

Methode B. In der unter Methode A beschriebenen Apparatur wurden 0,050 Mol 2,6-Dimethylbenzoylchlorid, in 50 ml trockenem Äther gelöst und mit einer Lösung von 0,10 Mol Halogenalkylamin in 50 ml Äther zur Reaktion gebracht. Die ätherische Lösung des N-Halogenalkyl-2,6-dimethylbenzamids wurde vom ausgefallenen N-Halogenalkylammoniumchlorid abgesaugt und das Filtrat bis zur Trockenen eingeengt. Das N-Halogenalkyl-2,6-dimethylbenzamid, das bei den durchgeführten Synthesen kristallin war, wurde durch Umkristallisieren gerenigt. 0,030 Mol N-Halogenalkyl-2,6-dimethylbenzamid wurden in 50 ml über Natrium getrocknetem Benzol mit 0,10 Mol Diäthylamin fünf Stunden unter Rückfluss gekocht. Nach dem Erkalten der Lösung wurde vom abgeschiedenen Diäthylammoniumchlorid oder -bromid abgesaugt und das benzolische Filtrat erschöpfend mit verdünnter Salzsäure extrahiert. Die Base wurde dann mit 30 % Natronlauge freigesetzt und in Äther aufgenommen. Nach dem Trocknen über K_2CO_3 und Abdampfen des Äthers wurde der Rückstand wie unter Methode A beschrieben weiterbehandelt.

Methode C. Die für die Reaktion erforderliche Menge Natriumamid wurde nach der von Vaughen, Vogt und Nieuwland [30] aus metallischem Natrium und flüssigem Ammoniak mit einer Spur von Eisen (III)-nitrat als Katalysator frisch bereitet. In einem 500 ml Dreihalskolben, der mit einem durch Quecksilberverschluss abgedichteten Hershberg-Rührer und Rückflusskühler (mit $CaCl_2$-Rohr) versehen war, wurden 0,11 Mol Natriumamid in 100 ml trockenem Toluol suspendiert, 0,050 Mol 2,6-Dimethylbenzamid eingetragen und unter kräftigem Rühren eine Stunde unter Rückfluss gekocht. Dann wurde das Halogenalkyldiäthylammoniumchlorid oder -bromid zugesetzt und unter fortgesetztem Rühren weitere vier Stunden unter Rückfluss gekocht. Fand die Alkylierung des 2,6-

* Alle Schmelzpunkte sind korrigiert, alle Siedepunkte unkorrigiert. — Wenn nicht anders vermerkt, wurden die Äquivalentgewichts-Bestimmungen der synthetisierten Basen in 30 % äthanol mit 0,1 N HCl durchgeführt. Mischindikator: 1 Teil 0,1 % äthanolische Methylenblaulösung und 1 Teil 0,2 % äthanolische Methylrotlösung.

Dimethylbenzamids mit Diäthylaminoalkylhalogenid statt, so wurden 0,050 Mol Benzamid und 0,055 Mol Natriumamid angewandt. Nach dem Abkühlen auf Zimmertemperatur wurde vom ausgefallenen Natriumchlorid oder -bromid abgesaugt und die Base in verdünnter Salzsäure aufgenommen. Die saure wässerige Phase wurde stark alkalisch gemacht. Die in Freiheit gesetzte Base wurde in Äther aufgenommen und die Ätherlösung über K_2CO_3 getrocknet. Nach dem Abdampfen des Äthers wurde die Base wie unter A weiterbehandelt.

N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid (I), *N-(2-Bromäthyl)-2,6-dimethylbenzamid* (VIII) *und 2-(2,6-Dimethylphenyl)-2-oxazolin* (IX). 2-(Diäthylamino)äthylamin wurde nach der von Amundsen und Krantz [31] beschriebenen Methode synthetisiert, d.h. N-(2-Chloräthyl)diäthylammoniumchlorid wurde mit alkoholischer Ammoniaklösung behandelt. Das Diamin wurde als farbloses Öl vom Kp. 80°/85 mm erhalten (in Übereinstimmung mit dem von Amundsen und Krantz [31] angegebenen Kp.); $n_D^{20} = 1{,}4380$; Ausbeute 65 % d.Th. Nach der Methode A (S. 1596) wurden 4,9 g (0,030 Mol) 2-(Diäthylamino)-äthylamin und 5,0 g (0,030 Mol) 2,6-Dimethylbenzoylchlorid zur Reaktion gebracht und durch Destillation 4,2 g (0,017 Mol) Base, entspr. 57 % d.Th. als leicht gelbliches Öl vom Kp. 149—150°/0,5 mm erhalten. Durch chromatographische Reinigung einer *Cyclo*hexanlösung mittels Al_2O_3 (Brockmann) wurden die spurenhaften Verunreinigungen entfernt und das *N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid* (I) als farbloses Öl erhalten; $n_D^{20} = 1{,}5194$. (Gef.: Äquiv.-Gew. 249. Ber. für $C_{15}H_{24}N_2O$: 248.) N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid (I) wurde auch nach Methode B (S. 1596) synthetisiert, wobei als Nebenprodukt das 2-(2,6-Dimethylphenyl)-2-oxazolin (IX) isoliert wurde. — Nach der Vorschrift von Cortese [32] wurde Aminoäthanol durch Behandeln mit Bromwasserstoffsäure in N-(2-Bromäthyl)ammoniumbromid überführt. Die aus 29 g (0,11 Mol) des Salzes frisch bereitete Base wurde mit 8,4 g (0,050 Mol) 2,6-Dimethylbenzoylchlorid zur Reaktion gebracht. Nach dem Umkristallisieren des Rückstandes der ätherischen Lösung aus 96 % Äthanol wurde das *N-(2-Bromäthyl)-2,6-dimethylbenzamid* (VIII) in Form farbloser, blättchenförmiger Kristalle vom Fp. 126° erhalten. Ausbeute 12 g (0,047 Mol), entspr. 94 % d.Th. (Gef.: C 51,4; H 5,60; Br 31,4; N 5,57. Ber. für $C_{11}H_{14}BrNO$ (256,15): C 51,6; H 5,51; Br 31,2; N 5,47.) Die Diäthylaminobase wurde durch Behandeln von 8,0 g (0,031 Mol) N-(2-Bromäthyl)-2,6-dimethylbenzamid (VIII) mit 7,3 g (0,10 Mol) Diäthylamin dargestellt. Bei der fraktionierten Destillation des Reaktionsproduktes wurden zwei Hauptfraktionen erhalten: 1) Kp. 90—93°/0,5 mm; $n_D^{20} = 1{,}5355$; Ausbeute 2,0 g. 2) Kp. 149—150°/0,5 mm; $n_D^{20} = 1{,}5192$; Ausbeute 3,8 g.

Die niedriger siedende Fraktion 1 erwies sich als das *2-(2,6-Dimethylphenyl)-2-oxazolin* (IX). (Gef.: C 75,4; H 7,63; N 8,18. Ber. für $C_{11}H_{13}NO$ (175,2): C 75,4; H 7,48; N 8,00.) Die oben angegebene Ausbeute von 2,0 g entspricht demnach 0,013 Mol oder 42 % d.Th. — Typisch für die Oxazoline ist die Reaktion mit Halogenwasserstoffsäuren. Das Oxazolin wird dabei unter Ringöffnung in das entsprechende N-Halogenalkylbenzamid verwandelt. Mit Bromwasserstoffsäure sollte demnach IX die Ausgangsverbindung VIII geben. Um dies zu bestätigen wurde IX mit konzentrierter Bromwasserstoffsäure im Überschuss bis zur Sirupkonsistenz auf dem Wasserbad eingeengt. Auf Zugabe von Wasser ging nur ein geringer Teil in Lösung. Der körnige Rückstand hatte einen Schmelzpunkt von 123° und nach dem Umkristallisieren aus 96 % Äthanol von 126°. Der Mischschmelzpunkt mit N-(2-Bromäthyl)-2,6-dimethylbenzamid (VIII) zeigte keine Depression.

Die höher siedende Fraktion 2 war mit dem nach Methode A (S. 1596) synthetisierten N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzamid (I) identisch. (Gef.: Äquiv.-Gew. 249. Ber. für $C_{15}H_{24}N_2O$: 248.) Die oben angegebene Ausbeute von 3,8 g entspricht demnach 0,015 Mol oder 50 % d.Th.

N-(2-Diäthylaminoäthyl)-N-methyl-2,6-dimethylbenzamid (II). Das N-(2-Diäthylaminoäthyl)methylamin wurde früher von Kermack und Wight [33] aus N-(2-Bromäthyl)-diäthylammoniumbromid und alkoholischer Methylaminlösung synthetisiert. Die Versuchsbedingungen wurden von den Autoren nicht näher beschrieben. Das Diamin wurde hier auf folgende Weise synthetisiert: Im Autoclaven wurden 45 g (0,18 Mol) N-(2-Bromäthyl)diäthylammoniumbromid mit 90 g einer 33 % Lösung von Methylamin in absolutem Äthanol 20 Stunden bei 100° erhitzt. Nach dem Ansäuern mit Salzsäure wurde bis zur Sirupkonsistenz im Vacuum eingeengt, die Base mit Natronlauge in Freiheit gesetzt, in

Äther aufgenommen und die Lösung über K_2CO_3 getrocknet. Nach dem Abdampfen des Lösungsmittels wurde durch Destillation eine farblose Flüssigkeit vom Kp. 44°/10 mm (lt.Lit.[33]: 158°/760 mm) erhalten; n_D^{20} = 1,4340; Ausbeute 7,2 g (0,056 Mol), entspr. 32 % d.Th. — Nach der Methode A wurden 4,2 g (0,025 Mol) 2,6-Dimethylbenzoylchlorid und 3,6 g (0,028 Mol) N-(2-Diäthylaminoäthyl)methylamin zur Reaktion gebracht. Durch fraktionierte Destillation wurde die Base als farbloses Öl vom Kp. 135—137°/0,6—0,7 mm erhalten; n_D^{20} = 1,5145; Ausbeute 4,3 g (0,016 Mol), entspr. 64 % d.Th. (Gef.: Äquiv.-Gew. 262. Ber. für $C_{16}H_{26}N_2O$: 262.)

N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III), *N-(3-Brompropyl)-2,6-dimethylbenzamid* (X) *und 2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin* (XI). 1-Amino-3-(diäthylamino)propan wurde durch Reduktion des nach Terent'ev und Kost [34] sowie Whitmore und Mitarbeiter [35] durch Addition von Diäthylamin und Acrylnitril dargestellten 3-Diäthylaminopropionitrils mit Natrium und Äthanol in 30 % Ausbeute erhalten. Die katalytische Hydrierung nach letzteren Autoren ergab eine Ausbeute von 45 %. Die Reduktion wurde auch mit Lithium-Aluminiumhydrid nach der von Nystrom und Brown [36] allgemein angegebenen Methode durchgeführt: Zu 300 ml über Natrium und Lithium-Aluminiumhydrid getrockneten Äther in einem Dreihalskolben, der mit einem durch Quecksilberverschluss abgedichteten Hershberg-Rührer, Rückflusskühler und Tropftrichter (beide mit $CaCl_2$-Rohren) versehen war, wurden 7,6 g (0,20 Mol) $LiAlH_4$ gegeben. 25 g (0,20 Mol) 3-Diäthylaminopropionitril, in 200 ml Äther gelöst, wurden unter Rühren so zugetropft, dass das Reaktionsgemisch in gelindes Sieden geriet. Nach einstündigem Sieden unter Rückfluss wurde das überschüssige $LiAlH_4$ mit 25 ml Äthylacetat und der graue Komplex mit 50 ml 10 N Natronlauge unter Eiskühlung zerlegt. Es wurde vom Salzrückstand abgesaugt, die ätherische Lösung über Na_2SO_4 getrocknet, das Lösungsmittel abgedampft und der Rückstand fraktioniert destilliert. Das Diamin ging als farblose Flüssigkeit bei 64°/22 mm über; n_D^{20} = 1,4445 (nach Shriner und Hickey [37]: n_D^{20} = 1,4437); Ausbeute 14 g (0,11 Mol), entspr. 50 % d.Th. Nach der Methode A wurde das *N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid* (III) synthetisiert und die Base durch Destillation und nachherige Reinigung einer *Cyclo*hexanlösung durch Schütteln mit Al_2O_3 (Brockmann) als farbloses Öl erhalten. Kp. 160—161°/0,6 mm; n_D^{20} = 1,5143. Das Öl erstarrte zu farblosen, nadelförmigen Kristallen, die nach dem Umkristallisieren aus Pentan einen Schmelzpunkt von 44° hatten. Ausbeute 75 % d.Th. (Gef.: Äquiv.-Gew. 263. Ber. für $C_{16}H_{26}N_2O$: 262.)

Die gleiche Verbindung wurde auch nach Methode B (S. 1596) synthetisiert, wobei als Nebenprodukt das 2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin(XI) isoliert wurde. N-(3-Brompropyl)ammoniumbromid wurde durch Gabriel-Synthese aus Kaliumphthalimid und 1,3-Dibrompropan und anschliessende Hydrolyse des N-(3-Brompropyl) phthalimids mit Bromwasserstoffsäure in Eisessig nach Hamer und Rathbone [38] synthetisiert. Fp. 163° (lt.Lit.[38]: 161—163°). Das Amid wurde durch Kupplung von aus 6,4 g (0,028 Mol) N-(3-Brompropyl)ammoniumbromid frisch bereiteter Base und 4,5 g (0,028 Mol) 2,6-Dimethylbenzoylchlorid gewonnen. Nach dem Umkristallisieren aus einer Mischung Methylalkohol-Wasser 3:1 (V/V) wurde das *N-(3-Brompropyl)-2,6-dimethylbenzamid* (X) in Form farbloser, fettigglänzender, blättchenförmiger Kristalle vom Fp. 86° erhalten. Ausbeute 4,4 g (0,023 Mol), entspr. 83 % d.Th. (Gef.: C 53,4; H 5,74; Br 29,9; N 5,31. Ber. für $C_{12}H_{16}BrNO$ (270,2): C 53,3; H 5,97; Br 29,6; N 5,19.) 4,4 g (0,023 Mol) N-(3-Brompropyl)-2,6-dimethylbenzamid (X) wurden mit 5,1 g (0,070 Mol) Diäthylamin zur Reaktion gebracht. Bei der fraktionierten Destillation des Reaktionsproduktes fielen zwei distinkt siedende Fraktionen an: 1) Kp. 95—98°/0,5 mm; n_D^{20} = 1,5395; Ausbeute 0,96 g. 2) Kp. 160°/0,5 mm; n_D^{20} = 1,5143; Ausbeute 3,8 g.

Bei der ersten Fraktion handelt es sich um das *2-(2,6-Dimethylphenyl)-5,6-dihydro-1,3,4*H*-oxazin* (XI). (Gef.: C 75,7; H 8,10; N 7,41. Ber. für $C_{12}H_{15}NO$ (189,3): C 76,1; H 7,99; N 7,40.) Die oben angegebene Ausbeute von 0,96 g entspricht demnach 0,0051 Mol oder 23 % d.Th. Diese Substanz gab ebenfalls die für die Oxazoline charakteristische Reaktion (vgl. unter Subst. IX, S. 1597). Es wurde also durch Behandeln mit Bromwasserstoffsäure im Überschuss das N-(3-Brompropyl)-2,6-dimethylbenzamid (X) zurückerhalten.

Die zweite Fraktion entsprach dem *N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid* (III) (vgl. oben). (Gef.: Äquiv.-Gew. 261. Ber. für $C_{16}H_{26}N_2O$: 262.) Die oben angegebene Ausbeute von 3,8 g entspricht demnach 0,015 Mol oder 65 % d.Th.

N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) wurde auch nach Methode C (S. 000) synthetisiert: N-(3-Brompropyl)diäthylammoniumbromid wurde früher von Marvel, Zartmann und Bluthardt [39] durch Spaltung des von Lohmann [40] synthetisierten 1-Diäthylamino-3-phenoxypropans mit Bromwasserstoffsäure dargestellt. Es wurde nun unter Modifizierung des von Cortese [32] zur Darstellung des N-(2-Bromäthyl)ammoniumbromids ausgearbeiteten Methode durch Behandeln des 3-Diäthylamino-1-propanols mit Bromwasserstoffsäure dargestellt. Das 3-Diäthylamino-1-propanol erhielt man durch Addition von Diäthylamin und Allylalkohol nach Gawron und Spoerri [41]. In einem 500 ml Rundkolben wurden 50 g (0,38 Mol) 3-Diäthylamino-1-propanol auf 0° gekühlt und unter kräftigem Rühren mit 350 ml (2,59 Mol) Bromwasserstoffsäure (d = 1,49) gemischt. Dann wurden über eine 45 cm hohe Widmer-Kolonne 90 ml Wasser abdestilliert und das Reaktionsgemisch anschliessend zwei Stunden bei gelindem Sieden unter Rückfluss gehalten. Weitere 35 ml, 30 ml und 20 ml Wasser wurden abdestilliert und zwischen den Destillationen zwei Stunden, am Ende der Reaktion noch drei Stunden unter Rückfluss gekocht. Nach Abdestillieren von 115 ml Bromwasserstoffsäure wurde der tiefbraune Rückstand mit Äther gewaschen und zur Entfärbung mit Aktivkohle (Norit) 15 Min. auf dem siedenden Wasserbad erhitzt. Das fast farblose Filtrat wurde im Vacuum bis zur Trockenen eingedampft. Der wachsartige, leicht gelbliche Rückstand wurde bei 40–45° in 100 ml Butanol gelöst und das Hydrobromid mit 300 ml Äther ausgefällt. Es wurde vom Lösungs- und Fällungsmittel abgesaugt und das fast farblose, stark hygroskopische Salz über P_2O_5 getrocknet. Fp. 91–92° (lt.Lit.[39]: 91–94°); Ausbeute 71 g (0,26 Mol), entspr. 68 % der Theorie. 7,5 g (0,050 Mol) 2,6-Dimethylbenzamid, 14,3 g (0,050 Mol) N-(3-Brompropyl)diäthylammoniumbromid und 4,4 g (0,11 Mol) Natriumamid wurden nach Methode C zur Reaktion gebracht. Die Base wurde durch fraktionierte Destillation und nachherige Reinigung einer *Cyclo*hexanlösung durch Schütteln mit Al_2O_3 (Brockmann) als farbloses Öl vom Kp. 158–160°/0,5 mm erhalten; n_D^{20} = 1,5145. Das Öl erstarrte bald und kristallisierte aus Pentan in Form nadelförmiger Kristalle vom Fp. 43–44°. Ausbeute 11,5 g (0,0438 Mol), entspr. 88 % d.Th. (Gef.: Äquiv.-Gew. 261. Ber. für $C_{16}H_{26}N_2O$: 262.)

N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamid (IV). Nach der von Goldfarb [42] abgeänderten Methode von Callsen und Hahl [43] wurde N-(2-Chlorpropyl)diäthylammoniumchlorid durch Addition von Diäthylamin an Propylenoxyd und anschliessende Behandlung des 1-Diäthylamino-2-propanols mit Thionylchlorid in 50 % Gesamtausbeute erhalten. Fp. 107° (lt.Lit.[42]: 107°). Nach der Methode C (S. 1596) wurden 7,5 g (0,050 Mol) 2,6-Dimethylbenzamid, aus 2,3 g (0,10 Mol) Natrium frisch bereitetes Natriumamid und 9,2 g (0,050 Mol) N-(2-Chlorpropyl)diäthylammoniumchlorid zur Reaktion gebracht. Die durch fraktionierte Destillation gewonnene Base ging als farbloses Öl vom Kp. 162°/0,5 mm über; n_D^{20} = 1,5165. Das Öl erstarrte bald zu einer Kristallmasse und wurde nach dem Umkristallisieren aus Pentan in Form weisser, blättchenförmiger Kristalle vom Fp. 56° erhalten. Ausbeute 6,3 g (0,024 Mol), entspr. 48 % d.Th. (Gef.: Äquiv.-Gew. 263. Ber. für $C_{16}H_{26}N_2O$: 262.) Die gleiche Verbindung wurde nach Methode A (S. 1596) erhalten: 1-Amino-2-(diäthylamino)propan wurde durch Reduktion des 2-Diäthylaminopropionitrils gewonnen. Das Nitril wurde nach der von Luten [25] angegebenen Methode durch Kondensation von Acetaldehyd, Diäthylamin und Kaliumcyanid synthetisiert. Bei der Reduktion mit Natrium und Äthanol wurde die von Magidson und Grigorowsky [44] für die Reduktion des 6-Diäthylaminocapronitrils angegebene Vorschrift befolgt. Das Diamin siedete bei 57°/16 mm; n_D^{20} = 1,4390; Ausbeute 55 % d.Th. (Die Verbindung ist früher [45] durch Behandeln von 1-Chlor-2-diäthylaminopropan mit methylalkoholischem Ammoniak dargestellt worden.) Das 1-Amino-2-(diäthylamino)propan und das 2,6-Dimethylbenzoylchlorid wurden dann nach Methode A zur Reaktion gebracht. Durch Destillation und darauffolgende Reinigung einer *Cyclo*hexanlösung durch Schütteln mit Al_2O_3 (Brockmann) wurde das *N-(2-Diäthylamino-2-methyläthyl)-2,6-dimethylbenzamid* (IV) als farbloses Öl vom Kp. 158–160°/0,4 mm erhalten; n_D^{20} = 1,5163. Das Öl erstarrte zu einem farblosen Kristallbrei. Nach dem Umkristallisieren aus Petroläther (Kp. 45–65°) wurden farblose, rhombische Kristalle vom Fp. 56–57° erhalten. Der Schmelzpunkt

stimmt also mit dem nach Methode C (S. 1596) synthetisierten Umlagerungsprodukt (IV) überein. Weiterhin zeigt der Mischschmelzpunkt mit beiden Substanzen keine Depression.

N-(2-Diäthylamino-1-methyläthyl)-2,6-dimethylbenzamid (V). Nach Breslow und Mitarbeitern [24] wurde 2-Amino-1-(Diäthylamino)propan durch reduktive Aminierung von Diäthylaminoaceton dargestellt. Aus diesem Diamin wurde nach Methode A (S. 1596) das *N-(2-Diäthylamino-1-methyläthyl)-2,6-dimethylbenzamid* (V) als farbloses Öl vom Kp. 152–153°/0,7 mm gewonnen; n_D^{20} = 1,5120; Ausbeute 67 % d.Th. Das Öl erstarrte zu farblosen Kristallen, die nach dem Umkristallisieren aus Pentan in Form weisser, blättchenförmiger Kristalle vom Fp. 50° erhalten wurden. (Gef.: Äquiv.-Gew. 262. Ber. für $C_{16}H_{26}N_2O$, 262.)

N-(2-Diäthylamino-1,2-dimethyläthyl)-2,6-dimethylbenzamid (VI), *3-Diäthylamino-2-butanon-Oxim* (XII) und *2-Amino-3-(diäthylamino)butan* (XIII). 3-Brom-2-butanon wurde nach der Vorschrift von Catch und Mitarbeitern [27] durch Bromierung von Methyläthylketon und anschliessende wiederholte fraktionierte Destillation dargestellt. 3-Diäthylamino-2-butanon wurde in der unterMethode B (S. 000) schon beschriebenen Weise durch Behandeln von 3-Brom-2-butanon mit Diäthylamin gewonnen. Es wurde als farblose Flüssigkeit vom Kp. 57–58°/12 mm erhalten; n_D^{20} = 1,4265; Ausbeute 83 % d.Th. (Gef.: Äquiv.-Gew. 145. Ber. für $C_8H_{17}NO$: 144.) Der hier gefundene Siedepunkt des Aminoketons stimmt mit denen von Rose und Weedon [26] angegebenen überein; sie erhielten die Verbindung durch Hydratisierung von 3-Diäthylamino-1-butin. Die Oximierung wurde in Anlehnung an eine Vorschrift von Shriner und Fuson [46] vorgenommen: Zu 15 g (0,11 Mol) Keton wurde unter Umschütteln und Eiskühlung eine Lösung von 10 g (0,15 Mol) Hydroxylaminhydrochlorid in 25 ml Wasser gegossen und über Nacht bei Zimmertemperatur stehen gelassen. Die Lösung wurde mit 50 ml Äther überschichtet und eine Lösung von 12 g Natriumhydroxyd in 12 ml Wasser und festes Kaliumkarbonat bis zur Sättigung zugefügt. Die wässerige Phase wurde dreimal mit je 50 ml Äther ausgeschüttelt und die vereinigten Ätherextrakte über K_2CO_3 getrocknet. Der Äther wurde abgedampft und das Oxim als farbloses Öl vom Kp. 113–114°/12 mm durch Destillation gewonnen; n_D^{20} = 1,4710. Das 3-Diäthylamino-2-butanon-Oxim (XII) kristallisierte bald in farblosen Nadeln vom Fp. 52°; Ausbeute 12 g (0,076 Mol), entspr. 69 % d.Th. (Gef.: Äquiv.-Gew. 158. Ber. für $C_8H_{18}N_2O$: 158.) In der gleichen Weise, wie unter Verbindung (III) (S. 000) beschrieben, wurden 11,5 g (0,073 Mol) Ketoxim mit 3,8 g (0,10 Mol) $LiAlH_4$ in Äther reduziert. Das *2-Amino-3-(diäthylamino)butan* (XIII) wurde als farblose Flüssigkeit vom Kp. 60°/12 mm erhalten; n_D^{20} = 1,4380; Ausbeute 5,5 g (0,038 Mol), entspr. 50 % d.Th. (Gef.: C 66,3; H 13,7; N 19,6. Ber. für $C_8H_{20}N_2$ (144,3): C 66,6; H 13,9; N 19,5.) Das Oxim wurde auch mit Natrium und Äthanol reduziert: In einem mit Quecksilberverschluss abgedichteten Hershberg-Rührer und mit Rückflusskühler versehenen Dreihalskolben wurden 46 g (2,0 Mol) Natrium in 100 ml trockenem Toluol fein verteilt und unter kräftigem Rühren eine Lösung von 28 g (0,18 Mol) Oxim in 250 ml absolutem Äthanol aus einem Tropftrichter unter Ausschluss der Luftfeuchtigkeit langsam zugetropft. Nach dem Eintragen des Oxims wurde das Reaktionsgemisch noch eine Stunde unter fortgesetzem Rühren in gelindem Sieden gehalten bis das Natrium vollständig in Lösung gegangen war. Es wurde unter Eiskühlung mit konzentrierter Salzsäure angesäuert, im Vacuum bis zur Sirupkonsistenz eingeengt und die Base, wieder unter Eiskühlung, mit festem Kaliumhydroxyd in Freiheit gesetzt. Sie wurde in Äther aufgenommen und in der gleichen Weise wie oben beschrieben behandelt. Ausbeute 16 g (0,11 Mol), entspr. 60 % d.Th. (Gef.*: Äquiv.-Gew. 72,6. Ber. für $C_8H_{20}N_2$ (144,3): 72,2.) 4,7 g (0,028 Mol) 2,6-Dimethylbenzoylchlorid und 5,5 g (0,036 Mol) 2-Amino-3-(diäthylamino)butan (XIII) wurden nach Methode A (S. 1596) zur Reaktion gebracht. Das *N-(2-Diäthylamino-1,2-dimethyläthyl)-2,6-dimethylbenzamid* (VI) wurde durch fraktionierte Destillation als leicht gelbliches Öl vom Kp. 162°/0,5 mm erhalten. Die spurenhaften Verunreinigungen wurden durch chromatographische Reinigung einer *Cyclo*hexanlösung mittels Al_2O_3 (Brockmann) entfernt und die Base als farb-

* Titration der Base in wasserfreiem Medium (Eisessig) mit 0,1 N Überchlorsäure, in wasserfreiem Eisessig gelöst, gegen den Indikator BZL-Blau (Ciba) nach der Methode von Riddick [47].

loses Öl, dass bald zu farblosen, prismenartigen Kristallen vom Fp. 39° erstarrte, fast quantitativ zurückerhalten. Aus Pentan kristallisierte die Verbindung in Form von Nadeln vom Fp. 40°. — Auf eine Trennung der beiden Racemate musste wegen Substanzmangel verzichtet werden. (Gef.: Äquiv.-Gew. 275. Ber. für $C_{17}H_{28}N_2O$: 276.)

N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid (VII). 3,5-Dimethylbenzoesäure wurde durch Oxydation von Mesitylen mit verdünnter Salpetersäure nach der von Snyder, Adams und McIntosh [48] beschriebenen Methode gewonnen. Fp. 165—166° (lt.Lit.[48]: 165—167°). Das 3,5-Dimethylbenzoylchlorid wurde in der üblichen Weise durch Behandeln mit Thionylchlorid erhalten. Kp. 116°/13 mm; n_D^{20} = 1,5465. (Kp. lt. Weiler [49]: 110°/10 mm) Nach der Methode A (S. 1596) wurden aus 6,7 g (0,040 Mol) 3,5-Dimethylbenzoylchlorid und 5,2 g (0,040 Mol) 1-Amino-3-(diäthylamino)propan 7,5 g (0,029 Mol) *N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid* (VII), entspr. 73 % d.Th., als farbloses Öl vom Kp. 174°/0,4 mm erhalten; n_D^{20} = 1,5200. (Gef.: Äquiv.-Gew. 261. Ber. für $C_{16}H_{26}N_2O$: 262.)

N,N-Dimethyl-2,6-dimethylbenzamid (XIV). In einem 250 ml Dreihalskolben, der mit einem durch Quecksilberverschluss abgedichteten Hershbergrührer, Rückflusskühler und Tropftrichter (beide mit $CaCl_2$-Rohren) versehen war, wurde eine Lösung von 4,5 g (0,062 Mol) Dimethylamin in 50 ml über Natrium getrocknetem Äther eingetragen. Unter kräftigem Rühren und Eiskühlung wurden 5,0 g (0,030 Mol) 2,6-Dimethylbenzoylchlorid, in 25 ml Äther gelöst, langsam zutropfen lassen. Es wurde vom Niederschlag des N,N-Dimethylammoniumchlorids abgesaugt und der Rückstand in 50 ml Wasser aufgenommen. Aus der mit Schwefelsäure schwach angesäuerten Lösung wurde das Amid im Perforator mit Äther extrahiert. Nach dem Trocknen der ätherischen Lösung über Na_2SO_4 und Abdampfen des Äthers wurde der zähflüssige, ölige Rückstand im Vacuum destilliert. Das leicht gelbliche Öl vom Kp. 118—119°/2,5 mm erstarrte bald. Es wurde in einer Mischung Petroläther (Kp. 50—60°)-Kohlenstofftetrachlorid 4:1 (V/V) aufgelöst und die Lösung dann auf eine Säule gegossen, die aus Al_2O_3 (Brockmann) und der Flüssigkeitsmischung bereitet war. Mittels dieser Mischung wurde dann das Chromatogramm entwickelt. Das N,N-Dimethyl-2,6-dimethylbenzamid lief ziemlich schnell durch, während die gelben Verunreinigungen sehr fest zurückgehalten wurden. Das Lösungsmittel wurde abgetrieben und der farblose Rückstand aus 96 % Äthanol umkristallisiert. Es wurden weisse, rhombische Kristalle erhalten. Fp. 62—63°; Ausbeute 4,3 g (0,024 Mol), entspr. 80 % d.Th. (Gef.: C 74,3; H 8,47; N 7,91. Ber. für $C_{11}H_{15}NO$: C 74,5; H 8,53; N 7,94.)

N-(2-Diäthylaminoäthyl)-2,6-dimethylbenzylidenimin (XV). In einem 15 ml Erlenmeyer-Kölbchen wurden 1,32 g (0,00985 Mol) 2,6-Dimethylbenzaldehyd mit 1,2 g (0,011 Mol) 2-(Diäthylamino)äthylamin 15 Min. auf siedendem Wasserbad erhitzt. Das Reaktionsgemisch trübte sich bald. Es wurde in Äther aufgenommen und über K_2CO_3 getrocknet. Nach dem Abdampfen des Lösungsmittels wurde die Schiff-Base durch Destillation als farblose Flüssigkeit vom Kp. 140—141°/0,5 mm erhalten; n_D^{20} = 1,5230; Ausbeute 1,28 g (0,00551 Mol), entspr. 50 % d.Th. (Gef.: C 77,9; H 10,5; N 12,2. Ber. für $C_{15}H_{24}N_2$ (232,3): C 77,6; H 10,4; N 12,1.)

N-(3-Diäthylaminopropyl)-2,6-dimethylbenzylidenimin (XVI). In der gleichen Weise wie unter Substanz (XV) beschrieben, wurden aus 0,65 g (0,0050 Mol) 1-Amino-3-(diäthylamino)propan und 0,65 g (0,0049 Mol) 2,6-Dimethylbenzaldehyd die Schiff-Base als farblose Flüssigkeit vom Kp. 129—130°/0,6 mm erhalten; n_D^{20} = 1,5200; Ausbeute 1,0 g (0,0039 Mol), entspr. 80 % d.Th. (Gef.: C 78,8; H 10,3; N 11,2. Ber. für $C_{17}H_{26}N_2$ (258,4): C 79,0; H 10,1; N 10,9.)

Vergleichender Hydrolyseversuch des N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamids (III) *und des N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamids* (VII). Es wurden 0,60 g (0,0023 Mol) N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) und 1,0 g (0,0038 Mol) N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid (VII) mit je 20 ml 10 % Schwefelsäure vier Stunden unter Rückfluss bei einer Ölbadtemperatur von 130° gekocht. Das Reaktionsgemisch, dass das N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamid (III) enthielt, war völlig klar. Die schwefelsaure Lösung wurde mit Äther ausgeschüttelt und der Äther abgedampft. Es hinterblieb nur ein spurenhafter Rückstand. Die wässerige Phase wurde stark alkalisch gemacht und die ausgefallene Base in Äther aufgenommen. Die ätherische Lösung wurde über K_2CO_3 getrocknet und das Lösungsmittel vollständig abgedampft. Der fast farblose ölige Rückstand hatte einen Brechungsindex n_D^{20} = 1,5140

(n_D^{20} des N-(3-Diäthylaminopropyl)-2,6-dimethylbenzamids (III) = 1,5144). Ausbeute 0,55 g (0,0021 Mol), entspr. 91 % d.Th. (Gef.: Äquiv.-Gew. 262. Ber. für $C_{16}H_{26}N_2O$: 262.)

Das im Rückflusskühler befindliche Sublimat und der im Kölbchen mit dem N-(3-Diäthylaminopropyl)-3,5-dimethylbenzamid (VII) befindliche Niederschlag wurden in Äther aufgenommen. Nach dem Trocknen der ätherischen Lösung über Na_2SO_4 konnten 0,49 g (0,0033 Mol), entspr. 85 % d.Th., 3,5-Dimethylbenzoesäure vom Fp. 164° erhalten werden. Der Mischschmelzpunkt mit der reinen 3,5-Dimethylbenzoesäure zeigte keine Depression.

PHARMAKOLOGISCHER TEIL

Die Verbindungen I—VII wurden auf ihre lokalanästhetische Wirksamkeit an der Kaninchencornea nach der Technik von Wiedling [50] geprüft und mit der Wirksamkeit des Xylocains verglichen. Um die Veränderung der in den Xylocainverbindungen vorkommenden Gruppierung Ar·NH·CO- (Anilid-Typus) in die in den hier aktuellen Verbindungen enthaltene Gruppierung Ar·CO·NH- (Benzamid-Typus) in ihrer pharmakologischen Auswirkung zu studieren, wurde auch das früher [5,8,51] beschriebene Xylocainanalog β-Diäthylamino-2,6-dimethylpropionanilid, $2,6\text{-}(CH_3)_2C_6H_3\cdot NH\cdot CO\cdot CH_2\cdot CH_2\cdot N(C_2H_5)_2$ mit untersucht. Diese Substanz unterscheidet sich von der Benzamid-Verbindung I, $2,6\text{-}(CH_3)_2C_6H_3\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot N(C_2H_5)_2$, lediglich durch die Umkehrung der Gruppierung -NH·CO-.

Für die Prüfung wurden sämtliche Basen in Wasser mittels Salzsäure gelöst. Diese Lösungen bestimmter Konzentration wurden alle auf ein pH von 5,5 eingestellt und die physiologische Menge Kochsalz zugesetzt.

Bei der Testung einer Substanz wurde die Konzentration aufgesucht, bei der die Lösung dieselbe Duration wie eine 0,0738 molare Lösung von Xylocainhydrochlorid (2 %-ige Lösung von Xylocain·HCl) aufwies. Die Aktivität letzterer Lösung wurde gleich eins gesetzt. Die *relative Aktivität*, RA, einer Substanz wurde berechnet als der Quotient zwischen der Molarität der Standard-Lösung von Xylocain (= 0,0738 M) und der gefundenen Molarität der Testlösung. Die Auswertung einer Substanz wurde mit 10—20 Tieren ausgeführt und der Mittelwert berechnet. Falls eine Substanz bei der Applikation einer 0,0738 M Lösung keine messbare Anästhesie hervorrief, so wurde die Verbindung als *inaktiv* bezeichnet.

Die Toxizität der Substanzen wurde mittels subcutaner Injektionen an weissen Mäussen bestimmt. Für jede Substanz wurden ungefär 50 Tiere verwendet und der LD_{50}-Wert berechnet. Die LD_{50}-Werte werden hier in zwei verschiedenen Massen angegeben, nähmlich (a) in g per kg Körpergewicht und (b) in Mol per kg Körpergewicht („molarer" LD_{50}-Wert).

Den Quotient aus dem *molaren* LD_{50}-Wert von Xylocain und dem entsprechenden Wert einer der anderen Substanzen, bezeichnen wir als die *relative Toxizität*, RT, der Substanz. Für den Quotienten RA/RT führen wir die Bezeichung *anästhetischer Index*, Q, ein.

In der Tabelle 2 sind die Resultate der pharmakologischen Messungen zusammengestellt, Ein Vergleich zwischen dem Xylocainhomolog B (Anilid-Typus) und der isomeren Substanz I (Benzamid-Typus) zeigt, dass die Umkehr der Gruppierung -NH·CO- in die Gruppierung -CO·NH-, mit einer

Tabelle 2. Lokalanästhetische Aktivität (Kaninchencornea) und subcutane Toxizität (weisse Maus) von neun Verbindungen. Für die Begriffe *relative anästhetische Aktivität* (RA), *relative Toxizität* (RT) und *anästhetischer Index* (Q), siehe S. 1602.

	Verbindung	Relative anästhetische Aktivität (RA) Xylocain = 1	Toxizität, LD_{50}		Relative Toxizität (RT) Xylocain = 1	Q
			g Base/kg	Mol/kg · 10^3		
A	2,6-$(CH_3)_2C_6H_3$·NH·CO·CH_2·N$(C_2H_5)_2$ (=Xylocain)	1,0	0,34	1,4	1,0	1,0
B	2,6-$(CH_3)_2C_6H_3$·NH·CO·$(CH_2)_2$·N$(C_2H_5)_2$	1,3	0,30	1,2	1,2	1,1
I	2,6-$(CH_3)_2C_6H_3$·CO·NH·$(CH_2)_2$·N$(C_2H_5)_2$	0,51	0,57	2,3	0,61	0,84
II	2,6-$(CH_3)_2C_6H_3$·CO·N(CH_3)·$(CH_2)_2$·N$(C_2H_5)_2$	inaktiv [a]	~1,0	~3,8	~0,4	—
III	2,6-$(CH_3)_2C_6H_3$·CO·NH·$(CH_2)_3$·N$(C_2H_5)_2$	inaktiv [a]	~0,9	~3,4	~0,4	—
IV	2,6-$(CH_3)_2C_6H_3$·CO·NH·CH_2·CH(CH_3)·N$(C_2H_5)_2$	1,8	0,23	0,88	1,6	1,1
V	2,6-$(CH_3)_2C_6H_3$·CO·NH·CH(CH_3)·CH_2·N$(C_2H_5)_2$	2,1	0,22	0,84	1,7	1,2
VI	2,6-$(CH_3)_2C_6H_3$·CO·NH·CH(CH_3)·CH(CH_3)·N$(C_2H_5)_2$	3,4	0,075	0,27	5,2	0,65
VII	3,5-$(CH_3)_2C_6H_3$·CO·NH·$(CH_2)_3$·N$(C_2H_5)_2$	0,88	0,71	2,7	0,52	1,7

[a] Hinsichtlich des Begriffes inaktiv, siehe S. 1602.

Erniedrigung der Aktivität (RA) um etwa 60 % einhergeht. Gleichzeitig wird die Toxizität (RT) um etwa 50 % herabgesetzt, so dass der anästhetische Index (Q) ungefähr 25 % niedriger wird. Wenn in I der Amidwasserstoff gegen eine Methylgruppe ausgetauscht wird (I → II), so ist die resultierende Verbindung II „inaktiv" * und hat im Vergleich mit I eine um etwa 30 % niedrigere Toxizität. Dieselben Veränderungen sowohl an Aktivität als auch an Toxizität erhält man bei Verlängerung der zweigliedrigen Methylenkette in I zu einer dreigliedrigen in III. Verzweigt man in I die Seitenkette, —CO·NH·CH_2·CH_2·N$(C_2H_5)_2$, mit einer Methylgruppe an einer der beiden Methylengruppen, so steigt in beiden Fällen (I → IV, I → V) die Aktivität um das drei- bis vierfache, während gleichzeitig die Toxizität ungefähr dreimal erhöht wird. Man beachte in diesem Zusammenhang, dass unter den drei isomeren Verbindungen III, IV und V, die eine Trimethylenkette, -CH_2·CH_2·CH_2-, enthaltende Verbindung III „inaktiv" ist, während die beiden anderen Verbindungen, die statt dessen eine methylsubstituierte Äthylenkette, -CH_2-CH(CH_3)-

* Hinsichtlich des Begriffes inaktiv — siehe oben.

oder $-CH(CH_3)-CH_2-$, besitzen, beide ungefähr 100 % aktiver sind als Xylocain. Die „inaktive" Verbindung III hat eine Toxizität, die mindestens um 50 % niedriger ist als die des Xylocains, während IV und V Toxizitäten haben, die im Vergleich mit Xylocain mindestens um 50 % höher sind. Führt man in I Verzweigungen an beiden Methylengruppen ein (I → VI), so wird die Aktivität ungefähr siebenmal und die Toxizität acht- bis neunmal gesteigert.

Schliesslich mag erwähnt werden dass, wenn man in der Verbindung III, die — wie oben besprochen — „inaktiv" ist, die 2,6-Stellung der Kernmethylgruppen zur 3,5-Stellung verändert, eine Verbindung VII resultiert, die beinahe so aktiv ist wie Xylocain. Im Vergleich mit III hat VII ungefähr dieselbe Toxizität, und mit Xylocain verglichen ist die Verbindung um etwa 50 % weniger toxisch.

Zwei von den synthetisierten Verbindungen, nähmlich I und VII wurden auf ihre infiltrationsanästhetische Wirksamkeit beim Menschen geprüft und mit Xylocain verglichen. Um auch hier den Anilid-Typus mit dem Benzamid-Typus zu vergleichen, wurde in den Messungen das Xylocainhomolog B mituntersucht (Vergleich von B und I). — Von sämtlichen vier Substanzen wurden eine 0.0214 molare Lösung * des Hydrochlorids bereitet. Diese Lösungen enthielten die physiologische Menge Kochsaltz, eine Adrenalinkonzentration von 1:100 000 und hatten ein pH von 5,0. Bei der Testung wurden die Lösungen subcutan in Mengen von 1 ml appliziert (hinsichtlich Einzelheiten in der Methodik — siehe Löfgren, Tegnér und Takman [1]). Die Latenzzeit und die Duration wurden beobachtet und die auf Xylocain bezogenen Relativwerte berechnet. In Tabelle 3 ist die *relative Latenzzeit* mit RL und die *relative Duration* mit RD bezeichnet.

In Übereinstimmung mit den Messungen an der Cornea findet man auch hier, dass der Übergang vom Anilid-Typus zum Benzamid-Typus (B → I) von einer Aktivitätserniedrigung begleitet ist ($(RD)_B > (RD)_I$). An der Cornea fanden wir, dass VII merklich aktiver war als I, während dagegen in der subcutanen Technik, VII im Vergleich zu I eine merklich geringere Aktivi-

Tabelle 3. Testung der Duration, der Latenzzeit und der Irritation von vier Verbindungen durch subcutane Injektion am Menschen. Für die Begriffe RD (relative Duration) und RL (relative Latenzzeit), siehe oben.

	Verbindung	RD	RL	Irritation
A	$2,6\text{-}(CH_3)_2C_6H_3\cdot NH\cdot CO\cdot CH_2\cdot N(C_2H_5)_2$ (= Xylocain)	1	1	0
B	$2,6\text{-}(CH_3)_2C_6H_3\cdot NH\cdot CO\cdot(CH_2)_2\cdot N(C_2H_5)_2$	0,9	3	+
I	$2,6\text{-}(CH_3)_2C_6H_3\cdot CO\cdot NH\cdot(CH_2)_2\cdot N(C_2H_5)_2$	0,7	3	+
VII	$3,5\text{-}(CH_3)_2C_6H_3\cdot CO\cdot NH\cdot(CH_2)_3\cdot N(C_2H_5)_2$	0,4	4	0

* Eine 0,0214 molare Lösung von Xylocain ist 0,5 %-ig in Bezug auf die Basenmenge.

tät besitzt. Löfgren und Mitarbeiter haben früher [1,52,53] solche Diskrepanzen zwischen Oberflächen- und Infiltrationsanästhesie gefunden. Neuerdings ist auch von Koelzer und Wehr [54] hervorgehoben worden, dass „bei den Aminoacetaniliden die Fähigkeit zur Oberflächenanästhesie * fast nie mit der Wirkung als Injektionsanästheticum ** parallel geht". Bedenkt man, dass die physiologischen Verhältnisse — wie z.B. Durchblutung und Enzymaktivität — denen eine Substanz bei der oberflächlichen Applikation an der Cornea, und andererseits bei der Injektion unter die Haut begegnet sehr verschieden sind, so ist es nicht erstaunlich, dass Diskrepanzen von ohen erwähnter Art existieren. Unserer Ansicht nach sollte es für eine grössere Anzahl Substanzen verschiedener Konstitution überhaupt unmöglich sein, eine Korrelation zwischen der oberflächenanästhetischen Wirkung an der Cornea und der Infiltrationsanästhetischen Wirkung zu finden.

Bei den subcutanen Testungen wurden, wie oben erwähnt, auch die Latenzzeiten bestimmt. Die Fehler dieser Messungen sind relativ gross, so dass die in der Tabelle 3 angeführten RL-Werte (siehe oben) nur als Grössenordnungen aufzufassen sind. Man kann aber mit Sicherheit sagen, dass sämtliche drei Verbindungen (B, I und VII) als Infiltrationsanästhetica im Vergleich mit Xylocain wesentlich grössere Latenzzeiten aufweisen. — Es wurde weiter beobachtet, dass Xylocain und VII keine Irritation hervorriefen, während B und I eine schwach schmerzerregende Wirkung ausübten.

Herrn Dr. S. Wiedling danken wir für die Ausführung der Toxizitätsbestimmungen und für die Messung der lokalanästhetischen Wirkung an der Kaninchencornea.

LITERATUR

1. Löfgren, N., Tegnér, C., und Takman, B. *Acta Chem. Scand.* **11** (1957) 1724.
2. Plieninger, H., und Werst, G. *Angew. Chem.* **67** (1955) 156.
3. Grundmann, C. *Ann.* **524** (1936) 31.
4. Weygand, F., Eberhardt, G., Linden, H., Schäfer, F. und Eigen, I. *Angew. Chem.* **65** (1953) 525.
5. Löfgren, N. *Studies on Local Anesthetics. Xylocaine a New Synthetic Drug.* Dissertation, Stockholm 1948.
6. Fischer, I. und Löfgren, N. *Acta Chem. Scand.* **4** (1950) 1408.
7. Löfgren, N. *Arkiv Kemi, Mineral. Geol.* **A22** (1946) no. 1.
8. Goldberg, L. *Acta Physiol. Scand.* **18** (1949) 1.
9. Lock, G. und Schmidt, K. *J. prakt. Chem.* **140** (1934) 229.
10. Clarke, H. und Read, R. *Org. Syntheses*, Coll. Vol. **1** (1946) 514.
11. Berger, G. und Olivier, S. *Rec. trav. chim.* **46** (1927) 600.
12. Hoffmann, A. *Ber.* **18** (1885) 1821.
13. Gabriel, S. und Heymann, Th. *Ber.* **23** (1890) 2495.
14. Gabriel, S. und Elfeldt, P. *Ber.* **24** (1891) 3213.
15. Hinshelwood, C. und Newling, W. *J. Chem. Soc.* **1936** 1360.
16. Ingold, C. und Rothstein, E. *J. Chem. Soc.* **1928** 1217.
17. Branch, G. und Calvin, M. *The Theory of Organic Chemistry*, 1. Aufl., Prentice-Hall, Inc., New York 1945, S. 261—264.
18. Lindström, S. *Acta Chem. Scand.* **11** (1957) 962.
19. Watson, H. *Modern Theories of Organic Chemistry*, 2. Aufl., Oxford University Press, Oxford 1941, S. 243.

* Oberflächenanästhesie der Kaninchencornea.
** Injektionsanästhesie des Ratten-Ischiadicus.

20. Schultz, E., Robb, C. und Sprague, J. *J. Am. Chem. Soc.* **69** (1947) 188, 2454.
21. Brode, W. und Hill, M. *J. Am. Chem. Soc.* **69** (1947) 724.
22. Ross, S. *J. Am. Chem. Soc.* **69** (1947) 2982.
23. Dahlbom, R. *Acta Chem. Scand.* **3** (1949) 247.
24. Breslow, D., Walker, H., Yost, R., Shivers, J. und Hauser, O. *J. Am. Chem. Soc.* **68** (1946) 100.
25. Luten, D. *J. Org. Chem.* **3** (1938) 588.
26. Rose, J. und Weedon, B. *J. Chem. Soc.* **1949** 782.
27. Catch, J., Elliott, D., Hey, D. und Jones, E. *J. Chem. Soc.* **1948** 272.
28. Rosenmund, K. *Ber.* **51** (1918) 585.
29. Arndt, F. und Amende, J. *Angew. Chem.* **43** (1930) 444.
30. Vaughen, T., Vogt, R. und Nieuwland, J. *J. Am. Chem. Soc.* **56** (1934) 2120.
31. Amundsen, L. und Krantz, K. *J. Am. Chem. Soc.* **63** (1941) 306.
32. Cortese, F. *Org. Syntheses* Coll. Vol. **2** (1946) 91.
33. Kermack, W. und Wight, T. *J. Chem. Soc.* **1935** 1421.
34. Terent'ev, A. und Kost, A. *J. Gen. Chem. (U.S.S.R.)* **16** (1946) 859.
35. Whitmore, F., Mosher, H., Adams, R., Taylor, R., Chapin, E., Weisel, C. und Yanko, W. *J. Am. Chem. Soc.* **66** (1944) 725.
36. Nystrom, R. und Brown, W. *J. Am. Chem. Soc.* **70** (1948) 3738.
37. Shriner, R. und Hickey, J. *J. Am. Chem. Soc.* **61** (1934) 888.
38. Hamer, E. und Rathbone, R. *J. Chem. Soc.* **1943** 246.
39. Marvel, C., Zartman, W. und Bluthardt, O. *J. Am. Chem. Soc.* **49** (1927) 2301.
40. Lohmann, J. *Ber.* **24** (1891) 2631.
41. Gawron, O. und Spoerri, P. *J. Am. Chem. Soc.* **67** (1945) 515.
42. Goldfarb, A. *J. Am. Chem. Soc.* **63** (1941) 2280.
43. Callsen, J. und Hahl, H. *D. R. P.* **43,960** (1926).
44. Magidson, O. und Grigorowsky, O. *Ber.* **69** (1936) 396.
45. Prelog, V., Rajner, E. und Stern, P. *Helv. Chim. Acta* **26** (1943) 1172.
46. Shriner, R. und Fuson, R. *Systematic Identification of Organic Compounds*, John Wiley and Sons, Inc., New York 1935, S. 145.
47. Riddick, J. *Anal. Chem.* **24** (1952) 41.
48. Snyder, H., Adams, R. und McIntosh, A. *J. Am. Chem. Soc.* **63** (1941) 3280.
49. Weiler, M. *Ber.* **32** (1899) 1908.
50. Wiedling, S. *Acta Pharmacol. Toxicol.* **8** (1952) 117.
51. Löfgren, N. und Lundqvist, B. *Svensk Kem. Tidskr.* **58** (1946) 206.
52. Löfgren, N. und Takman, B. *Acta Chem. Scand.* **6** (1952) 1010.
53. Löfgren, N. und Tegnér, C. *Acta Chem. Scand.* **8** (1954) 1806.
54. Koelzer, P. und Wehr, K. *Arzneimittel-Forsch.* **8** (1958) 550.

Eingegangen am 28. Mai, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1607–1622

Infrared and Raman Spectra of Some Systems Containing Thiocyanate Groups

PER-OLOF KINELL and BROR STRANDBERG

Institute of Physical Chemistry and Institute of Inorganic Chemistry, University of Uppsala, Uppsala, Sweden

Infrared spectra have been taken of solid silver thiocyanate and ammonium silver thiocyanate in the region 400–2 200 cm^{-1}. These spectra indicate that the latter compound is composed of silver thiocyanate and thiocyanate ions in agreement with X-ray data. The assignment of the fundamentals has not been possible because one of the fundamentals has evaded detection. A probable correspondence between the lines in the spectra of the thiocyanate ion, the silver thiocyanate and the ammonium silver thiocyanate has been given.

Raman spectra from solutions containing silver thiocyanate dissolved in ammonium and sodium thiocyanate have shown that these solutions do not contain spectroscopically detectable units having strong bonds between SCN groups and silver ions. The silver ion influences the CN stretching frequency. There is an increase in this frequency when going from the solutions to the solid compounds.

In the present paper some results from a study of systems containing silver and thiocyanate groups will be reported. The systems have been investigated by means of molecular spectroscopic methods. The principal aim of the work has been to compare the vibrational behaviour of the thiocyanate group in different environments. Therefore systems having a known crystal structure have been chosen, namely silver thiocyanate investigated by Lindqvist [1] and ammonium silver dithiocyanate studied by Lindqvist and Strandberg [2]. Furthermore, water solutions of sodium and silver thiocyanates (Leden and Nilsson [3]) and ammonium and silver thiocyanates (Nilsson [4]) were examined in order to elucidate possible differences between thiocyanate groups in a solid phase and in its corresponding mother liquid.

The vibrational behaviour of the thiocyanate ion, SCN^-, is characterized by three fundamental modes (*cf.* Herzberg [5]) either it is regarded as linear (point group $C_{\infty v}$) or bent (point group C_s). There has been some discussion about its proper structure (Saraf [6]), but for the sake of simplicity it will *pro tempore* be regarded as linear. Then the first of the normal vibrations, ν_1, corresponds to a stretching of the CN-bond. Being a triple bond it is strong

and, consequently, the vibrational frequency high, about 2 065 cm^{-1}. The second vibration, ν_2, is a bending of the molecule with a frequency of about 480 cm^{-1}. It is a doubly degenerate mode. The third vibration, ν_3, can essentially be looked upon as a stretching of the S—C-bond with a frequency of about 750 cm^{-1}. In the crystalline phase the vibrational state of the SCN^- ion corresponds in its main features to that in solution (Jones [7]). The degenerate mode, ν_2, is split.

In some previous works the change in the ν_1 mode frequency has been studied in order to elucidate the structure of various thiocyanate complexes. Thus Chatt *et al.*[8,9] have found a change in the infrared absorption in the region 2 100—2 170 cm^{-1} with the position of thiocyanate groups in platinum and palladium complexes containing tri-*n*-propyl phosphine or -arsine groups. They distinguish between terminal SCN groups having absorption in the range 2 100—2 120 cm^{-1} and bridging SCN groups giving rise to frequencies in the region 2 150—2 185 cm^{-1}. The difference has been attributed to increased bond order of the CN bond with increasing positive charge on the sulphur atom as a consequence of coordination to two metal atoms. However, the type of bridging is at present not definitely settled (*cf.* Ref.[9]).

Fujita, Nakamoto and Kobayashi [10] have explained the higher ν_1-stretching frequency in Cr^{3+} and Co^{3+} complexes as due to *sp* hybridization of the nitrogen atom compared to sp^2 hybridization in thio*iso*cyanic acid. The frequencies are 2 000—2 180 cm^{-1} and 1 963 cm^{-1}, respectively. The interaction between the sulphur atom and outer ions such as Hg^{2+} and Ag^+ shifts the CN stretching vibration to higher frequencies.

The crystal symmetry of the silver thiocyanate is according to Lindqvist's investigation [1] monoclinic with the space group $C2/c$ (C_{2h}^6). The unit cell contains eight silver thiocyanate molecules. The structure can best be described as built up by endless chains, with the composition $(AgSCN)_n$ formed by a covalent bond from a silver atom to the end sulphur of one thiocyanate group and a bond to the end nitrogen of another thiocyanate group. (The latter bond is not necessarily covalent.) The chains have a zig-zag shape and progress almost perpendicularly to the *bc* plane. There is a silver-sulphur interaction between different chains: the Ag-S distances are 2.997 ± 0.011 Å and 2.886 ± 0.012 Å compared to the Ag-S bond distance of 2.428 ± 0.001 Å and the Ag^+-S distance of 2.9—3.1 Å. No significant deviation from linearity in the SCN group has been found.

The symmetry of the ammonium silver dithiocyanate is monoclinic [2], belonging to space group $P2_1/n$ (C_{2h}^5). The unit cell contains four molecules. The crystal is built up by AgSCN molecules, NH_4^+ ions and SCN^- ions. The silver atom coordinates four sulphur atoms in a very distorted tetrahedron. The Ag-S distances are: 2.474 ± 0.020 Å within the AgSCN molecule, 2.654 ± 0.019 Å, 2.630 ± 0.027 Å and 2.742 ± 0.029 Å from one AgSCN molecule to the three surrounding SCN^- ions.

The state of the thiocyanate group in water solution at high contents of thiocyanate is, according to solubility and potentiometric data [3], characterized by a series of complexes of the type $Ag_m(SCN)_{2m+2}^{(m+2)-}$. The complex having $m = 1$ is the most abundant in solutions containing sodium thiocyanate.

From electron distribution functions [4] in ammonium silver thiocyanate systems it has been found that a model based on the $Ag_m(SCN)_{2m+2}^{(m+2)-}$ complexes gives one Ag-S distance, which does not correspond to a maximum of the distribution function. An idealized layer structure based upon the crystal structure of ammonium silver dithiocyanate gives Ag-S and Ag-Ag distances in agreement with the distribution curve.

EXPERIMENTAL

Material. The potassium, sodium and ammonium thiocyanates used in the present investigation were of analytical grade. The ammonium salt was recrystallized because it was slightly reddish, probably due to some iron impurities. All the solutions were prepared from distilled water and were carefully filtered. Silver thiocyanate was precipitated from silver nitrate and potassium thiocyanate solutions. It was always used just after preparation. Ammonium silver dithiocyanate was crystallized from mother liquid M I prepared from 100.0 g AgSCN, 98.8 g NH_4SCN and 102.5 g H_2O. The composition corresponds to a point in the phase diagram of $AgSCN-NH_4SCN-H_2O$ situated in the liquid area but quite near to the phase limit of $NH_4Ag(SCN)_2(s)$ (*cf.* Occleshaw [11]). The molarities of the different ions are approximately $M_{SCN^-} = 9.5$, $M_{Ag^+} = 3.0$ and $M_{NH_4^+} = 6.5$. (M means here total concentration.) After about 30 g of $NH_4Ag(SCN)_2$ had crystallized, the supernatant liquid was separated from the crystals as mother liquid M II, having the approximate composition $M_{SCN^-} = 9$, $M_{Ag^+} = 2.5$ and $M_{NH_4^+} = 6$. Mother liquid M III was prepared from 11.5 g of AgSCN, 48.5 g of NaSCN and 40 g of H_2O in order to get a saturated solution without solid phase (Occleshaw [11]). The approximate composition is $M_{SCN^-} = 10$, $M_{Ag^+} = 1$ and $M_{Na^+} = 9$.

Infrared measurements. Infrared spectra were taken from the solid compounds using either the technique with paraffin oil mulls or with potassium bromide pellets. Spectra were run both in a Perkin-Elmer model 21 spectrophotometer equipped with NaCl, KBr and CsBr prisms and in a Beckman spectrophotometer IR-2 in the NaCl region. Difficulties arouse with the KBr pellets, for even when very high pressures were used, the prisms were slightly opaque. The reason for this is believed to be the difference in compressibilities of the crystals. The softer thiocyanate material cannot be sintered with the harder potassium bromide. Nevertheless quite good spectra could be obtained in all regions of interest for the present investigation. The accuracy in the wave number determination is $\pm$ 2—3 cm^{-1}.

Jones [7] has reported the appearance of anomalous absorption bands from potassium thiocyanate in potassium chloride and bromide disks. The type of anomaly is not stated. Furthermore, Jones [12] has observed foreign peaks from disks containing potassium silver dicyanide. As pointed out by Jones and Chamberlain [13], this can depend upon an exchange of ions or ion complexes between the different lattices. Such an exchange between K^+ and NH_4^+ has been observed by Pliskin and Eischens [14]. In the present case the differences between spectra from mulls and pellets generally are small.

Raman measurements. The raman spectra of the soluble salts and of the mother liquids were obtained using a raman spectrograph arranged for both photographic and photoelectric recording (Kinell [15]). The sample volume was about 20 ml. A saturated sodium nitrite solution was used to filter away the 4 046 and 4 077 Å lines. The temperature in the raman tube was kept at 20°C. Depolarization measurements were made using the polaroid sheet technique. The accuracy in the wave number determinations varied with the shape of the lines and also with their intensity. Some of the lines obtained were fairly broad. The accuracy is $\pm$ 2—3 cm^{-1}, and the spectral band width used was 5 — 60 cm^{-1}. Under the influence of the exciting light, the solution of ammonium thiocyanate and the mother liquids M I and M II slowly became coloured reddish-brown. This was probably due to the presence of minute amounts of ferric ions and could be remedied by adding traces of a reducing agent (ascorbic acid) to the solutions. All the solutions were filtered through a Jena G4 sintered disk filter which had been thoroughly washed

Table 1. Infrared absorption frequencies (in cm^{-1}) of the thiocyanate ion in solid systems, observed with different prisms and in various preparations (vs = very strong, m = medium).

Assignments Jones [7]	KSCN				NH_4SCN					Values given by Jones [7]	Intensity
	NaCl			KBr	NaCl		KBr		CsBr		
	Mull	Disk	Acetone soln	Disk	Mull	Disk	Mull	Disk	Mull		
ν_1	2 041	2 053	2 062	—	—	2 053	—	—	—	2 053	vs
$2\nu_{2a}$	967	969	—	—	935	970	970	—	—	968	m
$2\nu_{2b}$	946	948	—	—	930	951	—	—	—	951	m
ν_3	747	748	745	746	752	749	750	747	—	749	m
ν_{2a}	—	—	—	485.5	—	—	476	486	475	484	m
ν_{2b}	—	—	—	471	—	—	466	472	465	470	m

with distilled water. The solid ammonium thiocyanate and ammonium silver dithiocyanate were also run, but only the strong ν_1 vibration could be observed due to excessive scattering of mercury light. Also impurities in the mercury spectrum caused disturbances. In photographic recording, Ilford Zenith plates were used. They were processed in Kodak D-76 fine grain developer. For intensity comparisons the carbon tetrachloride line 459 cm^{-1} was used. The ratio of the heights of the actual line to the reference line was taken as a measure of the intensity (*cf.* Kinell [15]).

RESULTS

Infrared spectra. The absorption frequencies of the thiocyanate ion obtained from the potassium and ammonium salts in the solid state are collected in Table 1, together with the values of ν_1 and ν_2 from an acetone solution of the potassium salt. The assignments are those given by Jones [7]. His values

Table 2. Infrared absorption frequencies (in cm^{-1}) of silver thiocyanate in the solid state, observed with different prisms and in various preparations (vs = very strong, m = medium, w = weak). The assignments are only to be regarded as a classification.

Assignments	NaCl		KBr		CsBr	Intensity
	Mull	Disk	Mull	Disk	Mull	
ν_1	2 169	2 083	—	—	—	vs
ν_2 overtones	901	—	895	901	—	m
	861	—	855	—	—	m
ν_3	743	—	741	736	—	m
	723	715	720	717	—	w
ν_2	—	—	446.5	444.5	444.5	m
	—	—	423	421	423	m

Table 3. Infrared absorption frequencies (in cm^{-1}) of ammonium silver dithiocyanate in the solid state, observed with different prisms and in various preparations (vs = very strong, s = strong, m = medium, w = weak). The assignments are only to be regarded as a classification.

Assignments	NaCl		KBr		CsBr	Intensity
	Mull	Disk	Mull	Disk	Mull	
ν_1	2 120	2 119	—	—	—	s
	2 090	2 070	—	—	—	vs
ν_2 overtones	940	—	—	—	—	m
	909	—	—	—	—	m
	899	901	—	901	—	m
	887	—	877	—	—	w
ν_3	738	730	736	736	—	m
	722	717	720	717	—	m
ν_2	—	—	472	470.5	470	m
	—	—	454.5	454.5	454.5	m
	—	—	450.5	—	449.5	m
	—	—	443.5	442.5	442.5	m
?	—	—	420	419	420	w

are also given in the last column. The values from disks agree very well with Jones' values. The slightly changed frequencies in paraffin oil mulls of ammonium thiocyanate cannot at present be explained. The higher ν_3 value and lower ν_1 value in acetone solution correspond to similar results found for water solutions by Jones [7] and Gordy and Williams [16].

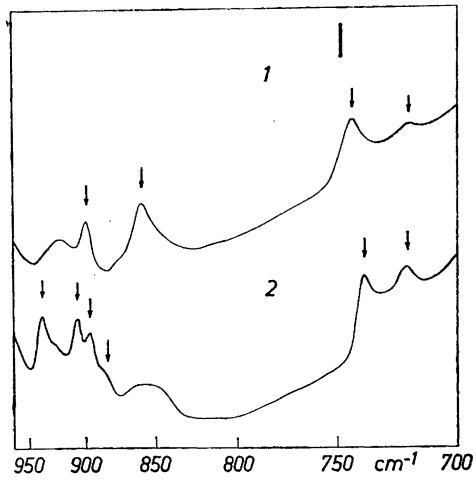


Fig. 1. Infrared spectra of silver thiocyanate (1) and ammonium silver dithiocyanate (2) showing the fundamentals in the region 700 – 750 cm^{-1} and the overtones in the region 850 – 950 cm^{-1}. All actual lines are indicated by arrows to distinguish them from the background. The position of the SCN^- ν_3 vibration frequency is shown by the dash in the upper part of the figure. Paraffin oil mulls were used. Prism: NaCl.

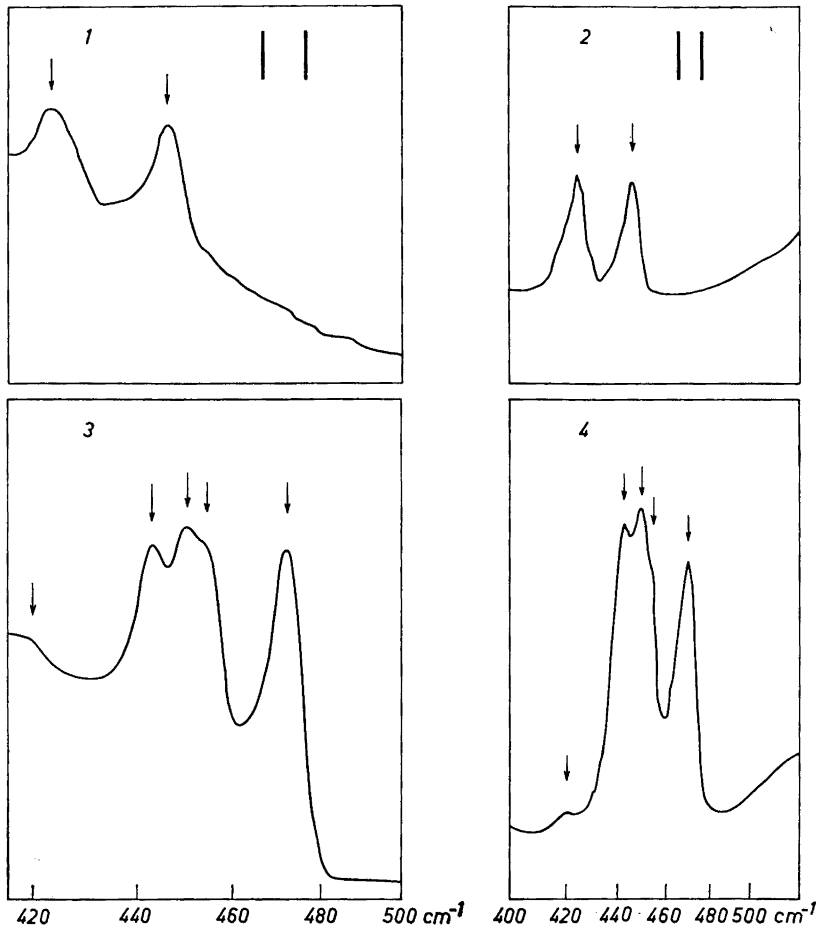


Fig. 2. Infrared spectra of thiocyanates: 1) AgSCN, KBr prism; 2) AgSCN, CsBr prism; 3) $NH_4Ag(SCN)_2$, KBr prism; 4) $NH_4Ag(SCN)_2$, CsBr prism. The actual lines are indicated by arrows. The positions of the ν_2 vibration frequencies of the SCN^- ion are shown by the dashes in the upper part of the figure. Paraffin oil mulls were used.

The absorption frequencies of solid silver thiocyanate are collected in Table 2. There are some disagreements between paraffin mull and potassium bromide disk values. The high frequency 2 160 cm^{-1} corresponds very well to the value 2 149 cm^{-1} reported by Chatt [9]. In potassium bromide disks a line at 455 cm^{-1} has been observed. It has a tendency to decrease with the age of the disks. As it does not appear in any of the other spectra, it has been discarded. No indications of any lines which could be associated with silver thiocyanate were found in the region 400—250 cm^{-1}.

Finally the absorption frequencies of solid ammonium silver dithiocyanate are given in Table 3. The ν_2 values at 450 and 455 cm^{-1} are from bands which

Table 4. Raman lines of the thiocyanate ion (in cm^{-1}) in water solutions of potassium and ammonium thiocyanate. The figures within parentheses under the wave numbers are the intensities of the lines compared to the 459 cm^{-1} carbon tetrachloride line as a reference.

Assignments	KSCN				NH_4SCN	
	Photoelectric	Photographic			Photoelectric	Photographic
		Light unpol.	Light pol. ∥	Light pol. ⊥		
ν_1	2 065 (170)	2 063	2 064	2 064	2 066 (120)	2 062
$2\nu_2$	—	940	—	—	—	—
ν_3	745 (20)	746	—	—	752 (15)	—
ν_2 ?	481 (2)	488	—	—	485 (2)	—

are not completely resolved. Not even in this case could any vibrations be found in the low frequency region 400—250 cm^{-1}.

The assignments in Tables 2 and 3 are to be regarded as a classification only.

The intensities given in the tables are estimations of the relative heights of the absorption bands. In Fig. 1 parts of the spectra from silver thiocyanate and ammonium silver dithiocyanate obtained in single beam runs (Beckman IR-2) are shown. The actual absorption maxima are indicated by arrows. The 450 cm^{-1} bands of the same compounds recorded in the double beam instrument (Perkin-Elmer model 21) using KBr and CsBr prisms respectively

Table 5. Raman lines of mother liquids M I, M II, M III. For explanation *cf.* Table 4.

Assignments	M I	M II	M I′	M II′	M III		
	Photoelectric		Photographic		Photographic		
					Light unpol.	Light pol. ∥	Light pol. ⊥
ν_1	2 093 (105)	2 089 (140)	2 086	2 082	2 075	2 073	2 074
$2\nu_2$	—	—	921	924	931	—	—
ν_3	771 ? 737 (10)	771 ? 735 (10)	732	735	746	—	—
ν_2	465 (6)	455 (4)	461	463	466	—	—

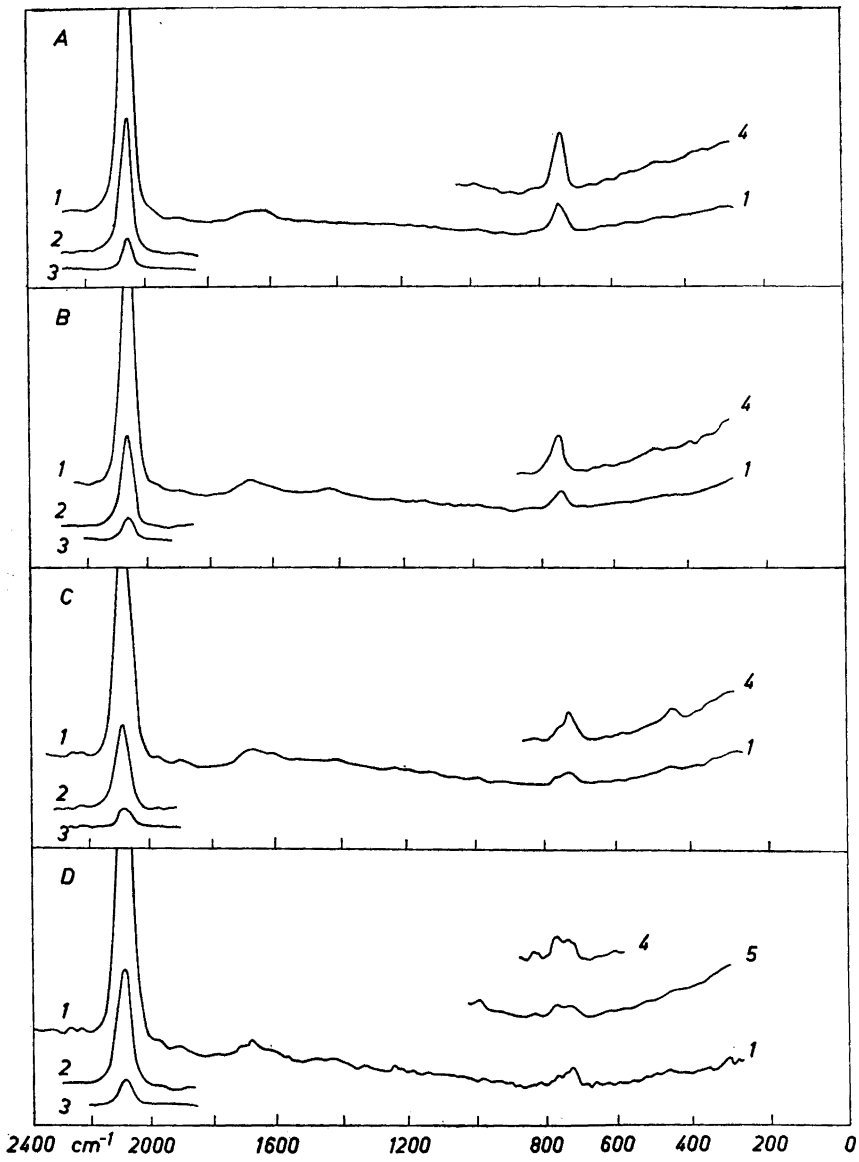


Fig. 3. Photoelectrically recorded raman spectra of A) water solution of KSCN; B) water solution of NH_4SCN; C) mother liquid M I and D) mother liquid M II. Spectra marked with the same figure are directly comparable.

are given in Fig. 2. In both figures the position of the corresponding bands of ammonium thiocyanate has been indicated.

For the present purpose the absorption bands due to the ammonium ion were not studied.

Raman spectra. The frequencies of the raman displacements observed in water solutions of the thiocyanate ion and in the mother liquids are given in Tables 4 and 5. A dash indicates that the line was observed but its frequency not estimated. The existence of the line 485 cm^{-1} as a fundamental mode excited from the Hg line 4 358 Å is dubious. The value is in agreement with the ν_1 mode excited by 4 077 Å. However, existence of a ν_2 mode is evident from the first harmonic at 940 cm^{-1}. Then the ν_2 frequency would be 470 cm^{-1}. This is in good agreement with the infrared data and with the value in water solution observed in the infrared by Jones [7]. The value given by Saraf [6], 499 cm^{-1}, seems too high. The corresponding line from the mother liquids is easily observed. The ν_1 mode excited from 4 077 Å would appear at about 500 cm^{-1}. In some of the spectra of the mother liquids a splitting of the ν_3 line was observed. In test runs (M I′, M II′) it could not be reproduced. No indications of a splitting of the other lines have been found in runs made and photographs taken at different slit widths. The ν_1 line is slightly skew towards shorter wave lengths.

The general appearance of the spectra is shown in Fig. 3. All the spectra No. 1 have been recorded under the same conditions. The bands between 1 400—1 800 cm^{-1} are due to the water molecules and to the ammonium ions. From spectra Nos. C:3 and D:3 the skewness of the ν_1 line can be seen. The results of the runs made on solid ammonium thiocyanate and ammonium silver dithiocyanate are given in Fig. 4. In the first case the existence of a line at about 2 065 cm^{-1} is quite clear from a comparison of the background spectrum (4) with the actual spectrum (5). In the last case both the background (1) and the actual curve (2) are reproducible in all essential details. The curve (3) representing the difference between (2) and (1) has two maxima indicating absorption at 2 085—2 090 cm^{-1} and at about 2 120 cm^{-1}. In Fig. 5 the ν_1 vibration of potassium thiocyanate is recorded at four different concentrations. No significant change in the position of the line can be found.

In order to find any possible differences between the state of polarization of the raman lines of the thiocyanate group in pure water solution and in the mother liquid, spectra with the incident light polarized in directions parallel and perpendicular to the direction of observation were taken. From these, the depolarization factors were found to be 0.58 and 0.65 for the ν_1 line in potassium thiocyanate solution and in mother liquid M III, respectively. The value 0.58 corresponds with the figure 0.55 given by Saraf [6]. Both of the values obtained here show that the ν_1 mode is polarized. There are no significant shifts in the position of the lines (*cf.* Tables 4 and 5), and thus it is not probable that the higher depolarization factor from M III should indicate the presence of a depolarized line quite near to a polarized one. The other lines were not measured, but the photographically obtained spectra showed that they are polarized. This is also the case with the $2\nu_2$ mode.

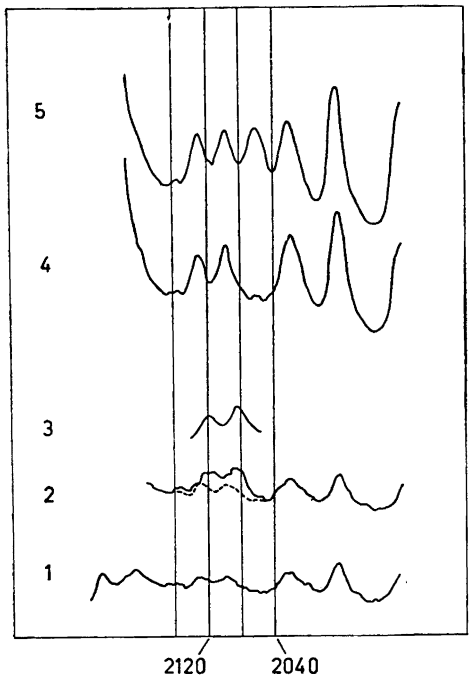


Fig. 4. Raman spectra of solid thiocyanates: 1) and 4) mercury lamp background; 2) background and superimposed spectrum of $NH_4Ag(SCN)_2$(s); 3) spectrum of $NH_4Ag(SCN)_2$(s); 5) background and spectrum of NH_4SCN(s).

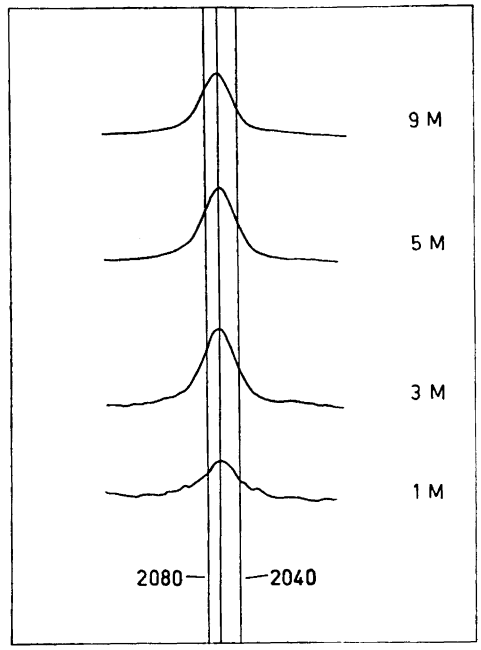


Fig. 5. The ν_1 line of SCN^- in water solutions of potassium thiocyanate of different concentrations.

DISCUSSION

Interpretation of spectra. In the following section some remarks concerning the interpretation of the spectra obtained will be made. The complete assignment of the frequencies must be postponed to a later occasion (Kinell, work in progress).

The only symmetry element of an isolated four atomic molecule of the type AgSCN is a plane of symmetry, *i.e.* the plane containing all the atoms. The number of normal modes is six. The molecule belongs to the point group C_s. This contains two species of normal vibrations; A', symmetric with respect to the plane of symmetry and, A'', antisymmetric with respect to this plane. Then, the representation which has the vibrational motions as its basis can be broken down into its irreducible components in the following way

$$\Gamma_{vib} = 5A' + A''.$$

All the vibrations belonging to A' must occur in the plane of symmetry and the only one of A'', at right angle to this plane. All six vibrations are active in both raman and infrared spectra.

In a crystal the field of force, in which the molecules are located, will cause a splitting of the internal vibrational modes. Furthermore, the classification and the selection rules of the modes will change because of the different symmetry properties of the molecule and the crystal. Applying the methods (*cf.* Bhagavantam and Venkatarayudu [17], Hornig [18], and Winston and Halford [19]) worked out for analysis of the normal vibrations of molecules in crystals, one finds for the silver thiocyanate that its factor group of the space group C_{2h}^6, having the invariant subgroup of the lattice translations as its identity element, is isomorphic to the point group C_{2h}. Then the following reduction of the representation which has the internal vibrational modes as its basis will be obtained

$$\Gamma_{vib} = 4A_g + 6B_g + 6A_u + 6B_u$$

Only the species symmetric with respect to inversion will be raman active, and the species antisymmetric with respect to the same operation infrared active. Thus, the six normal modes of the isolated AgSCN molecule will in the crystal be split into twelve vibrations.

In silver thiocyanate the crystal can be regarded as built up from AgSCN chains [1], and then the line group method of Tobin [20] can be used for the analysis of the normal modes. The chains are regarded as isolated and infinite. Their symmetry is described by a one dimensional space group, a line group. The symmetry of the repeating unit is characterized by the elements of the factor group of the line group, which has the invariant subgroup of the chain translations as its identity element. The repeating unit consists of two AgSCN molecules and the factor group is isomorphic to the point group C_s. Then we have for the internal vibrations

$$\Gamma_{vib} = 8A' + 8A''.$$

Both of the species A' and A'' are raman and infrared active. Thus even in this case more frequencies than for the isolated molecule will appear. Taking even lattice modes under consideration, one finds that each factor group mode of the line group corresponds to one infrared active and one raman active mode under the space group. The silver thiocyanate molecules are located on general points in the unit cell and thus no classifiction is obtained under the site group (*cf.* Hornig [18]).

The above analyses indicate that the silver thiocyanate in the solid state ought to have a rather complicated spectrum in the infrared. On the contrary, the observed spectrum contains only a few lines. If the 900 cm^{-1} lines are regarded as overtones, only five fundamental frequencies have been found. The silver atom is heavy compared to all the other atoms in the silver thiocyanate molecule, and therefore any frequency involving the stretching of the Ag-S bond or bending of the Ag-S-C angle will be low and probably evade observation with the instruments used. An investigation of the *iso*cyanic acid (Herzberg and Reid [21]) has shown that this molecule, which has the same geometrical shape as the silver thiocyanate molecule, has bending modes at 572, 670 and 797 cm^{-1}. The last value refers to the H-N-C angle. In addition, Eyster and Gillette [22] have shown that the frequency of the bending deforma-

tion of the angle in hydrazoic acid and methyl azide decreases from 1 153 cm^{-1} to 259 cm^{-1} when the methyl group is substituted for the hydrogen atom. Also in methyl *iso*cyanate and methyl *iso*cyanide the frequencies are low, 353 cm^{-1} and 290 cm^{-1}, respectively. A more thoroughgoing analysis of possible overtones and combination lines in the spectrum of AgSCN (except those already given here) has been started. It is hoped that the new data can give information about possible low frequencies.

The spectrum of the ammonium silver dithiocyanate is also fairly simple. In the case of completely free AgSCN and SCN^- units it should consist of nine fundamental frequencies, all of which are allowed in both the raman and infrared spectrum. The factor group analysis (the space group C_{2h}^5 of the crystal has a factor group having the invariant subgroup of the lattice translations as its identity element, which is isomorphic to the point group C_{2h}) gives for the internal vibrational modes

$$\Gamma_{vib} = 9A_g + 9B_g + 9A_u + 9B_u.$$

The gerade species are raman active, the ungerade infrared active. The observed spectrum may contain nine lines (the 900 cm^{-1} bands are tentatively regar-

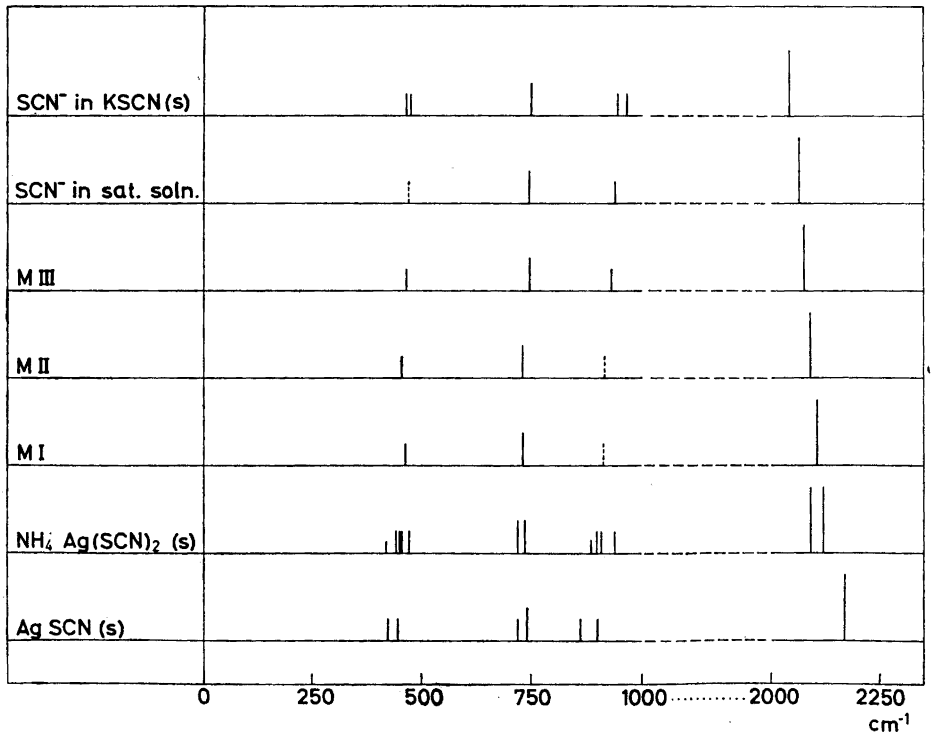


Fig. 6. Schematic raman and infrared spectra of systems containing thiocyanate groups. The intensities are only approximate.

ded as overtones), but only a more thorough study of possible overtones and combination bands in other regions will give a definite answer.

From Fig. 6, where all the spectra have been collected in a schematic diagram, it follows that the spectrum of ammonium silver dithiocyanate seems to be a superposition of the spectra of the SCN^- ion and the AgSCN molecule. This is in agreement with the structure given by Lindqvist and Strandberg [2]. If the lines 2 090 cm^{-1}, 738 cm^{-1}, 472 cm^{-1} and 454.5 cm^{-1} (Table 3) are assumed to belong to the thiocyanate ion, then the remaining lines 2 120 cm^{-1}, 722 cm^{-1}, 450.5 cm^{-1}, 443.5 cm^{-1} and 420 cm^{-1} should correspond to the silver thiocyanate lines (Table 2) except for one missing ν_3 line and one extra ν_2 line. The ν_3 line may not be resolved from the SCN^- line. The interpretation of the 420 cm^{-1} line as a fundamental may be somewhat doubtful because of its very low intensity. It could be an overtone of a low frequency vibration at about 200 cm^{-1}, which would evade detection by the equipment used. The 423 cm^{-1} of silver thiocyanate is most probably a fundamental. Then the following coordination between the spectral lines can be done on the basis of the present data:

	SCN^-	$NH_4Ag(SCN)_2$	AgSCN
Fundamentals	2 053	2 120	2 169
		2 090	
Overtones	968	940	
	951	909	
		899	901
		887	861
Fundamentals	749	738	743
		722	723
	484	472	
	470	454.5	
		450.5	446.5
		443.5	423
Overtone?		420	?

The raman spectra of the mother liquids are very similar to the spectrum of the SCN^- ion in water solution. Therefore it seems quite reasonable to make the same assignments of the lines in both cases. Due to the weakness of the 460 cm^{-1} band, no detailed study of its structure can be made. The broadening of the energy levels on account of the strong interaction in the solution may have smoothed out a possible splitting. On the other hand, bending modes are not very sensitive to changes in the symmetry of the local field (Bauer and Magat [23]). This has clearly been demonstrated in the case of the nitrate ion in water solutions, where at higher concentrations only one of the two doubly degenerate vibrations is split, the other one is not because it should approach a pure bending of the ion (Mathieu and Lounsbury [24]).

As a summary of this discussion, one could say (*cf.* Fig. 6) that irrespective of the environmental conditions, the vibrational frequencies of the SCN

unit fall into four groups, which are well separated from each other. The environmental circumstances influence the position of the lines in each group. Especially the displacement of the 2 050 cm^{-1} band to higher frequencies is noticeable. There may be a fifth group of frequencies, however, this one has most certainly to do with vibrations involving the silver atom.

The state of the SCN group in solution. From the foregoing discussion it is evident that the behaviour of the SCN group in the different environments can approximately be described by the three vibrational modes of the free SCN^- ion. The dependence of the ν_2 and ν_3 vibrations on the conditions of the solution is noticeable but not very pronounced and no conclusions of general value con be drawn.

The vibration of the CN group seems to be the most sensitive to differences in the composition of the solution. The spectra taken at various slit widths show that a splitting of the line is very unlikely. The polarization measurements indicate the improbability of the presence of different symmetry types of SCN groups having very nearly equal frequencies. The existence of complexes of the type $Ag(SCN)_4^{3-}$ (Leden and Nilsson [3]) with a strong bond between the silver and sulphur atoms should at the concentrations and exposure times used certainly have revealed new raman lines separated from those of the thiocyanate ion. Assuming I_d symmetry of the complex ion one gets the following reduction of the normal mode representation

$$\Gamma_{vib} = 3A_1 + 3E + 2F_1 + 6F_2$$

The totally symmetric A_1 species, the doubly degenerate E species and the triply degenerate F_2 species are raman active. The last species is the only one active in infrared. Of these the three totally symmetric lines can be expected to have rather high intensities. Complexes of the type $Ag_2(SCN)_6^{4-}$ have on the assumption of D_{2h} symmetry,

$$\Gamma_{vib} = 10A_g + 6B_{1g} + 5B_{2g} + 6B_{3g} + 3A_u + 9B_{1u} + 9B_{2u} + 6B_{3u}.$$

The gerade species are raman active and the ten totally symmetric modes are expected to have the highest intensity. However, Leden and Nilsson [3] have found that the concentration of this complex is much lower than that of the mononuclear complex, and therefore it is doubtful if any of its lines could have been found under the actual recording conditions.

The frequency of the CN stretching mode as a function of the molar ratio of Ag/SCN is given in Fig. 7. Evidently the displacement of the line is dependent upon the amount of silver present in the system. Furthermore, there is an increase of the frequency going from the various solutions to the solid compounds. The displacement cannot be an effect of the SCN^- concentration. This follows from the frequencies in the M I and M III solutions, which have almost the same SCN^- molarity, and also from the results given in Fig. 5 on the ν_1 frequency in potassium thiocyanate at different concentrations. It can also be concluded that the effect cannot be a first order electrostatic phenomenon because M I, M III and the KSCN solution have each the same molarity of positive ions. The differences in ionic radii of Ag^+ (1.26 Å),

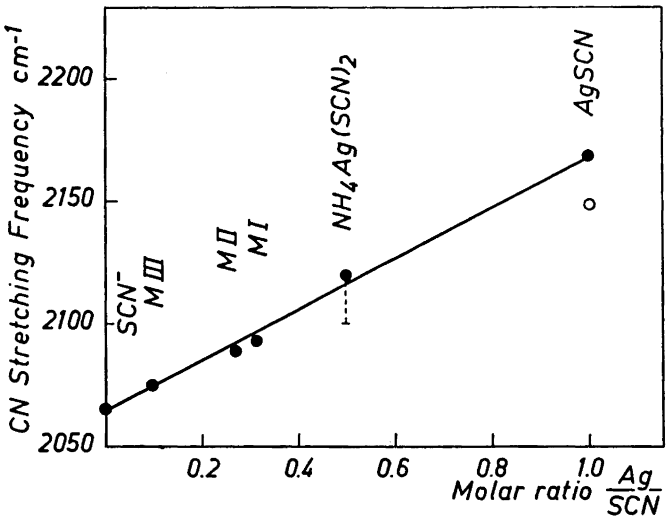


Fig. 7. The frequency of the CN stretching mode as a function of the molar ratio Ag/SCN. O at Ag/SCN = 1 is the value taken from Chatt's *et al.* work [9]. The dotted line at Ag/SCN = 0.5 corresponds to the frequencies given by Chatt *et al.*[8] for platinum and palladium complexes having terminal SCN groups.

Na^+ (0.95 Å), K^+ (1.33 Å) and NH_4^+ (1.48 Å) indicate that sodium could approach the SCN^- ion more closely than the ammonium ion, and consequently in the case of a pure electrostatic attraction, the M III solution would expect to give a higher displacement than the M I solution.

It is of interest to note that the platinum and palladium complexes studied by Chatt *et al.*[8], and having terminal SCN groups, fit into the pattern. The frequencies of complexes having molar ratios of Pt/SCN and Pd/SCN equal to unity and containing bridging SCN groups fall in the region 2 154—2 169 cm^{-1}. Thus they correspond to the behaviour of silver thiocyanate.

The possibility that the ν_1 line should consist of two unresolvable lines, one from the free SCN^- ion and the other from a SCN group bound to the silver ion, is not probable because the half intensity width of the line in M I (53 cm^{-1}) is only slightly higher than in a solution of potassium thiocyanate (47 cm^{-1}). The peak distance of such lines would be at least 50 cm^{-1} and thus the half intensity width of the sum line would be expected to be at least about twice this value.

The evidences given here indicate that the presence of the silver ions in the solutions influences all the thiocyanate groups to approximately the same amount. The displacements observed in the CN stretching vibration are directly dependent upon the amount of silver present per thiocyanate group. The binding of the sulphur atoms to the silver nucleus is not strong enough to give the complexes a character of independent units, which can be detected by spectroscopic means. The data also show that there is a difference between the structure of the solid ammonium silver thiocyanate and its mother liquid.

Acknowledgements. The authors are indebted to Professor Stig Claesson and Professor Gunnar Hägg for their interest in this work and for the ways in which they have facilitated its completion.

We thank Professor Arne Tiselius for his kindness to place an infrared spectrograph at our disposal.

We are very thankful to Dr. Ingvar Lindqvist for his interest in this study and for many stimulating discussions.

Grants from the *Swedish Natural Science Research Council* and from the *Knut and Alice Wallenberg Foundation* are gratefully acknowledged.

REFERENCES

1. Lindqvist, I. *Acta Cryst.* **10** (1957) 29.
2. Lindqvist, I. and Strandberg, B. *Acta Cryst.* **10** (1957) 173.
3. Leden, I. and Nilsson, R. *Z. Naturforsch.* **10 a** (1955) 67.
4. Nilsson, R. *Arkiv Kemi* **12** (1958) 513.
5. Herzberg, G. *Infrared and raman spectra of polyatomic molecules*, New York 1945. p. 174.
6. Saraf, J. R. *Science of light* **5** (1956) 23.
7. Jones, L. H. *J. Chem. Phys.* **25** (1956) 1069; **28**, 1234 (1958).
8. Chatt, J. and Duncanson, L. A. *Nature* **178** (1956) 997.
9. Chatt, L., Duncanson, L. A., Hart, F. A., and Owston, P. G., *Nature* **181** (1958) 43.
10. Fujita, J., Nakamoto, K. and Kobayashi, M. *J. Am. Chem. Soc.* **78** (1956) 3295.
11. Occleshaw, V. J. *J. Chem. Soc.* **134** (1932) 2404.
12. Jones, L. H. *J. Chem. Phys.* **26** (1957) 1578.
13. Jones, L. H. and Chamberlain, C. M. *J. Chem. Phys.* **25** (1956) 365.
14. Pliskin, W. A. and Eischens, R. P. *J. Phys. Chem.* **59** (1955) 1156.
15. Kinell, P.-O. *Arkiv Kemi* **14** (1959) 337. *Cf.* also Kinell, P.-O. *A spectrophotometric study of polymethylmethacrylate*, Uppsala 1953.
16. Gordy, W. and Williams, D. *J. Chem. Phys.* **3** (1935) 664.
17. Bhagavantam, S. and Venkatarayudu, T. *Theory of groups and its application to physical problems*, Andhra University, Waltair 1948.
18. Hornig, D. F. *J. Chem. Phys.* **16** (1948) 1063.
19. Winston, H. and Halford, R. S. *J. Chem. Phys.* **17** (1949) 607.
20. Tobin, M. C. *J. Chem. Phys.* **23** (1955) 891.
21. Herzberg, G. and Reid, C. *Discussions Faraday Soc.* **9** (1950) 92.
22. Eyster, E. H. and Gillette, R. H. *J. Chem. Phys.* **8** (1940) 369.
23. Bauer, E. and Magat, M. *J. phys. radium* **9** (7) (1938) 319.
24. Mathieu, J.-P. and Lounsbury, M. *Discussions Faraday Soc.* **9** (1950) 196.

Received June 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1623–1633

Studies on Local Anesthetics XVIII *

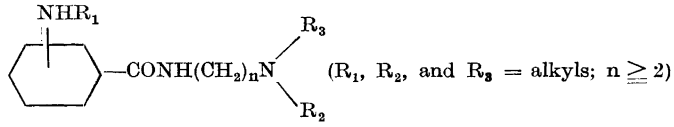
Synthesis of N-(Dialkylaminoalkyl)benzamides

BJÖRN LÜNING

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

Benzamides of the general structure $Ar-CO-NH-R_1-N(R_2)_2$, in which Ar is a methyl substituted benzene nucleus, have been synthesized. Ar contains 1–3 methyl groups in all possible positions. R_1 represents $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH(CH_3)CH_2-$, $-CH_2CH(CH_3)-$, $-CH(CH_3)CH(CH_3)-$, or $-CH_2C(CH_3)_2CH_2-$, and R_2 is a methyl or ethyl group. The number of new compounds which have been synthesized is 63. In one case a compound containing two asymmetric carbon atoms was separated into two racemates. The physical constants of 8 unknown benzoyl chlorides are also reported.

A well known local anesthetic is Nupercaine, *i.e.* N-(2-diethylaminoethyl)2-butoxycinchoninamide [1,2]. This compound was the first local anesthetic belonging to the amide type, that was introduced into the market. The discovery of nupercaine led to further research on amides as local anesthetics. Thus Eisleb [3] in a patent describes benzamides of the general structure:

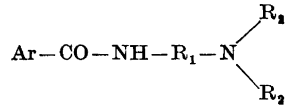


(R_1, R_2, and R_3 = alkyls; $n \geq 2$)

An amide of the anilide type is Xylocaine®, *i.e.* α-(diethylamino)-2,6-dimethylacetanilide which was introduced clinically in 1948 [4,5a]. Since then an extensive research work on anilides as local anesthetics has been carried out, and in the last few years anesthetics of the benzamide type have been subjected to much interest. Thus Hazard *et al.*[6,7], Hach *et al.*[8], Koelzer and Wehr [9], Moore [10], as well as Löfgren and Stoffel [11] have investigated benzamides in which the aryl group consists of a hydrocarbon residue.

* For paper XVII of this series, see Löfgren and Stoffel [11].

In the present work sixtythree new compounds of the benzamide type have been synthesized, and these will later be investigated for their local anesthetic action; all of these compounds are represented by the formula:



Ar is a methyl substituted benzene nucleus. The number of methyl groups in Ar is one, two, or three — all possible combinations, *viz.* fifteen, being included. R_1 is an alkylene radical presented in Table 1 ; R_2 is methyl or ethyl. Chemical data of the bases synthesized are given in Table 2.

To synthesize N-(dialkylaminoalkyl)benzamides, one mole of acid chloride and 1.6 mole of dialkylaminoalkylamine were mixed in ether, the excess of amine being used as a proton acceptor. If only one mole of amine per mole of acid chloride was used, the yield was considerably lower (*c.f.* p. 1631).

Most of the methyl substituted benzoic acids used in this investigation have recently been described by Smith and Stanfield [12]. The methods of synthesis reported by these investigators were not followed in all cases but the melting points of the acids are in agreement with the reported ones [12].

2,3,5-Trimethylbenzoic acid and 3,4,5-trimethylbenzoic acid were prepared in two different ways. The conventional route, which was first followed, involves the oxidation of *iso*durene with dilute nitric acid [13,14]. However, this process gives a mixture of three monobasic acids, four dibasic acids, nitro

Table 1. Alkylene radicals R_1 (*cf.* above).

Ethylene	$-CH_2-CH_2-$
Trimethylene	$-CH_2-CH_2-CH_2-$
1-Methylethylene	$-CH(CH_3)-CH_2-$
2-Methylethylene	$-CH_2-CH(CH_3)-$
1,2-Dimethylethylene	$-CH(CH_3)-CH(CH_3)-$
2,2-Dimethyl-1,3-propylene	$-CH_2-C(CH_3)_2-CH_2-$

acids, and an appreciable amount of nonacidic nitro compounds [14]. Only about 10 % of this mixture consists of monobasic acids. 3,4,5-Trimethylbenzoic acid and 2,3,5-trimethylbenzoic acid were isolated from this mixture according to a method described under 3,4,5-trimethylbenzoic acid (*cf.* p.1628). This process differs in several ways from that given by Jannasch and Weiler [14]. Because of the small yield (1 % of each acid) and since the 2,3,5-trimethylbenzoic acid prepared in this way is also very difficult to purify, other routes of synthesis of the two acids were devised. 2,3,5-Trimethylbenzoic acid was prepared by carbonation [15] of the Grignard reagent of 6-bromopseudocumene, which was obtained by a four step procedure from pseudocumene [16]. 3,4,5-Trimethylbenzoic acid was made from 5-bromohemimellitene *via* the Grignard reagent. The bromo compound was prepared by a Sandmeyer reaction [17] from 3,4,5-trimethylaniline, which was obtained by a nuclear methylation of 3,5-dimethylaniline according to a recently published method [18].

Some of the acid chlorides required for the synthesis of the amides have not been hitherto reported in literature; their data are found in Table 3.

Most of the amines used in this investigation have been described by Löfgren and Stoffel [11]. 1-Amino-3-diethylamino-2,2-dimethylpropane was obtained by $LiAlH_4$ reduction [19] of the oxime of diethylaminopivalaldehyde [20].

2-Amino-3-diethylaminobutane contains two asymmetric carbon atoms, and consequently each of the corresponding amides forms two racemates having different chemical properties. In fact, these amides appear as mixtures of crystals and oil. The values given in Table 2 refer to these mixtures. The two racemates were separated completely and collected only in one case, *viz.* the *o*-toluamide. Of the other racemates, only the solid forms were collected. They were obtained as crystalline substances after treating the semi-solids on porous plate. The melting points of these substances are given in brackets in Table 2.

Pharmacological data, ultraviolet spectra, and ionization constants will be published elsewhere, together with a discussion of the results.

EXPERIMENTAL *

o-, m-, and p-*Toluic acids* were all available commercially. *2,3-Dimethylbenzoic acid* **, *2,4-dimethylbenzoic acid*, *2,5-dimethylbenzoic acid*, and *3,4-dimethylbenzoic acid* were synthesized from the corresponding amino compounds by diazotization followed by treatment of the diazonium salt with potassium cyanocuprate(I) [21]: The nitriles formed were saponified by treatment with 50 % (vol/vol) sulfuric acid [22].

3,4-Dimethylbenzoic acid was also prepared in the following way: *o*-xylene was brominated [23] to form 3,4-dimethylbromobenzene, which was then converted to the acid by carbonation [15] of the corresponding Grignard reagent.

2,6-Dimethylbenzoic acid. To obtain this acid 2,6-dimethylbromobenzene was prepared by means of a Sandmeyer reaction [17] and then transformed to the acid *via* the Grignard compound [15].

3,5-Dimethylbenzoic acid was made from mesitylene by partial oxidation with dilute nitric acid [24].

* All melting points are corrected unless otherwise stated.

** The melting points of the acids were all in accordance with those given by Smith and Stanfield [12].

Table 2. Chemical data of N-dialkylaminoalkyl benzamides I—LXIV. Abbreviations for solvents: L.pet.,

Compound Ar—CO—NH—R_1—$N(R_2)_2$			
Number	Name	Ar	R_1
I	N-(2-Diethylaminoethyl)-2-methylbenzamide	2-$CH_3C_6H_4$	CH_2CH_2
II	N-(3-Diethylaminopropyl)-2-methylbenzamide	»	$CH_2CH_2CH_2$
III	N-(2-Diethylamino-1-methylethyl)-2-methylbenzamide	»	$CH(CH_3)CH_2$
IV	N-(2-Diethylamino-2-methylethyl)-2-methylbenzamide	»	$CH_2CH(CH_3)$
V*a* [c]	N-(2-Diethylamino-1,2-dimethylethyl)-2-methylbenzamide	»	$CH(CH_3)CH(CH_3)$
V*b* [c]	N-(2-Diethylamino-1,2-dimethylethyl)-2-methylbenzamide	»	»
VI	N-(3-Diethylamino-2,2-dimethylpropyl)-2-methylbenzamide	»	$CH_2C(CH_3)_2CH_2$
VII	N-(2-Diethylaminoethyl)-3-methylbenzamide	3-$CH_3C_6H_4$	CH_2CH_2
VIII	N-(3-Diethylaminopropyl)-3-methylbenzamide	»	$CH_2CH_2CH_2$
IX	N-(2-Diethylamino-1-methylethyl)-3-methylbenzamide	»	$CH(CH_3)CH_2$
X	N-(2-Diethylamino-2-methylethyl)-3-methylbenzamide	»	$CH_2CH(CH_3)$
XI	N-(2-Diethylamino-1,2-dimethylethyl)-3-methylbenzamide	»	$CH(CH_3)CH(CH_3)$
XII	N-(3-Diethylamino-2,2-dimethylpropyl)-3-methylbenzamide	»	$CH_2C(CH_3)_2CH_2$
XIII	N-(2-Diethylaminoethyl)-4-methylbenzamide	4-$CH_3C_6H_4$	CH_2CH_2
XIV	N-(3-Diethylaminopropyl)-4-methylbenzamide	»	$CH_2CH_2CH_2$
XV	N-(2-Diethylamino-1-methylethyl)-4-methylbenzamide	»	$CH(CH_3)CH_2$
XVI	N-(2-Diethylamino-2-methylethyl)-4-methylbenzamide	»	$CH_2CH(CH_3)$
XVII	N-(2-Diethylamino-1,2-dimethylethyl)-4-methylbenzamide	»	$CH(CH_3)CH(CH_3)$
XVIII	N-(3-Diethylamino-2,2-dimethylpropyl)-4-methylbenzamide	»	$CH_2C(CH_3)_2CH_2$
XIX	N-(2-Diethylaminoethyl)-2,3-dimethylbenzamide	2,3-$(CH_3)_2C_6H_3$	CH_2CH_2
XX	N-(3-Diethylaminopropyl)-2,3-dimethylbenzamide	»	$CH_2CH_2CH_2$
XXI	N-(2-Diethylamino-1-methylethyl)-2,3-dimethylbenzamide	»	$CH(CH_3)CH_2$
XXII	N-(2-Diethylamino-2-methylethyl)-2,3-dimethylbenzamide	»	$CH_2CH(CH_3)$
XXIII	N-(2-Diethylaminoethyl)-2,4-dimethylbenzamide	2,4-$(CH_3)_2C_6H_3$	CH_2CH_2
XXIV	N-(3-Diethylaminopropyl)-2,4-dimethylbenzamide	»	$CH_2CH_2CH_2$
XXV	N-(2-Diethylamino-1-methylethyl)-2,4-dimethylbenzamide	»	$CH(CH_3)CH_2$
XXVI	N-(2-Diethylamino-2-methylethyl)-2,4-dimethylbenzamide	»	$CH_2CH(CH_3)$
XXVII	N-(2-Diethylaminoethyl)-2,5-dimethylbenzamide	2,5-$(CH_3)_2C_6H_3$	CH_2CH_2
XXVIII	N-(3-Diethylaminopropyl)-2,5-dimethylbenzamide	»	$CH_2CH_2CH_2$
XXIX	N-(2-Diethylamino-1-methylethyl)-2,5-dimethylbenzamide	»	$CH(CH_3)CH_2$
XXX	N-(2-Diethylamino-2-methylethyl)-2,5-dimethylbenzamide	»	$CH_2CH(CH_3)$
XXXI	N-(3-Diethylamino-2,2-dimethylpropyl)-2,6-dimethylbenzamide	2,6-$(CH_3)_2C_6H_3$	$CH_2C(CH_3)_2CH_2$
XXXII	N-(2-Diethylaminoethyl)-3,4-dimethylbenzamide	3,4-$(CH_3)_2C_6H_3$	CH_2CH_2
XXXIII	N-(3-Diethylaminopropyl)-3,4-dimethylbenzamide	»	$CH_2CH_2CH_2$
XXXIV	N-(2-Diethylamino-1-methylethyl)-3,4-dimethylbenzamide	»	$CH(CH_3)CH_2$
XXXV	N-(2-Diethylamino-2-methylethyl)-3,4-dimethylbenzamide	»	$CH_2CH(CH_3)$
XXXVI	N-(2-Diethylaminoethyl)-3,5-dimethylbenzamide	3,5-$(CH_3)_2C_6H_3$	CH_2CH_2
XXXVII	N-(2-Diethylamino-1-methylethyl)-3,5-dimethylbenzamide	»	$CH(CH_3)CH_2$
XXXVIII	N-(2-Diethylamino-2-methylethyl)-3,5-dimethylbenzamide	»	$CH_2CH(CH_3)$
XXXIX	N-(2-Diethylamino-1,2-dimethylethyl)-3,5-dimethylbenzamide	»	$CH(CH_3)CH(CH_3)$
XL	N-(3-Diethylamino-2,2-dimethylpropyl)-3,5-dimethylbenzamide	»	$CH_2C(CH_3)_2CH_2$
XLI	N-(2-Diethylaminoethyl)-2,3,4-trimethylbenzamide	2,3,4-$(CH_3)_3C_6H_2$	CH_2CH_2
XLII	N-(3-Diethylaminopropyl)-2,3,4-trimethylbenzamide	»	$CH_2CH_2CH_2$
XLIII	N-(2-Dimethylaminoethyl)-2,3,4-trimethylbenzamide	»	CH_2CH_2
XLIV	N-(2-Diethylamino-1-methylethyl)-2,3,4-trimethylbenzamide	»	$CH(CH_3)CH_2$
XLV	N-(2-Diethylamino-2-methylethyl)-2,3,4-trimethylbenzamide	»	$CH_2CH(CH_3)$
XLVI	N-(2-Diethylaminoethyl)-2,3,5-trimethylbenzamide	2,3,5-$(CH_3)_3C_6H_2$	CH_2CH_2
XLVII	N-(3-Diethylaminopropyl)-2,3,5-trimethylbenzamide	»	$CH_2CH_2CH_2$
XLVIII	N-(2-Diethylaminoethyl)-2,3,6-trimethylbenzamide	2,3,6-$(CH_3)_3C_6H_2$	CH_2CH_2
XLIX	N-(3-Diethylaminopropyl)-2,3,6-trimethylbenzamide	»	$CH_2CH_2CH_2$
L	N-(2-Dimethylaminoethyl)-2,3,6-trimethylbenzamide	»	CH_2CH_2
LI	N-(2-Diethylamino-1-methylethyl)-2,3,6-trimethylbenzamide	»	$CH(CH_3)CH_2$

light petroleum b. p. 40°—60°; Pet., petroleum ether b. p. 60°—85°; *Iso*prop. eth., *iso*propyl ether.

R_2	Empirical formula	Yield %	B. p. °C, uncorr.	B. p. Pressure mm Hg	n_D^{25}	M.p., °C corr.	Solvent for recrystallization	Equivalent weight [a] Calc.	Equivalent weight [a] Found
C_6H_5	$C_{14}H_{22}N_2O$	63	132—134	0.10	1.5209	—	—	234.3	234
»	$C_{15}H_{24}N_2O$	76	167	0.05	1.5183	—	—	248.4	248
»	$C_{15}H_{24}N_2O$	89	135—137	0.10	1.5125	—	—	248.4	248
»	$C_{15}H_{24}N_2O$	95	146	0.10	1.5168	—	—	248.4	248
»	$C_{16}H_{26}N_2O$	21	123—124	0.20	—	52	L.pet.	262.4	262
»	$C_{16}H_{26}N_2O$	22	—	—	—	111.5—112	L.pet.-*Iso*prop. eth.	262.4	264
»	$C_{17}H_{28}N_2O$	83	152	0.08	1.5132	—	—	276.4	277
»	$C_{14}H_{22}N_2O$	75	163—165	0.10	1.5251	—	—	234.3	235
»	$C_{15}H_{24}N_2O$	90	156—158	0.10	1.5230	—	—	248.4	249
»	$C_{15}H_{24}N_2O$	89	142—143	0.10	—	50—51	—	248.4	248
»	$C_{15}H_{24}N_2O$	90	164	0.15	1.5200	—	—	248.4	249
»	$C_{16}H_{26}N_2O$	85	151—155	0.30	1.5176 [b]	(66—69)	—	262.4	261
»	$C_{17}H_{28}N_2O$	85	155	0.10	1.5177	—	—	276.4	278
»	$C_{14}H_{22}N_2O$	71	140—141	0.15	1.5268	36—37	—	234.3	234
»	$C_{15}H_{24}N_2O$	85	155—157	0.15	1.5253	—	—	248.4	248
»	$C_{15}H_{24}N_2O$	85	—	—	—	75—76	L.pet.-*Iso*prop. eth.	248.4	248
»	$C_{15}H_{24}N_2O$	93	149—150	0.10	—	62—63	—	248.4	248
»	$C_{16}H_{26}N_2O$	87	147—149	0.30	1.5190 [b]	(109—110)	—	262.4	262
»	$C_{17}H_{28}N_2O$	83	156—158	0.10	1.5193 [b]	38—39	—	276.4	277
»	$C_{15}H_{24}N_2O$	74	154	0.15	1.5220	—	—	248.4	249
»	$C_{16}H_{26}N_2O$	84	152—153	0.12	1.5197	—	—	262.4	263
»	$C_{16}H_{26}N_2O$	69	—	—	—	74—75	Pet.-*Iso*prop. eth.	262.4	262
»	$C_{16}H_{26}N_2O$	78	157	0.10	1.5190	—	—	262.4	262
»	$C_{15}H_{24}N_2O$	81	145—147	0.15	1.5202	—	—	248.4	249
»	$C_{16}H_{26}N_2O$	77	164	0.15	1.5184	—	—	262.4	262
»	$C_{16}H_{26}N_2O$	85	149	0.15	1.5127	—	—	262.4	262
»	$C_{16}H_{26}N_2O$	89	151	0.15	1.5172	—	—	262.4	262
»	$C_{15}H_{24}N_2O$	73	163—167	0.05	1.5189 [b]	49.5	—	248.4	250
»	$C_{16}H_{26}N_2O$	78	—	—	—	78—79	Pet.-*Iso*prop. eth.	262.4	265
»	$C_{16}H_{26}N_2O$	85	150	0.15	1.5113	—	—	262.4	262
»	$C_{16}H_{26}N_2O$	91	152	0.15	1.5152	23—24	—	262.4	262
»	$C_{18}H_{30}N_2O$	68	—	—	—	74—74.5	Pet.	290.4	289
»	$C_{15}H_{24}N_2O$	92	160—162	0.15	1.5299 [b]	65	—	248.4	248
»	$C_{16}H_{26}N_2O$	85	171	0.12	1.5280	—	—	262.4	263
»	$C_{16}H_{26}N_2O$	91	162	0.15	1.5241	—	—	262.4	261
»	$C_{16}H_{26}N_2O$	95	161—163	0.14	1.5250	—	—	262.4	261
»	$C_{15}H_{24}N_2O$	90	148—151	0.12	1.5250	—	—	248.4	249
»	$C_{16}H_{26}N_2O$	90	170—173	0.10	1.5182	—	—	262.4	261
»	$C_{16}H_{26}N_2O$	90	177—180	0.10	1.5195	—	—	262.4	262
»	$C_{17}H_{28}N_2O$	84	158—160	0.30	1.5173 [b]	(111—112)	—	276.4	277
»	$C_{18}H_{30}N_2O$	97	158—160	0.10	1.5174	—	—	290.4	292
»	$C_{16}H_{26}N_2O$	93	154	0.10	1.5246	—	—	262.4	259
»	$C_{17}H_{28}N_2O$	95	165—166	0.10	1.5227 [b]	37—38	—	276.4	273
CH_3	$C_{14}H_{22}N_2O$	85	152—154	0.12	1.5325 [b]	71—72	—	234.3	234
C_2H_5	$C_{17}H_{28}N_2O$	86	159—160	0.10	1.5169 [b]	38—39	—	276.4	273
»	$C_{17}H_{28}N_2O$	69	160	0.10	1.5228 [b]	35	—	276.4	277
»	$C_{16}H_{26}N_2O$	98	—	—	—	51—52	[c] —	262.4	263
»	$C_{17}H_{28}N_2O$	95	—	—	—	64—65	L.pet.-*Iso*prop. eth.	276.4	278
»	$C_{16}H_{26}N_2O$	87	—	—	—	75.5	Pet.-*Iso*prop. eth.	262.4	264
»	$C_{17}H_{28}N_2O$	72	—	—	—	84	» »	276.4	277
CH_3	$C_{14}H_{22}N_2O$	61	—	—	—	96—97	» »	234.3	236
C_2H_5	$C_{17}H_{28}N_2O$	87	—	—	—	96— 97	» »	276.4	277

Table 2 cont.

LII	N-(2-Diethylamino-2-methylethyl)-2,3,6-trimethylbenzamide	2,3,6-$(CH_3)_3C_6H_2$	$CH_2CH(CH_3)$
LIII	N-(2-Diethylaminoethyl)-2,4,5-trimethylbenzamide	2,4,5-$(CH_3)_3C_6H_2$	CH_2CH_2
LIV	N-(3-Diethylaminopropyl)-2,4,5-trimethylbenzamide	»	$CH_2CH_2CH_2$
LV	N-(2-Dimethylaminoethyl)-2,4,5-trimethylbenzamide	»	CH_2CH_2
LVI	N-(2-Diethylamino-1-methylethyl)-2,4,5-trimethylbenzamide	»	$CH(CH_3)CH_2$
LVII	N-(2-Diethylamino-2-methylethyl)-2,4,5-trimethylbenzamide	»	$CH_2CH(CH_3)$
LVIII	N-(2-Diethylaminoethyl)-2,4,6-trimethylbenzamide [d]	2,4,6-$(CH_3)_3C_6H_2$	CH_2CH_2
LIX	N-(3-Diethylaminopropyl)-2,4,6-trimethylbenzamide	»	$CH_2CH_2CH_2$
LX	N-(2-Dimethylaminoethyl)-2,4,6-trimethylbenzamide	»	CH_2CH_2
LXI	N-(2-Diethylamino-1-methylethyl)-2,4,6-trimethylbenzamide	»	$CH(CH_3)CH_2$
LXII	N-(2-Diethylamino-2-methylethyl)-2,4,6-trimethylbenzamide	»	$CH_2CH(CH_3)$
LXIII	N-(2-Diethylaminoethyl)-3,4,5-trimethylbenzamide	3,4,5-$(CH_3)_3C_6H_2$	CH_2CH_2
LXIV	N-(3-Diethylaminopropyl)-3,4,5-trimethylbenzamide	»	$CH_2CH_2CH_2$

[a] Titration of the base in 30 % ethanol with 0.1 N HCl; mixed indicator methylene blue — methyl red.
[b] Supercooled.
[c] Purified *via* the hydrochloride, see Table 3.

2,3,4-Trimethylbenzoic acid was synthesized from 2,6-dimethylaniline, which was first converted to 2,6-dimethyliodobenzene by means of a Sandmeyer reaction [25]. 2,6-Dimethyliodobenzene was converted to hemimellitene by treatment of the corresponding Grignard reagent with dimethyl sulfate according to a general method [26]. The hemimellitene was brominated to form 4-bromohemimellitene [27] from which the acid was obtained by carbonation [15] of the Grignard reagent.

2,3,5-Trimethylbenzoic acid was synthesized by oxidation of *iso*durene as is described under *3,4,5-trimethylbenzoic acid (cf.* below). The 2,3,5-trimethylbenzoic acid was also made by nitration of pseudocumene, and the 5-nitropseudocumene thus formed was then reduced with iron and acetic acid [5b] to pseudocumidine. This was brominated in concentrated hydrochloric acid [16,27] giving 2-bromo-3,4,6-trimethylanilinium chloride, which was then de-aminated by treatment with ethyl nitrite and concentrated sulfuric acid in ethanol solution [28]. The acid was obtained from the bromo compound by carbonation [15] of the corresponding Grignard reagent; yield 33 %; m. p. 126°—127°. (Jannasch and Weiler [14] state m. p. 127° for this acid.)

2,3,6-Trimethylbenzoic acid was made in a good yield from pseudocumene according to Smith and Stanfield [12].

2,4,5-Trimethylbenzoic acid was obtained by partial oxidation of durene with dilute nitric acid [30].

2,4,6-Trimethylbenzoic acid was prepared from mesitylene by bromination [23] and subsequent carbonation [15] of the corresponding Grignard reagent.

*3,4,5-Trimethylbenzoic acid. Iso*durene, 50 g (0.37 mole), was added to 225 ml of water and 75 ml of nitric acid (d = 1.40; 1.15 mole) and the mixture was refluxed for 6 h. After cooling, the reaction products were taken up in ether, which was then extracted with potassium carbonate solution. The oily residue remaining after evaporation of the ether was subjected to another oxidation with nitric acid. The potassium carbonate solutions obtained from the two runs were combined and acidified. After cooling to 0°, the aqueous layer was decanted off and rejected. The residue was dissolved in 100 ml of glacial acetic acid, 5 g of zinc powder were added, and the mixture boiled for 30 min. Water, 700 ml, was added and the aqueous solution containing acetic acid was decanted off from the sticky yellow residue, which was dissolved in 375 ml of ethanol. To this solution, dry hydrogen chloride was introduced at a "fairly rapid rate" [31] for 30 min. A large excess of water was added and the esters formed were taken up in ether, which was then washed three times with dilute sodium carbonate solution. The ether was evaporated, and the residual esters were saponified on the steam bath with 5 % potassium hydroxide in

Table 2 cont.

I_5	$C_{17}H_{28}N_2O$	88	160—162	0.12	1.5163 [b]	39— 40		—	276.4	276
	$C_{16}H_{26}N_2O$	71	—	—	—	62	Pet.-*Iso*prop. eth.		262.4	263
	$C_{17}H_{28}N_2O$	81	—	—	—	73— 74	»	»	276.4	278
I_3	$C_{14}H_{22}N_2O$	68	—	—	—	80— 81	»	»	234.3	236
I_5	$C_{17}H_{28}N_2O$	93	155—157	0.12	—	69		—	276.4	274
	$C_{17}H_{28}N_2O$	96	168—171	0.10	1.5205 [b]	44— 45		—	276.4	276
	$C_{16}H_{26}N_2O$	70	150—151	0.10	—	71— 72	Pet.-*Iso*prop. eth.		262.4	263
	$C_{17}H_{28}N_2O$	76	—	—	—	67— 68	»	»	276.4	277
3	$C_{14}H_{22}N_2O$	61	—	—	—	86— 87	»	»	234.3	236
I_5	$C_{17}H_{28}N_2O$	85	164	0.10	1.5063 [b]	52— 53		—	276.4	275
	$C_{17}H_{28}N_2O$	84	165—167	0.10	—	46— 48		—	276.4	277
	$C_{16}H_{26}N_2O$	95	—	—	—	74— 75	[c]	—	262.4	262
	$C_{17}H_{28}N_2O$	97	—	—	—	53— 54	[c]	—	276.4	276

[d] During the course of this work compound LVIII was synthesized by Hach *et al.*[8] and studied pharmacologically by Koelzer and Wehr [9]. Hach *et al.*[8] describe the compound as melting at 64°. Moreover, they did not succeed in preparing the hydrochloride of LVIII. (*cf.* Table 3.)

[e] *cf.* p. 1632.

ethanol. After precipitation of the "*iso*durylic" acids with dilute hydrochloric acid, they were dissolved in thionyl chloride. The solution was refluxed for 5 h, and after removing excess thionyl chloride the residue was distilled at 10 mm Hg. A fraction boiling at 120°—135° was collected and hydrolyzed at room temperature with 30 % sodium hydroxide. The mixture of acids was precipitated and dissolved in an equivalent amount of 10 % sodium hydroxide. The solution was washed with ether, and an excess of saturated barium chloride solution added. A voluminous precipitate separated from the solution, and was collected. The acid, liberated from this slightly soluble barium salt, is 3,4,5-trimethylbenzoic acid; m. p. 215°; yield (based on 450 g, *i. e.* 3.36 mole of *iso*durene): 7.3 g (0.044 mole, 1.3 %); recorded m. p. [13,14] 215°. The filtrate which was separated from the barium salt precipitate (*cf.* above) gave another acid, which after several recrystallizations from different solvents (*e. g.* ethanol, light petroleum) melted at 108°. This must be impure 2,3,5-trimethylbenzoic acid since dry distillation with soda lime gave an oil, which after sulfonation yielded a sulfonamide of m. p. 172°—174°. Very probably this compound was identical with pseudocumenesulfonamide with a recorded m. p. at 174° [13,32] *.

The yield of acid was 4.8 g (0.030 mole, 0.9 %). (Melting point of pure 2,3,5-trimethylbenzoic acid, *cf.* above.) (Found: equiv. wt.** 161. Calc. for $C_{10}H_{12}O_2$: 164.)

3,4,5-Trimethylbenzoic acid was also obtained by carbonation of the Grignard reagent of 5-bromohemimellitene, this compound being prepared from 3,4,5-trimethylaniline.

3,4,5-Trimethylaniline ***. 3,5-Dimethylaniline, 47 g (0.39 mole), was dissolved in 30 ml of 37 % HCl (0.36 mole) and 200 ml of water. After cooling to 0°, 75 ml of 37 % (1.0 mole) formaldehyde solution was added under stirring. The temperature was kept at 0° for 60 min and was then allowed to reach 20°. After adding a large excess of sodium carbonate solution, the orange coloured sludge was filtered and the collected solid was dried at 80°. The resulting yellow powder, after mixing with 60 g (0.81 mole) of calcium hydroxide, was dry distilled in a wide necked distillation apparatus, heated by means of a metal bath which was kept at 500°. A semi-solid distillate formed which was taken up in a mixture of ether and 20 % hydrochloric acid. Following removal of the ether phase,

* No mixed melting point could be determined because of lack of substance.

** Titration of the acid with NaOH, using phenolphtalein as an indicator.

*** This compound has been obtained previously but in a very low yield [33,34]. The method applied here is a modification of one described by Barclay *et al.* [18], and has given a relatively good yield.

Table 3. Data of acid chlorides synthesized in this investigation.

Acyl radical	B. p.		M. p.	n_D^{25}	Yield	Cl	
	°C, uncorr.	Pressure mm Hg	°C, corr.		%	Calc.	Found
2,3-Dimethylbenzoyl	122–123	15	+2	1.5530	63	21.0	20.9
2,5-Dimethylbenzoyl	112–114	13	—	1.5490	81	21.0	21.0
3,4-Dimethylbenzoyl	122	10	−3–−1	1.5563	78	21.0	21.0
2,3,4-Trimethylbenzoyl	138–140	14	—	1.5614	84	19.5	19.3
2,3,5-Trimethylbenzoyl	126–127	10	35–37	1.5515[a]	65	19.5	19.3
2,3,6-Trimethylbenzoyl	116–117	10	—	1.5295	85	19.5	19.1
2,4,5-Trimethylbenzoyl	142–145	15	20–21	1.5595	85	19.5	19.5
3,4,5-Trimethylbenzoyl	137–139	10	44–46	—	86	19.5	19.6

[a] Measurement made on supercooled compound.

the base was liberated from the aqueous acid solution and distilled. Yield of 3,4,5-trimethylaniline boiling at 238°–248° was 11.3 g (0.084 mole, 22 %); m. p. 68°–72°. Acetyl derivative recrystallized from methanol; m. p. 163°–164°. (Recorded melting point [33,34] of base is 76°, and of acetyl derivative 164°.)

5-Bromohemimellitene. 3,4,5-Trimethylaniline was converted to 5-bromohemimellitene by a Sandmeyer reaction [17]. This compound appeared as an orange coloured oil; b. p. 109°–114°/12 mm Hg; n_D^{25} 1.5560; yield 34 % (*cf.* Ref.[35]). The low yield is probably due to instability, even at 0°, of the diazonium salt. 3,4,5-Trimethylbenzoic acid was obtained from the bromo compound by carbonation [15] of the corresponding Grignard reagent; yield 64 %; m. p. 214° (*cf.* above).

Acid chlorides. The acids were converted to the corresponding acid chlorides by treatment with thionyl chloride and subsequent distillation [36]. In Table 3 the physical constants, yields, and analyses are given for those acid chlorides which have not been described previously.

1-Amino-2-dimethylaminoethane was prepared by a Mannich reaction between dimethylammonium chloride, paraformaldehyde, and cyanide ion [37] and subsequent reduction of the condensation product according to a general method with $LiAlH_4$ [19]: b. p. 103°–108°; yield 36 % (*cf.* Ref.[37]).

1-Amino-2-diethylaminoethane was available commercially.

1-Amino-3-diethylaminopropane has been obtained by cyanoethylation of diethylamine [38], and subsequent reduction of the cyano group with $LiAlH_4$ [19] (*cf.* Refs.[11,38]).

2-Amino-1-diethylaminopropane. From diethylaminoacetone the oxime was prepared, the general method given by Vogel [39] being followed; yield 80 %; b. p. 98°–100°/10 mm Hg; m. p. 40°. (Found: equiv. wt.* 143. Calc. for $C_7H_{16}N_2O$: 144.2.) The desired amine was obtained from the oxime in accordance with a method given for the reduction of nitriles with $LiAlH_4$ [19]; yield 53 % (*cf.* Ref.[11]).

1-Amino-2-diethylaminopropane was prepared *via* the nitrile [40] (Mannich reaction), reduction being carried out with $LiAlH_4$ [19] (*cf.* Ref.[11]).

2-Amino-3-diethylaminobutane was made [11] by bromination of ethyl methyl ketone, and subsequent separation of the isomeric bromo ketones [41]. The resulting 3-bromo-2-butanone was treated with diethylamine to form 3-diethylamino-2-butanone; this was converted to the corresponding oxime which was then reduced with $LiAlH_4$ [19]; yield 58 % (*cf.* Ref.[11]).

*1-Amino-3-diethylamino-2,2-dimethylpropane. Iso*butyraldehyde, paraformaldehyde, and diethylammonium chloride were condensed by a Mannich reaction to diethylamino-

* Titration with 0.1 N HCl in 30 % ethanol; mixed indicator methylene blue — methyl red.

Table 4. Chemical data of hydrochlorides prepared. Abbreviations for solvents: *iso*Prop. eth., di-*iso*propyl ether; *iso*Prop.-ol, *iso*propanol; Me.am.ket., methyl amyl ketone; Me.et.ket., methyl ethyl ketone.

Compound *B* · *HCl*		M.p., °C	Solvent for recrystallization	Analyses Cl	
B	Empirical formula	corr.		Calc.	Found
I	$C_{14}H_{23}ClN_2O$	105	*iso*Prop.eth.-*iso*Prop.-ol	13.1	12.9
V*b*	$C_{16}H_{27}ClN_2O$	165—166	» »	11.9	11.8
VII	$C_{14}H_{23}ClN_2O$	106	» »	13.1	13.0
XIII	$C_{14}H_{23}ClN_2O$	99—100	» »	13.1	13.1
XIX	$C_{15}H_{25}ClN_2O$	121—122	» »	12.4	12.3
XXIII	$C_{15}H_{25}ClN_2O$	122—123	» »	12.4	12.3
XXVII	$C_{15}H_{25}ClN_2O$	106—107	» »	12.4	12.3
N-(2-Diethylaminoethyl)--2,6-dimethylbenzamide [a]	$C_{15}H_{25}ClN_2O$	139—140	» »	12.4	12.4
XXXII	$C_{15}H_{25}ClN_2O$	150—151	» »	12.4	12.4
XXXVI	$C_{15}H_{25}ClN_2O$	117—118	» »	12.4	12.4
N-(3-Diethylaminopropyl)--3,5-dimethylbenzamide [a]	$C_{16}H_{27}ClN_2O$	117—118	» »	11.9	11.9
XXXVIII	$C_{16}H_{27}ClN_2O$	139—140	» »	11.9	11.8
XLI	$C_{16}H_{27}ClN_2O$	154—155	» »	11.9	11.9
XLVI	$C_{16}H_{27}ClN_2O$	139—140	» »	11.9	11.8
XLVIII	$C_{16}H_{27}ClN_2O$	138—139	» »	11.9	11.9
XLIX	$C_{17}H_{29}ClN_2O$	142—143	» »	11.3	11.2
L	$C_{14}H_{23}ClN_2O$	163—164	» »	13.1	13.1
LIII	$C_{16}H_{27}ClN_2O$	132—133	» »	11.9	11.8
LV	$C_{14}H_{23}ClN_2O$	171	» »	13.1	12.9
LVIII	$C_{16}H_{27}ClN_2O$	114—115	Me.am.ket.-*iso*Prop.-ol	11.9	11.8
LX	$C_{14}H_{23}ClN_2O$	152—154	*iso*Prop. eth.-*iso*Prop.-ol	13.1	13.1
LXIII	$C_{16}H_{27}ClN_2O$	207	Me.et.ket.-*iso*Prop.-ol	11.9	11.9
LXIV	$C_{17}H_{29}ClN_2O$	175	» »	11.3	11.5

[a] For the preparation of these bases see Löfgren and Stoffel [11].

pivalaldehyde [20]. From this aldehyde the oxime was prepared [39]; yield 89 %; b. p. 132°—133°/22 mm Hg; n_D^{20} 1.4649. Without further characterization this compound was reduced according to general methods by (a) sodium in ethanol [42] (b) $LiAlH_4$ [19]. The desired amine was obtained in a yield of 42 % and 45 %, respectively; b. p. 105°/52 mm Hg; n_D^{20} 1.4442. (Found: equiv. wt.* 78.8. Calc. for $C_9H_{22}N_2$: 79.1.)

Preparation of dialkylaminoalkylamides of methyl substituted benzoic acids. The appropriate dialkylaminoalkylamine (0.040 mole) was dissolved in 50 ml of highly purified ether in a 300 ml three necked round bottomed flask, equipped with a sealed Hershberg stirrer, funnel, and a condenser ($CaCl_2$). The desired acid chloride (0.025 mole) was dissolved in 50 ml of ether. Stirring was started, and the acid chloride solution was added to the amine solution during a period of 30 min, agitation being maintained for another 2 h. Then 100 ml of 2 N NaOH were added. The ethereal layer was separated off and washed twice with water. After drying (Na_2SO_4), the ether was driven off and the base was recrystallized or distilled. Characterization of the compounds is found in Table 2.

In order to study the same reaction but with the reactants in another proportion an experiment was carried out using 3,5-dimethylbenzoyl chloride and 1-amino-2-diethyl-

* Titration with $HClO_4$ in glacial acetic acid; indicator BZL-BLUE (Ciba) [43].

aminoethane in the ratio 1:1.1; the yield of N-(2-diethylaminoethyl)3,5-dimethylbenzoyl chloride was 53 %. When using the molar proportion 1:1.6 as is described in the general method above the yield of the same product was 90 %.

Hydrochlorides were prepared from the bases by dissolving them in dry ether and adding an appropriate amount of dry hydrogen chloride in dry ether. The colourless microcrystalline precipitates were recrystallized from suitable solvents. Melting points, solvents for recrystallization, and analytical data are found in Table 4.

Separation of the racemates of N-(2-diethylamino-1,2-dimethylethyl)2-methylbenzamide (V). From the oily crude base which remained after evaporation of the ether, crystals slowly deposited, at room temperature resulting in a semi-solid mass. The two phases were separated from each other according to the excellent method described by Morton [44] and Hicks [45]. The solid component was recrystallized twice from *iso*propyl ether containing light petroleum. The separated oil was then distilled. The oil is called V*a* and the solid V*b*. For further characterization *the picrate of V*a was made, and after two recrystallizations from *iso*propanol and one from dioxane, yellow rhombic plates of m. p. 146° were obtained. (Found: C 54.0; H 6.22; N 14.24; O 26.06. Calc. for $C_{22}H_{29}N_5O_8$: C 53.8; H 5.95; N 14.25; O 26.04.) The salt (2 g) was dispersed in 50 ml of ethanol and 175 ml of water. To the suspension thus formed, 150 ml * of moist Amberlite IRA 400 anion exchanger (chloride form) was added, the mixture being stirred for 6 h. From the colourless solution the base was liberated and taken up in ether. The solvent was evaporated leaving V*a* as a crystalline solid which was recrystallized once from light petroleum. Characterizations of the bases are found in Table 2.

*Di-picrate of V*b; needles from *iso*propanol of m. p. 126°. (Found: C 47.0; H 4.47; N 15.75; O 32.60. Calc. for $C_{28}H_{32}N_8O_{15}$: C 46.7; H 4.48; N 15.55; O 33.30.)

N-(2-Diethylamino-1,2-dimethylethyl)3-methylbenzamide (XI), N-(2-diethylamino-1,3-dimethylethyl)4-methylbenzamide (XVII), and N-(2-diethylamino-1,2-dimethylethyl)-3,5-dimethylbenzamide (XXXIX). After distillation of these bases, all of them appeared as semi-solid mixtures of crystals and oil. The refractive indices of these bases, given in Table 2, refer to the super-cooled mixtures. A small portion of each of the three bases was left on porous plate for three days. The crystals were scraped off and the melting points determined. These melting points are given in brackets in Table 2 (concerning theory see Morton [44]).

I wish to express my sincere gratitute to *Aktiebolaget Astra*, Södertälje for financial support during the course of this work.

REFERENCES

1. Soc. Anon. pour l'Ind. Chim. à Bâle. *Ger.* 540 697 (1933).
2. Miescher, K. *Helv. Chim. Acta* **15** (1932) 163.
3. Eisleb, O. *U. S.* 2 073 100 (1937).
4. Löfgren, N. M. *Arkiv Kemi, Mineral. Geol.* **22 A** (1946) No. 18.
5a. Löfgren, N. M. *Studies on Local Anesthetics. Xylocaine a New Synthetic Drug.* Diss. Stockholm 1948.
5b. *Ibid.* p. 27.
6. Hazard, R., Giudicelli, R., Thuillier, G. and Beauvallet, M. *Compt. rend. soc. biol.* **147** (1953) 755.
7. Hazard, R., Chabrier, P., Giudicelli, R., Beauvallet, M. and Thuillier, G. *Compt. rend. soc. biol.* **147** (1953) 1207.
8. Hach, V., Horáková, Z., Reichelt, J. and Havlova, D. *Chem. Listy* **51** (1957) 547.
9. Koelzer, P. P. and Wehr, K. H. *Arzneimittel-Forsch.* **8** (1958) 708.
10. Moore, M. B. *J. Am. Pharm. Assoc.* **40** (1951) 388.
11. Löfgren, N. M. and Stoffel, W. *Acta Chem. Scand.* **13** (1959) 1585.
12. Smith, H. A. and Stanfield, J. A. *J. Am. Chem. Soc.* **71** (1949) 81.
13. Jacobsen, O. *Ann.* **184** (1877) 184.
14. Jannasch, P. and Weiler, M. *Ber.* **27** (1894) 3441.

* A moist ion exchanger is most accurately measured by volume.

15. Bowen, D. M. *Org. Syntheses, Coll. Vol.* **3** (1955) 553.
16. Carpenter, M. S. and Easter, W. M. *J. Org. Chem.* **19** (1954) 85.
17. Hartwell, J. L. *Org. Syntheses, Coll. Vol.* **3** (1955) 185.
18. Barclay, M. G., Burawoy, A. and Thomson, G. H. *J. Chem. Soc.* **1944** 109.
19. Amundsen, L. H. and Nelson, L. S. *J. Am. Chem. Soc.* **73** (1951) 242.
20. Mannich, C., Lesser, B. and Sitten, F. *Ber.* **65 B** (1932) 378.
21. Clarke, H. T. and Read, R. R. *Org. Syntheses, Coll. Vol.* **1** (1941) 514.
22. Scholl, R. and Kačer, F. *Ber.* **36** (1903) 328.
23. Smith, L. I. *Org. Syntheses, Coll. Vol.* **2** (1943) 95.
24. Snyder, H. R. Adams, R. R. and Mc Intosh jr, A. V. *J. Am. Chem. Soc.* **63** (1941) 3280.
25. Lucas, H. J. and Kennedy, E. R. *Org. Syntheses, Coll. Vol.* **2** (1943) 351.
26. Smith, L. I. *Org. Syntheses, Coll. Vol.* **2** (1943) 360.
27. Smith, L. I. and Moyle, C. L. *J. Am. Chem. Soc.* **58** (1936) 9.
28. Schultz, G. *Ber.* **42** (1909) 3606.
29. Mayer, F. *Ber.* **20** (1887) 971.
30. Nef, A. *Ann.* **237** (1887) 7.
31. Meyer, V. *Ber.* **27** (1894) 510.
32. Jacobsen, O. *Ber.* **15** (1882) 1853.
33. Nölting, E. and Forel, S. *Ber.* **18** (1885) 2668.
34. Limpach, L. *Ber.* **21** (1888) 643.
35. Porowska, N. *Roczniki Chem.* **31** (1957) 677.
36. Barnes, R. P. *Org. Syntheses, Coll. Vol.* **3** (1955) 555.
37. Turner, R. A. *J. Am. Chem. Soc.* **68** (1946) 1607.
38. Kost, A. N. *Vestnik Moskov. Univ.* **1947 No. 2** 141.
39. Vogel, A. I. *A Textbook of Practical Organic Chemistry.* Longmans Green and Co. London 1948, p. 341, method B'.
40. Luten, D. B. *J. Org. Chem.* **3** (1938) 588.
41. Catch, J. R. Elliot, D. F. Hey, D. H. and Jones, E. R. H. *J. Chem. Soc.* **1948** 272.
42. Lycan, W. H. Puntambeker, S. V. Marvel, C. S. *Org. Syntheses, Coll. Vol.* **2** (1943) **318.**
43. Riddick, J. *Anal. Chem.* **24** (1952) 41.
44. Morton, A. A. *Laboratory Technique in Organic Chemistry.* McGraw-Hill Book Company Inc. New York 1938, p. 163.
45. Hicks, M. M. *Bur. Standards J. Research* **2** (1929) 484, method 4.

Received June 23, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1634–1638

An Initiating Mechanism of Color Formation and Decomposition in Polyvinyl Chloride

SVEN SÖNNERSKOG

A-B Svenskt Konstsilke, Borås, Sweden

The thermal instability of polyvinyl chloride, prepared in emulsion systems, is related to the presence of peroxides, formed during the polymerization process, or later, by autoxidation. The rate of color formation and decomposition is preferably studied in pyridine below 100°C. Redox systems, where the polymer takes part as an active component, are effective inhibitors of discoloration and degradation. Graft copolymers can be prepared by a seeding technique, based upon the same principle.

Polymerization of vinyl chloride is usually carried out in emulsion systems. The reaction takes place in water, containing ammonium or sodium salts of higher, saturated fatty acids. The aqueous phase is saturated with monomer under pressure at moderate temperatures (40—50°C). Persulphate ions are the preferred initiators. Sulphate ion radicals are generated by thermal dissociation, followed by diffusion into soap micelles, where solubilized monomer enters into reaction. The general picture of the kinetic scheme at steady state conditions is well-known from the fundamental work of Smith and Ewart [1,2].

The degree of conversion of monomer into polymer may be varied by release of pressure at different times of reaction. This is actually used in practice as a versatile method influencing upon the solubility characteristics of the final polymer; other working variables, such as added amounts of initiator, soap and modifiers being unconsidered.

A critical state arises in the polymerising system when remaining monomer is suddenly removed. Growing polymer radicals have to find a way of deactivation or propagation. Traces of molecular oxygen may act as comonomer: *

$$R\cdot + O_2 = R{-}O{-}O\cdot$$

* The occurrence of an induction period in vinyl chloride polymerization indicates formation of peroxide radicals at this early stage of the process, but this does not exclude the presence of oxygen at high degrees of conversion, as monomer is preferably added continually to the reacting system.

especially when the concentration of vinyl chloride is low. Radicals, trapped in the polymer particle, will react in the same way, when exposed to air at (spray) drying or grinding operations. Deactivation occurs by hydrogen abstraction from the surrounding medium. The net result is a polymeric hydroperoxide. High degree of stability and bad accessibility may cooperate to render its detection difficult by standard reagents.

The dry, purified polymer is soluble in pyridine at room temperature, or slightly above, at moderate degrees of polymerization. Such a solution remains colorless at 0—5°C in the absence of light, but discoloration rapidly takes place in sunlight or merely by heating, even in an inert atmosphere. The following changes of color are observed at 100°C (Table 1).

Table 1. Color formation in a solution of polyvinyl chloride in pyridine at 100°C. Concentration of solute: 10^{-2} g · ml^{-1}.

Time of heating, min	Color
0	Water white
10	Pale brown
25	Pale red brown
145	Red brown
300	Deeply red
330	Dusky brown
Blank of solvent after 330 min : water white	

The color changes indicated above are accompanied by a gradually decreased solubility, easily seen by cooling back to room temperature, when a gel is obtained from deeply colored solutions. This sol-gel transformation is reversible within the period of heating, found in Table 1. At continued thermolysis, approximately during 10—12 h, the hot solution shows increasingly viscoelastic properties, apparently due to formation of an infinite network. The dusky brown color is further enhanced.

It deserves to be noticed that similar results are obtained when the polymer is purified by reprecipitation from dioxane or separated into two fractions by soaking in acetone. The instability in pyridine seems to be a property inherently related to molecular structure.

Identical changes of color are observed when polyvinyl chloride is milled with plasticizers at elevated temperature in the absence of stabilizers. Extensive trial and error has been a requisite of finding adequate structures of such additives. The work has partly been guided by the current opinion that stabilizers principally act as scavengers for hydrogen chloride [3–6]. This is an oversimplification, as recently pointed out [7,8], as hydrogen chloride is a secondary product from a transfer reaction between a chlorine atom and a macromolecule:

$$\sim\sim\sim CH_2-CHCl-CH_2-CHCl\sim\sim + Cl\cdot = HCl + \sim\sim CH_2-CHCl-\dot{C}H-CHCl\sim\sim$$

leaving the question open in what way elementary chlorine is liberated. Earlier studies on dehydrochlorination of polyvinyl chloride [7-9] suggest the existence of thermolabile chlorine-carbon bonds in special chain sections of the macromolecules, for instance at branching units (chlorine bonded to a tertiary carbon atom) or in the vicinity of unsaturated end groups. These groups would be formed when termination occurs by disproportionation or by chain transfer to monomer. Many explanations merely rest on the incidental presence of initiator residues in the primary polymer particles. Such impurities dissociate on heating into free radicals and initiate degradation and decomposition by a chain reaction.

In the opinion of the writer no imperfections of these kinds are indispensable to explain the thermal instability of polyvinyl chloride, prepared in emulsion systems, as the presence of (hydro) peroxides can be varified experimentally in a very simple manner. Even a peroxide of unusual stability would be decomposed in a basic medium as pyridine by the formation of a redox system with said peroxide as active component. This can be done in many ways, but it is exceedingly important to find such coreactants that deactivate the released radicals without generation of a new population attacking the polymer. It also deserves to be noticed that free radicals and elementary chlorine mostly show identical type reactions, why a distinction between primary and secondary steps would be solely obtained at such a low reaction temperature, where thermal dissociation of C—Cl bonds is insignificant. Fortunately many redox systems are worked out with a view of acting at room temperature or below. Table 2 shows some reduction activators, inhibiting color formation in solu-

Table 2. Inhibitors of color formation in solutions of polyvinyl chloride in pyridine.

Coreactant(s)	Added amount to 1 g polymer	Suggested type reactions
$H_2N-OH \cdot HCl$	0.01—0.1 g	$ROOH \rightarrow RO\cdot + HO\cdot$ $HO\cdot + H_2N-OH \rightarrow H_2O + \dot{H}N-OH$ $RO\cdot + H_2N-OH \rightarrow ROH + \dot{H}N-OH$ $2\ \dot{H}N-OH \rightarrow N_2 + 2\ H_2O$
H_2N-NH_2 + $FeCl_2$	0.01—0.1 g 10 mg	$ROOH \rightarrow RO\cdot + HO\cdot$ $Fe^{2+} + HO\cdot \rightarrow Fe^{3+} + HO:^-$ $RO\cdot + H_2N-NH_2 \rightarrow ROH + \dot{H}N-NH_2$ $Fe^{3+} + H_2N-NH_2 \rightarrow Fe^{2+} + \dot{H}^+ + \dot{H}N-NH_2$ $H^+ + HO:^- \rightarrow H_2O$ $2\ \dot{H}N-NH_2 \rightarrow 2\ NH_3 + N_2$
$H_2N-NH_2 \cdot H_2O$	0.1 g	$N_2H_4 \cdot H_2O \rightleftharpoons H_2N-NH_3^+ + HO:^-$ $ROOH \xrightarrow{(HO:^-)} RO\cdot + HO\cdot$ $RO\cdot + N_2H_4 \rightarrow ROH + \dot{N}_2H_3$ $HO\cdot + N_2H_4 \rightarrow H_2O + \dot{N}_2H_3$ $2\ \dot{N}_2H_3 \rightarrow 2\ NH_3 + N_2$

tions of polyvinyl chloride in pyridine below 100°C. Inhibition has been considered as satisfactory when no color formation is visible during the schedule of observation, used in Table 1.

Several reducing agents behave as strong retarders of color formation and gelation. Paraformaldehyde, hydroquinone and *p*-hydroxybenzoic acid are a few of them. Classical inhibitors of vinyl polymerization, nitrobenzene and picric acid, show a similar effect. Unsaturated compounds, known to polymerize in a neutral aqueous medium by initiation of radicals, inhibit the later steps of the color formation cycle (red and dusky brown). This is especially valid for N,N′-methylene-bis(acrylamide).

The prehistory of the polymer is rather important, quite apart from the very method of preparation. It has been repeatedly observed that technical grades of polyvinyl chloride with a very low particle size are able to oxidise *p*-aminophenol, metol and pyrogallol at room temperature in the absence of air (suspension in methanol-water-reagent). A reprecipation from pyridine reduces the rate of oxidation, — indicated by the brown/black color in the liquid — but this improvement is gradually lost by repeated drying in air. Hydroperoxydation of polyvinyl chlroide would seem to be partly reversible.

Present experience on the properties of polyvinyl chloride (emulsion type) supports the idea that it might serve as an initiator of vinyl polymerization, provided the reaction medium is selected with care. This was proved by making a graft copolymer with acrylonitrile according to a "seeding" technique. The reacting mixture consisted of:

Polyvinyl chloride (5 g), dispersed in 500 g water,
Antarox G 100 (a nonionic detergent, 1 g)
Sodium pyrosulphite (1 g) and ferrous chloride (0.1 g)
Acrylonitrile (25 g)
Temp. of reaction: 32°C, time of reaction: 240 min, rate of stirring: 50 rpm.

Yield of pure, dry copolymer: 22 g.

It was easily verified that a true graft copolymer had been produced. Contrary to homopolymers of acrylonitrile dissolution can be carried out at 15—20°C, using only pyridine as solvent. Such a solution shows a very low viscosity and phase separation takes place on heating. Stable solutions are found in dimethyl formamide and dimethyl sulfoxide.

The high reactivity of polyvinyl chloride in the presence of reduction activators and a comonomer emphasises its character of peroxide or polymer radical. The inherent thermal instability of technically produced material is self-evident from this point of view. It also explains why a minor amount of (elementary) chlorine is stripped from the polymer already during its synthesis. After a long induction period due to oxygen, polymerization sets in at a rate far exceeding that working at standard conditions in an inert atmosphere. Polymeric peroxides at arbitrary degrees of polymerization are apparently decomposed into free radicals. Their primary constitution may be written

$$\sim\sim\sim\sim CH_2-\underset{\displaystyle Cl}{\underset{|}{CH}}-O\cdot \rightarrow \sim\sim\sim CH_2-\underset{\displaystyle \cdot}{CH}-O\cdot + Cl\cdot$$

Such a radical would be easily deactivated, if its reactivity is too low to initiate propagation, as the chlorine atom is in a labile position, compared to a regular sequence. Final polymer would show the presence of carbonyl groups in its infrared spectrum. This has been confirmed [9].

REFERENCES

1. Smith, W. V. and Ewart, R. H. *J. Chem. Phys.* **16** (1948) 592.
2. Smith, W. V. *J. Am. Chem. Soc.* **70** (1948) 3695.
3. Drusedow, D. and Gibbs, C. F. *Modern Plastics* **30** (1953) 123.
4. Arlman, E. J. *J. Polymer Sci.* **12** (1954) 543.
5. Fox, V. W., Hendricks, J. G. and Ratti, H. J. *Ind. Eng. Chem.* **41** (1949) 1774.
6. Smith, H. V. *Brit. Plastics* **25** (1952) 304.
7. Winkler, D. E. *J. Polymer Sci.* **35** (1959) 3.
8. Stromberg, R. R., Strauss, S. and Achhammer, B. G. *J. Polymer Sci.* **35** (1959) 355.
9. Baum, B. and Wartman, L. H. *J. Polymer Sci.* **28** (1958) 537.

Received June 23, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1639–1652

Addition Compounds of Olefins with Metal Salts

II. The Reaction of Ethylene with Silver Ions in Aqueous Solution

PAUL BRANDT

Chemistry Department A, Technical University, Copenhagen, Denmark

The reaction between silver ions and ethylene is bimolecular. The reaction is reversible, but the equilibrium is not as strongly displaced towards the reaction product as that of the equilibrium of mercuric ions and ethylene. The former process takes place as a simple addition of ethylene without formation of hydrogen ions.

The equilibrium formation constants were determined at seven temperatures between 17.5°C and 32.5°C. The dependence of the equilibrium constant upon the temperature was determined in 1 M trifluoracetate solution as:

$$\log_{10} K_1 = -3.42 + \frac{7.37}{2.3 \cdot RT}$$

The relation between the rate constant (time in sec.) and the absolute temperature for the reaction between the aquo silver ion and ethylene in 1 M trifluoracetate solution was found to be:

$$\log_{10} k_{+1} = 14.88 - \frac{20.36}{2.3 \cdot RT}$$

from determination of the rate constant at five temperatures between 15.0°C and 25.0°C.

In solutions containing triethanolamine(Am) the constants of the following equilibria were determined at 25.0°C:

$AgAm^+ + C_2H_4 \rightleftharpoons AgAm,C_2H_4^+$, $K_3 = 57.0 \pm 0.5$ and

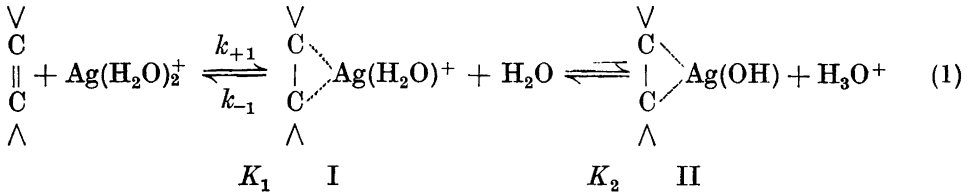
$AgAm_2^+ + C_2H_4 \rightleftharpoons AgAm,C_2H_4^+ + Am$, $K_4 = 2.65 \pm 0.15$.

In contrast to the addition product of ethylene and mercuric ion the corresponding ethylene silver ion was found to be a weak acid (pK about 8.5).

In the present work the reaction between silver ions and ethylene has been studied. The reaction is fast and an equilibrium is established in about 20 min. The equilibrium is obtainable from both sides.

The only silver salt which could be used was the trifluoroacetate. The nitrate and perchlorate ethylene salts turned out to be unsoluble. In all experiments a constant salt medium of 1 M sodium trifluoroacetate was used.

Trueblood and Lucas [2] have shown that only one ethylene molecule combines with the silver ion. In the present work it has been shown manometrically that the reaction is bimolecular with respect to the diaquo silver ion and to ethylene. The following equilibria are assumed:



(1)

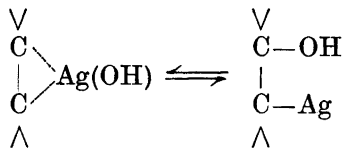
The equilibrium constants are,

$$K_1 = \frac{[\mathrm{I}]}{[\mathrm{Ag^+}][\mathrm{C_2H_4}]} \tag{2}$$

and

$$K_2 = \frac{[\mathrm{II}][\mathrm{H^+}]}{[\mathrm{I}]} \tag{3}$$

It has not been possible to prove the existence of an organic form of the ion II:



(4)

The results deviate in some respects from those of the corresponding reaction between ethylene and mercuric ions investigated by Brandt and Plum [1]. The time of establishing this equilibrium is about 10 h. However, the equilibrium is much more displaced towards the reaction product than that of the silver ion-ethylene reaction.

It is assumed that no measurable amounts of the organic form are formed with silver, while the main product formed with mercury is assumed to be the corresponding organic form. It has been shown that the complex ion I is a weak acid ($pK = 8.5$), while that of the corresponding mercuric ion is a strong acid ($pK = -0.5$), but this is understandable as the acidity constants of the corresponding aquo ions differ about 10^8 times.

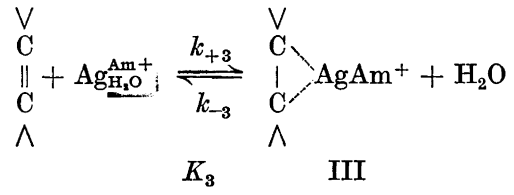
The equilibrium constant K_1 was determined at seven temperatures in the range from 17.5°C to 32.5°C. The solution was 0.025 M with respect to free hydrogen ions. The dependence of K_1 upon temperature was found to be:

$$\log_{10} K_1 = -3.42 + \frac{7.37}{2.3 \cdot RT} \tag{5}$$

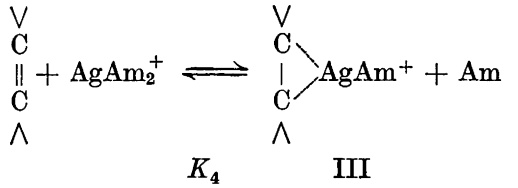
where T is the absolute temperature.

At 25°C, K_1 is 94.0 ± 0.5. Trueblood and Lucas [2] have determined the same equilibrium constant by measuring the partition coefficient of ethylene between silver nitrate solution and carbon tetrachloride, and found the value 85.3.

The acid dissociation constant K_2 of the complex ion I has been determined in solutions containing triethanolamine. In these solutions the following equilibria are assumed together with (1):



(6)



(7)

where Am stands for triethanolamine. The equilibrium pressure of ethylene was measured together with pH. From these measurements, from the previously found value of K_1 and from the known stoichiometry of the solutions the concentrations of all species of the equilibria could be calculated using the equilibrium constants determined by Bjerrum and Refn [3] for the silver-triethanolamine system.

The values of K_2 and K_3 together with the corresponding values of the concentrations of the ions II and III were calculated by successive approximations. The results are, $K_2 = 2.2 \times 10^{-9}$ and $K_3 = 57.0$ at 25°C.

Finally the value of K_4 was calculated to 2.65 ± 0.15 at 25°C.

The rate constant, k_{+1}, depends upon temperature as,

$$\log_{10} k_{+1} = 14.88 - \frac{20.36}{2.3 \cdot RT} \tag{8}$$

where k_{+1} is measured in mole per liter per sec. and T is the absolute temperature. k_{+1} was determined at five temperatures in the range from 15°C to 25°C. The value at 25°C was found to be, 0.875 mole per liter per sec.

The rate constant, k_{+3}, was determined at one temperature only, 25°C, and found to be, 0.355 mole per liter per sec.

The heat of formation of the ethylene-silver complex was determined as −7.37 kcal/mole as expressed in (5).

The activation energy of + 1 was found to be 20.36 kcal/mole as shown in (8).

Consequently the energy of activation of −1 is 27.73 kcal/mole.

EXPERIMENTAL

Apparatus. All the experiments were carried out in a Brønsted shaking apparatus similar to that described by Hofman-Bang [4] and Brandt & Plum [1] with the following alteration: Somewhat larger flasks were used as the consumption of ethylene was much less than obtained with mercuric salts. The total volume of the reaction vessel was usually 175—250 ml.

Materials and solutions. The triethanolamine was of pure grade and was distilled once (bp. 277—279°C at 150 mm Hg). All other chemicals were of analytical grade.

The ethylene was taken from a steel cylinder and purified before being transferred to the apparatus by passing it through six Friedrichs washing bottles, three of which contained concentrated sulphuric acid and three others alkaline pyrogallol solution.

The pure silver perchlorate and trifluoroacetate solutions (only used in preliminary experiments) were prepared by dissolving a known amount of freshly prepared silver oxide in a known amount of free acid in a volumetric flask and diluting with water containing suffient sodium hydroxide to obtain a total anion concentration of 1.000 mole per liter and a hydrogen ion concentration of 0.025 mole per liter.

In most of the experiments the solutions used were prepared by weighing a known amount of silver nitrate and a known amount of trifluoroacetic acid and diluting with water containing suffient sodium hydroxide as above. The stoichiometric concentrations of the solutions were: 0.100 silver nitrate; 0.900 trifluoroacetic acid and 0.975 sodium hydroxide which, for the ion concentrations, corresponds to: 0.100 silver ions; 0.025 hydrogen ions and 1.000 for the anions.

The alkaline solutions containing triethanolamine were prepared by weighing the silver nitrate, triethanolamine, trifluoroacetic acid and sodium hydroxide, dissolving each of them in a little water in a volumetric flask; thereafter diluting with water. A survey of these solutions is given in Table 2.

The exact concentration of silver ion was determined by a Volhard titration in the acid solutions, whereas for the alkaline solutions it was calculated from the known amount of silver nitrate weighed.

In the present work, square brackets are used to denote true concentrations, while C indicates total concentrations. All the concentrations are in mole per liter, and all pressures are in mm Hg.

The constants given here are therefore not the 'activity constants' but 'concentration constants', valid only under these specific conditions. In the calculation of the constants only concentration in mole per liter are used.

The hydrogen ion concentration, $[H^+]$, was measured with an ordinary glass electrode connected with a 'Radiometer' valve potentiometer, model PHM 3i. $[H^+]$ was determined in every case by standardizing against solutions of well-defined hydrogen ion concentrations having as nearly as possible the same values as the solutions investigated.

Preliminary experiments. Some preliminary experiments were carried out using pure silver nitrate solution, but the solution became opaque within a few minutes and it was impossible to obtain reproducible results. The following values were found for K_1: 64.7; 67.2; 43.6; 64.2; 77.0 and 84.3.

Other experiments were carried out with a solution of pure silver perchlorate, but the same difficulties occurred again, as a white precipitate was formed within a few minutes. The following values for the equilibrium constant were ascertained: 94.0; 92.1 and 94.2. The average value 92.9 agrees very well with the results obtained in the main experiments (see Table 1), whereas they differ a little from the value found by Trueblood and Lucas [2].

A few experiments were carried out with other cations, *i. e.*, K^+ and NH_4^+ instead of Na^+, but in every case a white precipitate occurred within a few minutes.

No precipitation occurs either with pure silver trifluoroacetate solution or with a solution containing 20 % nitrate ions and 80 % trifluoroacetate ions.

The experiments mentioned above were carried out in neutral solution or a solution with a hydrogen ion concentration of 0.025.

Some experiments were carried out in weakly alkaline solutions containing ammonia and ammonium nitrate, ammonium perchlorate or ammonium trifluoroacetate, but a white precipitate occurred in each case. With triethanolamine no precipitation was observed.

Table 1. Results of experiments at various temperatures for determination of the equilibrium constant K_1 in 1 M sodium trifluoroacetate solution. The stoichiometric concentration of the solutions used were:

$C_{AgNO_3} = 0.1000$, $C_{CF_3COOH} = 0.025$ and $C_{CF_3COONa} = 0.875$ mole per liter.

Expt. No.	Temperature in °C	K_1	K_1 average	K_1 calculated from (5)
1	17.5	129.1	129.3	129.4
2	17.5	129.4		
3	20.0	114.3		
4	20.0	118.4	116.1	116.1
5	20.0	118.6		
6	20.0	113.1		
7	22.5	100.4		
8	22.5	103.0		
9	22.5	105.9	103.4	103.8
10	22.5	106.0		
11	22.5	101.9		
12	25.0	92.9		
13	25.0	92.1		
14	25.0	96.3		
15	25.0	95.0		
16	25.0	92.8		
17	25.0	92.3		
18	25.0	93.0	94.0	94.0
19	25.0	95.1		
20	25.0	95.0		
21	25.0	96.0		
22	25.0	95.4		
23	25.0	95.3		
24	25.0	92.9		
25	27.5	85.0		
26	27.5	84.2	84.7	84.7
27	27.5	85.0		
28	30.0	74.1		
29	30.0	76.0	77.2	76.2
30	30.0	78.5		
31	30.0	80.2		
32	32.5	68.7		
33	32.5	68.3	68.5	69.2

When employing a great excess of triethanolamine (solution F in Table 2), the uptake of ethylene after 24 h corresponded only to the ethylene dissolved in water. In this solution the concentrations of the monoamine ion and the free silver ion are negligible compared to the concentration of $AgAm_2^+$. Thus it is evident that the equilibrium constant K_4 for the reaction between ethylene and the diamine ion, can not be determined directly.

DETERMINATION OF THE EQUILIBRIUM CONSTANTS

The experimental results for the determination of the equilibrium constant K_1 are shown in Table 1. The results are summarized in eqn. (5). Graphical methods were used to obtain the numerical data of the equation.

The concentration of the silver ethylene ion is calculated, using the assumption that the concentration of the ion II is negligible, as being equal to the uptake of ethylene,

$$[\mathrm{I}] = \beta(P_0 - P_\infty) - \alpha \cdot P_\infty \tag{9}$$

where P_0 is the initial pressure, P_∞ the pressure of ethylene when equilibrium is established (both expressed in mm Hg), α the molar solubility of ethylene in water at 1 mm Hg pressure, and β an apparatus constant.

As the solubility of ethylene is slight, it may be assumed that Henry's law is valid, and the concentration of free ethylene may therefore be calculated from the equation,

$$[\mathrm{C_2H_4}] = \alpha \cdot P \tag{10}$$

where the value of α is 6.34×10^{-6} at 25°C when P is expressed in mm Hg.

Experiment No. 22 in Table 1 may be taken as an example. The volume V of the reaction vessel was 226.4 ml and the volume v of the solution was 100 ml. The value of β is 0.681×10^{-4}. By Volhard titration the initial concentration of the silver ion was found to be 0.0992. The initial pressure P_0 was 258.4 mm Hg and the equilibrium pressure P_∞ was 135.4 mm Hg. From eqn. (12) the value of [I] is calculated to be 0.00752 and the concentration of the free silver ions is thus, $[\mathrm{Ag^+}] = 0.0992 - 0.0075 = 0.0917$. The equilibrium constant, K_1, is,

$$K_1 = \frac{0.00752}{0.0917 \times 6.34 \times 10^{-6} \times 135.4} = 94.4$$

The constancy of the expression of K_1 shows that the assumptions made are resonable. Perturbations of equilibrium mixtures caused by changes of ethylene pressure lead to reestablishments of equilibrium, independently of the sign of the perturbation.

In order to determine the second equilibrium constant K_2 experiments were carried out in weakly alkaline solutions containing triethanolamine. In Table 2 the compositions of these solutions are stated, and Table 4 gives the results of the absorbtion of ethylene in these solutions.

From the pH measurements the figures of Table 2 were calculated in the following way. The total amount of triethanolamine, Am, is distributed over the following four species: Am, $\mathrm{AmH^+}$, $\mathrm{AgAm^+}$, and $\mathrm{AgAm_2^+}$ each of which can be calculated by means of the following euqations,

$$C_{\mathrm{Am}} = [\mathrm{Am}] + [\mathrm{AmH^+}] + [\mathrm{AgAm^+}] + 2[\mathrm{AgAm_2^+}] \tag{11}$$

$$\bar{n} \cdot C_{\mathrm{Ag}^+} = [\mathrm{AgAm^+}] + 2[\mathrm{AgAm_2^+}] \tag{12}$$

$$[\mathrm{AmH^+}] = \frac{[\mathrm{Am}][\mathrm{H^+}]}{K} \tag{13}$$

Table 2. Solutions containing triethanolamine. The stoichiometric concentrations of the solutions were: $C_{AgNO_3} = 0.2000$ and $C_{CF_3COOH} = 0.800$. In solutions A, B, D and F: $C_{NaOH} = 0.700$. In solutions C and E: $C_{NaOH} = 0.780$.

Solution	A	B_1	B_2	C	D_1	D_2	E_1	E_2	F
pH (measured)	7.10	8.18	8.40	8.90	7.69	7.83	8.45	8.78	9.20
$\bar{n}$	0.85	1.76	1.79	1.75	1.37	1.41	1.62	1.65	2.00
pAm	2.017	0.791	0.721	0.814	1.395	1.363	1.060	1.015	—
[Am]	0.0096	0.1625	0.1844	0.1533	0.0403	0.0434	0.0912	0.0966	1.81
$[AmH^+]$	0.0626	0.0851	0.0579	0.0153	0.0640	0.0509	0.0263	0.0127	0.09
$\bar{n} \cdot C_{Ag^+}$	0.1602	0.3524	0.3576	0.3506	0.2740	0.2810	0.3240	0.3304	0.40
$C_{Am}^{calc.}$	0.2375	0.6000	0.5998	0.5192	0.3783	0.3753	0.4415	0.4397	2.30
$C_{Am}^{weighed}$	0.2380	0.6000	0.6000	0.5200	0.3760	0.3760	0.4400	0.4400	2.30

Bjerrum and Refn [3] determined corresponding values of pAm and $\bar{n}$ (the average number of Am molecules bound to one silver ion) in 0.5 M $NaClO_4$ solution. It is permissable to use these values in our solutions (1 M medium) as the dependence of the equilibrium constants upon the concentration of the inert salt is small as shown by Bjerrum [6]. The value of $\bar{n}$ was found by trial and error.

As an example of the calculations solution D_1 may be used. For the values 1.3 and 1.4 of $\bar{n}$, the C_{Am} is calculated to be 0.3401 and 0.3918, respectively. As it should be 0.3760, the correct value of $\bar{n}$ must be close to 1.4. Using the value 1.37 for $\bar{n}$ we get 0.3783 for the total Am concentration, which is less than 1 % from the correct value.

Ethylene reacts with the silver diaquo ion and with the silver monoamine ion, whereas no measurable reaction takes place between ethylene and the diamine silver ion as proved by the experiments with solution F. The experiments in alkaline solution were carried out at one temperature only, *i.e.*, 25°C.

From the total uptake of ethylene the sum of concentrations of the complex ions I, II, and III can be calculated by means of eqn. (9),

$$C_x = [\mathrm{I}] + [\mathrm{II}] + [\overline{\mathrm{III}}] \tag{14}$$

The concentration of the ion I is calculated from the expression of K_1 (2). The concentration of the free silver ion is estimated in the following way,

$$C_{Ag^+} = [Ag^+] + [AgAm^+] + [AgAm_2^+] + C_x \tag{15}$$

denoting $C_{Ag^+} - C_x = a$ we obtain

$$a = [Ag^+] + [AgAm^+] + [AgAm_2^+] \tag{16}$$

$$[Ag^+] = \alpha_0 \cdot a \; ; \; [AgAm^+] = \alpha_1 \cdot a \; ; \; [AgAm_2^+] = \alpha_2 \cdot a \tag{17}$$

where

$$\alpha_0 + \alpha_1 + \alpha_2 = 1 \tag{18}$$

The equilibrium constants for the mono- and diamine silver ions are

$$K_1' = \frac{[AgAm^+]}{[Ag^+]\cdot[Am]} = 10^{2.30} \text{ and } K_{12}' = \frac{[AgAm_2^+]}{[Ag^+][Am]^2} = 10^{3.64} \quad (19)$$

From eqns. (16), (17), (18), and (19) we obtain,

$$\alpha_0 = \frac{1}{1 + K_1'[Am] + K_{12}'[Am]^2}, \quad \alpha_1 = \frac{K_1'[Am]}{1 + K_1'[Am] + K_{12}'[Am]^2},$$

$$\alpha_2 = \frac{K_{12}'[Am]^2}{1 + K_1'[Am] + K_{12}'[Am]^2} \quad (20)$$

C_x is always a small fraction of the total silver concentration. The ratio C_x/C_{Ag^+} varies between 2 % and 6 %. In the first approximation we can therefore assume that the change of [Am] caused by uptake of ethylene is negligible. Consequently, approximate values of α_0, α_1 and α_2 can be evaluated, and knowing $[Ag^+]$, $[C_2H_4]$ and K_1 an approximate value of [I] is obtained. $K_2 = \frac{[II][H^+]}{[I]}$ and $K_3 = \frac{[III]}{[AgAm^+][C_2H_4]}$ are determined by successive approximations. It is assumed that [II] is small compared to [III] in solu-

Table 3. Examples of the iteration procedure for the determination of the constants K_2 and K_3 at 25°C. The stoichiometric concentrations of the solutions are given in Table 2.

Solution	Expt.	Values of K_3 calculated from the values of $K_2 \times 10^9$ =			
No.	No.	0	1.6	2.1	2.2
B_1	35	59.4	58.8	58.5	58.5
B_2	36	54.8	53.8	53.5	53.4
B_2	37	58.6	57.6	57.3	57.2
D_1	41	59.0	58.1	57.7	57.7
D_1	42	57.4	56.5	56.3	56.2
D_1	43	56.6	55.6	55.4	55.3
D_2	44	61.9	60.7	60.3	60.3
D_2	45	59.1	57.9	57.6	57.5
Average values:		58.3	57.4	57.1	57.0

Solution	Expt.	Values of $K_2 \times 10^9$ calculated on the basis of the value of K_3:		
No.	No.	58.3	57.4	57.1
C	38	2.1	2.5	2.5
C	39	1.9	2.2	2.3
C	40	1.2	1.6	1.6
E_1	46	3.1	3.7	3.9
E_1	47	1.4	2.1	2.3
E_1	48	0.7	1.3	1.5
E_1	49	3.0	3.7	3.9
E_2	50	0.2	0.5	0.6
E_2	51	1.1	1.4	1.5
Average values:		1.6	2.1	2.2

Table 4. Experiments in alkaline solutions containing triethanolamine at 25°C. P_0 and P_∞ are the initial and equilibrium pressure of ethylene, respectively, in mm Hg. The values of [Ag+], [AgAm+], [$AgAm_2$+], C_x, [I], [II] and [III] are all to be multiplied by 10^{-4}.

Expt. No.	Solution No.	P_0	P_∞	[Ag+]	[AgAm+]	[$AgAm_2$+]	C_x	[I]	[II]	[III]	K_4
34	A	217.6	145.2	567.0	1 090.0	228.0	116.0	47.6	12.8	55.5	2.80
35	B_1	220.1	177.4	13.4	432.5	1 534.0	30.3	1.4	0.5	28.4	2.70
36	B_2	231.9	207.3	10.6	390.0	1 569.4	29.4	1.3	0.7	27.3	2.44
37	B_2	229.2	203.9	10.6	390.0	1 569.4	30.8	1.3	0.7	28.8	2.62
38	C	219.1	191.1	14.7	450.9	1 494.7	36.3	1.7	2.8	31.8	2.69
39	C	219.5	191.6	14.7	450.9	1 494.7	36.1	1.7	2.8	31.6	2.59
40	C	221.7	194.0	14.7	450.9	1 494.7	35.6	1.7	2.9	31.0	2.58
41	D_1	225.7	175.6	120.0	960.0	840.0	75.5	13.0	1.4	61.2	2.62
42	D_1	221.3	174.2	120.0	960.0	840.0	73.2	12.9	1.3	59.0	2.55
43	D_1	216.3	172.8	120.0	960.0	840.0	71.7	12.7	1.3	57.6	2.51
44	D_2	214.1	163.8	114.0	987.0	830.0	75.6	11.6	1.6	62.4	(2.97)
45	D_2	225.1	174.5	114.0	987.0	830.0	76.4	12.3	1.7	62.4	2.77
46	E_1	204.1	172.4	36.0	652.0	1 262.0	48.3	3.8	2.1	42.4	2.79
47	E_1	200.6	170.6	36.0	652.0	1 262.0	46.1	3.7	2.1	40.3	2.67
48	E_1	206.7	175.7	36.0	652.0	1 262.0	46.7	3.8	2.2	40.7	2.62
49	E_1	201.5	170.0	36.0	652.0	1 262.0	47.6	3.7	2.1	41.8	2.78
50	E_2	226.7	191.6	32.5	626.0	1 291.7	48.5	3.8	2.2	42.5	2.60
51	E_2	245.7	206.6	32.5	626.0	1 291.7	54.5	4.1	2.3	48.1	2.73

tions B and D. This gives a rough determination of K_3. From this value of the constant, [III] is calculated for solutions C and E and estimate is obtained for the concentration [II] and the constant K_2. Using this value of K_2, [II] is calculated in solution B and D and the assumption that [II] is small compared to [III] is seen to be justified. The concentration [III] and K_3 are now adjusted by aid of the correction, and new values of [II] and K_2 are obtained. After a few iterations K_2 and K_3 became really constant.

In Table 3 examples of the iteration procedure are given. From K_3 and the equilibrium constants of Bjerrum and Refn [3] (19) K_4 could be calculated. The values for K_4 are shown in Table 4. The small calculated value, 2.65, is in agreement with the experimental fact that solutions in which the ion $AgAm_2^+$ is predominating do not take up ethylene.

MEASUREMENT OF THE RATE OF REACTION WITH THE SILVER AQUEOUS ION

In Table 6 a representative run is shown. It is seen that it takes about one minute before stationarity is established between the rate of solution of ethylene and the velocity of the reaction with silver ions. In this first minute, there is not enough ethylene in the solution (even though the solubility rate

is very high), as the rate of reaction is very great. Then for about 2 min the reaction takes place at a rate that permits of measuring the pressure exactly at the desired time. The rate constant in this interval is almost a true constant. In the first minute nearly 70 % of the complex is formed, and in the following 2 min about 26 %. After about 20 min the equilibrium is established, and in this last interval the remaining 4 % of the complex is formed.

The calculation of the rate constant, on the basis of the assumption that the reaction is bimolecular, is made by means of the equation:

$$-\frac{d[C_2H_4]}{dt} = k_{+1}[Ag^+][C_2H_4] - k_{-1}[Ag(C_2H_4)^+] \quad (26)$$

The integrated form of this equation is:

$$\frac{K \cdot P_\infty}{P_0 - 2P_\infty - K\alpha^2 P_\infty^2 \log e} \log \frac{K\alpha P_\infty(P_0 - P) - (P_0 - P_\infty)}{K\alpha P_\infty(P_0 - 2P_\infty) + P_\infty} = k_{+1} \cdot t \quad (27)$$

where
$$K = \frac{\alpha \cdot P_\infty - \beta(P_0 - P_\infty)}{\alpha \cdot P_\infty(\alpha \cdot P_\infty + \beta(P_0 - P_\infty) - C_{Ag^+})} \quad (28)$$

As a rule the rate constant was determined from the slope of the straight line plot of logarithmic expression (27) against t, usually simplified to:

$$\frac{K}{\sqrt{q} \cdot \log e} \log \frac{x + A}{x + B} = k_{+1} \cdot t \quad (29)$$

where: $a = K \cdot \alpha \cdot P_0 \cdot C_{Ag^+}$; $b = -1 - c \cdot C_{Ag^+} - \frac{a}{C_{Ag^+}}$;

$$c = \frac{K\alpha(P_0 - P_\infty)}{C_x} \; ; \quad q = b^2 - 4ac \; ; \quad A = \frac{b - \sqrt{q}}{2c} \; ;$$

$$B = \frac{b + \sqrt{q}}{2c} \text{ and } x = \frac{C_x(P_0 - P)}{P_0 - P_\infty}$$

(x is the concentration of the complex ion at the time t).

In all the tables in this paper the rate constants are given in mole per liter per min.

As small amounts of atmospheric air are always present in ethylene, the concentration of the air was determined by reaction with a mercuric salt solution and a deficit of ethylene. The value of the pressure when equilibrium had been established was used to calculate how much atmospheric nitrogen was mixed with the ethylene (the oxygen was absorbed in the basic pyrogallol before the ethylene was transferred into the reaction chamber). Usually the nitrogen content was about 3—5 %.

Some experiments were carried out in the opposite direction, by starting with a solution in equilibrium with ethylene at high pressure. By decreasing the pressure with about 200 mm Hg, a reaction occurs in the reverse direction, as ethylene is now given off. In Table 7 the calculation of one of these

Table 5. Results of experiments for determination at various temperatures of the rate constant in 1 M trifluoroacetate solution of the reaction between silver diaqueous ions and ethylene. The stoichiometric concentrations of the solution are the same as described in Table 1.

Expt. No.	Temperature in °C	k_{+1}	k_{+1} average	k_{+1} calculated	forward (ordinary) or reciprocal
52	15.0	16.2	16.2	15.9	o
53	17.5	18.4			r
54	17.5	23.5	21.6	21.7	r
55	17.5	22.1			r
56	17.5	22.6			r
57	20.0	29.2			o
58	20.0	27.6			r
59	20.0	29.5	29.2	29.2	r
60	20.0	30.4			r
61	20.0	29.0			o
62	22.5	37.3			o
63	22.5	38.9			o
64	22.5	32.0	39.1	39.4	r
65	22.5	43.7			o
66	22.5	37.1			r
67	22.5	45.1			r
68	25.0	52.9			o
69	25.0	52.9			r
70	25.0	49.0			o
71	25.0	53.1			r
72	25.0	49.5			o
73	25.0	53.4			r
74	25.0	54.6	52.7	52.5	o
75	25.0	50.4			r
76	25.0	53.2			o
77	25.0	55.2			r
78	25.0	56.2			o
79	25.0	50.9			r
80	25.0	52.5			o

experiments is shown, and it is seen that the pressure increases about 50 mm Hg in the first 3 min, and only 2 mm Hg in the following 20 min, thereafter the equilibrium is again established.

The calculation is carried out by means of the same equation as above (26), but as the initial concentration of the complex ion is not at zero, the integrated expression differs slightly from eqn (27):

$$\frac{K}{\sqrt{q} \cdot \log e} \left(\log \frac{y + A}{y + B} - \log \frac{y_0 + A}{y_0 + B} \right) = k_{+1} \cdot t \qquad (30)$$

Table 6. Measurement and calculation of an ordinary experiment. This experiment is the same as No. 68 in Table 5. $V = 226.4$ ml; $v = 100$ ml; $\beta = 0.681 \times 10^{-4}$; $a = 5.96$; $b = -2.122$; $c = 9.76$; $\sqrt{q} = 1.978$; $A = -0.210$; $B = -0.0075$.

Time in min	P; $\log\frac{x+A}{x+B}$	$-\log\frac{A}{B}$; $k_{+1} = \frac{K_1}{\sqrt{q}\cdot\log e}\cdot\frac{\log\frac{x+A}{x+B}-\log\frac{A}{B}}{t}$	
0.00	260.6	0.000	
0.25	224.6	0.114	49.9
0.50	203.6	0.232	50.8
0.75	187.6	0.350	51.1
1.00	173.6	0.490	53.7
1.25	165.1	0.606	53.1
1.50	159.6	0.701	51.2
1.75	154.0	0.832	52.0
2.00	150.5	0.968	53.0
2.25	148.1	1.031	50.2
2.50	145.4	1.159	50.8
2.75	143.7	1.284	51.1
3.00	142.6	1.356	49.7
5.00	138.8	1.972	(43.2)
20.00	137.8		

where y_0 is the initial concentration of the complex ion and y its concentration at time t. The expressions for b, q, A and B are the same as above, while a and c are:

$$a = K \cdot \alpha \cdot P \cdot C_{Ag^+} + c \cdot y_0 \cdot C_{Ag^+} \; ; \qquad c = \frac{K \cdot \alpha(P_\infty - P_0)}{y_0 - y_\infty}$$

A summary of the results of the experiments is given in Table 5 (in the last column an 'o' or 'r' indicate whether the experiment is a forward or a

Table 7. Measurement and calculation of the reciprocal reaction. This experiment is the same as No. 71 in Table 5. $V = 226.4$ ml; $v = 100$ ml; $\beta = 0.681 \times 10^{-4}$; $a = 0.0177$; $b = -1.899$; $c = 7.26$; $\sqrt{q} = 1.758$; $A = -0.2510$; $B = -0.0097$.

Time in min	P ; $\log\frac{y+A}{y+B} - \log\frac{y_0+A}{y_0+B}$; $k_{+1} = \frac{K_1}{\sqrt{q}\cdot\log e}\cdot\frac{\log\frac{y+A}{y+B}-\log\frac{y_0+A}{y_0+B}}{t}$	
0.00	129.1	0.000	
0.50	152.1	0.251	61.7
1.00	164.6	0.479	58.9
1.17	168.0	0.572	60.3
1.33	170.0	0.646	59.6
1.50	171.0	0.673	55.2
1.67	172.9	0.751	55.0
1.83	175.0	0.860	57.7
2.00	175.6	1.005	61.8
2.50	177.9	1.090	53.6
3.00	179.0	1.195	49.0
5.00	179.9	1.320	(32.4)
20.00	180.9		

reciprocal reaction). Employing Arrhenius' equation in 1 M trifluoroacetate solution all the results are expressed as follows:

$$\log_{10}k_{+1} = \log_{10}A - \frac{E_A}{2.2 \cdot RT} = 14.88 - \frac{20.36}{2.3 \cdot RT} \quad (31)$$

The value of the energy of activation (expressed in kcal) and the value of the frequency exponent are in good agreement with other values found in rapid complex reactions [5]. The value of the rate constant given in the last column is calculated from this equation and is in very good conformity with the experimental average value.

The value of the rate constant in dilute aqueous solution must be of the same order of magnitude as the one found in 1 M salt solution, because the reaction occurs between an ion and a neutral molecule. From the equation:

$$\log K_1 = \log \frac{k_{+1}}{k_{-1}} \quad (32)$$

the dissociation velocity constant k_{-1} for the mono ethylene complex silver ion can be calculated, and from the equation:

$$Q = E_A - E_{-A} \quad (33)$$

the energy of activation for the dissociation reaction of the complex ion can also be calculated; the value of E_{-A} is found to be 27.73 kcal.

MEASUREMENT OF THE RATE OF REACTION WITH SILVER TRIETHANOLAMINE ION

In Table 8 a survey is given of the results for determination of the rate constant for the reaction between the silver triethanolamine ion and ethylene (5). The value found is about half of that found for the aquo ion, *i.e.*, it is of the same order of magnitude as for the aqueous ion.

As previously mentioned the hydrogen ion concentration is the same before and after reaction with ethylene. As not more than about 2 % of the total

Table 8. Results of experiments for determination of the rate constant at 25°C for the reaction between the silver mono triethanolamine ion and ethylene.

Expt.No.	Solution	k_{+1}	k_{+1}(average)
81	B_1	21.3	
82	B_1	21.4	
83	B_1	21.0	21.3
84	B_1	22.1	
85	C	20.5	

amount of silver ions reacts with ethylene, the calculation of these experiments can be made using the assumption that the free silver ion concentration as well as the concentration of the mono triethanolamine silver ion are both

constant, *i.e.*, $\bar{n}$ is constant. As the concentration of the ion I is only about 5 % of the total amount of the complex ions formed, the calculation can be made under the assumption that a reaction with ethylene occurs only with the mono triethanolamine silver ion. Eqn. (27) can therefore be simplified to:

$$\frac{\beta(P_0-P_\infty) - a \cdot P_\infty}{a \cdot a \cdot P_0 \cdot \log e} \log \frac{P_0-P}{P_0-P_\infty} = k_{+1} \cdot t \qquad (34)$$

where 'a' is the concentration of the silver mono triethanolamine ion.

The author takes the opportunity to thank Professor J. Bjerrum for his interest, advice and helpful discussions during the course of this work, as well as *Statens almindelige Videnskabsfond* for financial support.

REFERENCES

1. Brandt, P. and Plum, O. *Acta Chem. Scand.* **7** (1953) 97.
2. Trueblood, K. N. and Lucas, H. J. *J. Am. Chem. Soc.* **74** (1952) 1338.
3. Bjerrum, J. and Refn, S. *Suomen Kemistilehti* **B 29** (1956) 68.
4. Hofman-Bang, N. *Acta Chem. Scand.* **3** (1949) 874.
5. Bjerrum, J. and Poulsen, K. G. *Nature* **169** (1952) 463.
6. Bjerrum, J. *Metal Ammine Formation in Aqueous Solution* (1941) 136. (Reprinted: P. Haase and Son, Copenhagen 1957.)

Received June 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1653–1658

The Acid Soluble Nucleotides of *Vicia Faba*

M. F. ABDEL-WAHAB and S. A. EL-KINAWI

From the Isotope Centre, A.E.E., Cairo, Egypt and Institute of Biological Chemistry, University of Copenhagen, Denmark

The use of small doses of ^{32}P has been found to stimulate the synthesis of the acid soluble nucleotides of *Vicia Faba*. A suitable incorporated activity has been found to be 0.1 μC per seed in a 48 h period, while higher and lower doses seemed to be less effective.

The acid soluble nucleotides of *Vicia Faba* were extracted with 10 % perchloric acid, and fractionated by ion exchange chromatography. They were shown to include the 5′-triphosphates of adenosine, guanosine, cytidine and uridine as well as mono- and diphosphate.

The identification of ten nucleotides was mainly based on the ultraviolet absorption measurements on the extract and pure nucleotides, radioactivity measurements together with chemical and enzymatic analyses. The extraction was carried out on plants exposed to ^{32}P before sowing.

While heavy radiation is damaging to plant life, some authors have claimed that moderate exposure would stimulate plant growth and accelerate the metabolic processes [1]. Bean plants were grown in solutions in which the ^{32}P concentration was varied. The plants were later radiographed, (that is, laid over sheets of X-ray film) showing that the ^{32}P uptake increased within but not beyond a very low concentration range of phosphorus in the soil [1].

Small doses of ^{32}P have been shown to increase the production of nucleoproteins in plants [2].

In the last few years many reports have appeared concerning the metabolism of the acid soluble nucleotides, in yeast [3–5], wheat, oats and barley [6]. As a further continuation of the study of the metabolism of acid soluble nucleotides in different plants, we present here the first results of experiments carried out on *Vicia Faba*.

The main difficulties encountered in the demonstration of these compounds in plants have centred on the problem of their separation from interfering substances and their isolation in pure form. Methods developed primarily for the isolation of the nucleotides from animal tissues have proved to be unsatisfactory for the isolation of these compounds from plants [7].

Cohn was one of the pioneers in using the chromatographic methods for the estimation of the acid soluble nucleotides in yeast [7]. Potter and coworkers [8]

have improved the method of separation on the column and shortened the time for the estimation of the acid soluble nucleotides by the so called gradient elution method.

An improved method for the isolation of the acid soluble nucleotides from plant tissues has been described by Bergkvist [6]. The application of this method to *Vicia Faba* established the occurrence of all the four ribonucleotides, *viz.* the adenosine, guanosine, cytidine and uridine derivatives. Such nucleotides exist in the form of acid soluble 5-ribonucleotides at different levels of phosphorylation. Of great interest is the occurrence of uridine, besides adenosine derivatives in *Vicia Faba*, in contrast to the dominating role of the latter in animal tissues.

In the present paper we have tried to give a complete picture of the acid soluble nucleotides obtained by cold 10 % perchloric acid (PCA) extraction of 250 g of *Vicia Faba* plant, just before fruiting. The yield was found to be low; however, the nucleotides isolated were similar to those which have previously been obtained from yeast under anaerobic conditions [5]. The results given are the average of ten experiments. The seeds of the plants used for the extraction were first exposed to the beta-radiation emitted from small doses of ^{32}P. In a previous paper we have estimated the suitable dose for irradiation to produce no harmful effect on the plant. The morphological appearance of the plant and the analyses of total nitrogen, soluble nitrogen, and protein nitrogen were taken as criteria for the estimation of the suitable dose (Abdel-Wahab and El-Kinawi, unpublished work). A suitable incorporated activity has been found to be 0.1 μC per seed and a 48 h period of exposure.

Both radioactivity and ultraviolet adsorption were measured on the extract. The results obtained for the acid soluble extract of the *Vicia Faba* nucleotides from ultraviolet absorption spectrum, and from radioactivity measurements, were found similar (see Figs. 1 and 2).

Autoradiogaphic experiments have shown that the ^{32}P-uptake is more pronounced in leaves than in shoot or root systems. They have shown also that plants grown in pots afford better material than those grown in the field. In our experiments we have used pot plants four weeks after sowing. At the end of such a period the plant has begun to fruit.

The acid soluble nucleotides were isolated by homogenising the plant material in ice-cold 10 % PCA and freed from other phosphorus-containing or ultraviolet-absorbing substances according to the method described by Bergkvist [6]. After adsorption on a strong base anion exchanger in the formate form, the individual nucleotides were eluted with formic acid solutions containing increasing concentration of sodium formate. Elution of the nucleotides was followed by the change in optical density of the effluent fractions at 260 mμ and 270 mμ. The optical densities of the effluent fractions at 260 mμ were plotted against the effluent volume to obtain the chromatograph shown in Fig. 1. The elution positions as well as the absorbance ratios gave a preliminary identification of the individual fractions. Resolution of the elution position was achieved by plotting the radioactivity against the tube number. The activity was measured in counts per min and gave rise to the results shown in Fig. 2. Figs. 1 and 2 are nearly similar. From these figures one can conclude also that no reduced nucleotides are detected under our

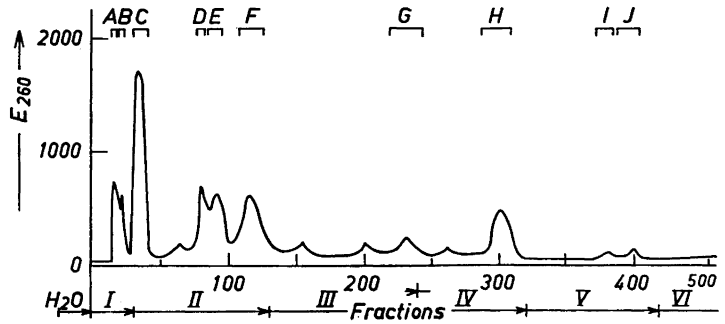


Fig. 1.

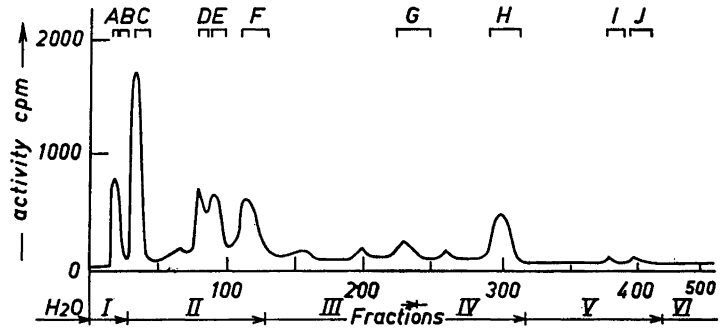


Fig. 2.

experimental conditions in *Vicia Faba*. An attempt to demonstrate such reduced nucleotides in yeast under anaerobic conditions has been carried out by us [5], and proved also their absence in yeast. In case such nucleotides are present in *Vicia Faba* they should only appear in Fig. 2 (radioactivity), but not in Fig. 1 (ultraviolet-absorption), because of the fact that such reduced nucleotides have no absorption in the ultraviolet spectrum.

The R_F values obtained from the paper chromatograms in different solvent systems and the density curves of the free nucleotides eluted from the paper chromatograms were also studied and compared to standard pure nucleotides. Since the nucleotides obtained from the column were dissolved in large volumes of formate buffer, it was desirable to concentrate them and to get rid of the excess salts. In order to remove the excess of salts, the individual peaks were separated, readsorbed on norite and then eluted with ammoniacal ethanol. The solutions thus obtained were used to identify the nucleotides and establish the purity of the peaks by comparison of their chromatographic behaviour with those of the corresponding authentic samples.

Further characterization of the nucleotides was obtained by the following procedures:

a. Ultraviolet absorption spectra in 0.1 N HCl and 0.1 N NaOH.
b. Determination of the total phosphorus content and of the phosphorus liberated after hydrolysis for 10 min in 1 N HCl.
c. Determination of the sugar content in each sample.
d. Paper chromatographic identification of the nucleotides and comparing their R_F values in different solvents with authentic samples.
e. Comparing the optical density curves of the nucleotides with standard curves over a range, 220—320 mμ, in intervals of 5 mμ.
f. The position of the phosphate group on the different nucleotides was confirmed by oxidation with periodate and the action of rattle snake venom [6].

Fig. 1 shows the ultraviolet extinction of 260 mμ of the fractions collected by an automatic fraction collector against the tube number. The results are obtained from 250 g *Vicia Faba* (whole plant), extracted by means of ice cold 10 % PCA and chromatographed on Dowex 1, formate form. The extinction values were obtained by the use of a Beckman spectrophotometer. The formate system used gave rise to peaks up to buffer solution V (*cf.* Experimental). Buffer solutions VI, VII, and VIII did not give rise to new peaks. Each tube represents 5 ml effluent. The diagram indicated the presence of ten distinct peaks as described at the bottom of Table 1 and are arranged in order of appearance.

In Fig. 2 the radioactivity is plotted against the tube number. The activity was measured as counts per min. Solutions used for measurements are those used in Fig. 1. The absence of extra peaks in Fig. 2 denotes that no reduced nucleotides are detected.

Table 1. R_F values of the ten peaks obtained from Fig. 1, compared with the R_F values of the standard pure nucleotides.

	R_F for the peaks appearing in Figs. 1 and 2. / R_F for the pure standard nucleotides									
System 1	0.22 0.20	0.38 0.38	0.45 0.43	0.22 0.21	0.26 0.24	0.29 0.28	0.08 0.07	0.20 0.20	0.20 —	0.05 —
System 2	0.46 0.46	0.40 0.39	0.66 0.67	0.32 0.31	0.18 0.19	0.20 0.20	0.40 —	0.19 0.18	0.62 —	0.50 —
System 3	0.76 0.76	0.28 0.28	0.70 0.70	— —	— —	— —	0.70 —	— —	0.78 —	0.50 —
System 4	0.30 0.29	— —	— —	0.40 0.40	0.26 0.27	— —	— —	— —	— —	— —
System 5	0.88 0.87	0.70 0.69	0.85 0.86	— —	— —	— —	— —	— —	— —	— —
Identified nucleotides	A CMP	B DPN	C AMP	D GMP	E UMP	F ADP	G UDPX*	H ATP	I UTP	J GTP

* UDPX is a sugar derivative of uridine diphospate.

Comparison of the R_F values obtained showed the existence of ten nucleotides identified in Table 1. The density curves obtained for the peaks in acid and alkaline medium and those of the ten pure standard nucleotides, together with the extinction ratios E_{250}/E_{260} and E_{270}/E_{250}, confirmed the presence of ten nucleotides.

EXPERIMENTAL

Irradiation of seeds. 250 g = 400 seeds of *Vicia Faba*, variety Rebaia 34, kindly supplied by the Egyptian Botanical Adminstration, were soaked in 2 l of tap water containing 40 μC ^{32}P. After 48 h, the seeds were removed from the soaking medium washed twice with tap water and used for sowing.

Sowing. Seeds were sowed in pots (3 per pot) and left to grow out of doors. The soil used for sowing was that used for sowing beans and other plants in Egypt. The plants were irrigated daily and after the first week the seedlings began to appear. At the end of four weeks the plants began to fruit. A control experiment was carried out using the same procedure but without using ^{32}P in the soaking medium.

Separation of the acid soluble nucleotides. The whole mature plant, which had begun to fruit, was ready for the extraction at the end of four weeks. 250 g of fresh whole plant were homogenised in 1 000 ml ice-cold 10 % PCA in a Waring Blender and reextracted with 250 ml 5 % PCA. The nucleotides were isolated from the PCA extract by ether extraction. The etherial layer containing the nucleotides was separated and left to evaporate by passing a stream of air through it. The oily residue left after aeration was dissolved in 500 ml CO_2-free redistilled water and brought to pH 8 by dilute ammonium hydroxide.

Fractionation of the nucleotides. Dowex 1 in the formate form was used as fractionating system. The individual nucleotides were eluted with increasing concentrations of formic acid or a mixture of formic acid and sodium formate. The following eluting system and the type of column described, were used.

Eluting Buffers	Formic acid, M		Sodium formate, M
I	0.02		—
II	0.10		—
III	0.10	+	0.05
IV	0.10	+	0.30
V	0.10	+	0.40
VI	0.10	+	0.60
VII	0.20	+	0.80
VIII	0.50	+	1.00

Exchanger. Dowex 1—X 10, 200—400 mesh, strong base anion exchanger, 1.2 × 20 cm formate column, effluent rate 1 ml per min.

The nucleotides were recovered from the pooled fractions by norite adsorption. For each peak the paper chromatographic procedure was carried out as is explained elsewhere.

Ultraviolet absorption. The chromatographic fractions were analysed by measuring their extinction at 250, 260 and 270 mμ in a spectrophotometer, Beckman Model DU with photomultiplier. The extinctions at 260 mμ were plotted against tube number as shown in Fig. 1. Each peak was also further studied by determining its optical density over a range of 220 to 320 mμ in intervals of 5 mμ, in 0.1 N HCl and 0.1 N NaOH solutions. The absorption ratios E_{250}/E_{260} and E_{270}/E_{260} were determined as well and used as criteria for the identification of the different nucleotides.

Phosphorus determination. Each sample was evaporated to a very small volume to remove the solvent used for elution. The samples were then heated with perchloric acid in 25 ml micro-Kjeldahl flasks till they turned colourless. The samples were left to cool and phosphorus was determined by the method of Allen [9], adapted to a total volume of 5 ml, and the acid labile phosphorus was determined after 10 min hydrolysis in 1 N HCl at 100°C.

Pentose estimation. Both samples from *Vicia Faba* extract and standard pure nucleotides were subjected to pentose estimation using the orcinol method of Albrum and Umbereit [10]. The results were calculated by comparison with the standards.

Periodic oxidation. Oxidation with periodate was performed according to Dixon and Lipkin [11], to confirm the position of the phosphate group in the nucleotides present.

Action of snake venom. The action of rattle snake venom was studied according to the method of Bergkvist [6].

Paper chromatography. Schleicher and Schüll, No. 204, 3b, filter paper was used. The Markham and Smith method [12], for localisation of the spots on the paper chromatogam was used. Identification of the R_F of nucleotides present, was achieved by using pure nucleotides and comparing the R_F values obtained (see Table 1).

Both the descending and ascending methods for paper chromatography were used to resolve the nucleotides.

Descending systems.

1. *Iso*butyric acid/NH_3/H_2O (60/1/33).
2. Ethanol/M ammonium acetate (aqueous) (70/30).
3. 0.1 M phosphate buffer (pH 6.8)/saturated ammonium sulphate/*n*-propanol (1/60/40).
4. Butanol/glacial acetic acid/water (40/11/50).

Ascending system.

5. *Iso*amylalcohol/water/citric acid (10/50/0.3 g).

The sample tubes belonging to one peak in the extinction curve (Fig. 1) were collected, shaken with 2 g norite for 20 min, then centrifuged and the supernatent fluid was discarded. The adsorbed nucleotides were then eluted successively with 10 and 5 ml of the ammoniacal ethanol (50 % aqueous ethanol containing 0.5 % ammonium hydroxide, *d* 0.91). The eluent was separated by centrifugation and lyophilised. The dry residues, dissolved in a minimum amount of distilled water (40—80 μl), were used to spot the chromatograms.

Acknowledgment. One of the authors (Abdel-Wahab) is most grateful to prof. Dr. Phil. Hans H. Ussing for his continuous interest and helpful criticism; he wishes also to thank the IAEA in Vienna for the maintenance award which enabled him to visit the Institute of Biological Chemistry in Copenhagen where he continued his work in this field.

REFERENCES

1. Some Applications of the Atomic Energy in Plant Science (USA, A.E.C.J. 1952).
2. Darlington, C. D. and Laqour, L. F. *J. Genet.* **46** (1945) 180.
3. Hoffmann-Ostenhof, O., Kenedy, J., Keck, K. and Gabriel, O. *Biochim.et Biophys. Acta* **14** (1954) 285.
4. Hoffmann-Ostenhof, O., Klima, A., Kenedy, J. and Keck, K. *Monatsh.* **86** (1955) 406, 616.
5. Abdel-Wahab, M. F. and Hashish, S. E. *J. Biol. Chem. In press.*
6. Bergkvist, R. *Acta Chem. Scand.* **10** (1956) 1303; **11** (1057) 1457, 1465; **12** (1958) 555, 752.
7. Cohn, W. E. *J. Am. Chem. Soc.* **72** (1950) 1471.
8. Hurlbert, R. B., Schmitz, H., Brum, A. F. and Potter, V. R. *J. Biol. Chem.* **209** (1954) 23.
9. Allen, R. J. L. *Biochem. J.* **34** (1940) 858.
10. Albaum, H. G. and Umbereit, W. W. *J. Biol. Chem.* **167** (1947) 369.
11. Dixon, J. S. and Lipkin, D. *Anal. Chem.* **26** (1954) 1092.
12. Markham, R. and Smoith, J. D. *Biochem. J.* **45** (1949) 294.

Received May 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1659–1667

Characterization of Intestinal Invertase as a Glucosido-Invertase

II. Studies on Transglycosylation by Intestinal Invertase

ARNE DAHLQVIST and BENGT BORGSTRÖM

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Purified hog intestinal invertase produced a number of oligosaccharides in a concentrated sucrose solution. Since no oligosaccharides were produced from a mixture of glucose and fructose under the same conditions, the mechanism for oligosaccharide formation is a transglycosylation.

At the initial stage of the reaction more fructose than glucose was liberated. Intestinal invertase therefore transfers *glucose*, which also appears from the structure of the oligosaccharides produced. This is in accordance with the classification of intestinal invertase as a glucosido-invertase (α-D-glucopyranosidase).

The origin and structure of some of the oligosaccharides produced were studied. The product by transfer of glucose to sucrose was identified as α-D-glucopyranosyl (1 → 4) α-D-glucopyranosyl (1 → 2) β-D-fructofuranoside. The name *maltosucrose* is proposed for this trisaccharide.

Maltosucrose was rapidly hydrolyzed by intestinal invertase. During this hydrolysis, sucrose was formed as an intermediate product. The maltose linkage of the trisaccharide is therefore hydrolyzed before the sucrose linkage.

No resynthesis of sucrose could be demonstrated during the hydrolysis of sucrose in the presence of ^{14}C-labelled glucose or fructose. The ^{14}C-hexoses were, however, incorporated into other oligosaccharides.

It is generally assumed that two different kinds of invertases exist, namely, fructosido-invertases (β-D-fructofuranosidases) and glucosido-invertases (α-D-glucopyranosidases). It has been suggested that intestinal invertase is a glucosido-invertase. The experimental evidence for this theory is, however, incomplete [1]. For this reason, a study of the transglycosylation by intestinal invertase was performed, the results of which are reported in the present paper.

During the action of glycosidases upon a *concentrated* substrate solution, a number of oligosaccharides are usually * formed by a process called transglycosylation [3]. Since this phenomenon occurs with highly purified glycosidase preparations as well, it is assumed that hydrolysis and transglycosylation are catalyzed by the same enzyme [3].

With invertases two different kinds of transglycosylation have been observed, namely *transfructosylation* and *transglucosylation* **. It has been demonstrated that yeast invertase [3] and invertase from *Penicillium spinulosum* [3,4], which undoubtedly are *fructosido*-invertases, have trans-*fructosyl*ase activity. Honey invertase [3,5,6] on the other hand, which is a *glucosido*-invertase, has trans*glucosyl*ase activity. The hypothesis that these enzymes are fructosido- and glucosido-invertases is therefore supported by the transglycosylase activities, which seem to provide a valuable basis for the classification of invertases.

MATERIALS AND METHODS

Purified intestinal invertase (from pig) was obtained by mutual displacement chromatography on TEAE-cellulose [2]. The preparation used had a maltase/invertase activity quotient of 0.6 and an invertase activity of 250 units per mg of protein [8]. It contained no trehalase or maltase III activity [7].

Sucrose, glucose and fructose had analytical grade purity. Sucrose and glucose were obtained from J. T. Baker Co., fructose from Pfanstiehl Chemical Inc.

^{14}C-Labelled glucose and fructose (uniformly labelled) were obtained from The Radiochemical Centre (England).

Paper chromatography of sugars was performed by the descending technique on Whatman No. 1 paper. The lower end of the paper was serrated so as to allow the solvent to drip off (Fig. 1). 2—5 μl spots of the solution to be analyzed were applied to the paper, and after drying the chromatogram was equilibrated and run with ethyl acetate:acetic acid:water 9:2:2 (v/v). For separation of the oligosaccharides produced by transglycosylation, the chromatogram was run for 3 days. Under these conditions the sucrose spot travelled about 15 cm.

As *general reagent* for the detection of the sugar spots, the *periodate* reagent described by Movery [9], was used. This reagent had the great advantage over the more commonly used aniline phthalate reagent that it had the same sensitivity to non-reducing oligosaccharides as to reducing sugars.

For detection of free *reducing groups* in oligosaccharides ammoniacal silver nitrate [10] was used. This reagent reacted with glucose, fructose and maltose, but did not produce any colour even with great amounts of non-reducing oligosaccharides such as sucrose, raffinose, melezitose or trehalose.

Fructose-containing oligosaccharides were detected with the modified Seliwanoff reagent described by Hattori and Shiroya [11]. This reagent is specific for ketoses. Fructose, sucrose, raffinose and melezitose produced brilliantly red spots with this reagent, while glucose, maltose and trehalose did not produce any colour at all.

For the chromatography of ^{14}C-labelled sugars papers were used which had previously been cut into 1.5 cm broad strips, 1 cm apart, held together by intact paper [12]. The lower end of these papers, too, was serrated. One sugar spot was applied to each strip. After the run, the distribution of the radioactivity was measured for each strip with a Geiger-Müller counter. The paper strip automatically moved in front of the tube, and the time for 100 counts was recorded for each cm length of the strip. No back-ground correction was made (Figs 2 and 3). After counting, the sugar spots were localized with the periodate reagent.

* No transglycosylation could be demonstrated with intestinal trehalase [2].

** Trans*glycos*ylation is the name for the transfer of any sugar residue while trans*glucos*ylation means the transfer of *glucose*.

For the *quantitative determination* of glucose and fructose, papers were used which had been cut in the same way as in the case of ^{14}C-labelled sugars. The chromatograms were run for 20 h only. The sugar spots were localized by marker sugars run on parallel strips. 3 cm long pieces of the paper containing the sugars to be determined were eluted in small beakers with 5 ml of water at room temperature for 20 min with occasional shaking. A sample of the eluate, containing 1 – 9 μg of glucose or fructose, was used for quantitative determination with the highly sensitive method of Park and Johnson [13]. The colour produced was measured at 690 mμ with a Beckman B spectrophotometer.

Chromatography of sugars on carbon-celite columns was performed as described by Whistler and Durso [14]. 300 g each of Darco G-60 and celite were mixed and packed to a column 9 × 25 cm. The column was washed with 1 l of 0.1 N HCl, 3 – 4 l of 50 % (by vol.) ethyl alcohol in water and finally with water. After adsorption of the sugar mixture to the column, the monosaccharides were eluted with 0 – 2.5 %, the disaccharides with 5 – 7.5 % and the trisaccharides with 10 – 15 % ethyl alcohol. The amount of sugar in the effluent was measured with the anthrone method, performed as described by Scott and Melvin [15], but with heating for 7.5 min at 100°C instead of for 16 min at 90°C. The column had a capacity of 10 g of sugar (determined with maltose). 10 – 15 l of solvent was used for the elution of each class of sugar.

Raybin's diazouracil test [16]. 10 mg of the sugar to be tested was dissolved in 0.5 ml of 0.1 N NaOH. 0.5 ml of a freshly prepared 0.4 % solution of diazouracil in water was added. If the reaction was positive, a *green colour* developed in a few minutes at room temperature. After 10 min 1 drop of 0.1 M $MgCl_2$ was added. When the reaction was positive, a stable *blue precipitate* was formed. If the reaction was negative, no colour developed, and after addition of $MgCl_2$ a red precipitate was formed.

Sucrose and raffinose gave positive Raybin's test, while glucose, fructose, maltose and melezitose gave negative reaction, in accordance with earlier findings [16,17]. The reaction is specific for the sucrose structure [17].

Determination of reducing sugars produced from oligosaccharide A. The enzymatic digestion of oligosaccharide A was performed in 0.1388 M (7 %) solution with 0.05 M maleate buffer pH 6.5. The reducing sugars produced were determined with the method of Somogyi and Nelson [18,19], after precipitation of the proteins with the Zn-Ba-reagent of Somogyi [20].

RESULTS AND DISCUSSION

Demonstration of oligosaccharide formation. In a solution containing 30 %* sucrose, 0.05 M maleate buffer pH 6.5 and 38 units of purified intestinal invertase per ml, glucose and fructose were rapidly formed at 37°C. During the course of the reaction, however, a number of oligosaccharides were formed, too, all of which moved slower than sucrose on the paper chromatogram with the solvent used.

One of these, oligosaccharide A, was formed very early in the reaction (Fig. 1). It has the R_s value (*i.e.* mobility/mobility of sucrose) 0.38. Later in the reaction two oligosaccharides were formed with mobility intermediate between oligosaccharide A and sucrose, namely, oligosaccharides B_1 and B_2 with R_s values 0.83 and 0.70, respectively. In addition one oligosaccharide with low mobility, oligosaccharide C with R_s 0.17, was formed.

When a 30 % solution of invert sugar was incubated during the same conditions, only glucose and fructose were found on the paper chromatogram. Intestinal invertase therefore has no demonstrable synthetic action. The oligosaccharides formed from sucrose must therefore be formed by *transglycosylation.*

* Per cent means g of solute per 100 ml of solution.

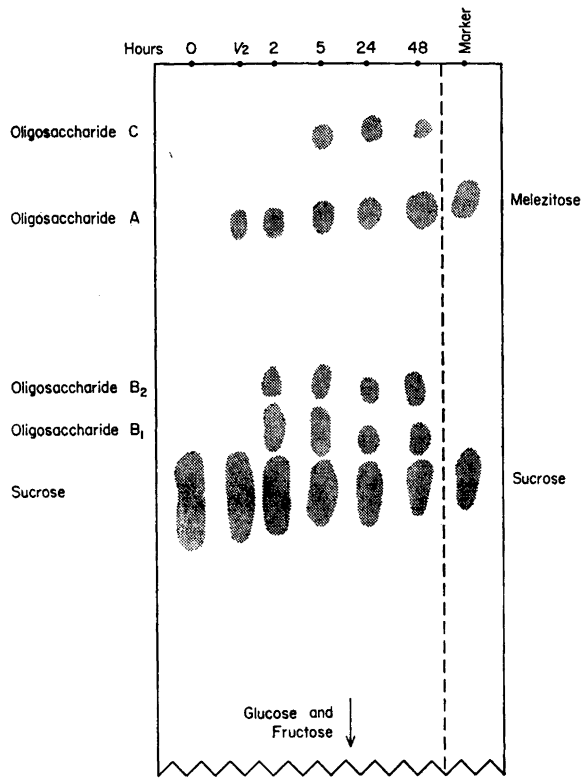


Fig. 1. Formation of oligosaccharides by transglycosylation. Purified intestinal invertase (38 units/ml) in 30 % sucrose with 0.05 M maleate buffer pH 6.5 at 37°C. Chromatogram developed during 3 days.

Oligosaccharides formed by transglycosylation are usually hydrolyzed when the reaction has been in progress for a sufficiently long time. In the present case, the reaction was followed for 14 days. Although the oligosaccharide spots had become smaller after this time, they were still clearly visible. This fact may be explained by inhibition of the enzyme brought about by the great amounts of monohexoses present. Pure oligosaccharide A was found to be rapidly hydrolyzed by intestinal invertase.

Amounts of glucose and fructose produced. The relative amounts of glucose and fructose produced by an invertase preparation during the action on a concentrated sucrose solution are dependent upon the nature of the transglycosylation. For each fructosyl group transferred one glucose molecule is liberated, and for each glucosyl group transferred, one fructose molecule is liberated.

During the action of intestinal invertase on a 30 % sucrose solution, the amount of fructose liberated at the initial stage of the reaction (5—10 %

Table 1. Properties of sugars produced by action of intestinal invertase in 30 % sucrose solution at pH 6.5 and 37°C.

	Reaction with ammoniacal $AgNO_3$ (reducing power)	Reaction with Seliwanoff reagent (fructose)
Fructose	pos.	pos.
Glucose	pos.	neg.
Sucrose	neg.	pos.
Oligosaccharide B_1	pos.	pos.
Oligosaccharide B_2	pos.	neg.
Oligosaccharide A	neg.	pos.
Oligosaccharide C	neg.	pos.

hydrolysis) was 1.5 times the amount of glucose liberated, determined by quantitative paper chromatography. The transglycosylation by intestinal invertase must therefore be a transfer of *glucose*. Since it has earlier been found that fructosido-invertases transfer fructose, while glucosido-invertases transfer glucose [3], this fact affords strong evidence that intestinal invertase is a glucosido-invertase. This conclusion implies, of course, that hydrolysis and transglycosylation are catalyzed by the same enzyme. This, however, seems probable since purified intestinal invertase (with 250 times increased specific activity) was found to produce the same oligosaccharide spots as a crude preparation of intestinal glycosidases.

Properties of the oligosaccharides produced. Some information about the properties of the different sugars formed may be obtained on the paper chromatogram by use of specific spray reagents. In the present case ammoniacal silver nitrate was used for the demonstration of reducing end groups, and a modified Seliwanoff reagent for the demonstration of fructose in oligosaccharides. The results with these reagents are listed in Table 1.

Oligosaccharide A is non-reducing and contains fructose. This may be expected if oligosaccharide A is formed by transfer of a glucosyl radical to sucrose. This is the probable way for the formation of oligosaccharide A, since it appears very early in the reaction. The isolation of oligosaccharide A and the determination of its structure are described below.

Oligosaccharide C is also non-reducing and contains fructose. Since this sugar has a low chromatographic mobility, it is probably a tetrasaccharide, possibly formed by transglycosylation to oligosaccharide A. This theory is supported by the fact that a sugar with the same mobility as oligosaccharide C is produced when intestinal invertase acts upon a solution of pure oligosaccharide A.

Oligosaccharides B_1 and B_2 are reducing sugars. Their relatively high chromatographic mobility indicates that they may be disaccharides. This theory is supported by the fact that they were eluted (together with sucrose) by 5 % ethyl alcohol from a carbon-celite solumn. The slowest moving one, oligosaccharide B_2, contains no fructose, and has the same chromatographic mobility as maltose. It may be identical with this sugar.

If oligosaccharides B_1 and B_2 are disaccharides, they may be formed either by transfer of glucose to free fructose and glucose, or by partial hydrolysis of a trisaccharide. By the use of ^{14}C-labelled fructose and glucose it was possible to identify oligosaccharides B_1 and B_2 as the products of transglucosylation to free fructose and glucose, respectively (see below).

Isolation and structure of oligosaccharide A. 60 g of sucrose were dissolved in 0.05 M maleate buffer pH 6.5 to a final volume of 200 ml and 1 ml of a purified invertase preparation, containing 600 units of invertase and 3 mg of protein, was added. As preservative, 5 ml of toluene was added. After 7 days at 37°C the solution had a reducing power corresponding to 25 % hydrolysis of the sucrose. The reaction was then interrupted by boiling for 5 min.

By paper chromatography the digest was found to contain fructose, glucose, sucrose and the series of oligosaccharides described above. The whole solution was filtrated through a 9 × 25 cm carbon-celite column. The amount of sugar was about 6 times the capacity of the column. Because greater molecules are adsorbed with greater force, however, the solution passing through the column contained only monosaccharides, sucrose and oligosaccharide B_1 and B_2, while all the oligosaccharide A was adsorbed to the column. The disaccharides adsorbed to the column were eluted with 15 l of 7.5 % ethyl alcohol. Oligosaccharide A was then eluted with 8 l of 12.5 % ethyl alcohol. This fraction was evaporated *in vacuo*, lyophilized and dried *in vacuo* at 50°C over P_2O_5. In this way 1.66 g of chromatographically pure oligosaccharide A was obtained (when 1 mg of the preparation was chromatographed it formed only one spot, which had the same mobility as oligosaccharide A).

The preparation did not reduce Somogyi's copper reagent. During total acid hydrolysis (boiling with 1 N HCl for 15 min) a mixture of glucose and fructose was obtained. With the aid of quantitative paper chromatography the mixture was found to contain two parts glucose and one part fructose. By cryoscopy (in water) the molecular weight of oligosaccharide A was determined at 470 (calculated for a trisaccharide 504.4). The sugar is therefore a non-reducing trisaccharide, composed of one fructose and two glucose molecules.

Raybin's diazouracil test was positive for oligosaccharide A, which demonstrates that fructose and one of the glucose molecules are joined by a sucrose link. This was later supported by the observation of a non-reducing, fructose-containing sugar, with the same mobility as sucrose, as intermediate product during the hydrolysis of oligosaccharide A by intestinal invertase.

Since the fructofuranoside link of sucrose is very easily split by acids, it was thought possible to split this link by mild acid hydrolysis while preserving the other link of the trisaccharide. When the trisaccharide was boiled with 0.05 N HCl for 15 min only fructose and a reducing disaccharide, which did not contain fructose, and which had the same chromatographic mobility as maltose, were found. 500 mg of oligosaccharide A were partially hydrolyzed by 0.05 N HCl and applied to a 3.5 × 20 cm carbon-celite column. The fructose was eluted with 2 l of 2.5 % ethyl alcohol and discarded. The disaccharide was eluted with 2 l of 7.5 % ethyl alcohol, evaporated *in vacuo* and lyophilized. The yield was 193 mg of chromatographically pure sugar.

This sugar was identified as maltose. It had the same chromatographic mobility as maltose, $[\alpha]_D^{20}$ + 130.0° (5 % in water) after slight downwards mutarotation (maltose $[\alpha]_D^{20}$ + 130.4° [17]). It gave a phenylosazone which (crystallized from ethyl alcohol) had the same crystal form as maltosazone. Acetylation with sodium acetate as catalyst yielded a β-octaacetate with m.p. 156—159°C (first crystallization from ethyl alcohol) maltose α-octaacetate 157—159°, mixed m.p. 156—158°).

Oligosaccharide A is therefore α-D-glucopyranosyl (1→ 4) α-D-glucopyranosyl (1→ 2) β-D-fructofuranoside. A sugar with this structure has earlier ben isolated from the oligosaccharides produced from sucrose by honey invertase [6]. The properties of our preparation agreed well with those reported for this trisaccharide. $[\alpha]_D^{20}$ + 122.1° (2.7 % in water) (reported $[\alpha]_D^{25}$ + 121.8°) [6] Acetylation with pyridine as catalyst [6,21] yielded a hendecaacetate with $[\alpha]_D^{20}$ + 83° (amorphous, 1.18 % in $CHCl_3$) (reported $[\alpha]_D^{25}$ + 86.0°) [6]. A suitable name for α-D-glucopyranosyl (1→ 4) α-D-glucopyranosyl (1→ 2) β-D-fructofuranoside should be *maltosucrose*.

Enzymatic hydrolysis of oligosaccharide A. In a 0.1388 M (7 %) solution of oligosaccharide A (maltosucrose), containing 0.05 M maleate buffer pH 6.5 and 600 units of purified intestinal invertase per ml, the oligosaccharide was rapidly hydrolyzed. After 30 min at 37°C, glucose, fructose and one intermediate disaccharide with the same chromatographic mobility as sucrose, were formed. The disaccharide was non-reducing and contained fructose, which supports the hypothesis that it is sucrose, formed by partial hydrolysis of oligosaccharide A.

A faint spot with the same chromatographic mobility as oligosaccharide C was also observed after 30 min. This supports the belief that oligosaccharide C is formed by transglycosylation to oligosaccharide A.

After 5 h at 37°C, only glucose and fructose were found on the paper chromatogram. The hydrolysis of oligosaccharide A therefore goes on until completed.

The *rate* of hydrolysis of oligosaccharide A in 0.1388 M solution was studied with more dilute enzyme solutions. The amount of reducing sugars produced from oligosaccharide A was 0.24 times the amount produced from sucrose during the same conditions. Calculated as the number of glycosyl linkages hydrolyzed per time unit, the rate for hydrolysis of oligosaccharide A is 0.48 times the rate for sucrose hydrolysis when calculated as hydrolysis to glucose and sucrose, or 0.32 times when calculated as complete hydrolysis into glucose and fructose.

Transglycosylation to free hexoses. The reaction mixture contained 30 % sucrose, ^{14}C-labelled fructose or glucose (about 1 mg/ml) which gave 1 250 000 cpm/ml with the counter used, 0.05 M maleate buffer pH 6.5 and 50 units/ml of purified intestinal invertase. After different times of reaction at 37°C, 5 μl samples were applied to a paper chromatogram. Each spot thus contained 6 250 cpm of ^{14}C-labelled sugar.

As is seen in Figs. 2—3 fructose and glucose are incorporated into oligosaccharides B_1 and B_2, respectively. This reaction is very rapid during the first hour, but then proceeds much slower since the ^{14}C-labelled monohexoses

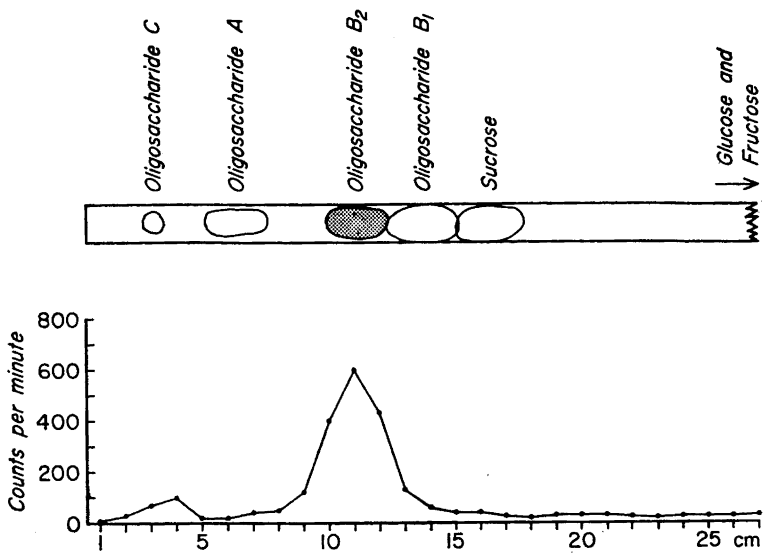


Fig. 2. Transglycosylation by intestinal invertase with ^{14}C-glucose as receptor. Incubated at 37°C for 23 h. For experimental conditions, see text. Glucose is incorporated into oligosaccharide B_2. The spot applied to the chromatogram contained 6 250 cpm of ^{14}C-labeled sugar. 25 % of the activity is localised in the oligosaccharide B_2 spot.

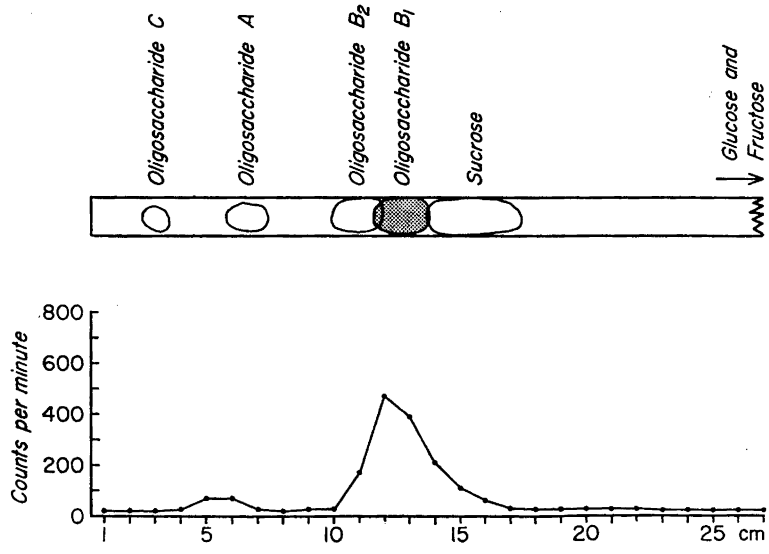


Fig. 3. Transglycosylation by intestinal invertase with ^{14}C-fructose as receptor. Incubated at 37°C for 5 h. For experimental conditions, see text. Fructose is incorporated into oligosaccharide B_1. The spot applied to the chromatogram contained 6 250 cpm of ^{14}C-labeled sugar. 20 % of the activity is localised in the oligosaccharide B_1 spot.

are diluted with inactive hexoses produced by hydrolysis. In both cases a very small peak of activity is also found, with chromatographic mobility intermediate between oligosaccharides A and C. Since no sugar spot could be detected in this place, these peaks must be caused by sugars which are formed only in very small amounts.

The incorporation of fructose and glucose into oligosaccharides B_1 and B_2, respectively, is in accordance with the fact that oligosaccharide B_1 contains fructose while oligosaccharide B_2 does not (see above), since intestinal invertase transfers *glucose*. The results show that oligosaccharides B_1 and B_2 are formed by transfer of glucose to free fructose and glucose respectively.

No resynthesis of sucrose by transglycosylation could be demonstrated. Resynthesis of sucrose (with glucose as receptor) has been observed with mold invertase, but could not be demonstrated with yeast invertase [3].

Acknowledgments. This work has been supported by grants from the *Medical Research Council* and the *Medical Faculty of the University of Lund.*

The technical assistance of Miss M. Florath, Mr. U. Telenius and Mr. T. Nilsson is gratefully acknowledged.

REFERENCES

1. Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 2012.
2. Dahlqvist, A. *To be published.*
3. Edelman, J. *Advances in Enzymol.* **17** (1956) 189.
4. Bealing, F. J. *Biochem. J.* **55** (1953) 93.
5. White, J. W. Jr. and Maher, J. *Arch. Biochem. Biophys.* **42** (1953) 360.
6. White, J. W. Jr. and Maher, J. *J. Am. Chem. Soc.* **75** (1953) 1259.
7. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 945.
8. Borgström, B. and Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 1997.
9. Mowery, D. F. *Anal. Chem.* **29** (1957) 1560.
10. Partridge, S. M. *Biochem. J.* **42** (1948) 238.
11. Hattori, S. and Shiroya, T. *Arch. Biochem. Biophys.* **34** (1951) 121.
12. Sjövall, J. *Arkiv Kemi* **8** (1955) 317.
13. Park, J. T. and Johnson, M. J. *J. Biol. Chem.* **181** (1949) 149.
14. Whistler, R. L. and Durso, D. F. *J. Am. Chem. Soc.* **72** (1950) 677.
15. Scott, T. A. and Melvin, E. H. *Anal. Chem.* **25** (1953) 1656.
16. Raybin, H. W. *J. Am. Chem. Soc.* **55** (1933) 2603.
17. Pigman, W. Ed. *The Carbohydrates*, New York 1957.
18. Nelson, N. *J. Biol. Chem.* **153** (1944) 375.
19. Somogyi, M. *J. Biol Chem* **195** (1952) 19.
20. Somogyi, M. *J. Biol. Chem.* **160** (1945) 69.
21. Barker, S. A. and Bourne, E. J. *J. Chem. Soc.* **1952** 209.

Received June 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1668–1670

The Synthesis of 1-Phenyl-3-methyl-4-acyl-pyrazolones-5

BROR SKYTTE JENSEN

Danish Atomic Energy Commission, RISØ, Denmark

A new synthesis of 4-acyl-1-phenyl-3-methyl-pyrazolones-5 is presented. Condensations of acid chlorides or anhydrides with 1-phenyl-3-methyl-pyrazolone-5 in dioxane, catalyzed by suspended calcium hydroxide, have been found to produce the 4-acylated derivatives directly and in good yields.

The following new compounds have been prepared: 4-propionyl-, 4-butyryl-, 4-trifluoroacetyl-, 4-chloroacetyl,- 4-ethoxycarbonyl-, 4-*p*-bromobenzoyl-, 4-*p*-nitrobenzoyl-1-phenyl-3- methyl-pyrazolone-5 and 4-benzoyl-1-*p*-nitrophenyl-3-methyl-pyrazolone-5.

The compounds thus prepared are promising chelating agents for the extraction of metal ions.

During a search for new chelating agents for the extraction of metal ions it was found [1] that 1-phenyl-3-methyl-4-acetyl-pyrazolone-5 possessed valuable properties as such. Hence the syntheses of other 4-acyl substituted pyrazolones were planned for an investigation of their properties.

Previously the 4-acetyl-, 4-benzoyl- and 4-oxalyl-1-phenyl-3-methyl-pyrazolones [2–4] have been synthetisized by conventional Claisen condensations of esters with the pyrazolone, the reaction being catalysed by the sodium salt of the acid or sodium ethoxide. The yield was not reported for the acetyl derivative, it was low for the benzoyl derivative and was exellent for the oxalyl derivative.

A new method of synthesis of these compounds has been evaluated. Recognizing the phenolic character of the rather acid 1-phenyl-3-methyl-pyrazolone-5 a Fries rearrangement of the 5—O acylated derivative to the 4—C acylated derivative by anhydrous zink chloride was tried. The 5—O acylated pyrazolones were easily prepared by a Schotten-Baumann acylation reaction. In fact the expected product was obtained by the reaction, however, accompanied by large amounts of coloured by-products. Further experiments showed that a suspension of calcium hydroxide in dioxane, with a small amount of water added, served equally well or better as a catalyst for the acyl migration from position 5 to position 4. Finally it was found that a direct one step synthesis was effected by treating a solution of 1-phenyl-3-methyl-pyrazolone-5 in dioxane containing suspended calcium hydroxide as a catalyst

with an acid chloride or anhydride. A rapid reaction led directly to the calcium complex of the 4-acylpyrazolone which is stable under the alkaline conditions, and which is expected to protect the wanted derivative from further reactions.

This method was successfully used to prepare the acetyl-, propionyl-, butyryl-, valeryl-, chloroacetyl-, benzoyl-, *p*-bromobenzoyl-, *p*-nitrobenzoyl- and ethoxycarbonyl derivatives of 1-phenyl-3-methyl-pyrazolone-5 and to prepare the benzoyl derivative of 1-*p*-nitrophenyl-3-methyl-pyrazolone-5. It failed to give a product capable of metal extraction with pivalyl chloride. An oily product was isolated which was not further characterized.

When an attempt was made to prepare the 5-trifluoroacetate of the pyrazolone by reacting trifluoroacetic anhydride with the pyrazolone in dry pyridine, a vigorous reaction led directly to the 4-trifluoroacetyl-pyrazolone. It was not tried to prepare this compound by the present method.

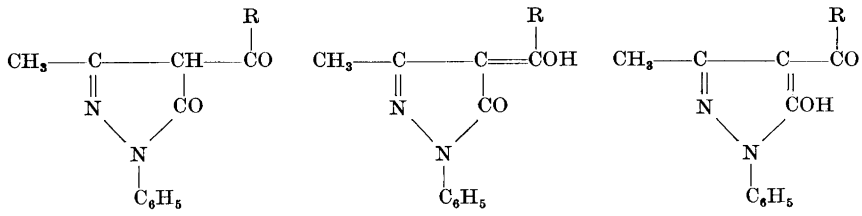


Fig. 1.

The 4-acyl-pyrazolones are good crystallizing compounds often obtainable both in the keto form (Fig. 1 a) by recrystallization from polar solvents as alcohol-water or dioxane-water mixtures, and in the enol form (Fig. 1 b, c) by recrystallization from nonpolar solvents as ligroin, chloroform *etc.* The enol forms are yellowish coloured solids, whereas the keto forms are colourless. Michaelis and Engelhardt also reported the isolation of both the keto and enol forms of 1-phenyl-3-methyl-4-benzoyl-pyrazolone-5.

The Schotten-Baumann 5—O acylation was found to be endothermic indicating a large heat absorption in the enolization of the 5-keto group. This indicates that in the unsubstituted pyrazolone, the keto form is much more stable than the enol form and that the resonance energy of the pyrazole nucleus is small. The enolization of the 4-acylated pyrazolones is believed to occur in the side chain. In this way energy is gained due to the extended conjugation which arises (Fig. 1 b). If the 5-keto group enolizes, the gain in energy will be smaller as the system becomes cross-conjugated (Fig. 1 c). This is because the pyrazole nucleus as indicated above is best represented as a cyclic unsaturated hydrazone.

The acid strength of the 4-acyl-pyrazolones is of the same magnitude as that of carboxylic acids, *i.e.* their pK values vary from 3—5. Their molecular-weights were obtained by titration in water-alcohol mixtures with phenolphtalein as indicator [5].

EXPERIMENTAL

The procedure for the preparation of the different compounds is the same. One typical synthesis is described in detail. For the other compounds prepared, only the solvent for recrystallization is noted together with the constants of the compounds.

1-Phenyl-3-methyl-4-benzoyl-pyrazolone-5. 15 g of 1-phenyl-3-methyl-pyrazolone were placed in a flask equipped with a stirrer, separatory funnel and a reflux condenser and dissolved in 60–80 ml dioxane (Merck reagent grade) by application of heat. Twelve g of calcium hydroxide were added, and 9.9 ml of benzoylchloride were added dropwise within 1 min. The reaction mixture became a thick paste and the temperature increased during the first few minutes. The mixture was heated to reflux for 30 min. The calcium complex in the flask was decomposed by pouring the mixture into dilute hydrochloric acid (200 ml, 2 N) which caused cream coloured crystals to separate. The crystals were collected on a Büchner funnel. They were recrystallized from methanol-water slightly acidified to destroy any undecomposed calcium-complex. Yield of pure product 70 %. m. p. keto 122°C, enol 92°C. Literature [3] m. p. 120°C.

1-Phenyl-3-methyl-4-acetyl-pyrazolone-5. Recryst. MeOH–H_2O. Yield 56 %, m. p. keto 58°C, Lit. 58°C. $C_{12}H_{12}N_2O_2$ (216.2), found 218.

1-Phenyl-3-methyl-4-propionyl-pyrazolone-5. Recryst. MeOH–H_2O. Yield 50 %, m. p. keto 62°C. $C_{13}H_{14}N_2O_2$ (230.3), found 236. (Found: C 67.80; H 6.14. Calc.: C 67.7; H 6.14.)

1-Phenyl-3-methyl-4-butyryl-pyrazolone-5. Recryst. MeOH–H_2O. Yield 57 %, m. p. keto 80°C. (Found: C 68.75; H 6.58. Calc.: C 68.8; H 6.60.) $C_{14}H_{16}N_2O_2$ (244.3), found 245.

1-Phenyl-3-methyl-4-chloroacetyl-pyrazolone-5. Recryst. MeOH–H_2O. Yield 73 %, m. p. keto 88°C, enol 140°C. $C_{12}H_{11}N_2O_2Cl$, H_2O (268.7), found 267. (Found: C 53.75; H 5.01. Calc.: C 53.8; H 4.87.)

1-Phenyl-3-methyl-4-ethoxycarbonyl-pyrazolone-5. Recryst. MeOH–H_2O. Yield 46 %, m. p. keto 122°C, enol 72°C. $C_{13}H_{14}N_2O_3$, H_2O (245.3), found 248. (Found: C 63.08; H 5.92. Calc.: C 62.9; H 6.48.)

1-Phenyl-3-methyl-4-p-bromobenzoyl-pyrazolone-5. Recryst. Dioxane-water. Yield 82 %, m. p. keto 167 C, enol 122°C. $C_{17}H_{13}N_2O_2Br$ (357.3), found 357. (Found: C 57.03; H 3.57. Calc.: C 57.1; H 3.67.)

1-Phenyl-3-methyl-4-p-nitrobenzoyl-pyrazolone-5. Recryst. Dioxane-water. Yield 79 %, m. p. keto 200°C, enol 192°C. $C_{17}H_{13}N_3O_4$ (323.3), found 324. (Found: C 63.37; H 4.49. Calc.: C 63.1; H 4.05.)

1-p-Nitrophenyl-3-methyl-4-benzoyl-pyrazolone-5. Recryst. Dioxane-water. Yield 50 %, m. p. keto 174°C, enol 224°C. $C_{17}H_{13}N_3O_4$ (323.3), found 323. (Found: C 63.17; H 4.13. Calc.: C 63.1; H 4.05.)

1-Phenyl-3-methyl-4-trifluoroacetyl-pyrazolone-5. To a solution of 8.5 g 1-phenyl-3-methyl-pyrazolone in 50 ml anhydrous pyridine 10 g trifluoroacetic anhydride were added dropwise. Heat was evolved and the flask was cooled under tap water. The reaction mixture became red brown. After one hour at room temperature the contents of the flask were poured into 200 ml of water whereby no precipitate was formed. After acidification the compound crystallized. Recrystallization from ethanol-water mixture gave 78 % yield of pure product, m. p. keto 144°C, enol 132°C. $C_{12}H_9N_2O_2F_3$ (273.3), found 273. (Found: C 53.27; H 3.47. Calc.: C 53.3; H 3.36.)

All melting points were measured on a Kofler hot bench. Microanalyses have been performed by Mr. P. Hansen at the Chemical Laboratory of the University of Copenhagen, and the syntheses by Mr. O. Jørgensen at the Chemical Laboratory of the Danish Atomic Energy Research Establishment, Risø.

REFERENCES

1. Skytte Jensen, B. *Acta Chem. Scand.* **13** (1959) 1347.
2. Stoltz, Fr. *J. Prakt. Chem.* (2) **55** (1897) 145.
3. Michaelis, A. and Engelhardt, F. *Ber.* **41** (1908) 2668.
4. Wislicenus, W. and Elvert, H. *Ber*, **46** (1913) 3395.
5. Veibel, S. *The Identification of Organic Compounds*, Gad, Copenhagen 1954.

Received June 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1671–1679

The Influence of Nitrocatechol and Sulphate Ions on Ox Liver Arylsulphatase A at 40° C

S. O. ANDERSEN*

Universitetets Fysisk-Kemiske Institut, Copenhagen, Denmark

Experiments are reported which show that when ox liver arylsulphatase A hydrolyses nitrocatechol sulphate at pH 5.0 and 40°C there is a distinct fall in the reaction velocity during the first 10–15 min. Later the reaction velocity increases and, eventually, it reaches a constant value. It is shown that this increase in velocity is due to the liberated nitrocatechol, whereas the liberated sulphate ions tend to inhibit the reaction, if they are not precipitated. A reaction scheme is proposed and an expression is derived which can describe the reaction progress under the condition of the experiments.

In 1953 Roy[1] observed that the kinetics of the enzymatic hydrolysis of the potassium salt of nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) by partially purified preparations of ox liver arylsulphatase A are of an anomalous type. Since then the kinetics of this system have been investigated by several groups,[2-6] but the problem cannot be considered as solved.

Both Roy [2] and Baum, Dodgson and Spencer [4] found that when the reaction takes place at 37°C and pH 5.0, the reaction progress can be divided in three stages. In stage I and stage III the velocity is rather high, but is low in stage II; the duration of the stages depends on substrate and enzyme concentrations. In contrast to this the present author has reported some experiments performed at pH 5.0 and 20°C, in which only stage I and II were found.[6] The cause of this discrepancy was not found. Experiments have now been performed at 40°C in an attempt to clarify this problem, and they are reported here together with a proposed mechanism, which explains the kinetics found at this temperature.

* Present address: Universitetets Zoofysiologiske Laboratorium B, Copenhagen, Denmark.

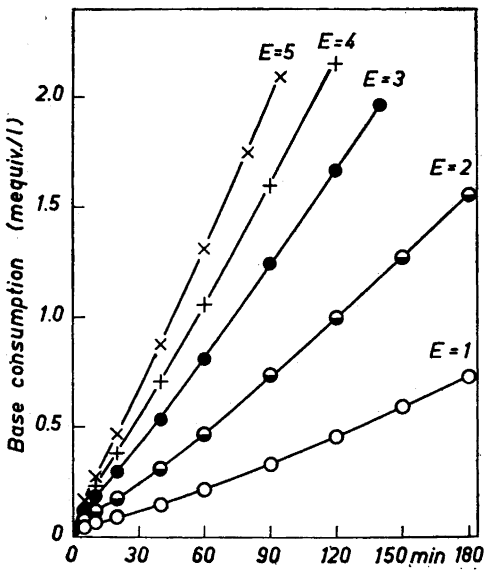


Fig. 1. Reaction progress of the enzymatic hydrolysis of nitrocatechol sulphate at pH 5.0 and 40°C. Substrate concentration was kept constant at 0.003 M in 30 ml 0.05 M $SrCl_2$. The relative enzyme concentrations are indicated on the figure.

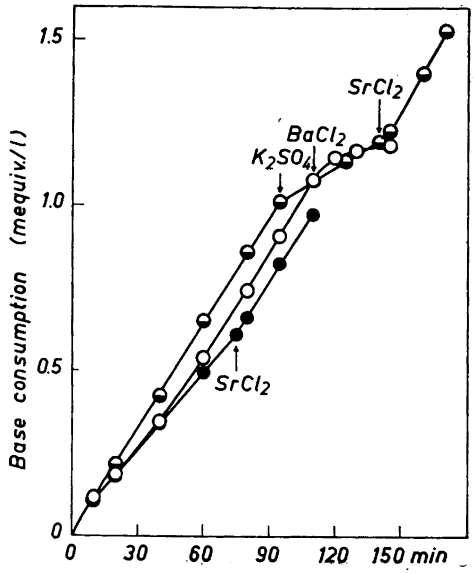


Fig. 2. Reaction progress of the enzymatic hydrolysis of nitrocatechol sulphate at pH 5.0 and 40°C. Substrate concentration was kept constant at 0.003 M in 30 ml 0.10 M KCl. ◒—◒—◒: 0.002 M nitrocatechol added before start of experiment, 1.0 ml M K_2SO_4 added at 95 min, 3 ml 0.5 M $SrCl_2$ added at 140 min. ●—●—●: 0.002 M K_2SO_4 added before start of experiment, 3 ml 0.5 M $SrCl_2$ added at 75 min. O—O—O: no addition before experiment, 20 mg $BaCl_2$ added at 110 min.

EXPERIMENTAL

The enzyme and the nitrocatechol sulphate were prepared as described earlier [6]. Nitrocatechol was prepared from its sulphate ester by acid hydrolysis.

The enzymatic hydrolysis of the sulphate ester was followed by continued automatic titration in a pH-stat, as already described [6]. The substrate concentration was kept constant during the experiments and the liberated sulphate was precipitated as $SrSO_4$ instead of as $BaSO_4$, since it has been found that excess Ba++ has a pronounced effect on the enzyme activity [7].

The experiments were performed at 40.0°C and at pH 5.0 in a medium, which consisted of 0.05 M solution of $SrCl_2$. A small amount of $SrSO_4$ was added to prevent supersaturation of this substance during the experiments. When the influence of sulphate ions was investigated 0.10 M KCl was used instead of 0.05 M $SrCl_2$.

RESULTS

The experiments in Fig. 1 were all performed at the same substrate concentration and at different enzyme concentrations. In contrast to the experiments at 20°C the three stages described by Roy and Dodgson are clearly seen

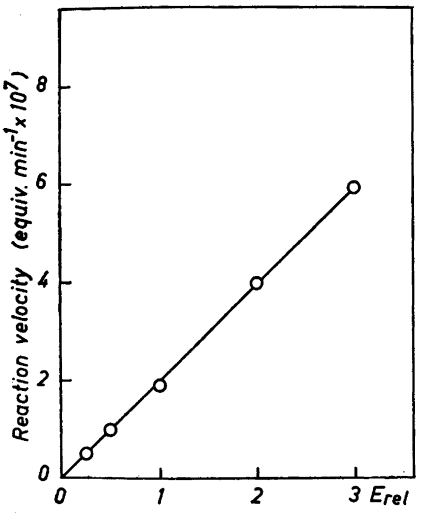


Fig. 3. Reaction velocity in stage III *versus* the relative enzyme concentration. Experiments with added nitrocatechol (0.002 M) and constant substrate concentration (0.003 M).

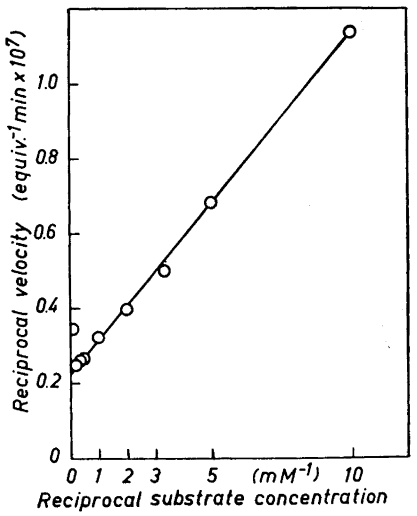


Fig. 4. Reciprocal reaction velocity in stage III *versus* reciprocal substrate concentration. Experiments with added nitrocatechol (0.002 M).

at 40°C. As the substrate concentration was kept constant during the experiments and the sulphate formed during the reaction was precipitated, the only factor known to change during the experiments is the liberated nitrocatechol. So nitrocatechol was added to the reaction mixture, before an experiment was started, in order to see, if it would have any influence on the reaction progress. The result was that stage II disappeared completely, so that stage I went directly into stage III. (◒—◒—◒ of Fig. 2). It made no difference to the reaction progress whether the enzyme was incubated for some hours with nitrocatechol, or it was added just at the beginning of the experiment.

Experiments with different enzyme concentrations and with 0.002 M nitrocatechol added were then performed and the reaction velocity in stage III determined. This velocity is plotted against the enzyme concentration in Fig. 3, and it is seen that under these circumstances the velocity is proportional to the enzyme concentration. Experiments with the same enzyme concentration but different substrate concentrations and added nitrocatechol were also performed. In Fig. 4 the reciprocal of the velocity in stage III is plotted against the reciprocal substrate concentration, according to Lineweaver and Burk [8]. This shows that below a substrate concentration of 0.005 M the kinetics of the reaction are in accordance with the Henry-Michaelis scheme with K_m equal to 0.0004 M. Inhibition occurred at the highest substrate concentration. The influence of sulphate ions can be seen in Fig. 2. 0.002 M nitrocatechol was added to one of the experiments and 0.002 M K_2SO_4 to another. The

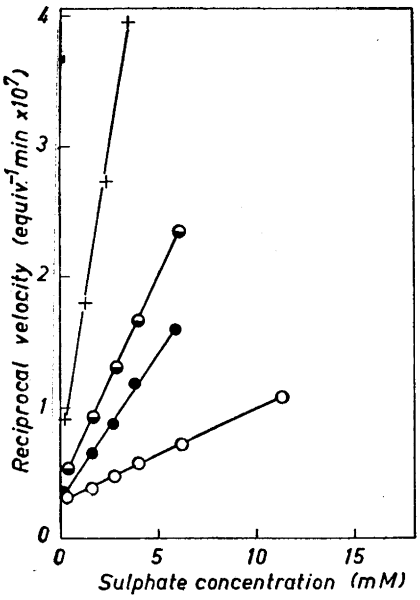


Fig. 5. Effect of sulphate ions on the reaction velocity in stage III in experiments with added nitrocatechol (0.002 M) and constant substrate concentration. O—O—O: 3 mM nitrocatechol sulphate, ●—●—●: 1 mM nitrocatechol sulphate, ◒—◒—◒: 0.5 mM nitrocatechol sulphate, +—+—+: 0.3 mM nitrocatechol sulphate.

reaction progress was followed for some time, whereafter it was tried, if addition of K_2SO_4, $SrCl_2$ or $BaCl_2$ would have any effects. It is seen that sulphate ions inhibit the reaction and that strontium ions can release the enzyme from this inhibition. Barium ions inhibit the reaction too. To investigate the influence of sulphate ions experiments were performed with excess of nitrocatechol and addition of varying amounts of K_2SO_4. The rate of the reaction was determined, and the concentration of SO_4^{--} was calculated as the sum of the sulphate added and the sulphate liberated during the reaction. The reciprocal velocity is plotted against the sulphate concentration for four different substrate concentrations in Fig. 5, which shows that the inhibition is of the competitive type.

REACTION MECHANISM

These experiments show that the kinetics of the enzyme at 40°C follow a simple scheme, when the reaction has reached a steady state, but that this is only the case when there is excess of nitrocatechol present, and when the liberated sulphate ions are removed. It has been shown previously that the kinetics at 20°C, where no reactivation was found, could be explained by supposing the following scheme:

$$A + X_1 \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P_1 + P_2 \qquad (+2)$$
$$X_2 \rightleftharpoons X_3 \qquad (\pm 3)$$

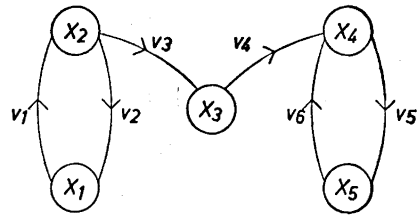


Fig. 6.

where X_1, X_2 and X_3 are different forms of the enzyme. A is the substrate, and P_1 and P_2 are the reaction products, nitrocatechol and sulphate ion respectively. The formation of X_3 is supposed to be rather slow.

To explain the reactivation found at 40°C the following reactions must be added to the scheme:

$$P_1 + X_3 \rightleftharpoons X_4 \qquad (\pm 4)$$
$$X_4 \rightarrow X_5 + P_1 + P_2 \qquad (+5)$$
$$A + X_5 \rightleftharpoons X_4 \qquad (\pm 6)$$

Nitrocatechol reacts in some way with the inactive form of the enzyme, X_3, and converts it to a new active form, X_4. The whole scheme is essentially two Henry-Michaelis schemes connected through the X_3-form. In the manner of Christiansen [9,10] it can be expressed as in Fig. 6.

If we make the simplifying assumption that reaction (3) only goes from the left to the right, so that there is no reverse reaction, then it is possible to derive from this scheme an expression for the reaction progress, which can be tested on the experiments. This assumption is not quite unjustified, as the equilibrium of the reaction must be far to the right. This follows from the results of experiments with very low enzyme concentration, where the velocity in stage II is less than a tenth of the initial velocity.

It is further assumed that the formation of X_3 is rather slow in comparison with all the other reactions in the scheme, so we have:

$$x_1 = (K_1/a)x_2 \qquad (7)$$
$$x_5 = (K_2/a)x_4 \qquad (8)$$
$$-\mathrm{d}(x_1 + x_2)/\mathrm{d}t = k_3 x_2 \qquad (9)$$

x_i is used for the concentration of the enzyme form X_i, and a and p_1 for the concentration of A and P_1, k_i is the velocity constant of reaction (i) and K_1 and K_2 are the Michaelis constants, $(k_{-1} + k_2)/k_1$ and $(k_{-6} + k_5)/k_6$, of the first and second cycle in the reaction scheme.

Further we have

$$E = x_1 + x_2 + x_3 + x_4 + x_5 \qquad (10)$$
$$x_3 p_1 = K_3 x_4 \qquad (11)$$

and

$$\mathrm{d}\xi/\mathrm{d}t = k_2 x_2 + k_5 x_4 \qquad (12)$$

where ξ is the amount of substrate split at time t. When no nitrocatechol has been added to the reaction mixture ξ will be equal to p_1. From (7), (8), (10), and (11) we get

$$E = (K_1/a + 1)x_2 + (K_3/\xi + K_2/a + 1)x_4 \tag{13}$$

and from (7) and (9) we get

$$x_2 = \frac{E}{(K_1/a + 1)} e^{-t/(K_1/a + 1)}$$

or setting $1/(K_1/a + 1) = \lambda$

$$x_2 = E\lambda e^{-\lambda t} \tag{14}$$

From (13) and (14) we can now get an expression for x_4

$$x_4 = E(1-e^{-\lambda t})/(K_3/\xi + K_2/a + 1) \tag{15}$$

Substitution of the values of x_2 and x_4 in (12) gives

$$d\xi/dt = Ek_2\lambda e^{-\lambda t} + Ek_5(1-e^{-\lambda t})/(K_3/\xi + K_2/a + 1) \tag{16}$$

This equation can be rewritten as

$$\left(\frac{K_3}{\xi} + \frac{K_2}{a} + 1\right) d\xi = \frac{Ek_2K_3\lambda e^{-\lambda t}}{\xi} dt + Ek_2\left(\frac{K_2}{a} + 1\right)\lambda e^{-\lambda t}dt + Ek_5(1-e^{-\lambda t})dt \tag{17}$$

This equation can be integrated directly apart from the term

$$Ek_2K_3\lambda(1/\xi)e^{-\lambda t}dt \tag{18}$$

but if we consider that this term will be insignificant in the later parts of the reaction when we no longer have small values of ξ and t, then we can transform it to an integrable form. We have for the first part of the reaction, before considerable amounts of x_4 have been formed:

$$\begin{aligned} d\xi/dt &= k_2x_2 = k_2E\lambda e^{-\lambda t} \\ \xi &= k_2E(1-e^{-\lambda t}) \end{aligned} \tag{19}$$

Substitution of ξ in (18) transforms it to

$$\frac{K_3\lambda e^{-\lambda t}}{1-e^{-\lambda t}} dt, \text{ or in integrated form } K_3\ln(1-e^{-\lambda t}) \tag{20}$$

Eqn. (17) can now be integrated:

$$K_3\ln\frac{\xi}{\xi_0} + \left(\frac{K_2}{a} + 1\right)\xi = K_3\ln\frac{1-e^{-\lambda t}}{1-e^{-\lambda t_0}} + E\left(\left(\frac{K_2}{a} + 1\right)k_2 - \frac{k_5}{\lambda}\right)(1-e^{-\lambda t}) + Ek_5t \tag{21}$$

As ξ_0 and t_0, the values of ξ and t at zero time, both are zero, ln ξ_0 and $\ln(1-e^{-\lambda t_0})$ become $-\infty$, but their difference has a finite value. From (19) we get

$$\begin{aligned}(1-e^{-\lambda t})/\xi &= k_2E \\ \ln(1-e^{-\lambda t_0}) - \ln\ \xi_0 &= \ln\ (k_2E)\end{aligned} \qquad (22)$$

Eqn. (21) can now be written

$$t + A(1-e^{-\lambda t}) + B\ln(1-e^{-\lambda t}) = C\xi + B\ln\ \xi - D \qquad (23)$$

where $A = (K_2/a + 1)k_2/k_5 - (K_1/a + 1)$
$B = K_3/Ek_5$
$C = (K_2/a + 1)/Ek_5$
$D = \dfrac{K_3\ln(k_2E)}{k_5E}$

That this expression is in close agreement with the experiments is shown in Table 1. The values of A, B, C, D, and λ which were used in the calculations are given in Table 2.

Table 1. The table indicates the amount of substrate, ξ, hydrolysed at the time t for different enzyme concentrations (E). The experimental conditions are given in the text. t is in min and ξ in mequiv/l. Δ is the difference between corresponding values of the terms $t + A(1-10^{-\lambda t}) + B\log(1-10^{-\lambda t})$ and $C\xi + B\log\xi - D$. These terms have been calculated from the values of A, B, C, D, and λ given in Table 2.

t	$E_{rel} = 1$		$E_{rel} = 2$		$E_{rel} = 3$		$E_{rel} = 4$		$E_{rel} = 5$	
	ξ	Δ	ξ	Δ	ξ	Δ	ξ	Δ	ξ	Δ
2	23	−1.3	33	2.2	53	0.7	67	0.2	77	0.1
5	41	−0.3	75	−0.3	116	−0.4	139	−0.1	161	−0.1
10	62	0.0	116	−0.9	185	−0.6	230	−0.4	274	−0.2
15	77	−0.5	146	−0.5	241	−0.5	304	−0.2	371	−0.1
20	89	−0.3	175	−0.1	295	−0.2	378	0.0	467	0.0
30	116	0.0	240	0.2	409	0.2	537	0.1	667	0.0
40	146	0.1	310	0.6	535	0.4	703	0.1	876	0.0
50	179	0.1	385	1.0	669	0.3	881	−0.1	1 097	−0.2
60	214	0.2	467	0.8	808	0.2	1 055	0.0	1 309	0.0
70	251	0.1	553	0.5	951	0.0	1 233	0.0	1 527	0.2
80	292	−0.4	641	0.2	1 096	−0.3	1 415	0.0	1 745	0.3
90	330	0.0	735	−0.7	1 241	−0.4	1 596	0.1	1 973	0.2
100	371	−0.1	825	−0.9	1 395	−1.0	1 779	0.2	2 087	0.1
110	413	−0.1	913	−0.7	1 531	−0.3	1 963	0.2		
120	455	0.0	997	−0.1	1 663	0.7	2 153	−0.1		
140	544	−0.3	1 186	−0.8	1 966	0.3				
150	590	−0.6	1 272	−0.1						
180	726	−0.7	1 554	−0.5						
210	865	−0.7	1 837	−0.6						
230	955	−0.1	2 019	0.0						
270	1 145	0.0								

Table 2. The values of the parameters A, B, C, D, and λ used to calculate the results in Table 1.

E_{rel}	A min	B min	C min/mM	D min	λ min^{-1}
1	8.6	40.7	194.0	54.2	0.12
2	3.4	22.6	100.8	23.0	0.12
3	4.0	15.8	63.8	13.8	0.12
4	3.6	11.5	51.5	9.0	0.12
5	3.8	9.36	42.5	7.1	0.12

A and λ are independent of the enzyme concentration, and B and C are inversely proportional to it as shown in Fig. 7. From Fig. 8 it can be seen that DE is linearly related to log E, which is in accordance with the derived expression.

DISCUSSION

The results presented in this paper show that when the reaction is performed at 40°C the reaction progress can be divided into three stages with different velocities in accordance with the results of Roy [2] and Baum, Dodgson and Spencer [4]. In contrast to these authors it is found that sulphate ions do not have any activating effect, but inhibit the enzyme at all concentrations investigated. This means that the activation from stage II to stage III is due only to the liberated nitrocatechol. Apart from this difference the scheme proposed

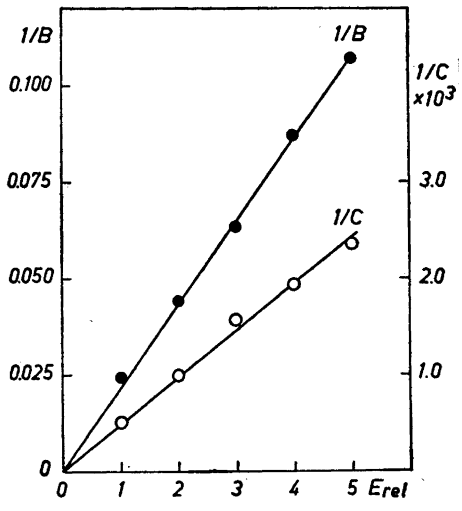


Fig. 7. Variation of the calculated values of 1/B and 1/C with the relative enzyme concentration.

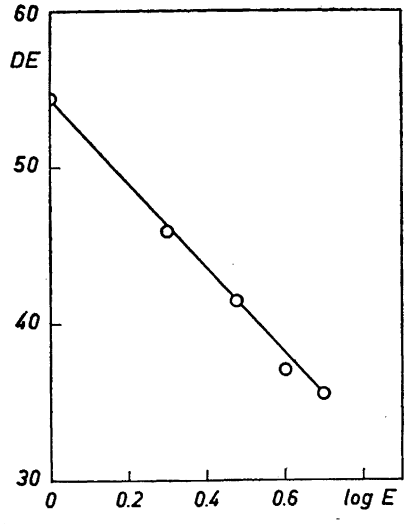


Fig. 8. Variation of the calculated value of DE with the logarithm of the relative enzyme concentration.

in this paper corresponds closely to the scheme proposed by Baum and Dodgson [5]. It is planned to investigate whether this difference could be due to acetate in the medium as used by the other groups, in contrast to chloride in this work.

The reaction scheme here proposed must only be regarded as tentative, though it is in accordance with the experiments. Minor variations in the reaction scheme are possible without the form of the final expression being effected. It is possible that reaction (3) should be $X_2 \rightleftharpoons X_3 + P_1 + P_2$, so that the inactive X_3-form of the enzyme does not contain substrate.
Then we should get further

$$X_3 + P_1 \rightleftharpoons X_4 \qquad (4a)$$
$$X_4 + A \rightleftharpoons X_5 \qquad (5a)$$
$$X_5 \rightarrow X_4 + P_1 + P_2 \qquad (6a)$$

This will give an expression for the reaction progress which will have the same form as (23), apart from the slight contribution to the reaction products from $X_2 \rightleftharpoons X_3 + P_1 + P_2$. But this contribution will be stoichiometrically equal to the enzyme concentration and far smaller than what can be measured.

Acknowledgements. My thanks are due to professor J. A. Christiansen for his interest and advice. Financial support from *Statens almindelige Videnskabsfond* is gratefully acknowledged.

REFERENCES

1. Roy, A. B. *Biochem. J.* **55** (1953) 653.
2. Roy, A. B. *Experientia* **13** (1957) 32.
3. Dodgson, K. S. and Spencer, B. *Biochem. J.* **62** (1956) 30P.
4. Baum, H., Dodgson, K. S. and Spencer, B. *Biochem. J.* **69** (1958) 567.
5. Baum, H. and Dodgson, K. S. *Biochem. J.* **69** (1958) 573.
6. Andersen, S. O. *Acta Chem. Scand.* **13** (1959) 120.
7. Andersen, S. O. *Acta Chem. Scand.* **13** (1959) 884.
8. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
9. Christiansen, J. A. *Acta Chem. Scand.* **3** (1949) 439.
10. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.

Received June 27, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1680–1686

The Kinetics of Crystal Growth in Barium Sulfate Precipitation

III. Mixed Surface Reaction and Diffusion Controlled Rate of Growth

ARNE E. NIELSEN

Universitetets fysisk-kemiske institut, Copenhagen, Denmark

The chronometric integrals for precipitation processes where the crystal growth velocity is controlled simultaneously by fourth-order surface reaction and diffusion are calculated numerically and found to agree with the experiments.

The method reported constitutes a new experimental method for obtaining rate-constants and diffusion coefficients.

In a previous article [1] the chronometric integrals for the pure cases of diffusion or p-th order surface reaction rate control of crystal growth in precipitation processes were expressed in terms of known functions and tabulated (for $p = 1, 2, 3$ and 4), and the general differential equation between the degree of advancement α and time t was derived also for the case where both types of rate control were effective at once. This equation has previously [2] been solved in terms of known functions for $p = 1$ only.

In the following we restrict the treatment to cases where the solubility of the substance is so small that it can be neglected ($s/c_0 < 0.05$). The crystals are assumed spherical. Only in cases of very flat or long crystals this will give errors larger than 10–20 %. We may write the solution to the "pure cases" thus

1. Diffusional rate control:

$$t = K_D \int_0^\alpha \frac{\mathrm{d}\alpha}{\alpha^{1/3}(1-\alpha)} = K_D I_D(\alpha)$$

2. p-th order surface reaction:

$$t = K_p \int_0^\alpha \frac{\mathrm{d}\alpha}{\alpha^{2/3}(1-\alpha)^p} = K_p I_p(\alpha)$$

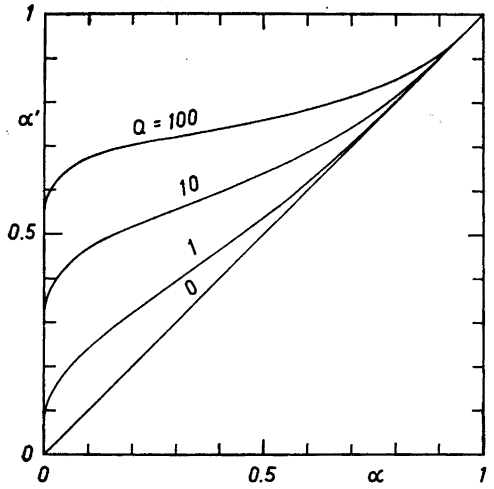


Fig. 1. The relation between the "effective" degree of advancement at the crystal surface α', and α, for different values of Q. The difference between α and α' is caused by the slowdown of the reaction by diffusion. Q is a measure of this influence; Q is a constant during the reaction.

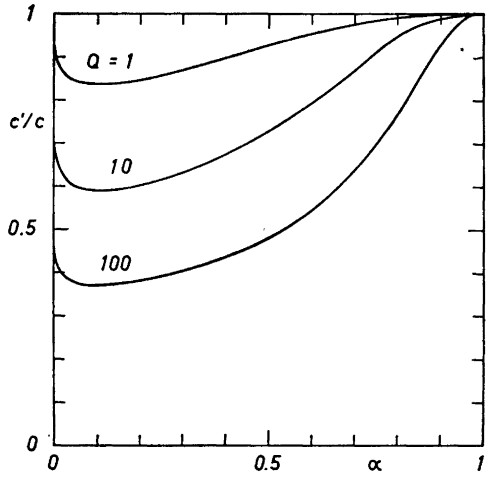


Fig. 2. The ratio between the concentration at the surface and in the bulk solution, when both diffusion and fourth-order surface reaction are rate determining.

where $K_D = r_1^2/3vDc_0$, $K_p = r_1/3vk_pc_0^p$, r_1 = particle radius when $\alpha = 1$, v = molar volume of precipitate, D = diffusion coefficient, c_0 = concentration when $t = 0$ (and $\alpha = 0$), k_p = p-th order surface reaction rate constant.

For $p = 1$ the solution to the "mixed case" is simply [2]

$$t = K_D I_D(\alpha) + K_1 I_1(\alpha)$$

For $p > 1$ the solution has not been found explicitely. In order to get a numerical solution we use the equation derived in the previous paper [1]

$$\frac{dr}{dt} = \frac{vD(c-c')}{r} = vk_p(c')^p$$

where c' is the concentration at the surface of the crystal. We define α' by $c' = c_0(1-\alpha')$ and derive

$$\alpha' - \alpha = (K_D/K_p)(1-\alpha')^p\alpha^{1/3}$$

The dimensionless quantity $Q \equiv K_D/K_p = r_1k_pc_0^{p-1}/D$ is constant during each single experiment (when the number of crystals is constant and they are all alike), and if we solve, for fixed p and Q, the equation

$$\alpha' - \alpha = Q(1-\alpha')^p\alpha^{1/3}$$

with respect to α' for a series of values of α, (see Figs. 1 and 2), we can calculate the following integrals

$$t = K_D \int_0^\alpha \frac{d\alpha}{\alpha^{1/3}(\alpha' - \alpha)} = K_p \int_0^\alpha \frac{d\alpha}{\alpha^{2/3}(1-\alpha')^p}$$

$$= K_D I_{DpQ}(\alpha) = K_p I_{pDQ}(\alpha)$$

The result of this procedure is given in Table 1 for $p = 4$ and for several values of Q. Since

$$I_{pDQ}/I_{DpQ} = K_D/K_p = Q = \text{a constant}$$

it is not necessary to tabulate both the integrals.

In order to compare experimental results with these integrals it is probably best to plot α as a function of log t on transparent paper and slide this over a plot of α as a function og log I_{pDQ} or I_{DpQ} (see Fig. 3).

If the kinetics is of the kind discussed here, one may find Q and for instance the value $t_{0.5}$ of t for $\alpha = 0.5$. This has been done for experiments on $BaSO_4$[1] and from the results Fig. 4 was drawn, to show a typical exemple of how Q varies with c_0.

In case of very exact measurements it may be necessary to use Q-values corresponding to curves not given in Fig. 3. For this purpose another method is suggested. The shape of the experimental curve may be expressed by the ratio

$$\Theta = t_{0.75}/t_{0.25}$$

From Fig. 5 the corresponding Q is found.

Table 1. The precipitation chronomals I_{4DQ}.

Q =	0.1	0.2	0.5	1	2	5	10	20	50	100
α										
0.001	0.30	0.31	0.33	0.36	0.40	0.54	0.73	1.07	1.95	3.32
0.01	0.69	0.71	0.79	0.91	1.10	1.60	2.46	3.81	7.59	13.3
0.05	1.27	1.35	1.57	1.90	2.46	3.90	6.12	9.88	20.3	36.2
0.10	1.72	1.84	2.19	2.70	3.59	5.87	9.29	15.25	31.7	57.0
0.15	2.10	2.26	2.72	3.40	4.57	7.58	12.07	20.0	41.7	75.1
0.20	2.49	2.70	3.26	4.09	5.54	9.27	14.8	24.6	51.4	92.7
0.25	2.90	3.14	3.82	4.81	6.69	10.94	17.5	29.1	61.0	109.9
0.30	3.37	3.65	4.44	5.59	7.59	12.6	20.4	33.9	70.9	128
0.35	3.92	4.23	5.14	6.46	8.75	14.6	23.4	38.8	81.0	146
0.40	4.60	4.96	5.98	7.48	10.09	16.8	26.7	44.3	91.9	165
0.45	5.46	5.86	7.02	8.70	11.65	19.2	30.4	50.2	103.7	186
0.50	6.60	7.05	8.34	10.23	13.5	22.1	34.6	56.9	117.0	209
0.55	8.18	8.67	10.12	12.21	15.9	25.5	39.6	64.6	132	234
0.60	10.45	11.02	12.6	15.0	19.1	30.0	46.0	74.3	150	264
0.65	14.0	14.6	16.4	19.0	23.6	36.0	54.1	86.0	170	298
0.70	20.1	20.8	22.7	25.7	30.9	45.2	65.9	102.3	198	343
0.75	31.5	32.1	34.2	37.5	43.5	60.0	83.6	125.4	236	401
0.80	55.0	56.4	58.9	62.3	69.2	88.6	116.5	166	295	489
0.82	72.8	73.3	75.3	79.4	86.5	107.2	138	193	331	539
0.84	100.0	100.5	102.3	105.9	114.8	137	171	232	380	604
0.86	145	146	148	152	160	183	221	284	450	696
0.88	223	224	226	230	239	264	305	380	561	829
0.90	376	377	378	384	394	422	464	543	749	1053
0.92	716	718	721	724	736	767	818	912	1131	1400

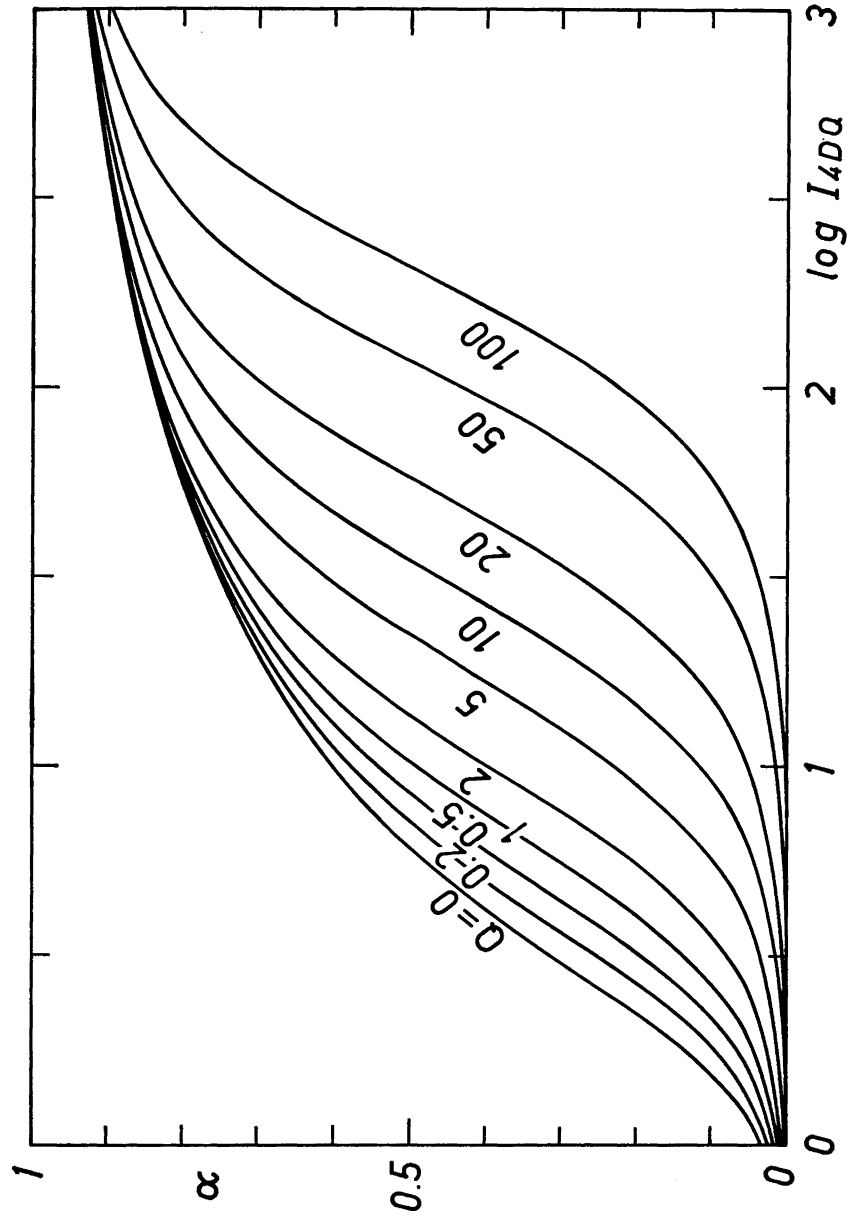


Fig. 3. A semi-logarithmic plot of α *versus* the chronomals I_{4DQ}. For check of kinetics and determination of Q: plot α *versus* log t on transparent paper and slide over this figure.

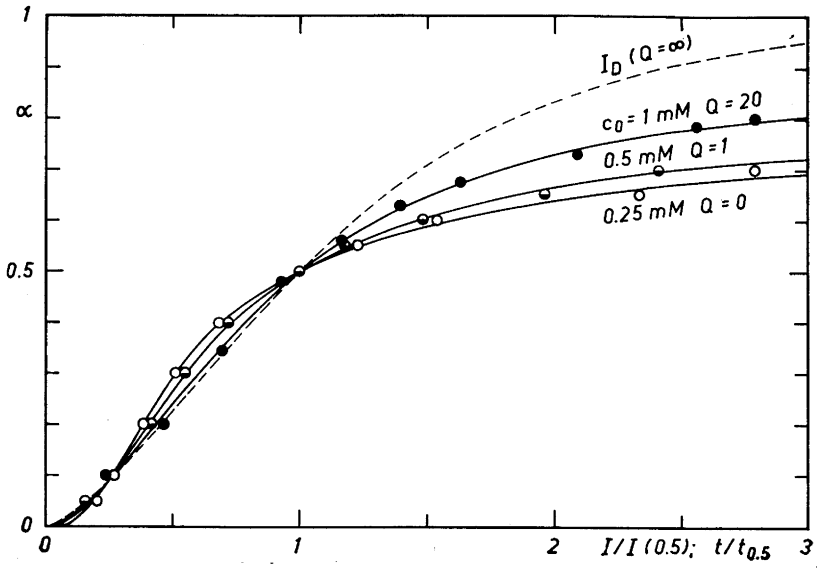


Fig. 4. Experimental values from $BaSO_4$-precipitations with different initial concentration c_0, and theoretical I_{4DQ}-curves. All t- and I-values are divided by the corresponding value at $\alpha = 0.5$ in order to make it easier to see the characteristic differences in shape of the curves with different values of Q. Large c_0 corresponds to large Q, *i. e.* diffusional slow-down is more important at high than at small c_0.

In Fig. 6 is shown how the concentration c' at the surface varies with time and how its deviation from c influences the kinetics.

If r_1 or N (the number of particles per unit volume) is known one may derive D and k_p from Q and $t_{0.5}$ by means of

$$\begin{aligned} K_p &= t_{0.5}/I_{pDQ}(0.5) \\ K_D &= t_{0.5}/I_{DpQ}(0.5) = QK_p \\ r_1 &= (3vc_0/4\pi N)^{1/3} \\ k_p &= r_1/3vc_0^pK_p = 1/(36\pi v^2N)^{1/3}c_0^{p-1/3}K_p \\ D &= r_1^2/3vc_0K_D = 1/(48\pi^2vN^2c_0)^{1/3}K_D \end{aligned}$$

$I_{pDQ}(0.5)$ may be found from Fig. 5 also for values of Q not given in Table 1.

In the previous paper D was found for $BaSO_4$ from the initial slope of a plot of I_D against t, and k_4 was found as the final slope ($0.70 < \alpha < 0.80$) of I_4 against t. In this way the diffusion coefficient of $BaSO_4$ at 25°C was found to be 3.0×10^{-6} cm^2/s.

If we plot α as a function of log t and determine Q and $t_{0.5}$ from Fig. 3 as described above, we get the values for D and k shown in Fig. 7. At 25°C, $D = 1.7 \times 10^{-5}$ cm^2/s.

The diffusion coefficient of $BaSO_4$ in aqueous solution has never been measured before (as far I know) because of the low solubility of this substance,

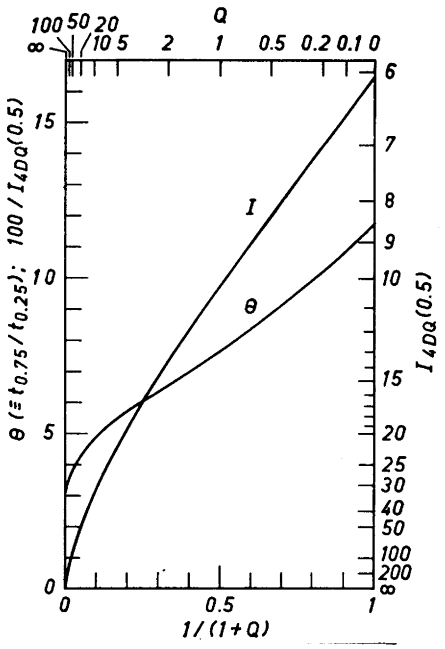


Fig. 5. Diagram for finding Q and I_{4DQ} (0.5) from the ratio $\Theta \equiv t_{0.75}/t_{0.25}$.

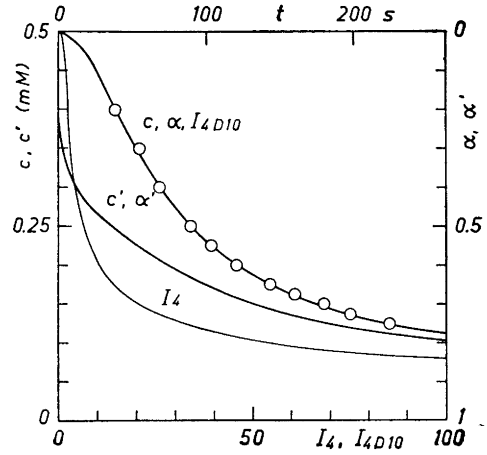


Fig. 6. The circles are experimental points and I_{4D10} is the corresponding theoretical curve. c' is the concentration at the surface, calculated for the actual experiment. I_4 is the hypothetic curve obtained if diffusion did not slow down the reaction (*i. e.* $D = \infty$), for the same value of k_4.

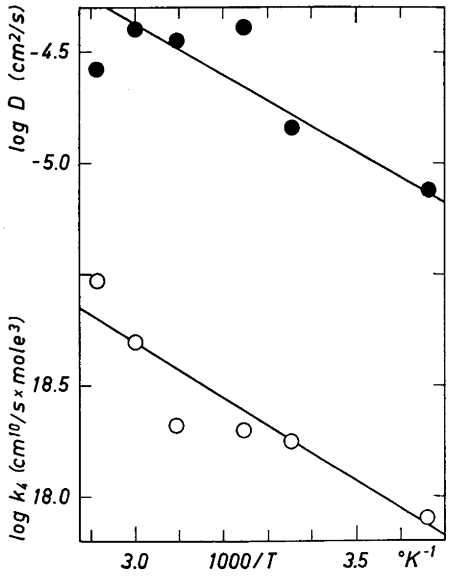


Fig. 7. The fourth-order rate constant of the crystal growth surface reaction and the diffusion coefficient plotted logarithmically as functions of the reciprocal temperature. The slopes give the heats (or energies) of activation, $-2.30\ R\mathrm{d}\log k_4/\mathrm{d}(1/T) = 5\,000$ cal/mole for each of the curves.

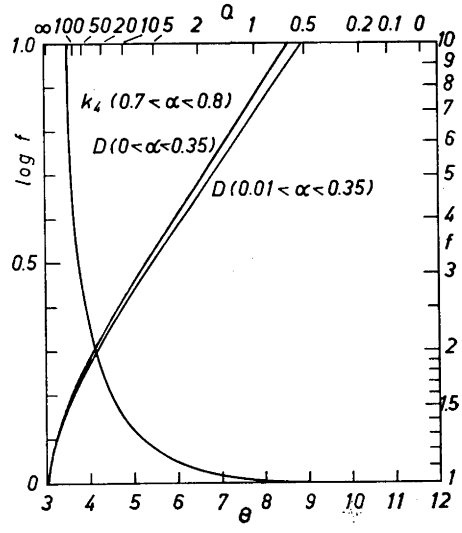


Fig. 8. The correction factors to be applied to the approximate values of D and k_4 obtained from the initial and final slopes on plots of t *versus* I_D and I_4, respectively. The two curves for D correspond to drawing the straight line through origo and to approximating the points around the point of inflexion (practically all of the points with $0.01 < a < 0.35$).

but it may be estimated rather accurately from the electric mobilities [3], which gives $D = 9.4 \times 10^{-6}$ cm^2/s. The value found by means of I_{pDQ} is seen to be better than the one obtained in the previous paper from I_D.

In Fig. 8 are given the correction factors to be applied to the (approximate) values determined by the method of the previous paper in order to get the correct ones. We observe that the correction factors are both equal to 2 for $Q = ca.$ 30, and when one of them is smaller than 2 the other one is larger.

The error introduced by treating the crystals geometrically as spheres is not serious compared with the uncertainty of the experiments reported. When the measuring technique has been improved it may be necessary to correct for this shape-error through solving the Laplace equation of diffusion in the space around the crystal.

REFERENCES

1. Nielsen, A. E. *Acta Chem. Scand.* **13** (1959) 784.
2. Frisch, H. L. and Collins, F. C. *J. Chem. Phys.* **21** (1953) 2158.
3. Nielsen, A. E. *Acta Chem. Scand.* **12** (1958) 951.

Received June 1, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1687–1692

Proton Magnetic Resonance of Thiophenes

III. Splittings of Side-chain Resonance Peaks

SALO GRONOWITZ and RAGNAR A. HOFFMAN

Institute of Chemistry and Institute of Physics, University of Uppsala, Uppsala, Sweden

Splittings of the aldehydic hydrogen resonance lines of the order of 1 c/s have been observed in certain thiophenealdehydes and oximes. This has been shown to be due to spin-spin coupling with the 5-hydrogen. No splittings have been observed when this position is blocked.

In connection with our work on the NMR-spectra of thiophenes [1,2], we have studied the splitting of side-chain hydrogens in methylthiophenes, thiophenealdehydes and thiophenealdoximes.

In the present paper we wish to communicate some of our results. These findings may provide a foundation for a discussion of some remarkable features in the NMR spectra of these compounds.

In an earlier communication [1] we discussed the NMR spectra of the ring hydrogens in several monosubstituted thiophenes. In this connection we observed the splittings ($\sim$ 1 c/s) of the resonance lines of the aldehydic hydrogens in 2- and 3-thiophenealdehyde.

These splittings could possibly be due to restricted rotation of the aldehyde group; conjugation between the aldehyde group and the thiophene ring giving rise to C—C double bond character [3] *. The aldehydic hydrogen might have different chemical shifts in the two possible rotational isomers thus giving rise to two hydrogen lines. The splittings then should be field as well as temperature dependent. Such chemical shifts due to hindered rotation have been observed, *e.g.* in amides and nitroso compounds [5].

Another possible explanation of the splittings is that they are caused by spin-spin coupling of the aldehyde proton to one or more of the ring protons. In 2-thiophenealdehyde the ring hydrogen spectrum was not well enough

* We obtained no splittings of the aldehyde resonance line in benzaldehyde or 1-naphthaldehyde, but this fact might be explained by the greater conjugation in the thiophene compounds as compared to the benzene analogues [4].

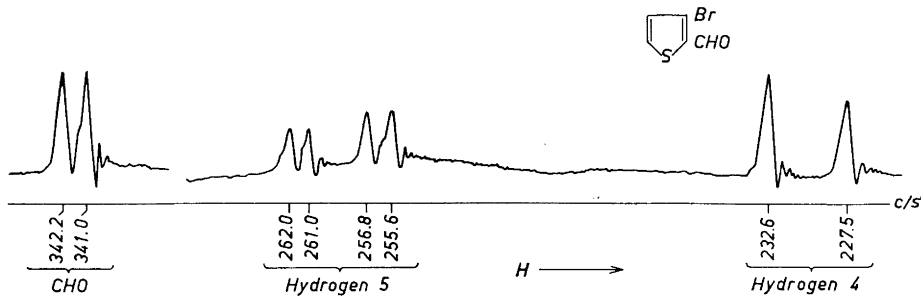


Fig. 1. NMR-spectrum of 3-bromo-2-thiophenealdehyde in *cyclo*hexane (internal reference) at 40 Mc/s.

resolved to make a definite choice between the two alternative explanations possible. However, some preliminary experiments indicated that the splittings were temperature independent. In disubstituted thiophenes the ring hydrogen spectra are simpler, and therefore such spin-spin splittings should be observed in the ring hydrogen resonance band as well.

This was the case in 3-bromo-2-thiophenealdehyde. The spectrum of this compound with *cyclo*hexane as the internal reference (Fig. 1) has the aldehydic hydrogen resonance at 342 c/s. This consists of a doublet with a splitting of 1.2 c/s. The two ring hydrogens give rise to a quartet and a doublet. The splittings in the quartet are 5.3 c/s and 1.1 c/s and in the doublet 5.1 c/s. This latter is the known J_{45} coupling constant for thiophenes [1,2]. The lower field quartet lies in the same region as the 5-hydrogen resonance in 2-thiophenealdehyde and the doublet has a shift similar to that of the 4-hydrogen of 2-thiophenealdehyde [1]. These results thus show that coupling occurs between the 5-hydrogen and the aldehydic hydrogen of this compound. Splittings of the aldehydic hydrogen in doublets were also observed in 4-substituted 2-thiophenealdehydes such as 4-bromo-2-thiophenealdehyde (0.9 c/s) and 4-methyl-2-thiophenealdehyde (1.3 c/s). In the first mentioned compound, however, the chemical shift between the 3- and 5-hydrogens is so small, (*cf.* 2-thiophenealdehyde[1]), that splittings due to coupling with the aldehydic hydrogen could not be observed in the ring spectrum. In the second compound the spectrum was complicated through additional coupling of the ring hydrogens with the methyl group.

No splitting of the aldehydic hydrogen resonance was however observed in 5-substituted 2-thiophenealdehydes such as 5-bromo-2-thiophenealdehyde and 5-methoxy-2-thiophenealdehyde.

These facts show that the splitting of the aldehydic hydrogen resonance line is caused by a coupling with the 5-hydrogen. These observations are quite remarkable since the coupling hydrogens are separated by as much as five bond distances, but consistent with Leane and Richards' interpretation [6] of the spectrum of furfural at 30 Mc/s. We have obtained a well resolved spectrum of furfural at 40 Mc/s and obtained a splitting of the aldehydic hydrogen equal to 0.8 c/s. Since they obtained a splitting of 0.9 c/s it is clear that these

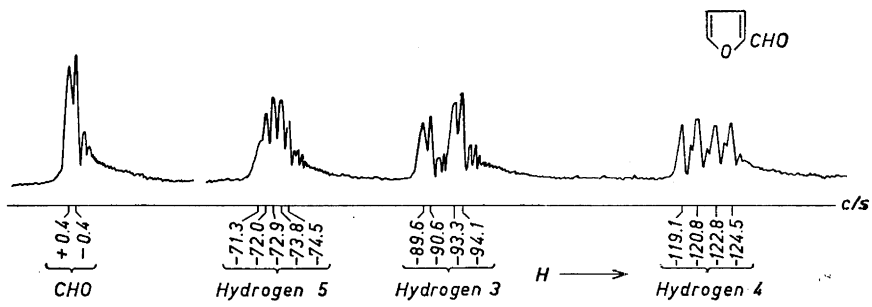


Fig. 2. NMR-spectrum of furfural at 40 Mc/s. The shifts are given relative to the aldehyde proton resonance line. The assignments are those of Refs.[6,7]

splittings are caused by spin-spin interactions. A chemical shift should have given a splitting at 30 Mc/s equal to 3/4 of that at 40 Mc/s.

The spectrum of the ring proton, which occurs at the lowest magnetic field (Fig. 2), consists of a quintet. This could be interpreted as a superposition of the patterns arising from three spin-spin couplings, two of which are nearly equal, and about half the magnitude of the third. This additional splitting of the lowest field ring proton resonance was also observed by Leane and Richards. They assign this multiplet to the five hydrogen as did Corey *et al.*[7] Apparently these assignments were based solely on the observed chemical shifts. If correct, it should then be pointed out that the relative magnitudes of J_{45} and J_{34} in furans are opposite to those in thiophenes. (In furans $J_{34} > J_{45}$ in thiophenes $J_{34} < J_{45}$ [1,8]). Leane and Richards [6] assumed that $J_{34} > J_{45}$ for thiophenes. Their interpretations of the spectra of 2-nitrothiophene and 2-thiophenecarboxylic acid thus would seem to be incorrect. This difference in relative magnitudes of the coupling constants in thiophenes and furans, if correct, is of considerable theoretical interest. It should be of great value therefore to obtain spectra of 2,3-disubstituted and 2,5-asymmetrically

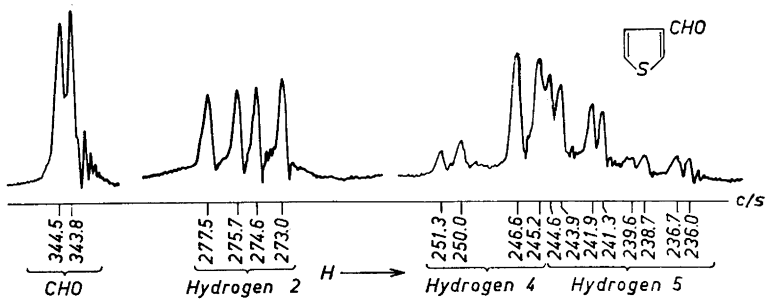


Fig. 3. NMR-spectrum of 3-thiophenealdehyde in *cyclo*hexane (internal reference) at 40 Mc/s.

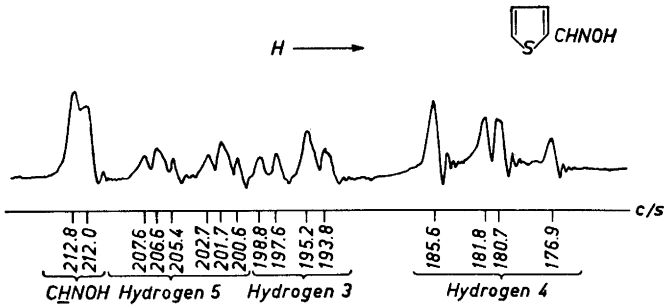


Fig. 4. NMR-spectrum of 2-thiophenealdoxime in dimethyl sulphoxide (internal reference) at 40 Mc/s.

disubstituted furans in order to confirm the assignments of coupling constants in furans.*

The observed splitting of the aldehydic hydrogen resonance in 3-thiophenealdehyde (Fig. 3) may be caused by spin-spin interaction with the five hydrogen since the resonance lines in the 2 × 2 × 2 multiplet have splittings equal to 2.7, 5.2 and 0.7 c/s, respectively [1]. We intend to confirm this interpretation by studying the spectra of substituted 3-thiophenealdehydes. We have shown through a study of several substituted 3-methylthiophenes [9] that the splitting of the methyl resonance in 3-methylthiophene is caused by coupling with the 2-hydrogen. This indicates that the mechanism of the spin-spin coupling between side-chain protons and protons of the ring is different in thiophenealdehydes and methylthiophenes.**

An investigation of the aldoxime hydrogen splitting in aromatic aldoximes is complicated by the fact that those compounds exist in *syn* and *anti* isomers. We have investigated a sample of 2-thiophenealdeoxime, m.p. 130—131°, which was predominantly the anti form [10]. Its spectrum in dimethyl sulphoxide (Fig. 4) showed a doublet at 212 c/s from the solvent peak with a splitting of 0.8 c/s. Because of its shift [5] and because it is absent in the spectrum of 2-acetothienone oxime, this doublet is assigned to the aldoxime hydrogen. The spectrum of its ring hydrogens is well resolved; consisting of one sextet and two quartets. The hydrogen causing the high field quartet has couplings to the other ring hydrogens equal to 3.8 and 4.9 c/s and is thus [1] hydrogen 4. The next quartet has splittings equal to 1.3 and 3.7 c/s and is therefore assigned [1] to hydrogen 3. The band from the 5-hydrogen at the lowest field consists of two triplets. This can be interpreted as arising from a proton coupled to three other hydrogens with two couplings being nearly equal and much smaller than the third. Thus the aldoxime hydrogen in 2-thiophenealdoxime

* The NMR spectrum of 5-chloromethyl furfural has been obtained by R. J. Abraham and H. J. Bernstein. *Canad. J. Chem.* **37** (1959) 1056.

** Smaller spin couplings (≈ 0.4 c/s) between the methyl group and the 4- and 5-hydrogens in 3-methylthiophene have been observed by P. L. Corio and I. Weinberg. *J. Chem. Phys.* **31** (1959) 569.

is coupled to the 5-hydrogen as is the aldehydic hydrogen in 2-thiophenealdehyde. The aldoxime hydrogen as well as the ring hydrogens in 3-thiophenealdoxime gave complex spectra probably due to small relative shifts of the ring hydrogens. The splittings of the aldehyde and oxime hydrogen resonances are listed in Table 1.

Table 1. Observed splittings of aldehyde and oxime hydrogen resonance lines.

Compound	B.p./M.p.	Ref.	Splitting c/s
Furfural	63— 64° (20 mm)		0.8
2-Thiophenealdehyde	85° (20 mm)	11	0.9
3-Thiophenealdehyde	86— 87° (20 mm)	12	0.7
5-Bromo-2-thiophenealdehyde	105—107° (11 mm)	13	≈0
5-Methoxy-2-thiophenealdehyde	79— 81° (0.9 mm)	14	≈0
3-Bromo-2-thiophenealdehyde	109—110° (10 mm)	9	1.2
4-Bromo-2-thiophenealdehyde	114—115° (11 mm)	9	0.9
	44— 45° (m. p.)		
4-Methyl-2-thiophenealdehyde	95— 96° (11 mm)	15	1.3
2-Thiophenealdoxime	130—131° (m. p.)	16	0.8

We note that long-range splittings of aldehydic hydrogen resonance lines have not as yet been observed in either the benzene or the α,β-unsaturated aldehyde series [9]. It is suggested that there might be a connection between the magnitude of the splittings and the degree of conjugation in the aldehydes. This would, however, require some interaction between the extended π-orbitals and the σ type C—H bond in the aldehyde group which is rather unexpected. We are continuing our studies in this field.*

EXPERIMENTAL

The spectra were obtained with a Varian Associates Model V-4300 B high resolution Spectrometer and a flux-stabilized 12″ magnet model V-4012-HR from the same company. The magnet sweep was calibrated by the familiar modulation side-band technique and the shifts from the internal standard were obtained by interpolation on the recorder chart. The modulation frequency obtained from the power line was 50 c/s.

References to the methods of preparation of the compounds investigated are given in Table 1. 4-Bromo-2-thiophenealdehyde and 3-bromo-2-thiophenealdehyde have been obtained from 4-bromo-2-lithiothiophene and 3-bromo-2-lithiothiophene, respectively, through the reaction with N,N-dimethyl formamide. Details of the preparation of these new compounds will be given later.

Acknowledgements. The authors wish to express their thanks to professor Arne Fredga and to professor Kai Siegbahn for their interest in this work and for all facilities put at the authors' disposal. We are indebted to professor J. Sicé (Chicago) for a generous gift of 5-methoxy-2-thiophenealdehyde. One of us (R.A.H.) thanks the *State Technical Research Council* (of Sweden) for a grant.

* Splittings of the aldehyde peak in ortho-substituted benzaldehydes (~ 0.5 c/s) have recently been observed in these laboratories by Drs. D. and V. J. Kowalewsky (private communication).

REFERENCES

1. Gronowitz, S. and Hoffman, R. A. *Arkiv Kemi* **13** (1958) 279.
2. Hoffman, R. A. and Gronowitz, S. *Arkiv Kemi. In print.*
3. Gronowitz, S. and Rosenberg, A. *Arkiv Kemi* **8** (1955) 23.
4. Gronowitz, S. *Arkiv Kemi* **13** (1958) 295.
5. Phillips, W. D. *Ann. New York Acad. Sci.* **70** (1958) 817.
6. Leane, J. B. and Richards, R. E. *Trans. Faraday Soc.* **55** (1959) 518.
7. Corey, E. J., Slomp, G., Dew, S., Tobinaga, S. and Glazier, R. E. *J. Am. Chem. Soc.* **80** (1958) 1204.
8. Takahashi, K., Matsuki, Y., Mashiko, T. and Hazato, G. *Bull. Chem. Soc. Japan* **32** (1959) 156.
9. Hoffman, R. A. and Gronowitz, S. *To be published.*
10. Hartough, H. D. *Thiophene and its derivatives*, New York 1952, p. 313.
11. Weston, A. W. and Michaels Jr. R. J. *J. Am. Chem. Soc.* **72** (1950) 1422.
12. Gronowitz, S. *Arkiv Kemi* **8** (1955) 441.
13. Gronowitz, S. *Arkiv Kemi* **8** (1955) 87.
14. Sicé, J. *J. Am. Chem. Soc.* **75** (1953) 3697.
15. Sicé, J. *J. Org. Chem.* **19** (1954) 70.
16. Goldschmidt, H. and Zanoli, E. *Ber.* **25** (1892) 2573.

Received June 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1693–1698

Uptake of Orthophosphate by the Pea Plant (*Pisum sativum*)

II.* Identification of the Organic Phosphate Esters Formed

JORMA K. MIETTINEN and TOIVO SAVIOJA

Biochemical Institute, Helsinki, Finland

The main phosphate esters formed in assimilation of orthophosphate in the pea plant are identified by two-dimensional paper chromatography using co-chromatography and radioautography. They are ** ATP, UTP, GTP, ADP, UDP, AMP, F6P, G6P, F1,6P, and an unknown compound marked No. 10. In the root several additional unidentified esters are formed, which are not found in the shoot extracts.

The general pattern of esterification of orthophosphate is similar in intact and detached pea root. No noticeable qualitative differences are found in the esterification pattern between pea, rye, oat, and barley plants, but some difference between these and the yeast *Torulopsis utilis* is noticeable.

In the first part [1] of this research project it was established that assimilation of orthophosphate by intact pea roots is a very rapid process. After 10 sec of assimilation, 2 organic phosphates — ATP and a diphosphate, probably ADP — showed labelling, and after 2 min, labelling was found in 4 triphosphates — ATP, GTP, UTP and an unidentified compound — and in 3 spots in the diphosphate area and 2 spots in the monophosphate area of the two-dimensional chromatogram. A dynamic equilibrium was reached in 5 min, after which time chromatograms from both roots and shoots were qualitatively very similar. The results with phosphorus-deficient pea plants (total P 30—50 % of normal) were not essentially different. The labelling obtained in the

* The first paper in this series is Ref.[1]

** The following abbreviations are used: AMP, ADP, ATP = adenosine-5'-mono-, -di- and -triphosphate, resp.; CMP, CDP, CTP = cytidine-5'-mono-, di- and -triphosphate, resp.; GMP, GDP, GTP = guanosine-5'-mono-, di- and -triphosphate, resp., and UMP, UDP, UTP = uridine-5'-mono-, di- and triphosphate, resp. 2-PGA = 2-phosphoglyceric acid, 3-PGA = 3-phosphoglyceric acid, PCHO = phosphorylcholine, G1P = glucose-1-phosphate, G6P = glucose-6-phosphate, F6P = fructose-6-phosphate, F1,6P = fructose-1,6-diphosphate, UDPG = uridine-diphosphoglucose, TCA = trichloroacetic acid.

short reaction times was relatively weak. Therefore identification of most of the spots was not achieved in the above study.

The main goal of the present study was the identification of the labelled phosphates formed. For this purpose somewhat longer assimilation times were used than in the above study to get stronger labelling in the spots. By the application of a new solvent combination [2] satisfactory two-dimensional paper chromatograms are obtained from the original extracts after removal of TCA, without any further purification, and this has made possible chromatographic identification of 10 of the labelled compounds formed. Though the time scale of the present experiment (1—30 min) extends far beyond the most interesting biokinetical interval of the primary phases of assimilation (1—60 sec), the activity of the separated spots was determined to give an idea of the quantitative changes taking place in longer times in the relative activities of the compounds formed. Stringent conclusions can hardly be drawn from figures like these, but the scale on which the experiment was carried out was too small to make determinations of specific activities possible. This will be tried later on and will require working on a larger scale and using different techniques, probably ion exchange columns, which may also provide conclusive identification of the isolated compounds. The present identifications by co-chromatography are only preliminary, although the probability that they are correct is relatively high, since a total of about one hundred two-dimensional co-chromatograms were made.

METHODS

Plant material. The pea plants *(Pisum sativum, var.* Torsdag) were grown in a sterilized nutrient solution inoculated with an efficient strain of *Rhizobium*, the bottles being closed with cotton wool. The green parts were free in the greenhouse air. The nutrient solution was similar to that used in the previous study [1] for "high-phosphorus" plants. The plants were 23 days old when the experiments were made.

Extraction and chromatography were also made as mentioned previously [1] except for the first solvent in paper chromatography. All extracts were diluted with water to 12 ml after removal of the TCA and 0.5 ml of the diluted extract was used for each chromatogram. The solvent used in the present study for the first dimension was *t*-butanol:formic acid (98 %): water, 8:3:4 (Miettinen [1]). In the second dimension the mixture of Hanes, *n*-propanol:NH_4OH (25 % NH_3):water, 6:3:1, was used.

Performance of the experiment. About 10 mC of carrier-free orthophosphate (PBS 1, Radiochemical Centre, Amersham, England), neutralized to pH 7.0, was added to 150 ml of phosphate-free but otherwise complete nutrient solution [1].

This solution was then evenly divided into 5 small beakers. Each sample consisted of a bunch of 5 plants, the roots of which were dipped into the isotope solution for the proper time (1, 5, 15 or 30 min), at the end of which the roots were rapidly washed in cold water, separated from the shoot and crushed in 8 % TCA. A fifth sample (30 min) was taken to study the effect of detachment (of the roots from the shoot) on the uptake rate of the phosphate ion. The detachment was made just before dipping. Of the roots all except the cutting place was dipped, of the shoot about 1 cm of the lower end only. The detached roots were dipped into the same solution as the 1 min sample of the intact plants. The detached shoots were dipped into a separate solution.

During the experiment the plants were, in addition to diffuse daylight, illuminated with one "Photolita" SM lamp at 70 cm distance.

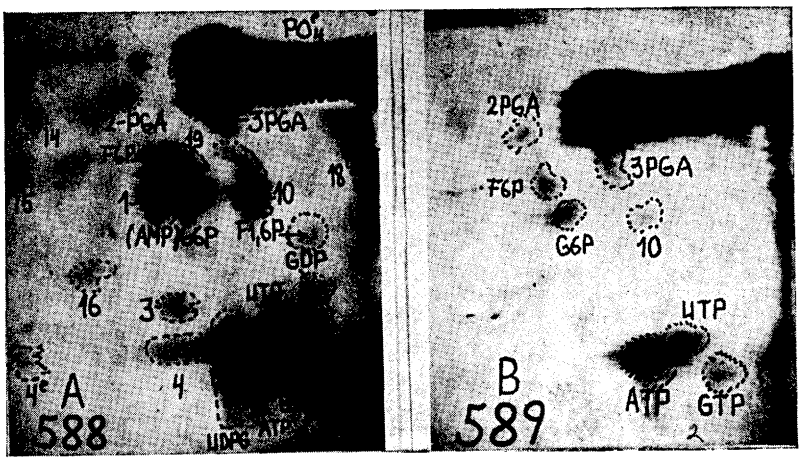


Fig. 1. Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in intact (A) and detached (B) pea roots after 1 min uptake of radioactive orthophosphate.

RESULTS

The following compounds were found to be labelled *in the roots of intact plants* after 1 min assimilation: ATP, UTP, GTP, UDPG, AMP(?), G6P (?), F6P, 2-PGA, 3-PGA, unknowns Nos 1, 3, 4, 10, 13—16, 18, 19, and traces of F1,6P and GDP (Fig. 1, A). The unknown No. 1 may be identical with G1P, but this is still uncertain because the coloured spot of added authentic compound did not exactly match the radioactive spot in one case. In the *roots detached from the shoot* (Fig. 1, B) only weak labelling was found in ATP, UTP and GTP and in traces of extremely faint spots of the other compounds. Total activity of the root extracts was: intact roots 2.0×10^7, detached roots 0.77×10^7 c.p.m. Because of their low activity the individual spots of these chromatograms were not counted.

In the 5 — 30 min samples PCHO and the nucleotide diphosphates ADP, UDP and GDP became labelled in addition to the compounds mentioned above. Chromatograms of the root (A) and shoot (B) extracts of the 15 min sample are presented in Fig. 2. The same spots are found labelled in both cases except for Nos 1, 3, 4, 18 and 19, which do not appear in the shoot (Fig. 2, B). The total activities of the extracts and relative activities of some individual compounds are reported in Table 1. The total activity of the shoots is *ca.* 2—3 % of the activity of the roots, but the relative activity of the organic phosphates, compared with the inorganic spot, is much higher in the shoot.

The composition of the extracts of the intact and the detached shoots after 30 min assimilation is illustrated in Fig. 3. Again, both extracts are qualitatively similar but the labelling of all compounds is very much stronger in the detached shoot which was able to absorb radioactive nutrient solution without the "sieving" effect of the roots. As the only unknowns in both of

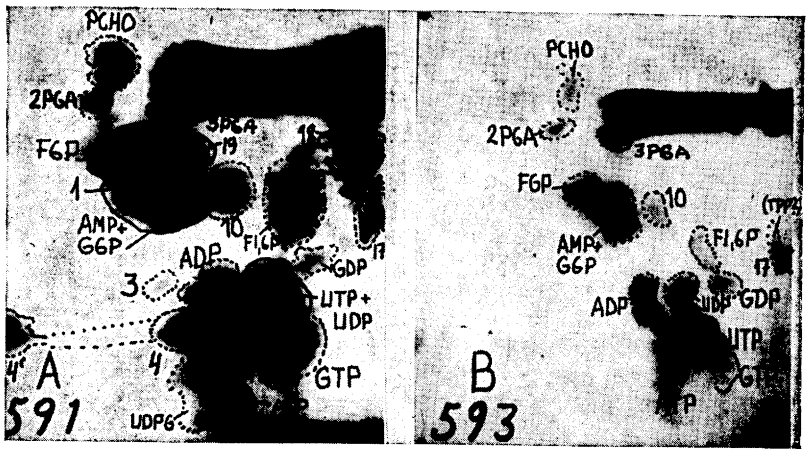


Fig. 2. Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in the root (A) and the shoot (B) of intact pea plants after 15 min uptake of radioactive orthophosphate.

these extracts, spots Nos 10, 17 and 18 show labelling, of which the two latter ones well match with inorganic pyrophosphate (P_2O_7) and thiamine pyrophosphate (TPP). The identity of these two spots is not yet fully established, however.

DISCUSSION

The main purpose of this study was the identification of the acid-soluble phosphates formed during phosphorus assimilation in the pea plant. A number of acid soluble phosphates have been chromatographically identified by earlier workers in the pea and in other higher plants. Rowan [3] identified in pea seed-

Table 1. Total acid soluble radioactivity and relative activities of orthophosphate and the main phosphate esters, separated by paperchromatography.

Sample	Total acid soluble activity c/min in millions	Relative activity, %									
		ATP	ADP	AMP + G6P	UTP	UDP	GTP	F1,6P	F6P	10	PO_4
Root											
5 min.	23.0	0.40	0.11	0.16	0.15	0.05	0.07	0.26	0.05	0.06	98.6
15 »	32.0	1.97	0.30	2.80	0.17	0.35	0.08	0.53	0.60	0.18	93.0
30 »	27.2	0.50	0.83	1.00	0.33	0.19	0.13	0.04	0.22	0.06	96.8
Shoot											
15 min.	0.58	3.84	1.60	2.78	(1.17)	1.40	—	0.87	1.83	0.44	86.1
30 »	0.88	2.91	2.86	4.12	0.97	3.24	0.32	0.89	2.10	0.32	82.5
30 min. detached	4.56	1.49	1.79	4.27	0.58	1.55	0.45	0.79	1.17	0.29	87.5

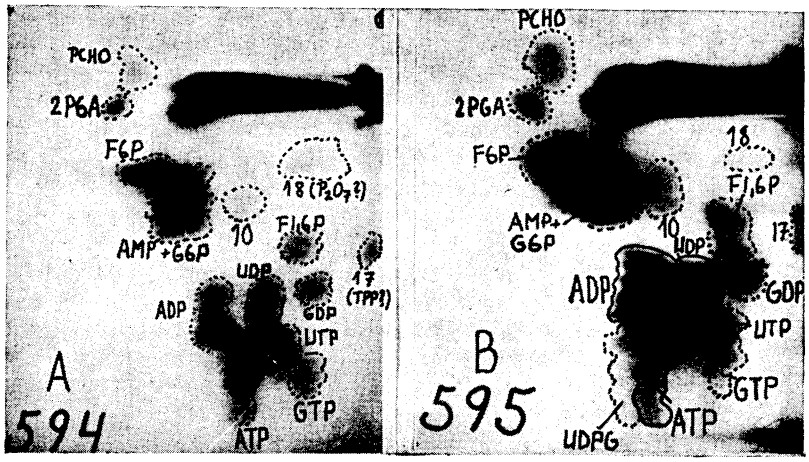


Fig. 3. Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in intact (A) and detached (B) pea shoots after 30 min uptake of radioactive orthophosphate.

lings ATP, ADP, UTP, UDP, UMP and a dinucleotide containing both adenosine and guanosine. In another paper [4] he identified G1P, G6P and F1,6P. Loughman and Scott Russell [5] found in barley roots 5 nucleotides, of which they identified ATP and ADP. Of sugar phosphates they mention G6P, F6P and F1,6P. Ginsburg, Stumpf and Hassid [6] isolated UDPG from mung bean seedlings. According to Burma and Mortimer [7] UDPG is synthetized in the leaves of sugar beet from G1P and UTP. Maizel, Benson and Tolbert [8] have found PCHO to be the main organic transport form of phosphate in higher plants. Lindeman [9] identified in *Chlorella* ATP, ADP, UTP, UDPG, AMP, F6P, G6P, F1,6P and 3PGA. We have found these same compounds among several other ones labelled in the pea plant in the present study.

Aronoff [10], when studing phosphate assimilation in the roots of soybean, concluded that the first compound to become labelled was F1,6P. Mitsui, Aso and Ishizuka [11], on the other hand, consider G6P and F6P to be the primary labelled phosphate esters in wheat seedlings. From these the labelling is transferred to nucleotides. Contrary to the above results we found in one of our earlier investigations [1] labelling earliest in nucleotides, primarily in ATP. Nearly simultaneously a weaker labelling appears, however, in three sugar phosphates, G6P, F6P, and F1,6P [12]. ATP is without doubt a primary assimilation product, but any of the other ones mentioned above may also be formed independently of and simultaneously with ATP [12].

All identifications in the present study are based on several tests by cochromatography. The nucleotide nature of spots so identified was further confirmed in an unpublished experiment in which the acid soluble phosphates were fractionated by means of Norite active charcoal. All spots identified as nucleotides and the unknowns 3 and 4 were adsorbed by the charcoal (and re-

eluted by pyridine), whereas the spots identified as sugar phosphates passed through the column unabsorbed.

In another unpublished experiment we compared acid soluble phosphates of pea, rye, oat and barley plants after 10 min's orthophosphate assimilation. The same and only the same labelled compounds were found in all these plants. However, comparison of pea and yeast [2] shows slight differences, *i.e.* first, the yeast does not show labelling in 2-PGA and second, two compounds in yeast, which have been preliminarily identified as phosphoserine and phosphothreonine, do not show labelling in the roots of higher plants.

In yeast extracts one of the strongest labelled phosphates is the unknown No. 3, which is absorbed by Norite like a nucleotide and is located on the two-dimensional chromatograms in the region of nucleotide triphosphates. It becoms faintly labelled after about 1 min's assimilation, but after 10—30 min it becomes one of the strongest spots. We are presently working on the identification of this compound.

An interesting difference between the root and shoot of the pea plant is, that the root extracts contain several unidentified phosphate esters which are not found in the shoot.

Acknowledgements. This study has been performed under the tenure of the *FAO André Mayer Fellowship* by one of the authors (JKM). A grant from *Suomen Luonnonvarain Tutkimussäätiö* is also gratefully acknowledged. We are greatly indebted to Prof. A. I Virtanen for his interest and encouragement and for the working facilities he has generously made available to us.

REFERENCES

1. Miettinen, J. K. and Savioja, T. *Suomen Kemistilehti* **B 31** (1958) 84.
2. Miettinen, J. K. *Proc. 2nd Intern. Conf. Peaceful Uses Atomic Energy*, Geneve 1958, paper No. 1102.
3. Rowan, K. S. *J. Exptl. Botany* **8** (1957) 256.
4. Rowan, K. S. *J. Exptl. Botany* **9** (1958) 436.
5. Loughman, B. C. and Scott Russel, R. *J. Exptl. Botany* **8** (1957) 280.
6. Ginsburg, V., Stumpf, P. K. and Hassid, W. Z. *Federation Proc.* **15** (1956) 162.
7. Burma, D. P. and Mortimer, D. C. *Plant Physiol.* **30** (1955) Proc. III.
8. Maizel, J. V., Benson, A. A. and Tolbert, N. E. *Plant Physiol.* **31** (1956) 407.
9. Lindeman, W. *Proc. 2nd Intern. Conf. Peaceful Uses Atomic Energy*, Geneve 1958, paper No. 558.
10. Aronoff, S. *Ann. Rev. Plant Physiol.* **2** (1951) 8.
11. Mitsui, S., Aso, S. and Isbizuka, K. *Soil and Plant Food, Tokyo* **3** (1957) 65.
12. Miettinen, J. K. and Savioja, T. *Suomen Kemistilehti* **B32** (1959) 128.

Received May 29, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1699–1702

An Electron Diffraction Investigation of the Molecular Structure of Allene

A. ALMENNINGEN, O. BASTIANSEN and M. TRÆTTEBERG

Universitetets kjemiske institutt, Blindern-Oslo;
Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The electron diffraction sector method was used to determine the structure of allene vapour. The observed molecular parameters are the following: C—H: 1.081_6 Å (u = 0.0795 Å), C=C: 1.311_6 Å (u = 0.0390 Å), C_1C_3: 2.617_3 Å (u = 0.0519 Å) and <H—C=C: 120.8°. The C_1C_3 distance was found to be shorter than twice the C=C distance. This can be explained by out-of-linearity vibrations.

The molecular structure of allene was determined by the electron diffraction sector method, using a modified s^3 sector. The electron diffraction diagrams were taken at three different distances between the scattering point and the photographic plate (approximately 48, 19 and 12 cm). The photometer curves were read off and the usual corrections were carried out.

The subtraction of the background, *viz.* atomic and incoherent scattering, was carried out by fitting a theoretical background to the intensity curve by means of a least square treatment giving the intensity zero line and the intensity scale. An example of the correspondence between the background and the intensity curve is demonstrated in Fig. 1 for the average of the 48 cm plates. The correspondence is good except for the innermost part. As a general rule, the middle parts of the intensity curves fit well with the theoretical background. At the innermost and outermost parts corrections generally have to be made. The short distance curves (12 cm) were all treated separately. Because of the low intensity values the correspondence is less satisfactory. A small error in the readings gives raise to a relatively large error in the amplitudes. The background was fitted to each of the experimental curves and the difference curves were averaged. The curves from the various distances were normalized, and after the averaging of the overlapping regions the first total molecular intensity curve was obtained extending from s = 1.25 to s = 65 Å^{-1}.

By Fourier transformation of the damped molecular intensity curve (damping factor $e^{-0.0009\ s^2}$) the corresponding radial distribution (R.D) curve was obtained. In this curve the Gaussian shaped peaks corresponding to the interatomic distances were completely resolved except for some unavoidable overlap of the C—H and C=C double bond peaks.

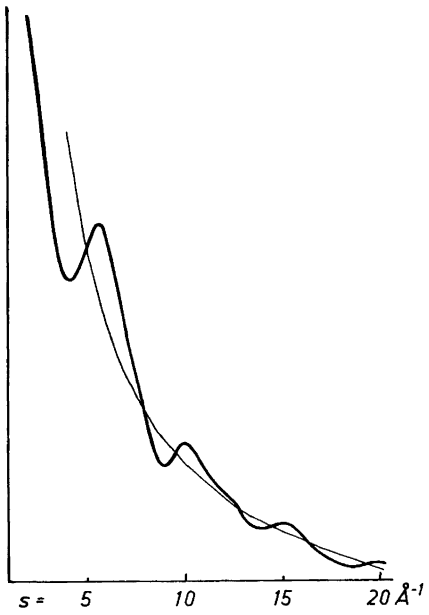


Fig. 1. Allene. Intensity curve from the 48 cm photographic plates with the theoretical background fitted in.

From this first R.D. curve, the most probable model of the molecule was determined and a theoretical intensity curve corresponding to this model computed. By means of the theoretical intensity curve it is possible to correct for the most serious errors in the background. In this case only small corrections were necessary for obtaining the final molecular intensity curve (Fig. 2) upon which the final conclusions were based.

A series of R.D. curves was calculated from the final intensity curve using different damping constants ($k = 0$, 0.0009 and 0.0015) and Δs values of 1/2 and 1/4 Å^{-1}. Three of these curves are presented in Fig. 3.

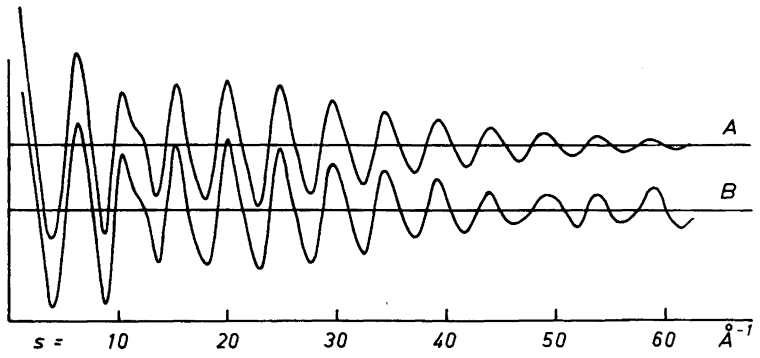


Fig. 2. Allene. Theoretical (A) and experimental (B) intensity curves.

Table 1. Allene. Internuclear distances and mean amplitudes of vibrations.

Distances	Observed r values	Observed u values	u values calculated from spectroscopic data
C—H	1.0816	0.0795	0.0772
C=C	1.3116	0.0390	0.0401
C_2H_1	2.0840	0.1046	0.1015
C_1C_3	2.6173	0.0519	0.0451
C_3H_1	3.2963	0.1272	0.1144
<H—C=C: 120.8°			

These R.D. curves were also resolved into Gaussian peaks. The internuclear distances and mean amplitudes of vibration ($u = \sqrt{\overline{(r - r_0)^2}}$) are presented in Table 1. If a linear carbon skeleton is assumed the structure is determined from three parameters. (The question of the relative position of the HCH planes has been ignored).* The R.D. curves give altogether five distances, and the best set of parameters was calculated using the method of least squares. The parameters thus obtained are: C—H: 1.083 Å, C=C: 1.308 Å and H—C=C angle 120.6°.

It is worth noting that the C=C value obtained by this procedure is 0.003 Å smaller than that computed directly from the C=C peak of the R.D. curves. This of course corresponds to the fact that the C_1C_3 distance as found from the R.C. curves is about 0.006 Å smaller than twice the C=C distance obtained in the same way. Even if this effect is very small we are inclined to believe that it is real. The effect can easily be explained by an out-of-linearity vibration. If this argument holds the best C=C distance should be 1.312 Å instead of 1.308 Å.

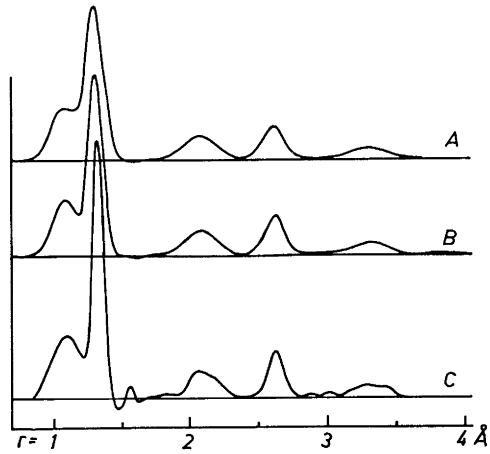


Fig. 3. Allene. Radial distribution curves with different damping constants (A: $k = 0.0015$, B: $k = 0.0009$, and C: $k = 0$).

* *Added in proof:* A closer examination of the R. D. curves reveals the presence of HH distance peaks beyond the largest CH peaks, corresponding to an angle of 90° between the two HCH planes.

Table 2. Allene. Theoretical and experimental values for areas under the peaks in the radial distribution curves.

Distances	Theoretical areas	Experimental areas
C—H C=C	54.80	54.5
C_2H_1	9.54	9.6
C_1C_3	9.12	9.1
C_3H_1	6.03	4.7

A similar effect which can also be interpreted by the assumption of out-of-linearity vibrations was observed for dimethyldiacetylene [1].

The structure parameters obtained for allene are in accordance with the values obtained by Stoicheff from high resultion Raman spectroscopy [2]. Using both allene and allene-d_4 the C=C distance of 1.309 Å is obtained. Assuming a C—H distance of 1.07 ± 0.01 Å as found in an earlier work on ethylene [3], he obtains an HCH angle of 117° (H—C=C angle equal to 121.5°). We feel that Stoicheff's assumed C—H distance is a trifle too small.

It is of interest to compare our parameters with the recently obtained values for ethylene [4-6]. The data obtained from three indepentent methods are: C—H: 1.086 ± 0.003 Å, C=C: 1.337 ± 0.003 Å and <H—C=C: 121.5° ± 1°. This shows that the CH_2 group in ethylene, within rather narrow error limits, has the same structure as in allene. However, the C=C distance is, as pointed out by Overend and Thompson [7] and by Stoicheff [3], no doubt larger in ethylene than in allene.

In the last column of Table 1 the u values calculated from spectroscopic data by Cyvin [8] have been presented. The correspondence is satisfactory except for the C_1C_3 distance where a significant discrepancy seems to be present. This discrepancy can be satisfactorily explained by the mentioned out-of-linearity vibration. The spectroscopic calculations were based upon the assumption of a linear model. The out-of-linearity vibrations should not only lead to a decrease in the C_1C_3 distance but should also lead to an increase in the mean amplitude of vibration for this distance.

As a control of the reliability of the R.D. curves the measured areas have been compared with the theoretical ones on a relative scale. The values are given in Table 2.

This investigation has been sponsored by the *United States Air Force Development Command* under contract AF 61(052)—72.

REFERENCES

1. Almenningen, A., Bastiansen, O. and Munthe-Kaas, T. *Acta Chem. Scand.* **10** (1956) 261.
2. Stoicheff, B. P. *Can. J. Phys.* **33** (1955) 811.
3. Gallaway, W. S. and Barker, E. F. *J. Chem. Phys.* **10** (1942) 88.
4. Bartell, L. S. and Bonham, R. A. *J. Chem. Phys.* **27** (1957) 1414.
5. Allen, H. C. and Plyler, E. K. *J. Am. Chem. Soc.* **80** (1958) 2673.
6. Dowling, J. M. and Stoicheff, B. P. *Private communication* (1958).
7. Overend, J. and Thompson, H. W. *J. Opt. Soc. Am.* **43** (1953) 1065.
8. Cyvin, S. J. *J. Chem. Phys.* **29** (1958) 583.

Received June 20, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1703–1708

Phase Relations in the System Ag-Sb-S at 400° C

JAN BARSTAD*

Department of Metallurgy, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.

The coexisting phases in the three-phase fields of the system Ag—Sb—S at 400°C within the region Ag_2S—Sb_2S_3—Sb—Ag have been determined by means of X-ray identification in samples annealed at 400°C and quenched to room temperature. In addition, the reactions between pellets of silver-antimony alloys and silver-antimony sulfides at 400°C have been investigated. The results are in agreement with the isothermal section of the ternary phase diagram deduced from X-ray investigations.

In conjunction with kinetic investigations on solid state reactions in the quasi-binary system Ag_2S—Sb_2S_3, it was found desirable to reexamine the isothermal section of the phase diagram of the ternary system Ag—Sb—S at 400°C. Previously this ternary system was investigated by Schenck, Hoffmann, Knepper and Vögler [1] with the help of measurements of the H_2S/H_2 ratio during the reduction of sulfides of the quasi-binary system Ag_2S—Sb_2S_3 with H_2 at 400°C. From the obtained staircase curves the boundaries of two-phase and three-phase fields were deduced. As the H_2S/H_2 ratios for some of the steps of the staircase curves were close together, the reality of some steps may be questioned. The composition of the silver-antimony sulfide phases was given as $Ag_2S{\cdot}Sb_2S_3$, $4Ag_2S{\cdot}Sb_2S_3$, and $2Ag_2S{\cdot}Sb_2S_3$. This is in disagreement with thermal studies by Jaeger and van Klooster [2] and by Jensen [3] where only the phases $Ag_2S{\cdot}Sb_2S_3$ and $3Ag_2S{\cdot}Sb_2S_3$ were observed. The work was confined to the region Ag—Sb—Ag_2S—Sb_2S_3 of the system Ag—Sb—S. The nature of the coexisting phases was ascertained by the following methods.

1) Samples whose compositions were presumably in three-phase fields were prepared from the elements, annealed at 400°C, and quenched. In addition, two samples in two-phase fields were investigated. The phases present were identified by means of X-ray powder diagrams with the help of a General Electric diffraction unit with Cu-radiation and Ni-filter.

2) Pressed and sintered pellets of Ag_2S, Ag_3SbS_3, $AgSbS_2$ and Sb_2S_3 and pellets of Ag, Ag_3Sb, and Sb solidified from melts were prepared. Two pellets

* Present address: Forsvarets Forskningsinstitutt, Kjeller pr. Lillestrøm, Norway.

of different composition 2—3 mm thick were pressed together and heated at 400°C for several days in a N_2 atmosphere. Observation of a reaction zone involving new phases indicates that the phases initially present do not coexist under equilibrium conditions.

The following informations on the crystal structures and homogeneity ranges of the phases in the binary systems were used.

1) The binary system Ag—Sb

By means of metallographic examination and X-ray diffraction identification, Weibke and Effinger [4] studied the system Ag—Sb in detail. Their results agree in principle with previous X-ray diffraction investigations by Broderick and Ehret [5] and by Westgren, Hägg, and Eriksson [6]. At 400°C, the following phases exist:

α-Phase. Homogeneity range 0—6.2 atom % Sb[4]. Face-centered cubic structure with a lattice constant increasing from $a = 4.086$ Å for pure silver to $a = 4.121$ Å[6]. Lattice constant for pure silver is given on the ASTM data card [7] as $a = 4.0862$ Å at 27°C.

ζ-Phase. Homogeneity range 9.4—14.9 atom % Sb[4]. Hexagonal close-packed structure with lattice constants increasing from $a = 2.927$ Å, $c = 4.784$ Å, $c/a = 1.634$[6] to $a = 2.961$ Å, $c = 4.797$ Å, $c/a = 1.620$ [4].

ε-Phase. Homogeneity range 21.3—26.2 atom % Sb [4]. Orthorhombic structure which can be considered as a slightly distorted ζ-phase structure. The change in lattice constants within the homogeneity range for alloys annealed at 400°C is from $a = 2.986$ Å, $b = 5.180$ Å, $c = 4.774$ Å to $a = 2.996$ Å, $b = 5.246$ Å, $c = 4.854$ Å [6].

The lattice constants given by Westgren, Hägg, and Eriksson [6] for the lower limit of the homogeneity range of the ε-phase are considerably higher than those given by Weibke and Effinger [4]. Only the latter authors give the annealing temperature of the alloys and report differences in lattice constants for alloys annealed at 400°C and 500°C and thus their values are considered to be more accurate.

Sb-Phase. The dimensions of the hexagonal unit cell of pure Sb are given on the ASTM data card [8] as $a = 4.307$ Å, $c = 11.273$ Å. The solubility of Ag in Sb and its effect on the lattice constants are not yet known.

2) The binary system Sb—S

As shown already by Jaeger and van Klooster [2], Sb_2S_3 is the only stable compound in the system Sb—S. The compound Sb_2S_5 has been obtained only by precipitation from aqueous solutions. The ASTM data card [9] gives the following dimensions of the orthorhombic unit cell of Sb_2S_3: $a = 11.229$ Å, $b = 11.310$ Å, $c = 3.839$ Å.

The homogeneity ranges of the Sb phase and the Sb_2S_3 phase must be considered as very limited, but actual data are missing.

3) The binary system Ag—S

There is only one compound Ag_2S, which has a cubic structure at 400°C [10], called α Ag_2S or argentite. Transformation to the low temperature modification, called β Ag_2S or acanthite, takes place spontaneously near 178°C even on rapid quenching. According to Ramsdell [11] acanthite is monoclinic, $a = 9.47$ Å, $b = 6.92$ Å, $c = 8.28$ Å, $\beta = 124°$. The homogeneity range of α

Ag_2S was deduced by Wagner [12] at 200° and 300°C from electrochemical measurements and can be given as $Ag_{2.000}S$ to $Ag_{2.002}S$.

The solubility of S in Ag is negligible.

4) The quasi-binary system $Ag_2S-Sb_2S_3$

A thermal study of the system $Ag_2S-Sb_2S_3$ was first made by Jaeger and van Klooster [2]. The most detailed investigation has been made by Jensen [3]. Two intermediate compounds with a narrow homogeneity range are reported, $AgSbS_2$ and Ag_3SbS_3.

$\alpha AgSbS_2$. This modification is stable above 380°C. The crystal structure is cubic with a lattice constant $a = 5.653$ Å according to Graham [13]. The transformation to the low temperature modification β $AgSbS_2$, miargyrite, takes place on slow cooling whereas α $AgSbS_2$ is preserved on quenching.

Ag_3SbS_3, pyrargyrite. The structure of this phase was determined by Harker [14]. The dimensions of the hexagonal unit cell are $a = 11.06$ Å, $c = 8.74$ Å.

In addition, there have been reported the minerals polyargyrite, 11 $Ag_2S{\cdot}Sb_2S_3$, polybasite, $8Ag_2S{\cdot}Sb_2S_3$, pyrostilpnite, $3Ag_2S{\cdot}Sb_2S_3$, and stephanite, $5Ag_2S{\cdot}Sb_2S_3$. Polybasite and pyrostilpnite were prepared by Weil and Hoeart [15] by heating the components in glycerol.

IDENTIFICATION OF PHASES BY X-RAY DIFFRACTION

In Fig. 1 the compositions of the samples in the ternary system are indicated in the phase diagram. The exact composition and the phases identified in each sample are given in Table 1.

The interpretation of the X-ray diffraction diagrams was complicated because overlapping of diffraction lines from different phases often occurred. The β Ag_2S, Sb_2S_3 and Ag_3SbS_3 phases give very complex patterns. The complex β Ag_2S pattern could have been avoided by using a high temperature camera where the specimen was kept at a temperature above 178°C, but in the present investigation a high temperature camera was not used.

The accuracy of the lattice constants is estimated to be 0.2 % unless otherwise stated.

The lattice constants of the individual phases found in the various samples were compared with values available in the literature with the following results.

Sb_2S_3-Phase. Within the limits of error, complete agreement was found between the Sb_2S_3 phase in sample G and pure Sb_2S_3 [9].

Sb-Phase. Within the limits of error the lattice constants of Sb in the samples G, A, and B agreed with those of pure Sb[8].

α $AgSbS_2$-Phase. The samples G, A, B, K, and C contain α $AgSbS_2$ with a lattice constant equal to the value given for pure α $AgSbS_2$ [13].

ε-Phase. The calculated lattice constants in samples B and K are $a = 2.992$ Å, $b = 5.244$ Å, $c = 4.852$ Å. In sample C considerable overlapping from Ag_3SbS_3 interferes with an accurate evaluation of the lattice constants, but they are within a limit of error of $\pm$ 0.4 % the same as in samples B and K. Upon comparing these values with the lattice constants of the ε-phase in the system Ag—Sb[6] within the homogeneity range, nearly complete agreement with the values for the Sb-rich limit of the ε-phase is found. In sample L where

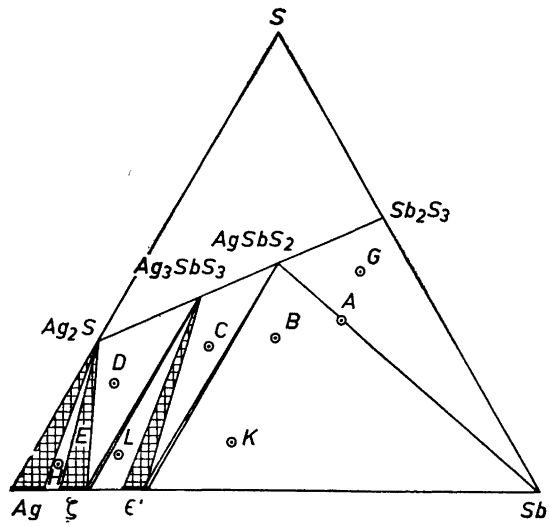


Fig. 1. Isothermal section of the phase diagram of the system Ag—Sb—S at 400°C for the region Ag—Sb—Ag_2S-Sb_2S_3.

both the ε-phase and the ζ-phase are present, nearly complete overlapping of the reflections from these phases is observed.

Ag_3SbS_3. Within an estimated error of ± 0.4 %, agreement of the lattice dimensions of the Ag_3SbS_3 phase in the samples C, L, D with synthetic Ag_3SbS_3 prepared from Ag_2S and Sb_2S_3 was obtained.

ζ-Phase. The following lattice constants were obtained:

Sample L: $a = 2.965$ Å, $c = 4.783$ Å
Sample D: $a = 2.963$ Å, $c = 4.780$ Å
Sample E: $a = 2.940$ Å, $c = 4.792$ Å
Sample H: $a = 2.924$ Å, $c = 4.763$ Å

Table 1.

Alloy	Atom % Ag	Sb	S	Phases present
G	9.4	41.9	48.7	Sb_2S_3, Sb, α $AgSbS_2$
A	18.8	43.8	37.4	Sb, α $AgSbS_2$
B	33.3	33.3	33.3	Sb, α $AgSbS_2$, ε
K	55.0	35.0	10.0	Sb, α $AgSbS_2$, ε
C	47.5	21.5	31.0	α $AgSbS_2$, ε, Ag_3SbS_3
L	76.5	16.5	7.0	ε, Ag_3SbS_3, ζ
D	69.3	7.7	23.0	Ag_3SbS_3, ζ, Ag_2S
E	79.0	5.3	15.7	ζ, Ag_2S
H	87.7	7.4	4.9	ζ, Ag_2S, Ag

α Ag_2S. The diffraction lines of Ag_2S in the alloys D, E, and H were identified by a comparison with those of pure Ag_2S. Due to the transformation to β Ag_2S on cooling, the lattice constant of α Ag_2S has not been obtained.

Ag-Phase. The lattice constant of the silver-phase in alloy H was found to be $a = 4.112$ Å compared with the value $a = 4.121$ Å given as the upper limit for the a phase in the system Ag—Sb.

The following conclusions can be drawn:

There is no indication of more than very limited homogeneity ranges of the phases Sb_2S_3, Sb, $AgSbS_2$, and Ag_3SbS_3 as deviations from the lattice dimensions of the pure stoichiometric phases have not been observed.

No significant solubility of sulfur in α Ag, ζ and ε seems to occur as the three-phase samples have lattice constants which do not differ from the lattice constants at the phase boundaries in the corresponding binary system Ag—Sb.

As sample D contains ζ-phase with lattice constants nearly equal to the lattice constants at the Sb-rich end of the ζ-phase in the system Ag—Sb, the tie lines from nearly the whole phase area must be directed towards the Ag_2S phase. The tie lines from the α Ag phase area are also directed towards the Ag_2S phase.

Sample C contains ε-phase with lattice constants nearly equal to the lattice constants at the Sb-rich end of the ε-phase in the system Ag—Sb. The tie lines from nearly the whole ε-phase area are thus directed towards the Ag_3SbS_3 phase.

There is no indication of a phase with the approximate composition $31\ Ag{\cdot}2Ag_2S{\cdot}Sb_2S_3$ as reported in the paper by Schenk *et al.*[1]

The experimental data given by Schenck *et al.*[1] are not in obvious conflict with the phase diagram in Fig. 1 although they have been interpreted differently. It must be recalled, however, that some of the reported steps of the H_2S/H_2 *vs.* composition plots are not very distinct and, therefore, do not permit a definite construction of the phase fields.

REACTION BETWEEN PELLETS OF DIFFERENT COMPOSITION

The pellet combinations Sb—$AgSbS_2$, Ag_3Sb—$AgSbS_2$, Ag_3Sb—Ag_3SbS_3 and Ag_3Sb—Ag_2S gave no indication of a reaction after heating 170 h at 400°C. Except for the combination Ag_3Sb—Ag_2S, the phases involved are connected by tie lines in the Fig. 1 and thus coexist at 400°C.

After 170 h at 400°, the pellets of the combinations Sb—Ag_3SbS_3 and Sb—Ag_2S were easily separated, but a thin reaction layer could be observed at the contact surfaces. The pellet combination Ag_3Sb—Sb_2S_3 showed a more pronounced reaction; the pellets stuck together. Reactions between the phases Ag and Ag_3SbS_3, Ag and $AgSbS_2$, and Ag and Sb_2S_3 take place very rapidly. After 100 h virtually only the reaction products could be observed on staining polished cross sections according to Gaudin and McGlashan [16].

In all the pellet combinations where a reaction was observed, the two initial phases do not coexist according to the phase diagram in Fig. 1.

Acknowledgement. The author wishes to express his thanks to Professor Carl Wagner for the interest in this work and for his valuable suggestions and to Professor John T. Norton for making X-ray equipment available. My work at M.I.T. was made possible by

a fellowship granted by Per Frølich. The author wishes also to express his thanks to *Norges Teknisk-Naturvitenskapelige Forskningsråd, Norge Amerika Foreningen* and *United States Educational Foundation* in Norway. The investigation has been sponsored by *Office of Ordnance Research, U.S. Army* under Contract DA-19-020-ORD-3661.

REFERENCES

1. Schenck, R., Hoffmann, I., Knapper, W. and Vögler, H. *Z. anorg. u. allgem. Chem.* **240** (1939) 173.
2. Jaeger, F. M. and van Klooster, H. S. *Z. anorg. u. allgem. Chem.* **78** (1912) 245.
3. Jensen, E. *Avhandl. Norske Vitenskapsakad. Oslo, I. Mat. Naturv. Klasse, No.* **2** (1947) 23.
4. Weibke, F. and Effinger, I. *Z. Elektrochem.* **46** (1940) 53.
5. Broderick, S. J. and Ehret, W. F. *J. Phys. Chem.* **35** (1931) 2631.
6. Westgren, A., Hägg, G. and Eriksson, S. *Z. physik. Chem.* **B 4** (1929) 461.
7. *ASTM X-ray Diffraction Data.* Swanson and Tatge, I. C. Fel. Reports, NBS, 1950.
8. *ASTM X-ray Diffraction Data.* Swanson and Fugat, NBS Circular 539 Vol. III (1953).
9. *ASTM X-ray Diffraction Data.* Swanson *et. al.* NBS Circular 539, Vol. V (1955).
10. Rahlfs, P. *Z. physik. Chem.* **B 31** (1935) 157.
11. Ramsdell, L. S. *Am. Mineralogist* **28** (1943) 401.
12. Wagner, C. *J. Chem. Phys.* **21** (1953) 1819.
13. Graham, A. R. *Am. Mineralogist* **36** (1951) 436.
14. Harker, D. *J. Chem. Phys.* **4** (1936) 381.
15. Weil, R. and Hocart, R. *Compt. rend. Congres soc. savantes, Toulouse* **1953**. *Sect. sci.*, 183.
16. Gaudin, A. M. and McGlashan, D. W. *Econ. Geol.* **31** (1938) 143.

Received June 19, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1709–1728

Short Communications

Lower Homologues of Thioctic Acid

GÖRAN CLAESON

Chemical Institute, University of Uppsala, Uppsala, Sweden

A few years ago I prepared 1,2-dithiolane-3-carboxylic acid[1], the lowest homologue of 6,8-thioctic acid [2-4]. It was my intention to prepare other homologues with side chains more like the biologically important acid. In 1956, however, Thomas and Reed prepared the nearest lower and higher homologues of 6,8-thioctic acid, 1,2-dithiolane-3-butyric acid and 1,2-dithiolane-3-caproic acid [5]. These homologues exhibited much lower biological activity than DL-6,8-thioctic acid in the acetate-replacing factor assay [6,7].

Preparation of the homologues between 1,2-dithiolane-3-carboxylic acid and 6,8-thioctic acid has thereby lost part of its biological interest. The acids with a carboxyl group in a position α to sulphur in the 1,2-dithiolane or 1,2-dithiane ring, however, show some interesting properties, which are not observed in the homologues where the carboxyl is screened by a side chain. This is a preliminary report of the syntheses and UV spectra of some carboxyl substituted cyclic disulphides.

The UV spectrum of 1,2-dithiane contains a characteristic absorption maximum at 290 mμ and that of 1,2-dithiolane a maximum at 330 mμ[8]. When a carboxylic group is substituted in a position α to the disulphide group, it causes a withdrawal of electrons from the disulphide group, resulting in a shift of the absorption band towards shorter wavelengths. This is observed for 1,2-dithiolane-3-carboxylic acid, which has an absorption peak at 280 mμ (Fig. 1) that is, a hypsochromic shift of 50 mμ. A still greater hypsochromic shift was observed by Schotte[9] for 1,2-dithiolane-3,5-dicarboxylic

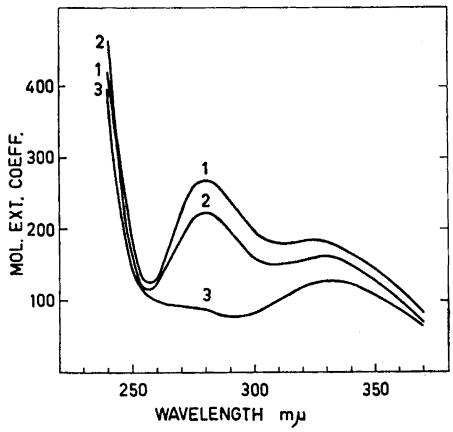


Fig. 1. UV absorption spectra of 1,2-dithiolane-3-carboxylic acid: (1) in 0.6 M HCl (solvent: alcohol); (2) in alcohol; (3) in 0.01 M NaOH (solvent: alcohol).

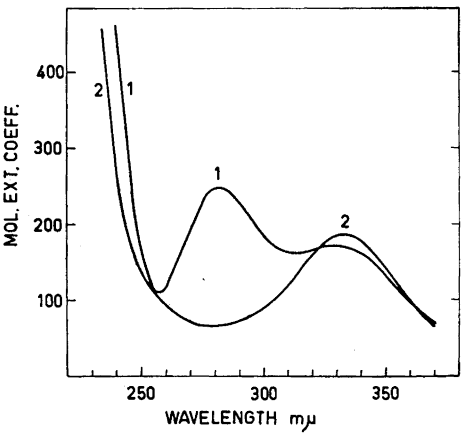


Fig. 2. UV absorption spectra of: (1) 5-methyl-1,2-dithiolane-3-carboxylic acid; (2) 5-methyl-1,2-dithiolane-3-acetic acid. Solvent: alcohol.

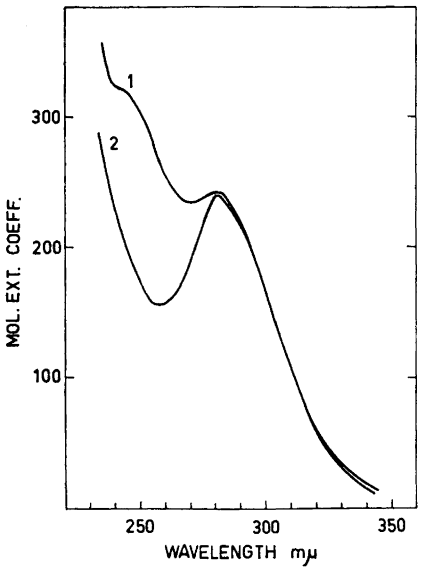


Fig. 3. UV absorption spectra of 1,2-dithiane-3-carboxylic acid: (1) in alcohol; (2) in 0.025 M KOH (solvent: alcohol).

acid (λ_{max} = 250 mμ). The spectrum of 5-methyl-1,2-dithiolane-3-carboxylic acid shows an absorption peak at 283 mμ (Fig. 2). The small bathochromic shift caused by methyl group in the latter acid will be discussed.[10]

In 6,8-thioctic acid the carboxylic group is too far away to have any influence on the -S-S-group, and the side chain therefore acts upon the disulphide group as an alkyl group, λ_{max} = 333 mμ[8]. Even if a carboxylic group is substituted in the β-position, it does not affect the disulphide group seriously. The spectrum of 5-methyl-1,2-dithiolane-3-acetic acid with an absorption maximum at 334 mμ, makes this clear (Fig. 2). The recently prepared 1,2-dithiolane-3-acetic acid also has an absorption maximum at the same wavelengths as 6,8-thioctic acid[11]. Schotte has shown that a carboxylic group in the 4-position, as well as other groups situated there, has no effect on the characteristic absorption peak of the five-membered ring[12,13]; *cf.* also Schotte.[14]

Fredga has prepared 1,2-dithiane-3,6-dicarboxylic acid[15,16], and Schotte has recorded its UV spectrum[9]. The carboxylic group shifts the absorption maximum to shorter wavelengths, but because the disulphide peak is so close to the absorption band at about 200 mμ the spectrum becomes nearly continuous above 200 mμ where the readings were made. 1,2-Dithiane-3-carboxylic acid has now been prepared. As could be expected, the hypsochromic shift of λ_{max} was here smaller. The spectrum is shown in Fig. 3. The inflexion at about 245 mμ corresponds to the 280 mμ maximum of 1,2-dithiolane-3-carboxylic acid, and the peak at 282 mμ corresponds to the absorption at 333 mμ in the dithiolane analogue. On the other hand, 1,2-dithiane-4-carboxylic acid gives nearly the same absorption band as the unsubstituted ring and 1,2-dithiane-3-butyric acid (5,8-thioctic acid)[17].

Figs. 1 and 2 show that the alkali salts of the two acids have absorption curves which are quite different in character from those of the free acids. The peaks attributed to the usual disulphide band have disappeared, but the lower intensity bands at longer wavelengths remain.

Thus it is clear from the spectra that 1,2-dithiolane-3-carboxylic acid and 1,2-dithiane-3-carboxylic acid show two maxima in the actual region for the disulphide absorption, and one of these maxima disappears when the acid is converted to its ionized form. Schotte has found that the absorption curves for 1,2-dithiolane-3,5-dicarboxylic acid and some other acids changed character in alkaline solution[9], but he did not obtain the pronounced maxima seen in Figs. 1 and 3. Such a split of the disulphide absorption band has not been previously reported in the literature. These double absorption bands may be explained by the assumption that the two sulphur atoms in the disulphide group need not be regarded as

Acid	M.p.	*Found* S	*Found* Eq.wt.	*Calc.* S	*Calc.* Eq.wt.
1,2-Dithiolane-3-carboxylic	81—82° *	42.61	150.6	42.69	150.2
5-Methyl-1,2-dithiolane-3-carboxylic	77—79°	39.14	164.7	39.05	164.3
5-Methyl-1,2-dithiolane-3-acetic	83—86.5°	35.67	177.8	35.97	178.3
1,2-Dithiane-3-carboxylic	75—76°	39.09	165.0	39.05	164.3

* This acid polymerizes easily and the polymer melts at about 147°.

a unit. In ultraviolet absorption the exited electron may originate from either of the two sulphur atoms, and in an electrically asymmetrical disulphide there must be two separate exitation energies for electrons from the two different sulphur atoms, resulting in two UV absorption bands.

Experimental. A Beckman model DU photoelectric quartz spectrophotometer equipped with a hydrogen discharge lamp was used for the ultraviolet absorption measurements.

The synthetic methods for the acids (*cf.* p. 1710) will be published later.

I wish to express my gratitude to Professor Arne Fredga for his great interest in this work and for many valuable discussions. I am also indebted to Dr. Lennart Schotte for interesting discussions.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

1. Claeson, G. *Acta Chem. Scand.* **9** (1955) 178.
2. Grisebach, H. *Angew. Chem.* **68** (1956) 554.
3. Reed, L. J. *Advances in Enzymol.* **18** (1957) 319.
4. Bergson, G. and Schotte, L. *Arkiv Kemi* **13** (1958) 43.
5. Thomas, R. C. and Reed, L. J. *J. Am. Chem. Soc.* **78** (1956) 6151.
6. Guirard, B. M., Snell, E. E. and Williams, R. J. *Arch. Biochem.* **9** (1946) 361.
7. Reed, L. J., De Busk, B. G., Johnston, P. M. and Getzendaner, M. E. *J. Biol. Chem.* **192** (1951) 851
8. Barltrop, J. A., Hayes, P. M. and Calvin, M. J. *J. Am. Chem. Soc.* **76** (1954) 4348.
9. Schotte, L. *Arkiv Kemi* **8** (1955) 579.
10. Claeson, G. *To be published.*
11. Wladislaw, B. *Chem. & Ind. London* **9** (1957) 263.
12. Schotte, L. and Ström, H. *Acta Chem. Scand.* **10** (1956) 687.
13. Schotte, L. *Arkiv Kemi* **9** (1955) 309.
14. Schotte, L. *Arkiv Kemi* **9** (1956) 441.
15. Fredga, A. *Ber.* **71** (1938) 289.
16. Fredga, A. *Arkiv Kemi, Mineral. Geol.* **12 A** (1938) No. 27.
17. Claeson, G. and Langsjoen, A. *Acta Chem. Scand.* **13** (1959) 840.

Received August 24, 1959.

On the Effect of Ionizing Radiations on Cyanocobalamin

MONICA SJÖSTEDT and L.-E. ERICSON

Division of Food Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The destruction of cyanocobalamin (vitamin B_{12}) by ionizing radiations has generally been estimated spectrophotometrically or microbiologically [1-4]. Smith [4] mentions in his recent article on the instability of radioactive vitamin B_{12} that absorption spectroscopy gives a moderately accurate measure of the residual vitamin B_{12}. He also states that a microbiological assay can be used as there is no evidence of a significant breakdown to other microbiologically active compounds. In our preliminary studies of the effect of γ-rays on cyanocobalamin we have been unable to correlate the changes in the absorption spectra with the microbiological activity of irradiated solutions of vitamin B_{12}. Moreover, evidence has been obtained for the formation of microbiologically active breakdown products.

Solutions of cyanocobalamin containing 5 mg per ml in a phosphate buffer of pH 5 were irradiated under aerobic conditions with γ-rays from a ^{60}Co source. The doses given were 2, 5 and 10 Mrad. The spectra of the irradiated and nonirradiated solutions of vitamin B_{12} were measured in a Beckman Model DU spectrophotometer. A tube assay with *Escherichia coli* 113—3 [5] was used for the microbiological tests. The assay media were those of Diding [6] and of Burkholder [7], to which 1.5 mg of KCN per l had been added. Growth was measured turbidimetrically after incubation of the tubes for 20 h at 37°C.

The spectra of the non-irradiated and of the irradiated solutions of cyanocobalamin are shown in Fig. 1. Concomitant with the decrease in the absorption maxima following irradiation, there was also a tendency for the peaks in the 360 and 550 mμ regions to shift towards lower wavelengths. Calculation of the degree of destruction by comparing the absorption at 361 mμ of irradiated and non-irradiated solutions indicated that 60 % of the vitamin originally present had been destroyed by a dose of 2 Mrad. According to the microbiological assay approximately 85 % was inactivated by this dose.

a unit. In ultraviolet absorption the exited electron may originate from either of the two sulphur atoms, and in an electrically asymmetrical disulphide there must be two separate exitation energies for electrons from the two different sulphur atoms, resulting in two UV absorption bands.

Experimental. A Beckman model DU photoelectric quartz spectrophotometer equipped with a hydrogen discharge lamp was used for the ultraviolet absorption measurements.

The synthetic methods for the acids (*cf.* p. 1710) will be published later.

I wish to express my gratitude to Professor Arne Fredga for his great interest in this work and for many valuable discussions. I am also indebted to Dr. Lennart Schotte for interesting discussions.

A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

1. Claeson, G. *Acta Chem. Scand.* **9** (1955) 178.
2. Grisebach, H. *Angew. Chem.* **68** (1956) 554.
3. Reed, L. J. *Advances in Enzymol.* **18** (1957) 319.
4. Bergson, G. and Schotte, L. *Arkiv Kemi* **13** (1958) 43.
5. Thomas, R. C. and Reed, L. J. *J. Am. Chem. Soc.* **78** (1956) 6151.
6. Guirard, B. M., Snell, E. E. and Williams, R. J. *Arch. Biochem.* **9** (1946) 361.
7. Reed, L. J., De Busk, B. G., Johnston, P. M. and Getzendaner, M. E. *J. Biol. Chem.* **192** (1951) 851
8. Barltrop, J. A., Hayes, P. M. and Calvin, M. J. *J. Am. Chem. Soc.* **76** (1954) 4348.
9. Schotte, L. *Arkiv Kemi* **8** (1955) 579.
10. Claeson, G. *To be published.*
11. Wladislaw, B. *Chem. & Ind. London* **9** (1957) 263.
12. Schotte, L. and Ström, H. *Acta Chem. Scand.* **10** (1956) 687.
13. Schotte, L. *Arkiv Kemi* **9** (1955) 309.
14. Schotte, L. *Arkiv Kemi* **9** (1956) 441.
15. Fredga, A. *Ber.* **71** (1938) 289.
16. Fredga, A. *Arkiv Kemi, Mineral. Geol.* **12 A** (1938) No. 27.
17. Claeson, G. and Langsjoen, A. *Acta Chem. Scand.* **13** (1959) 840.

Received August 24, 1959.

On the Effect of Ionizing Radiations on Cyanocobalamin

MONICA SJÖSTEDT and L.-E. ERICSON

Division of Food Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The destruction of cyanocobalamin (vitamin B_{12}) by ionizing radiations has generally been estimated spectrophotometrically or microbiologically [1-4]. Smith [4] mentions in his recent article on the instability of radioactive vitamin B_{12} that absorption spectroscopy gives a moderately accurate measure of the residual vitamin B_{12}. He also states that a microbiological assay can be used as there is no evidence of a significant breakdown to other microbiologically active compounds. In our preliminary studies of the effect of γ-rays on cyanocobalamin we have been unable to correlate the changes in the absorption spectra with the microbiological activity of irradiated solutions of vitamin B_{12}. Moreover, evidence has been obtained for the formation of microbiologically active breakdown products.

Solutions of cyanocobalamin containing 5 mg per ml in a phosphate buffer of pH 5 were irradiated under aerobic conditions with γ-rays from a ^{60}Co source. The doses given were 2, 5 and 10 Mrad. The spectra of the irradiated and nonirradiated solutions of vitamin B_{12} were measured in a Beckman Model DU spectrophotometer. A tube assay with *Escherichia coli* 113—3 [5] was used for the microbiological tests. The assay media were those of Diding [6] and of Burkholder [7], to which 1.5 mg of KCN per l had been added. Growth was measured turbidimetrically after incubation of the tubes for 20 h at 37°C.

The spectra of the non-irradiated and of the irradiated solutions of cyanocobalamin are shown in Fig. 1. Concomitant with the decrease in the absorption maxima following irradiation, there was also a tendency for the peaks in the 360 and 550 mμ regions to shift towards lower wavelengths. Calculation of the degree of destruction by comparing the absorption at 361 mμ of irradiated and non-irradiated solutions indicated that 60 % of the vitamin originally present had been destroyed by a dose of 2 Mrad. According to the microbiological assay approximately 85 % was inactivated by this dose.

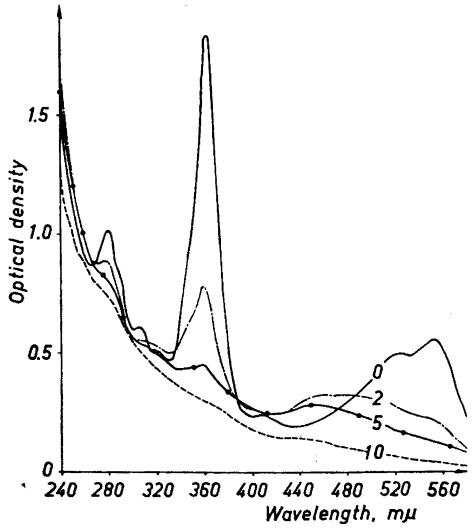


Fig. 1. Absorption spectra for solutions of non-irradiated cyanocobalamin (0), and for solutions irradiated with 2, 5 and 10 Mrad.

For the microbiological assays, series of dilutions were made of the irradiated cyanocobalamin solutions. In Fig. 2 the turbidity (optical density) of the tubes to which the solutions of irradiated vitamin B_{12} were added has been plotted against the degree of dilution. In the same figure

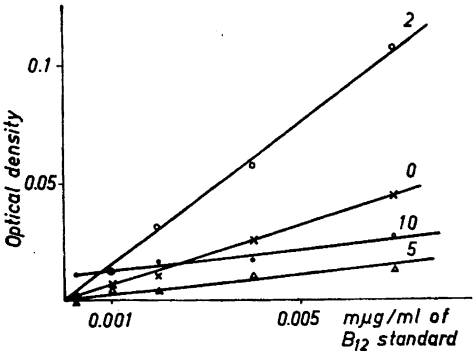


Fig. 2. The response of *E. coli* 113—3 to varying concentrations of non-irradiated cyanocobalamin (0) and to dilutions of cyanocobalamin solutions irradiated with 2, 5 and 10 Mrad.

the turbidity of the B_{12}-standards has been plotted against the concentration of the vitamin in mμg per ml. The slope of the line of the B_{12}-solution irradiated with 2 Mrad was twice as steep as that of the standard curve. The solution irradiated with 10 Mrad, however, gave a line with a slope that was less than that of the non-irradiated cyanocobalamin. As the retention of the vitamin was very low after irradiation with 10 Mrad, the latter line was obtained by adding increasing amounts of non-irradiated cyanocobalamin to the irradiated solution diluted $1:3.5 \times 10^6$. Vitamin B_{12}-solutions that had received a dose of 5 Mrad gave varying results but the dose-response line had in most cases (as in Fig. 2) a slope that was less than that for the non-irradiated vitamin. The same results were obtained irrespective of whether the simple medium of Diding or the more complex medium of Burkholder was used. If cyanocobalamin were the only microbiologically active compound remaining after irradiation one would expect the slopes of the lines to be identical.

The factor(s) responsible for the increased relative activity of the sample that had received a dose of 2 Mrad appears to be more heat labile than vitamin B_{12}.

Paper electrophoresis has revealed the presence of at least four compounds in the irradiated solutions which can support the growth of *E. coli* 113-3.

The authors would like to express their sincere thanks to Drs. P. Vidal and L. Brunelet, Conservatome, Lyon, France, for the irradiations of the solutions of cyanocobalamin. The financial support of the *Swedish Atomic Energy Commission*, the *Swedish Technical Research Council* and the *Swedish Agricultural Research Council* is also acknowledged.

1. Markakis, P. C., Goldblith, S. A. and Proctor, B. E. *Nucleonics* **9** (1951) 71.
2. Swallow, A. J. *Symposium on Processing of Foods with Ionizing Radiations*, Cambridge 1957, p. 16.
3. Horne, T. *Proc. Second Intern. Conf. Peaceful Uses Atomic Energy*, Geneva 1958, P/276.
4. Smith, E. Lester, *Lancet* **76** (1959) 387.
5. Davis, B. D. and Mingioli, E. S. *J. Bacteriol.* **60** (1950) 17.
6. Diding, N. *Scand. J. Clin. Lab. Invest.* **3** (1951) 215.
7. Burkholder, P. R. *Science* **114** (1951) 459.

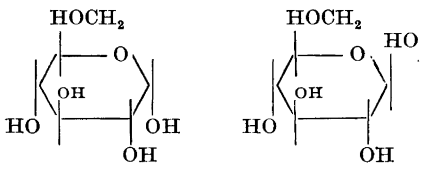
Received September 25, 1959.

The Structure of α- and β-Glucose

ANDERS KJÆR
Den kgl. Veterinær- og Landbohøjskoles organisk-kemiske laboratorium, Copenhagen, Denmark

BENGT LINDBERG
Institutionen för träkemi, Kungl. Tekniska Högskolan, and Träkemiska avdelningen, Svenska Träforskningsinstitutet, Stockholm, Sweden

The structures (I) and (II) are generally accepted for α- and β-D-glucose, respectively, on basis of chemical evidence (*cf. e.g.* Ref.[1]). In the energetically favoured chair form the hydroxyl group at $C_{(1)}$ assumes an axial orientation in (I), and an equatorial position in (II). This picture has been conclusively confirmed by complete X-ray structure determination of α-D-glucose [2]. A similar approach places the generally accepted structure of sucrose, representative of the α-D-glucopyranosides, beyond dispute [3]. Furthermore, the studies by Lemieux *et al.*[4] of proton magnetic resonance spectra of various pyranosides are entirely consistent with the commonly accepted structures.



I II

It hence appears surprising that Blom, in a recent communication to this journal [5], maintains the opposite view, that in the stable chair conformation the anomeric hydroxyl group assumes an equatorial orientation in α-D-glucose and α-D-galactose, and axial positions in the β-anomers. Without invalidating the evidence for the generally accepted structures, Blom presents four arguments in support of his revised formulation with its far-reaching consequences.

It is the purpose of the present communication to redress these arguments as either irrelevant or erroneous.

1. *Melting points.* Regarding a possible relationship between melting points and molecular structures in the penta- and hexa-substituted *cyclo*hexane series, Blom [5] states: "eine Verbindung mit einer höheren Anzahl von *para-trans*-Substituenten hat einen höheren Schmelzpunkt als die isomere Verbindung mit einer geringeren Anzahl".* This is definitely not the case. For example, *cis*-inositol, the sole stereoisomeride with no 1,4-*trans*-pair, melts higher (390° (corr.)) than any other inositol [6].

Blom extends the above "rule" to include the tetrahydropyran ring system and concludes, on basis of m. p. data for anomeric methyl and ethyl pyranosides of D-glucose and D-galactose, that the α-anomers contain an equatorial and the β-anomers an axial hydroxyl-group at $C_{(1)}$, contrary to the generally accepted structures. Unfortunately, crystalline preparations of both anomers of the methyl (and ethyl) D-aldohexopyranosides have, thus far, been recorded for only three (glucose, galactose and mannose) of the eight isomeric D-aldohexoses. However, closely analogous examples may serve to discredit Blom's suggestions. Thus, methyl β-D-xylopyranoside melts at 156—157°, the α-anomer at 91—92°, whereas the closely related methyl α-D-glucopyranoside melts at 166°, *i. e.* higher than the β-anomer (m. p. 105°).

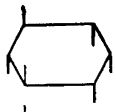
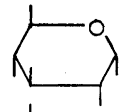
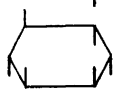
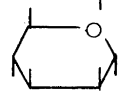
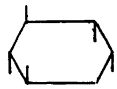
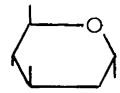
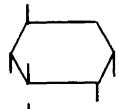
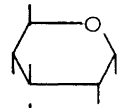
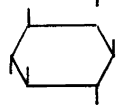
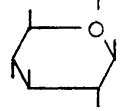
Melting points are of course dependent upon crystal properties which are again complicated functions of molecular structure. Hence, the utmost caution is urged in all attempts to attribute structural significance to melting point data, not least where strongly hydrogen-bonded molecules are involved as in the present case.

2. *Molecular rotations.* As support for his structural revision, Blom [5] quotes certain observed regularities between the molecular rotations of various cyclitols and formally similar glycosides. For the sake of clarity, some of Blom's examples are presented in Table 1, together with additional data pertinent for the following discussion.

On basis of the molecular rotations of the first three pairs of compounds, Blom concludes that introduction at $C_{(2)}$, in a $C_{(2)}$-

* This "rule" is presented as an extension of melting point regularities within the series of acyclic, unsaturated *cis-trans*-isomerides. It should be pointed out, however, that several examples are known of *cis*-compounds melting higher than the corresponding *trans*-isomerides within this class of compounds.

Table 1[a].

Cyclitol	Structure	Classification of substituents	M_D	Methyl Glycoside	Structure	Classification of substituents	M_D
Myo-inositol		*aeeeee*	0	α-D-Gluco-side		*aeeee*0	+309
L-Inositol		*aaeeee*	−117	α-D-Manno-side		*aaeee*0	+154
Myo-2-deoxy-inositol		*aCeeee*	−81	α-2-Deoxy-D-glucoside		*aCeee*O	+246
Myo-6-deoxy-inositol [b]		*aeeee*C	+81	α-D-Gluco-side		*aeeee*O	+309
Deoxy-scyllitol		*eeeee*C	0	β-D-Gluco-side		*eeeee*O	− 66

[a] Blom's nomenclature is employed but the generally accepted structures for the α- and β-glycosides are retained. In the schematic formulae no distinction is made between OH-, CH_2OH- and OCH_3-groupings.

[b] This compound is the antipode of myo-2-deoxy-inositol.

deoxy ring compound, of an equatorial hydroxyl group results in a shift to more positive values, whereas introduction of an axial substituent in the same position is accompanied by a change in rotation to more negative values. This rule is then extended to the anomeric $C_{(1)}$-carbon and used as an argument for reversing the generally accepted formulae for α- and β-glucose.

It should be borne in mind, however, that contributions from individual asymmetric centers to the total optical rotations are not additive. Accordingly, the $C_{(2)}$-rule may very well fail when the configuration of one of the asymmetric carbon atoms is changed. Extrapolation of the rule to include $C_{(1)}$-substituents is definitely impermissible. For example, the two last examples in Table 1 indicate that, by analogous reasoning, axial substitution at $C_{(1)}$ results in more positive rotations than introduction of the same substituent in an equatorial orientation, opposite to Blom's claim. It should be emphasized, however, that the last example is not regarded as support for the accepted structures of anomeric D-glucoses, but merely as a means of disproving the validity of Blom's argumentation.

3. *Oxidation.* It is generally agreed that, within the cyclitol series, axially located hydroxyl groups are preferentially oxidized. Again, it appears a well-established fact that β-glucose is oxidized at a higher rate than the anomeric α-glucose. Blom [5] seeks support for the proposed structural revision in these facts, apparently disregarding the semiacetalic character of the anomeric hydroxyl group in the pyranose system which may conceivably render the validity of such comparions more than questionable. Thus, both anomers of galactose, possessing an axial group at $C_{(4)}$, are oxidized more readily at $C_{(1)}$ than at $C_{(4)}$. A completely satisfactory interpretation of the different oxidation rates of pyranose anomers, as well as the structur-

ally analogous cyclitols, has yet to be given, but there is evidence to indicate that different reaction mechanisms may be involved in the two ring systems (*cf. e. g.* Ref.[7]).

The frequently observed enzymic catalysis of reactions different from those favoured in non-enzymic systems renders Blom's reference to the biological glucose oxidation irrelevant in the present context.

4. *1,2-Anhydro-sugars.* Blom's argumentation on basis of the ring-opening reactions of Briegl's anhydride reflects a fatal negligence of the current, well-established stereochemical course of such reactions (*cf. e. g.* Ref.[8]) and, therefore, requires no further discussion here. In fact, the conversion of such epoxides into glycosides affords about the best chemical evidence for the correctness of the generally accepted structures.

In conclusion, the character and validity of the argumentation by Blom, balanced against the bulk of evidence in favour of the commonly accepted structures for α- and β-glucose, is insufficient to warrant any revision of the latter.

1. Pigman, W. *The Carbohydrates*, Academic Press Inc., New York 1957.
2. McDonald, T. R. R. and Beevers, C. A. *Acta Cryst.* **5** (1952) 654.
3. Beevers, C. A., McDonald, T. R. R., Robertson, J. H. and Stern, F. *Acta Cryst.* **5** (1952) 689.
4. Lemieux, R. U., Kullnig, R. K., Bernstein, H. J. and Schneider, W. G. *J. Am. Chem. Soc.* **79** (1957) 1005; **80** (1958) 6098.
5. Blom, J. *Acta Chem. Scand.* **13** (1959) 388.
6. Angyal, S. I. and McHugh, D. I. *J. Chem. Soc.* **1957** 3682.
7. Bentley, R. *J. Am. Chem. Soc.* **79** (1957) 1720.
8. Newth, F. H. *Quart. Revs. (London)* **13** (1959) 30.

Received September 24, 1959.

The Synthesis of 4,4,7,7-Tetrabenzyl-1,2,3,5,6-pentathiepane

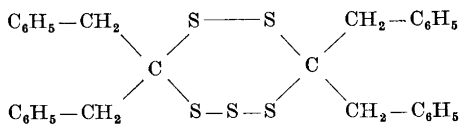
BENGT MAGNUSSON

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In connection with work on duplodithioketones [1,2], attempts have been made to prepare these by oxidation of *gem.*dithiols. These attempts were not successful. Methanedithiol gave only polymers even when the oxidation was carried out with air in high dilution at low temperature and in the presence of ferric chloride [3].

The products obtained by oxidation of *gem.*dithiols with iodine do not seem to be duplodithioketones [4].

The synthesis of a crystalline *gem.*dithiol, 1,3-diphenyl-2,2-dimercaptopropane, has recently been reported [5]. Attempts to oxidize it to a duplodithioketone was carried out by the autor, but the appropriate conditions have not yet been found. When the dithiol was treated with ammonium-polysulphide, 4,4,7,7-tetrabenzyl-1,2,3,5,6-pentathiepane (I) was obtained in 49 % yield. m.p. 130–131.5°C. (Found: C 65.52, 66.01; H 5.12, 4,96; S 29.09, 29.08; Mol.wt. 542, 550. Calc. for $C_{30}H_{28}S_5$: C 65.65; H 5.14; S 29.21; Mol.wt. 548.8.)



(I)

Further investigations of this reaction are in progress.

1. Fredga, A. *Acta Chem. Scand.* **12** (1958) 891.
2. Magnusson, B. *Acta Chem. Scand.* **13** (1959) 1031.
3. Magnusson, B. *To be published.*
4. Cairns, T. L., Evans, G. L., Larchar, A. W. and McKusick, B. C. *J. Am. Chem. Soc.* **74** (1952) 3982.
5. Berchold, G. A., Edwards, B. E., Campaigne, E. and Carmack, M. *J. Am. Chem. Soc.* **81** (1959) 3148.

Received September 28, 1959.

ally analogous cyclitols, has yet to be given, but there is evidence to indicate that different reaction mechanisms may be involved in the two ring systems (*cf. e. g.* Ref.[7]).

The frequently observed enzymic catalysis of reactions different from those favoured in non-enzymic systems renders Blom's reference to the biological glucose oxidation irrelevant in the present context.

4. *1,2-Anhydro-sugars.* Blom's argumentation on basis of the ring-opening reactions of Briegl's anhydride reflects a fatal negligence of the current, well-established stereochemical course of such reactions (*cf. e. g.* Ref.[8]) and, therefore, requires no further discussion here. In fact, the conversion of such epoxides into glycosides affords about the best chemical evidence for the correctness of the generally accepted structures.

In conclusion, the character and validity of the argumentation by Blom, balanced against the bulk of evidence in favour of the commonly accepted structures for α- and β-glucose, is insufficient to warrant any revision of the latter.

1. Pigman, W. *The Carbohydrates*, Academic Press Inc., New York 1957.
2. McDonald, T. R. R. and Beevers, C. A. *Acta Cryst.* **5** (1952) 654.
3. Beevers, C. A., McDonald, T. R. R., Robertson, J. H. and Stern, F. *Acta Cryst.* **5** (1952) 689.
4. Lemieux, R. U., Kullnig, R. K., Bernstein, H. J. and Schneider, W. G. *J. Am. Chem. Soc.* **79** (1957) 1005; **80** (1958) 6098.
5. Blom, J. *Acta Chem. Scand.* **13** (1959) 388.
6. Angyal, S. I. and McHugh, D. I. *J. Chem. Soc.* **1957** 3682.
7. Bentley, R. *J. Am. Chem. Soc.* **79** (1957) 1720.
8. Newth, F. H. *Quart. Revs. (London)* **13** (1959) 30.

Received September 24, 1959.

The Synthesis of 4,4,7,7-Tetrabenzyl-1,2,3,5,6-pentathiepane

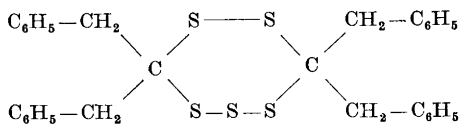
BENGT MAGNUSSON

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In connection with work on duplodithioketones [1,2], attempts have been made to prepare these by oxidation of *gem.*dithiols. These attempts were not successful. Methanedithiol gave only polymers even when the oxidation was carried out with air in high dilution at low temperature and in the presence of ferric chloride [3].

The products obtained by oxidation of *gem.*dithiols with iodine do not seem to be duplodithioketones [4].

The synthesis of a crystalline *gem.*dithiol, 1,3-diphenyl-2,2-dimercaptopropane, has recently been reported [5]. Attempts to oxidize it to a duplodithioketone was carried out by the autor, but the appropriate conditions have not yet been found. When the dithiol was treated with ammonium-polysulphide, 4,4,7,7-tetrabenzyl-1,2,3,5,6-pentathiepane (I) was obtained in 49 % yield. m.p. 130–131.5°C. (Found: C 65.52, 66.01; H 5.12, 4,96; S 29.09, 29.08; Mol.wt. 542, 550. Calc. for $C_{30}H_{28}S_5$: C 65.65; H 5.14; S 29.21; Mol.wt. 548.8.)



(I)

Further investigations of this reaction are in progress.

1. Fredga, A. *Acta Chem. Scand.* **12** (1958) 891.
2. Magnusson, B. *Acta Chem. Scand.* **13** (1959) 1031.
3. Magnusson, B. *To be published.*
4. Cairns, T. L., Evans, G. L., Larchar, A. W. and McKusick, B. C. *J. Am. Chem. Soc.* **74** (1952) 3982.
5. Berchold, G. A., Edwards, B. E., Campaigne, E. and Carmack, M. *J. Am. Chem. Soc.* **81** (1959) 3148.

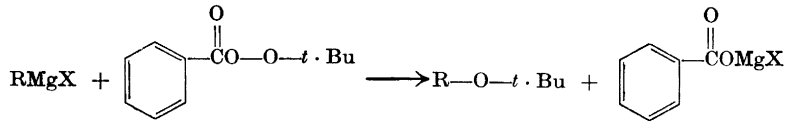
Received September 28, 1959.

Studies of Peroxycompounds II. * The Preparation of Diethyl-*t*-butoxy-malonate and Ethyl-*t*-butoxy-acetoacetate

SVEN-OLOV LAWESSON and CURT BERGLUND

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In the first paper of this series [1] a new method of preparing *t*-butylethers was described:



(1)

The reaction is very smooth and gives excellent yields in general, and seems further, at least in the laboratory scale, to be the best method to prepare aromatic *t*-butylethers.

It was then felt that a perester might react with sodium compounds of diethylmalonate or ethyl acetoacetate for instance. Earlier, Milas *et al.*[2] reacted *t*-butylperbenzoate with sodium methoxide in excess of methanol and obtained methylbenzoate and the sodium salt of *t*-butylhydroperoxide. In analogy with eqn. 1. the —O—O- bond could be split by certain types of organosodium compounds in inert solvents and an ether group introduced. We have now managed to introduce the *t*-butoxygroup into diethyl malonate and ethyl acetoacetate *via* the corresponding sodium compound. The perester used was *t*-butylperacetate, as the perbenzoate was difficult to separate from the new alkoxy-compound.

Experimentally the sodium compound was made in benzene from sodium hydride and the corresponding ester and to the preheated mixture *t*-butylperacetate was added dropwise. The reaction was exothermic but after the heat evolution ceased, the reaction flask was heated for some time. Diethyl-*t*-butoxy-malonate was isolated in a yield of 27 % (C 56.93; H 8.78. Calc. C 56.88; H 8.68) and the corresponding barbituric acid, m. p. 187—189°C, was also prepared (C 47,96; H 6.04. Calc. C 47,99; H 6.04). The sedative effect of the barbiturate was tested and found to be negligible. Compare the similar neopentylbarbituric acid prepared by Brändström [3]. Ethyl-*t*-butoxy-acetoacetate (C 59.47; H 8.85. Calc. C 59.38; H 8.97) was prepared in a similar way in a yield of 31 %.

Further research is planned in this field especially with peresters derived from stronger acids. Acknowledgement is expressed to *The Swedish Natural Science Research Council* for a grant and to Dr. R. Dahlbom *(AB Astra)* for the physiological test.

1. Lawesson, S.-O. and Yang, N. C. *J. Am. Chem. Soc.* **81** (1959) 4230.
2. Milas, N. A. and Surgenor, D. M. *J. Am. Chem. Soc.* **68** (1946) 642.
3. Brändström, A. *Acta Chem. Scand.* **13** (1959) 615.

Received September 28, 1959.

* Ref.[1] is considered as Part I of this series.

Studies of Peroxycompounds III. The Introduction of the Benzoyloxygroup into Diethylmalonate

SVEN-OLOV LAWESSON and TAMARA BUSCH

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In the reaction between *t*-butylperbenzoate and a Grignard reagent the O—O-bond is easily split and the corresponding *t*-butylether is formed [1]. Also in the case of benzoylperoxide [1] the O—O-bond is first attacked by a Grignard reagent but here the mechanism is probably not yet quite understood. When a *t*-butyl perester was treated with the sodium compound of diethylmalonate or ethyl acetoacetate [2] a very sluggish reaction took place and the *t*-butoxygroup was introduced into the corresponding esters, although in low yields. It was then quite natural to investigate the reaction between benzoyl-

peroxide and sodium salts of malonic ester derivatives. In that case it was found that in inert solvents the O—O-bond was split with ease and the benzoyloxy group introduced into the malonic ester derivatives in good yields. The new products were then also characterized by hydrolyzing them to tartronic acids.

As a full paper will soon be submitted for publication in this Journal we include here only one experiment. Additional work is under way to test more fully this reaction especially with derivatives of ethyl cyanoacetate [3], malononitrile [3], β-diketones [4], β-ketoesters [5] and other active methylene compounds.

Experimental. The sodium compound of diethyl ethylmalonate was prepared from 2.4 g of NaH and 18.8 g of diethyl ethylmalonate in 150 ml of dry benzene. Then 18 g of benzoylperoxide in 150—200 ml of dry benzene was added to the sodium compound, cooled in an ice-water bath. After standing over night the reaction mixture was worked up in the usual way. Distillation gave diethyl O-benzoylethyltartronate with b. p. 137°/0.15 mm Hg. Yield 75 % n_D^{20} 1.4890. (Found: C 62.23; H 6.50. Calc for $C_{16}H_{20}O_6$: C 62.32; H 6.54.)

The investigation has been partly supported by a grant from *The Swedish Natural Science Research Council*, which is gratefully acknowledged.

1. Lawesson, S.-O. and Yang, N. C. *J. Am. Chem. Soc.* **81** (1959) 4230.
2. Lawesson, S.-O. and Berglund, C. *Acta Chem. Scand.* **13** (1959) 1716.
3. Lawesson, S.-O. and Frisell, C. *Unpublished work.*
4. Lawesson, S.-O. and Taipale, J. *Unpublished work.*
5. Lawesson, S.-O. and Andersson, M. *Unpublished work.*

Received September 28, 1959.

Tetrahydrofuran as a New Solvent in Condensation Reactions

SVEN-OLOV LAWESSON and TAMARA BUSCH

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

Inspired by the work of H. Normant *et al.*[1,2] who succeded in preparing Grignard reagents from aryl chlorides and vinyl halides by running the reaction in tetrahydrofuran (THF) we have just began to study the usefulness of this solvent in certain condensation reactions. We now want to report very shortly some of our preliminary results. A full paper will be published at a later date.

It is known that potassium or sodium salts (enolates) of active methylene compounds in general are insoluble in such inert solvents as benzene, toluene, xylene, *etc.* Under these conditions further reactions are often very sluggish because of the inhomogeneous system. As magnesium ethoxide is soluble in inert solvents Lund's well-known method [3] offers some advantage in this case. An excellent review [4] has recently been published on this subject concerning these matters.

We have found that diethyl malonate reacts very vigorously with sodium hydride in THF and the corresponding sodium compound formed is completely soluble. Reactive halogen compounds as benzoylchloride, acetylchloride and ethylchloroformate react instantaneously and give diethyl benzoylmalonate, diethyl acetylmalonate and tricarbetoxymethane, respectively, in excellent yields. In the same way ethyl acetoacetate whose sodium compound is also soluble in THF, gives ethyl 2-benzoylacetoacetate and diethyl acetylsuccinate when treated with benzoylchloride and ethyl bromoacetate, respectively. The yields were in all cases higher than 70 % although we have not attempted to work out the conditions for maximum yields.

Experimental. All experiments were carried out in nitrogen atmosphere and in the usual type of apparatus. Preparation of diethyl benzoylmalonate may be taken as a typical example.

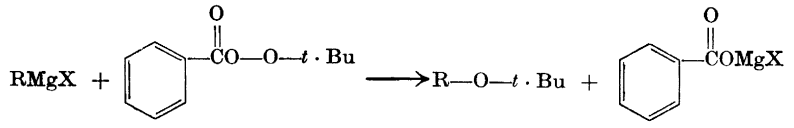
To sodium hydride (0.2 mole) covered with 200 ml of THF, was added 0.21 mole of diethylmalonate. In less than one hour the evolution of hydrogen had ceased and a clear solution was formed. The flask was cooled in ice-water and then 0.2 mole of redistilled benzoyl chloride was added all at once. Stirring was continued for another 5—10 min and then the mixture was poured into water and acidified. The aqueous solution was separated and extracted twice with ether. The organic layers were combined and washed with sodium bicarbonate solution and then with water, and finally dried over sodium sulphate. Distillation gave the main fraction at a b.p. of 137°C/0.5 mm Hg. Yield 72 %.

Studies of Peroxycompounds II. * The Preparation of Diethyl-*t*-butoxy-malonate and Ethyl-*t*-butoxy-acetoacetate

SVEN-OLOV LAWESSON and CURT BERGLUND

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In the first paper of this series [1] a new method of preparing *t*-butylethers was described:



(1)

The reaction is very smooth and gives excellent yields in general, and seems further, at least in the laboratory scale, to be the best method to prepare aromatic *t*-butylethers.

It was then felt that a perester might react with sodium compounds of diethylmalonate or ethyl acetoacetate for instance. Earlier, Milas *et al.*[2] reacted *t*-butylperbenzoate with sodium methoxide in excess of methanol and obtained methylbenzoate and the sodium salt of *t*-butylhydroperoxide. In analogy with eqn. 1. the —O—O- bond could be split by certain types of organosodium compounds in inert solvents and an ether group introduced. We have now managed to introduce the *t*-butoxygroup into diethyl malonate and ethyl acetoacetate *via* the corresponding sodium compound. The perester used was *t*-butylperacetate, as the perbenzoate was difficult to separate from the new alkoxy-compound.

Experimentally the sodium compound was made in benzene from sodium hydride and the corresponding ester and to the preheated mixture *t*-butylperacetate was added dropwise. The reaction was exothermic but after the heat evolution ceased, the reaction flask was heated for some time. Diethyl-*t*-butoxy-malonate was isolated in a yield of 27 % (C 56.93; H 8.78. Calc. C 56.88; H 8.68) and the corresponding barbituric acid, m. p. 187—189°C, was also prepared (C 47,96; H 6.04. Calc. C 47,99; H 6.04). The sedative effect of the barbiturate was tested and found to be negligible. Compare the similar neopentylbarbituric acid prepared by Brändström [3]. Ethyl-*t*-butoxy-acetoacetate (C 59.47; H 8.85. Calc. C 59.38; H 8.97) was prepared in a similar way in a yield of 31 %.

Further research is planned in this field especially with peresters derived from stronger acids. Acknowledgement is expressed to *The Swedish Natural Science Research Council* for a grant and to Dr. R. Dahlbom *(AB Astra)* for the physiological test.

1. Lawesson, S.-O. and Yang, N. C. *J. Am. Chem. Soc.* **81** (1959) 4230.
2. Milas, N. A. and Surgenor, D. M. *J. Am. Chem. Soc.* **68** (1946) 642.
3. Brändström, A. *Acta Chem. Scand.* **13** (1959) 615.

Received September 28, 1959.

* Ref.[1] is considered as Part I of this series.

Studies of Peroxycompounds III. The Introduction of the Benzoyloxygroup into Diethylmalonate

SVEN-OLOV LAWESSON and TAMARA BUSCH

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

In the reaction between *t*-butylperbenzoate and a Grignard reagent the O—O-bond is easily split and the corresponding *t*-butylether is formed [1]. Also in the case of benzoylperoxide [1] the O—O-bond is first attacked by a Grignard reagent but here the mechanism is probably not yet quite understood. When a *t*-butyl perester was treated with the sodium compound of diethylmalonate or ethyl acetoacetate [2] a very sluggish reaction took place and the *t*-butoxygroup was introduced into the corresponding esters, although in low yields. It was then quite natural to investigate the reaction between benzoyl-

peroxide and sodium salts of malonic ester derivatives. In that case it was found that in inert solvents the O—O-bond was split with ease and the benzoyloxy group introduced into the malonic ester derivatives in good yields. The new products were then also characterized by hydrolyzing them to tartronic acids.

As a full paper will soon be submitted for publication in this Journal we include here only one experiment. Additional work is under way to test more fully this reaction especially with derivatives of ethyl cyanoacetate [3], malononitrile [3], β-diketones [4], β-ketoesters [5] and other active methylene compounds.

Experimental. The sodium compound of diethyl ethylmalonate was prepared from 2.4 g of NaH and 18.8 g of diethyl ethylmalonate in 150 ml of dry benzene. Then 18 g of benzoylperoxide in 150—200 ml of dry benzene was added to the sodium compound, cooled in an ice-water bath. After standing over night the reaction mixture was worked up in the usual way. Distillation gave diethyl O-benzoylethyltartronate with b. p. 137°/0.15 mm Hg. Yield 75 % n_D^{20} 1.4890. (Found: C 62.23; H 6.50. Calc for $C_{16}H_{20}O_6$: C 62.32; H 6.54.)

The investigation has been partly supported by a grant from *The Swedish Natural Science Research Council*, which is gratefully acknowledged.

1. Lawesson, S.-O. and Yang, N. C. *J. Am. Chem. Soc.* **81** (1959) 4230.
2. Lawesson, S.-O. and Berglund, C. *Acta Chem. Scand.* **13** (1959) 1716.
3. Lawesson, S.-O. and Frisell, C. *Unpublished work.*
4. Lawesson, S.-O. and Taipale, J. *Unpublished work.*
5. Lawesson, S.-O. and Andersson, M. *Unpublished work.*

Received September 28, 1959.

Tetrahydrofuran as a New Solvent in Condensation Reactions

SVEN-OLOV LAWESSON and TAMARA BUSCH

Department of Organic Chemistry, Chemical Institute, University of Uppsala, Sweden

Inspired by the work of H. Normant *et al.*[1,2] who succeded in preparing Grignard reagents from aryl chlorides and vinyl halides by running the reaction in tetrahydrofuran (THF) we have just began to study the usefulness of this solvent in certain condensation reactions. We now want to report very shortly some of our preliminary results. A full paper will be published at a later date.

It is known that potassium or sodium salts (enolates) of active methylene compounds in general are insoluble in such inert solvents as benzene, toluene, xylene, *etc.* Under these conditions further reactions are often very sluggish because of the inhomogeneous system. As magnesium ethoxide is soluble in inert solvents Lund's well-known method [3] offers some advantage in this case. An excellent review [4] has recently been published on this subject concerning these matters.

We have found that diethyl malonate reacts very vigorously with sodium hydride in THF and the corresponding sodium compound formed is completely soluble. Reactive halogen compounds as benzoylchloride, acetylchloride and ethylchloroformate react instantaneously and give diethyl benzoylmalonate, diethyl acetylmalonate and tricarbetoxymethane, respectively, in excellent yields. In the same way ethyl acetoacetate whose sodium compound is also soluble in THF, gives ethyl 2-benzoylacetoacetate and diethyl acetylsuccinate when treated with benzoylchloride and ethyl bromoacetate, respectively. The yields were in all cases higher than 70 % although we have not attempted to work out the conditions for maximum yields.

Experimental. All experiments were carried out in nitrogen atmosphere and in the usual type of apparatus. Preparation of diethyl benzoylmalonate may be taken as a typical example.

To sodium hydride (0.2 mole) covered with 200 ml of THF, was added 0.21 mole of diethylmalonate. In less than one hour the evolution of hydrogen had ceased and a clear solution was formed. The flask was cooled in ice-water and then 0.2 mole of redistilled benzoyl chloride was added all at once. Stirring was continued for another 5—10 min and then the mixture was poured into water and acidified. The aqueous solution was separated and extracted twice with ether. The organic layers were combined and washed with sodium bicarbonate solution and then with water, and finally dried over sodium sulphate. Distillation gave the main fraction at a b.p. of 137°C/0.5 mm Hg. Yield 72 %.

A grant from *The Swedish Natural Science Research Council* is gratefully acknowledged.

1. Normant, H. *Compt. rend.* **239** (1954) 1510.
2. Normant, H. *Bull. soc. chim. France 1957* 728.
3. Lund, H. *Ber.* **67** (1934) 935.
4. Cope, A. C., Holmes, H. L. and House, H. O. in Adams, R. *Organic Reactions, IX*, Chapter 4, John Wiley and Sons, Inc, New York, N. Y. 1957, p. 107.

Received September 28, 1959.

Preparation and Properties of Glucoconringiin, the Precursor of the Thyreostatic 5,5-Dimethyl-2-Oxazolidinethione

ROLF GMELIN and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The mustard oil glucoside glucoconringiin is present according to Kjær *et al.*[1] in several *Cochlearia* spec. which are rather common in the Finnish flora. In the course of investigations with goitrogens in plants it seemed therefore of interest to isolate this glucosidic precursor of the thyreostatic and probably also goitrogenic 5,5-dimethyl-2-oxazolidinethione.

Some years ago Kjær *et al.*[1] and Schultz and Wagner [2] prepared and characterized glucoconringiin as its tetraacetyl derivative from seeds of *Conringia orientalis* (L.) Andrz. and showed that the deacetylated glucoside which they obtained in amorphous form is split by myrosinase into sulphate, glucose and 5,5-dimethyl-2-oxazolidinethione. This heterocyclic compound, isolated and identified by Hopkins [3] twenty years ago from *C. orientalis* seeds, is formed by spontaneous cyclization from an intermediate 2-hydroxy-2-methylpropyl *iso*thiocyanate. Its thyreostatic effect was established by Astwood *et al.*[4] and was found comparable to that of (−)-5-vinyl-2-oxazolidinethione (goitrin).

The purpose of this paper is to report briefly the method for preparation and some properties of crystalline glucoconringiin, which unlike its tetraacetyl derivative has the advantage of being split readily by myrosinase and is therefore more suitable for physiological investigations.

Glucoconringiin was prepared by ion exchange on Dowex 2-X 4 of an extract from seeds of *Conringia orientalis* (L.) Andrz. Upon elution with K_2SO_4 solution it was obtained as a colourless syrup which crystallized upon long standing in the refrigerator. Two recrystallizations from 90 % ethanol yielded pure glucoconringiin as anhydrous potassium salt in white, short needles. Elementary analysis agreed with the composition $C_{11}H_{20}NO_{10}S_2K$. F 168°C (decomp., uncorr.), $[\alpha]_D^{21}$ −10.87° in H_2O). In agreement with earlier results [1,2] the formation of glucose, sulfate and 5,5-dimethyl-2-oxazolidinethione could be observed during enzymatic cleavage. The enzymatic process could be followed by UV spectroscopy: with increasing cleavage the maximum absorbance shifts from 230.5 mμ to 240 mμ (ε = 15 400), the maximum of 5,5-dimethyl-2-oxazolidinethione.

Spectrophotometric assay of the enzymatic cleavage of glucoconringiin: 0.0155 g of glucoconringiin was dissolved in 2 ml of a mixture of equal parts of myrosinase solution and phosphate buffer pH 6.8. In certain intervals 0.1 ml portions were taken off, diluted to 50 ml with water and the UV absorption measured between 220 mμ and 260 mμ against an equal blank dilution of myrosinase solution and phosphate buffer solution.

t min	UV-maximum mμ	Optical density	Mol.* extinction, ε
1	230.5	0.245	6 760
20	233	0.258	7 140
90	235	0.310	8 560
180	237	0.430	11 900
270	238	0.516	14 250
360	239	0.562	15 500

* based on the initial substrate concentration.

By treatment of glucoconringiin with hydrochloric acid hydroxylamine was formed which was detected by the method of Blom [5]. These results indicate that glucoconringiin is of the same structural type as the other known mustard oil glucosides [6], and has the following structure (p. 1719).

85 g of finely ground seeds of *Conringia orientalis* (L.) Andrz. were defatted by petro-

A grant from *The Swedish Natural Science Research Council* is gratefully acknowledged.

1. Normant, H. *Compt. rend.* **239** (1954) 1510.
2. Normant, H. *Bull. soc. chim. France 1957* 728.
3. Lund, H. *Ber.* **67** (1934) 935.
4. Cope, A. C., Holmes, H. L. and House, H. O. in Adams, R. *Organic Reactions, IX*, Chapter 4, John Wiley and Sons, Inc, New York, N. Y. 1957, p. 107.

Received September 28, 1959.

Preparation and Properties of Glucoconringiin, the Precursor of the Thyreostatic 5,5-Dimethyl-2-Oxazolidinethione

ROLF GMELIN and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The mustard oil glucoside glucoconringiin is present according to Kjær *et al.*[1] in several *Cochlearia* spec. which are rather common in the Finnish flora. In the course of investigations with goitrogens in plants it seemed therefore of interest to isolate this glucosidic precursor of the thyreostatic and probably also goitrogenic 5,5-dimethyl-2-oxazolidinethione.

Some years ago Kjær *et al.*[1] and Schultz and Wagner [2] prepared and characterized glucoconringiin as its tetraacetyl derivative from seeds of *Conringia orientalis* (L.) Andrz. and showed that the deacetylated glucoside which they obtained in amorphous form is split by myrosinase into sulphate, glucose and 5,5-dimethyl-2-oxazolidinethione. This heterocyclic compound, isolated and identified by Hopkins [3] twenty years ago from *C. orientalis* seeds, is formed by spontaneous cyclization from an intermediate 2-hydroxy-2-methylpropyl *iso*thiocyanate. Its thyreostatic effect was established by Astwood *et al.*[4] and was found comparable to that of (−)-5-vinyl-2-oxazolidinethione (goitrin).

The purpose of this paper is to report briefly the method for preparation and some properties of crystalline glucoconringiin, which unlike its tetraacetyl derivative has the advantage of being split readily by myrosinase and is therefore more suitable for physiological investigations.

Glucoconringiin was prepared by ion exchange on Dowex 2-X 4 of an extract from seeds of *Conringia orientalis* (L.) Andrz. Upon elution with K_2SO_4 solution it was obtained as a colourless syrup which crystallized upon long standing in the refrigerator. Two recrystallizations from 90 % ethanol yielded pure glucoconringiin as anhydrous potassium salt in white, short needles. Elementary analysis agreed with the composition $C_{11}H_{20}NO_{10}S_2K$. F 168°C (decomp., uncorr.), $[\alpha]_D^{21}$ −10.87° in H_2O). In agreement with earlier results [1,2] the formation of glucose, sulfate and 5,5-dimethyl-2-oxazolidinethione could be observed during enzymatic cleavage. The enzymatic process could be followed by UV spectroscopy: with increasing cleavage the maximum absorbance shifts from 230.5 mμ to 240 mμ (ε = 15 400), the maximum of 5,5-dimethyl-2-oxazolidinethione.

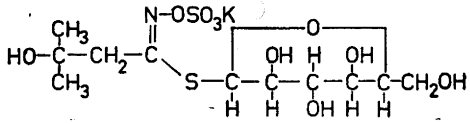
Spectrophotometric assay of the enzymatic cleavage of glucoconringiin: 0.0155 g of glucoconringiin was dissolved in 2 ml of a mixture of equal parts of myrosinase solution and phosphate buffer pH 6.8. In certain intervals 0.1 ml portions were taken off, diluted to 50 ml with water and the UV absorption measured between 220 mμ and 260 mμ against an equal blank dilution of myrosinase solution and phosphate buffer solution.

t min	UV-maximum mμ	Optical density	Mol.* extinction, ε
1	230.5	0.245	6 760
20	233	0.258	7 140
90	235	0.310	8 560
180	237	0.430	11 900
270	238	0.516	14 250
360	239	0.562	15 500

* based on the initial substrate concentration.

By treatment of glucoconringiin with hydrochloric acid hydroxylamine was formed which was detected by the method of Blom [5]. These results indicate that glucoconringiin is of the same structural type as the other known mustard oil glucosides [6], and has the following structure (p. 1719).

85 g of finely ground seeds of *Conringia orientalis* (L.) Andrz. were defatted by petro-



leum ether and extracted with two 1 l portions of 70 % methanol. The combined extracts were concentrated *in vacuo* to 300 ml and treated with a solution of lead acetate in order to precipitate undesired plant material. After separation of Pb-ions by H_2S the straw yellow filtrate was shortly boiled *in vacuo* until all H_2S had disappeared. The solution was filtered through a short column with 5 g of Al_2O_3 Merck and was then passed slowly through a column containing 10 ml Dowex 2-X 4 (chloride-form, 200 mesh). The effluent was free of glucoside and was discarded. After washing the resin with 200 ml of water the glucoside was eluted by 0.1 N K_2SO_4 solution. Fractions of 20 ml each were collected. The fractions 3—18 containing glucoconringiin as determined by the anthrone method and myrosinase test were brought to dryness *in vacuo*. The white residue was triturated with 3 portions of 30 ml CH_3OH at 40—50°C. The filtered methanolic solution left after evaporation *in vacuo* a colourless syrup which was dissolved in a small volume of hot 90 % ethanol. After cooling the glucoside separated partly as a viscous oil which crystallized after standing several weeks in the refrigerator. Two recrystallizations from 90 % ethanol yielded 585 mg glucoconringiin in short, white needles. F 168°C (decomp., uncorr.) $[\alpha]_D^{21}$ −10.87° (c = 3.68; in water). UV-maximum in H_2O 230.5 mμ (ε = 6 720), minimum 208 mμ (ε = 3 750). (Found: C 30.81; H 4.55; N 3.52; S 14.93. Calc. for $C_{11}H_{20}NO_{10}S_2K$ (429.51): C 30.75; H 4.69; N 3.27; S 14.93.)

The identification of the products formed by enzymatic cleavage was performed in the same way as described by Kjær *et al.*[1] for the crude glucoconringiin obtained from tetraacetylglucoconringiin. From 300 ml of glucoconringiin 82 mg of crude 5,5-dimethyl-2-oxazolidinethione were obtained which gave after two recrystallizations from benzene 32 mg of pure 5,5-dimethyl-2-oxazolidinethione, melting at 107°C alone or in mixture with a synthetic sample. Glucose was determined by paper chromatography in three solvent systems; sulfate was determined as $BaSO_4$.

For the detection of hydroxylamine as a product of hydrolysis with strong acids 10 mg of glucoconringiin were dissolved in 1 ml conc. hydrochloric acid and the solution was brought to dryness on the waterbath. The residue was taken up in 2 ml of water. After addition of about 200 mg of Na acetate the solution was further treated according to the procedure described by Blom [5].

This investigation is a part of a research project under U.S. Public Law No. 480, 83rd Congress.

1. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
2. Schultz, O. E. and Wagner, W. *Arch. Pharm.* **289** (1956) 597.
3. Hopkins, C. Y. *Can. J. Res.* **16 B** (1938) 341.
4. Astwood, E. B., Bissel, A. and Hughes, A. M. *Endocrinology* **37** (1945) 456.
5. Blom, I. *Ber.* **59** (1926) 121.
6. Ettlinger, M. G. and Lundeen, A. I. *J. Am. Chem. Soc.* **78** (1956) 4172.

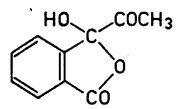
Received September 22, 1959.

2′,5′-Dihydroxyterphenyl-2,2″-dicarboxylic Acid Dilactone

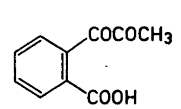
ERLING BERNATEK

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

Ozonolysis of 2-methylnaphthoquinone yields methylphenylglyoxal-*o*-carboxylic acid which has a cyclic lactol structure (I) rather than the free carboxyl structure (II) [1].

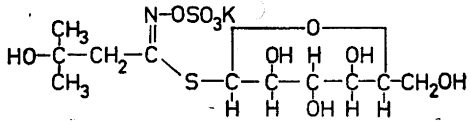


I



II

By briefly heating an alkaline solution of the acid a condensation product $C_{20}H_{10}O_4$ was formed in low yield. This product was sparingly soluble in most organic solvents and the melting point was very high (420—422°) without noticeable decomposition. Dissolution could be effected by ethanolic but not aqueous sodium hydroxide and



leum ether and extracted with two 1 l portions of 70 % methanol. The combined extracts were concentrated *in vacuo* to 300 ml and treated with a solution of lead acetate in order to precipitate undesired plant material. After separation of Pb-ions by H_2S the straw yellow filtrate was shortly boiled *in vacuo* until all H_2S had disappeared. The solution was filtered through a short column with 5 g of Al_2O_3 Merck and was then passed slowly through a column containing 10 ml Dowex 2-X 4 (chloride-form, 200 mesh). The effluent was free of glucoside and was discarded. After washing the resin with 200 ml of water the glucoside was eluted by 0.1 N K_2SO_4 solution. Fractions of 20 ml each were collected. The fractions 3—18 containing glucoconringiin as determined by the anthrone method and myrosinase test were brought to dryness *in vacuo*. The white residue was triturated with 3 portions of 30 ml CH_3OH at 40—50°C. The filtered methanolic solution left after evaporation *in vacuo* a colourless syrup which was dissolved in a small volume of hot 90 % ethanol. After cooling the glucoside separated partly as a viscous oil which crystallized after standing several weeks in the refrigerator. Two recrystallizations from 90 % ethanol yielded 585 mg glucoconringiin in short, white needles. F 168°C (decomp., uncorr.) $[\alpha]_D^{21}$ −10.87° (c = 3.68; in water). UV-maximum in H_2O 230.5 mμ (ε = 6 720), minimum 208 mμ (ε = 3 750). (Found: C 30.81; H 4.55; N 3.52; S 14.93. Calc. for $C_{11}H_{20}NO_{10}S_2K$ (429.51): C 30.75; H 4.69; N 3.27; S 14.93.)

The identification of the products formed by enzymatic cleavage was performed in the same way as described by Kjær *et al.*[1] for the crude glucoconringiin obtained from tetraacetylglucoconringiin. From 300 ml of glucoconringiin 82 mg of crude 5,5-dimethyl-2-oxazolidinethione were obtained which gave after two recrystallizations from benzene 32 mg of pure 5,5-dimethyl-2-oxazolidinethione, melting at 107°C alone or in mixture with a synthetic sample. Glucose was determined by paper chromatography in three solvent systems; sulfate was determined as $BaSO_4$.

For the detection of hydroxylamine as a product of hydrolysis with strong acids 10 mg of glucoconringiin were dissolved in 1 ml conc. hydrochloric acid and the solution was brought to dryness on the waterbath. The residue was taken up in 2 ml of water. After addition of about 200 mg of Na acetate the solution was further treated according to the procedure described by Blom [5].

This investigation is a part of a research project under U.S. Public Law No. 480, 83rd Congress.

1. Kjær, A., Gmelin, R. and Boe Jensen, R. *Acta Chem. Scand.* **10** (1956) 432.
2. Schultz, O. E. and Wagner, W. *Arch. Pharm.* **289** (1956) 597.
3. Hopkins, C. Y. *Can. J. Res.* **16 B** (1938) 341.
4. Astwood, E. B., Bissel, A. and Hughes, A. M. *Endocrinology* **37** (1945) 456.
5. Blom, I. *Ber.* **59** (1926) 121.
6. Ettlinger, M. G. and Lundeen, A. I. *J. Am. Chem. Soc.* **78** (1956) 4172.

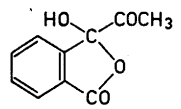
Received September 22, 1959.

2′,5′-Dihydroxyterphenyl-2,2″-dicarboxylic Acid Dilactone

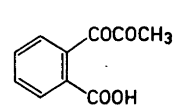
ERLING BERNATEK

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

Ozonolysis of 2-methylnaphthoquinone yields methylphenylglyoxal-*o*-carboxylic acid which has a cyclic lactol structure (I) rather than the free carboxyl structure (II) [1].



I

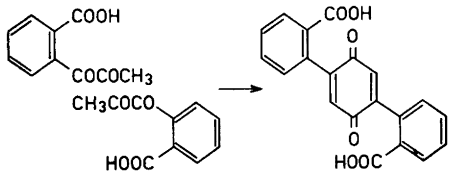


II

By briefly heating an alkaline solution of the acid a condensation product $C_{20}H_{10}O_4$ was formed in low yield. This product was sparingly soluble in most organic solvents and the melting point was very high (420—422°) without noticeable decomposition. Dissolution could be effected by ethanolic but not aqueous sodium hydroxide and

$C_{20}H_{10}O_4$ was precipitated unchanged after acidification. In concentrated sulphuric acid the substance dissolved easily with an intense light blue fluorescence.

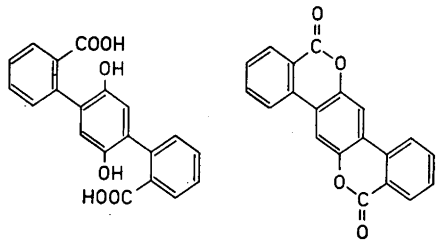
It is believed that $C_{20}H_{10}O_4$ is formed by condensation of two molecules of methylphenylglyoxal-*o*-carboxylic acid in the following way (writing the reactant in the open form for the sake of simplicity):



III

This is in close analogy to the condensation of methylphenylglyoxal in alkaline medium to 2,5-diphenylbenzoquinone [2].

In the hot alkaline medium some of the quinone III probably disproportionates giving the corresponding quinol (IV): 2′, 5′ - dihydroxyterphenyl - 2,2″ - dicarboxylic acid. On acidification this hydroxyacid is not stable, but lactonises immediately to $C_{20}H_{10}O_4$ (V).



IV *V*

The highly symmetrical structure for the condensation product is in accord with the high melting point and the low solubility of the substance. Further support comes from the spectroscopic data: A strong carbonyl band appears at 1 735 cm^{-1} which is in the lower part of the region for δ-lactones and consistent with the conjugation of the carbonyl groups with an aromatic nucleus. Aromatic absorption occurs strongly at 1 607 cm^{-1} and weakly at 1 582 cm^{-1}. The latter band must be due to the conjugated rings and is recognisable because of intensification by conjugation with carbonyl groups. Absorptions at 866 cm^{-1} and 767 cm^{-1} are probably caused by 1,2,4,5-tetrasubstitution and 1,2-disubstitution, respectively.

Our conclusion regarding the structure of $C_{20}H_{10}O_4$ is excellently borne out by X-ray crystallographic investigations [3].

Experimental. In the first experiments methylphenylglyoxal-*o*-carboxylic acid was isolated and purified before the alkali treatment. Later it was found more convenient to use directly the product from the ozonolysis in the following way:

2-Methylnaphthoquinone (5 g) in chloroform (150 ml) was ozonised at −5° until the solution became colourless. The ozonised solution was shaken with water (50 ml) and left overnight. Next day the chloroform was evaporated on a water-bath and the resulting aqueous solution filtered. To this was added aqueous sodium hydroxide (50 ml, 2 N) and the mixture boiled for a couple of minutes. In order to avoid deposition of tarry matter the alkaline solution was diluted with an equal volume of ethanol and thereafter acidified with 2 N hydrochloric acid. The dark red solution became yellow and a silky precipitate of fine needles separated (230 mg). The filtrate was made alkaline again (appr. 2 N) by the addition of solid sodium hydroxide and heated on the waterbath for 30 min. After addition of more ethanol and acidification an additional crop of needles (84 mg) was obtained. Recrystallisation was achieved with relatively large amounts of boiling glacial acetic acid. M. p. 420—422°, no decomp., crystallising again and giving the same m. p. (Found: C 76.4; H 3.3. Calc. for $C_{20}H_{10}O_4$: C 76.4; H 3.2.) The density was determined by the flotation method in mixtures of carbon tetrachloride and chloroform: $1.54 > d_{20} > 1.52$. Crystals large enough for this determination were obtained by recrystallisation from nitrobenzene.

The infrared solid-state spectra were recorded in Nujol-mull and KBr-discs with a Perkin-Elmer double beam spectrograph.

1. Bernatek, E. *Unpublished results.*
2. Müller, H. and v. Pechmann, H. *Ber.* **22** (1889) 2127.
3. Pedersen, B. *Acta Chem. Scand.* **13** (1959) 1721.

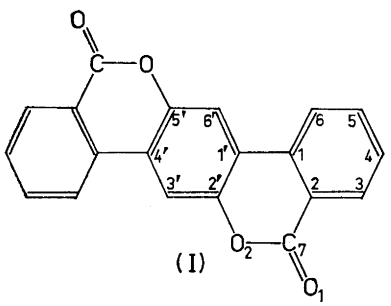
Received September 18, 1959.

The Crystal and Molecular Structure of 2′,5′-Dihydroxyterphenyl-2,2″-dicarboxylic Acid Dilactone

BJÖRN PEDERSEN

Kjemisk institutt, Universitetet i Oslo, Blindern, Norway

As shown by Bernatek [1] the compound, $C_{18}H_{10}O_4$, formed by briefly heating an alkaline solution of methylphenylglyoxal-*o*-carboxylic acid probably is 2′,5′-dihydroxyterphenyl-2,2″-dicarboxylic acid dilactone (I).



(I)

To get further support for the assumed formula an X-ray crystallographic study of the compound was undertaken. The crystals, recrystallised from nitrobenzene, were long, very thin needles. Oscillation and Weissenberg photographs taken with Cu*K*-radiation (λ = 1.540 Å) showed the crystals to be monoclinic with

$$a = 3.82 \text{ Å} \quad b = 14.11 \text{ Å} \quad c = 12.54 \text{ Å}$$
$$\beta = 94.0°$$

The needle axis of the crystals is parallel to the *a*-axis. Systematic absences. *h0l* when *l* is odd, *0k0* when *k* is odd. This unambiguously determines the space group as No. 14, $P2_1/c$. The observed density [1], 1.53 g/cm³, indicates the presence of two formula units in the unit cell (d_{calc} = 1.55 g/cm³). The space group symmetry requires that the molecule possesses a centre of symmetry. Furthermore the short *a*-axis shows that the molecule is nearly planar (van der Waals thickness of an aromatic nucleus is 3.70 Å [2]). All this fully supports the formula (I).

The molecule must consequently lie in the unit cell nearly parallel to the *a* plane with its centre of symmetry in the origin. The only parameter left to determine before the molecule is located completely in the unit cell is therefore the angle the molecule is turned out from the *b* or *c*-axis. Due to these simplifying features of the crystal structure a quantitative investigation of the [100]-projection could be carried out. The intensities of the *0kl*-reflexions (109 out of 258 theoretically accessible) were measured with a photometer from integrated Weissenberg photographs taken with Cu*K*-radiation. The intensities were corrected for Lorentz and polarisation effects but not for absorption and extinction. On the basis of the corrected $F(0kl)$ [2]-values a Patterson map was worked out. The map showed a marked peak near the origin, at $v = 0.131$, $w = 0.123$, which probably is due to the intramolecular vector between O_1 and O_2 and coinciding O—C and C—C vectors. This reduces the possible ways of placing the molecule in the projection to two: the benzene ring 1—6 may either be placed near the *b*- or the *c*′-axis. But as $|F(021)|$ is very large and almost twice $|F(012)|$ the benzene ring must be placed near the *c*′-axis. A set of signs were calculated on the basis of a planar molecule analogous to (I) with all atoms equal and all nearest neighbour distances

Table 1. Atomic coordinates as fractions of the corresponding cell edge and the temperature factor *B* (in $\exp(-B\sin^2\Theta/\lambda^2)$).

Atom	*y*	*z*	*B*
C_1	0.060	0.205	3.2
C_2	0.155	0.234	3.1
C_3	0.190	0.334	4.7
C_4	0.129	0.405	4.3
C_5	0.032	0.377	4.7
C_6	−0.004	0.278	3.7
C_7	0.223	0.157	3.9
C_1'	0.026	0.103	2.4
C_6'	−0.070	0.070	3.3
C_2'	0.092	0.033	3.5
O_1	0.305	0.174	3.3
O_2	0.186	0.062	2.8

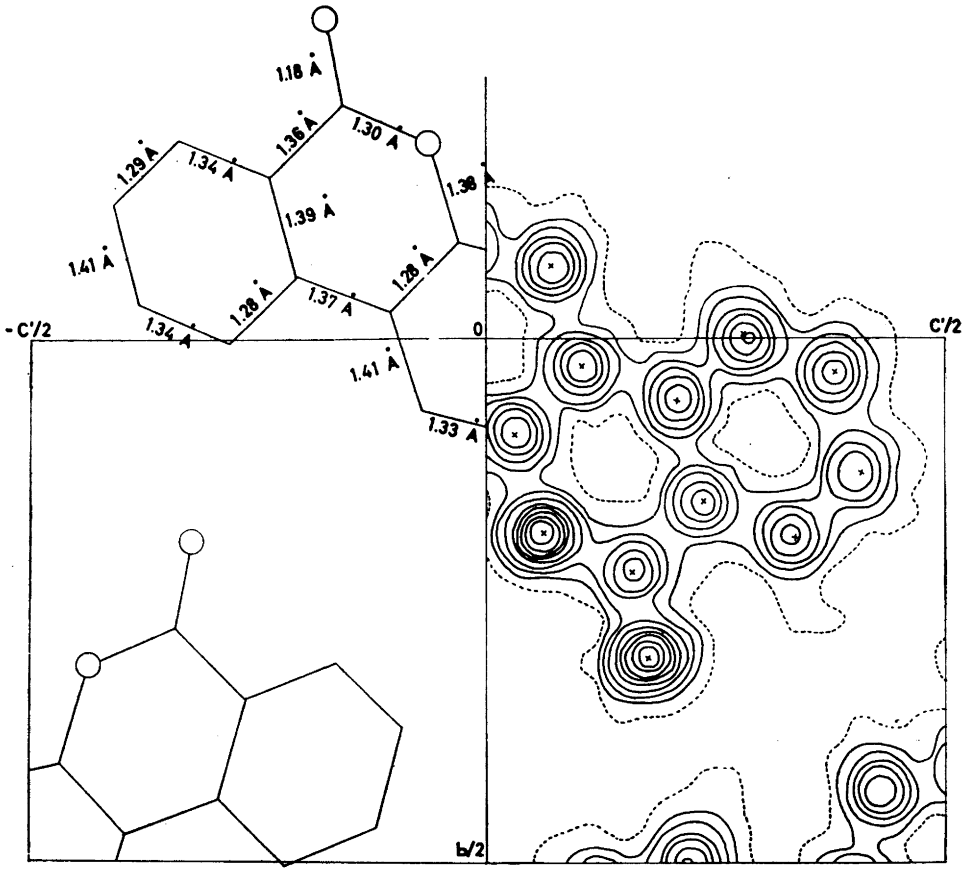


Fig. 1. *a*-Axis Fourier projection of $C_{20}H_{10}O_4$ with the found interatomic distances in the projection. Contours at intervals of 1 eÅ^{-2} with the 1 eÅ^{-2}-line broken.

equal to 1.40 Å in this position. A Fourier projection was evaluated, and this clearly showed all the atoms well resolved with the electron density in the oxygen positions distinctly higher than in the rest of the atomic positions. The atomic coordinates which were derived from the Fourier projection, were refined by the method of least squares on the Ferranti Mercury computer "Fredric" at the Norwegian Defence Research Establishment. The refinement was terminated after five cycles. The final coordinates and individual temperature factors, B, are given in Table 1. The reliability index, R, is 0.092 (unobserved reflexions not included).

The found interatomic distances in the projection are given in Fig. 1 together with the final Fourier map. The figure shows that the molecule is tilted somewhat out of the [100]-projection plane. The found projected bond distances indicate that the terphenyl part of the molecule is planar or nearly so with the carbonyl oxygen and lactone oxygen a little twisted out on each side of this plane. From such a model of the molecule and an assumed C—C distance in the terphenyl part of 1.40 Å the true C_2-C_7 and $C_1-C_{1'}$ distance are calculated as 1.48 Å and 1.43 Å, respectively. The former of these is in good agreement with what is found [3] for the corre-

sponding distance in benzoic acid, 1.48 Å. The Fourier map in Fig. 1 shows that the electron density in the carbon positions decreases with the distance from the origin, *i. e.* the centre of symmetry of the molecule. The temperature factors, B, increase in the same order. This is in accordance with what is found in other planar centrosymmetric molecules as naphthalene [4] and anthracene [5] and is due to the rigid-body vibrations of the molecules [6]. — The molecular packing is similar to the arrangement found in 1,2,5,6-dibenzanthracene [7].

1. Bernatek, E. *Acta Chem. Scand.* **13** (1959) 1719.
2. Ketelaar, J. A. A. *Chemical Constitution*, 2. Ed. Elsevier, Amsterdam 1958.
3. Sim, G. A., Robertson, J. M. and Goodwin, T. H. *Acta Cryst.* **8** (1955) 157.
4. Abrahams, S. C., Robertson, J. M. and White, J. G. *Acta Cryst.* **2** (1949) 238.
5. Cruickshank, D. W. J. *Acta Cryst.* **9** (1956) 915.
6. Cruickshank, D. W. J. *Revs. Modern Phys.* **30** (1958) 163.
7. Robertson, J. W. and White, J. G. *J. Chem. Soc.* **1947** 1001.

Received September 18, 1959.

The Fatty Acid Composition of Shea Butter and Olive Kernel Oil

AAGE JART

Danish Fat Research Institute, Copenhagen, Denmark

Shea butter. Shea butter (or karite butter) comes from the seeds of *Butyrospermum Parkii* of the family *Sapotaceae*. After the second world war shea nuts have been imported into Europe on a relatively small scale, but in Denmark the oil is used in appreciable quantities for certain types of margarines. It would therefore be of interest to know the composition of the fatty acids of this oil and especially the content of essential fatty acids.

For the present investigation shea nuts from West Africa were used. The fat in the nuts was isolated and analyzed, *i. a.* by alkali isomerization [1] and — in the form of methyl esters — by quantitative gas-liquid chromatography [2]. The chromatograph used was supplied by Griffin & George and had a 0.9 m column (25 % silicone grease on 40—60 mesh Firebrick). The temperature of the column was 215°C, the outlet pressure 1 atm, the katharometer current 100 mA, and the carrier gas used was helium (1.0 l/h). Using a speed of the recorder paper of 6 inches/h, the quantity M (in μl) of the individual components of the methyl ester mixture could be determined from the area A (in cm²) lying under the corresponding peaks in the chromatogram by applying the following formula (which has been derived by calibrating the apparatus by means of pure fatty acid methyl esters):

$$M_i = \log_{10}^{-1} (0.889 \times \log_{10} (A_i) - 0.058)$$

The calculated fatty acid composition (in weight per cent of the fatty acid mixture) and other results obtained from the analyses of the shea butter appear from Table 1; the content of oleic acid is found by determining the iodine value, while the stearic acid percentage is obtained as a difference. The results are in good agreement with those published in earlier works [3,4].

Olive kernel oil. Olive oil is obtained from the fruits of *Olea europea* which belongs to the family *Oleaceae*. The olive kernel contains about 1 % of the total amount of oil in the olive. Olive oil usually contains the kernel oil, as the olive stones are generally crushed during the milling procedure to which the olives are subjected in the factories. As adequate information concerning the linoleic acid content of olive kernel oil was not available from the literature, an investigation of this oil has been carried out.

The fatty acid composition *etc.* was determined in oil isolated from the kernels of olives obtained from Morocco. The investigation was made in the same manner as in the case of shea butter, and the results are summarized in the last column of Table 1. The oil examined showed a higher degree of unsaturation than a Portuguese olive kernel oil examined previously [5]. The composition ascertained is in fairly good agreement with that given for an olive kernel oil from the U.S.A. [6]

sponding distance in benzoic acid, 1.48 Å. The Fourier map in Fig. 1 shows that the electron density in the carbon positions decreases with the distance from the origin, *i. e.* the centre of symmetry of the molecule. The temperature factors, B, increase in the same order. This is in accordance with what is found in other planar centrosymmetric molecules as naphthalene [4] and anthracene [5] and is due to the rigid-body vibrations of the molecules [6]. — The molecular packing is similar to the arrangement found in 1,2,5,6-dibenzanthracene [7].

1. Bernatek, E. *Acta Chem. Scand.* **13** (1959) 1719.
2. Ketelaar, J. A. A. *Chemical Constitution*, 2. Ed. Elsevier, Amsterdam 1958.
3. Sim, G. A., Robertson, J. M. and Goodwin, T. H. *Acta Cryst.* **8** (1955) 157.
4. Abrahams, S. C., Robertson, J. M. and White, J. G. *Acta Cryst.* **2** (1949) 238.
5. Cruickshank, D. W. J. *Acta Cryst.* **9** (1956) 915.
6. Cruickshank, D. W. J. *Revs. Modern Phys.* **30** (1958) 163.
7. Robertson, J. W. and White, J. G. *J. Chem. Soc.* **1947** 1001.

Received September 18, 1959.

The Fatty Acid Composition of Shea Butter and Olive Kernel Oil

AAGE JART

Danish Fat Research Institute, Copenhagen, Denmark

Shea butter. Shea butter (or karite butter) comes from the seeds of *Butyrospermum Parkii* of the family *Sapotaceae*. After the second world war shea nuts have been imported into Europe on a relatively small scale, but in Denmark the oil is used in appreciable quantities for certain types of margarines. It would therefore be of interest to know the composition of the fatty acids of this oil and especially the content of essential fatty acids.

For the present investigation shea nuts from West Africa were used. The fat in the nuts was isolated and analyzed, *i. a.* by alkali isomerization [1] and — in the form of methyl esters — by quantitative gas-liquid chromatography [2]. The chromatograph used was supplied by Griffin & George and had a 0.9 m column (25 % silicone grease on 40—60 mesh Firebrick). The temperature of the column was 215°C, the outlet pressure 1 atm, the katharometer current 100 mA, and the carrier gas used was helium (1.0 l/h). Using a speed of the recorder paper of 6 inches/h, the quantity M (in μl) of the individual components of the methyl ester mixture could be determined from the area A (in cm²) lying under the corresponding peaks in the chromatogram by applying the following formula (which has been derived by calibrating the apparatus by means of pure fatty acid methyl esters):

$$M_i = \log_{10}^{-1} (0.889 \times \log_{10} (A_i) - 0.058)$$

The calculated fatty acid composition (in weight per cent of the fatty acid mixture) and other results obtained from the analyses of the shea butter appear from Table 1; the content of oleic acid is found by determining the iodine value, while the stearic acid percentage is obtained as a difference. The results are in good agreement with those published in earlier works [3,4].

Olive kernel oil. Olive oil is obtained from the fruits of *Olea europea* which belongs to the family *Oleaceae*. The olive kernel contains about 1 % of the total amount of oil in the olive. Olive oil usually contains the kernel oil, as the olive stones are generally crushed during the milling procedure to which the olives are subjected in the factories. As adequate information concerning the linoleic acid content of olive kernel oil was not available from the literature, an investigation of this oil has been carried out.

The fatty acid composition *etc.* was determined in oil isolated from the kernels of olives obtained from Morocco. The investigation was made in the same manner as in the case of shea butter, and the results are summarized in the last column of Table 1. The oil examined showed a higher degree of unsaturation than a Portuguese olive kernel oil examined previously [5]. The composition ascertained is in fairly good agreement with that given for an olive kernel oil from the U.S.A. [6]

Table 1. Fats of shea nuts and olive kernels.

	Butyrospermum Parkii	*Olea europea* (kernels)
Oil, % in nuts and kernels	46	41
Unsaponifiable matter, %	7.3	2.8
Free fatty acids, as % of oleic acid	3.4	3.9
Fatty acid composition, weight % of total fatty acids		
Palmitic acid	5.1	11.3
Stearic acid	43.5	1.2
Oleic acid	45.3	68.9
Linoleic acid	5.8	18.1
Linolenic acid	0.3	0.5

Experimental. Shea butter. The shea nuts were crushed in a hydraulic press and extracted with light petroleum (b.p. 50—70°C) in a large Soxhlet apparatus; after extraction for a couple of hours, the residue was ground and again extracted until all oil contained in the nuts had been isolated. The oil was saponified by means of ethanolic potassium hydroxide, and the unsaponifiable matter was extracted from an ethanolic/aqueous (50 % v/v) soap solution with several portions of ethyl ether. The fatty acids were isolated from the soap solution by dropwise addition of a 50 % solution of sulphuric acid and were converted into methyl esters by means of diazomethane.

Olive kernel oil. Air-dried olive stones from which the fruit coat had been removed almost completely were cracked carefully, and the individual kernels were removed by means of a pair of tweezers to avoid admixture of fruit coat oil. From 470 g of olive stones 40.0 g of kernels were obtained, and after crushing in an agate mortar and treatment with solvent naphtha in a Soxhlet apparatus these yielded 16.4 g of straw-coloured, crude olive kernel oil. A part of this oil was saponified, and the fatty acids obtained were converted into methyl esters by refluxing with excess of methanol and a little sulphuric acid to act as catalyst.

1. American Oil Chemists' Society. *Official and Tentative Methods of Analysis*, 2nd Ed. Rev. to 1958. Chicago 1946—58. Cd 7—58.
2. Jart, Aa. *Fette, Seifen, Anstrichmittel* **61** (1959) 541.
3. Hilditch, T. P. and Saletore, S. A. *J. Soc. Chem. Ind. London* **50** (1931) 468 T.
4. Green, T. G. and Hilditch, T. P. *J. Soc. Chem. Ind. London* **57** (1938) 49.
5. Klein, O. *Z. angew. Chem.* **12** (1898) 847. (Referred to in Hilditch, T. P. *The Chemical Constitution of Natural Fats*, London, 1956, p. 182.)
6. United States Department of Agriculture, *Fatty Acids in Animal and Plant Products*, Washington, May 1959 (Original paper not cited).

Received October 7, 1959.

Isolation of an Anti-*Fusarium* Substance Present in Intact Rye Seedlings

ÖRN WAHLROOS and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

After it was shown in this laboratory that 6-methoxybenzoxazolinone is formed in wheat and maize plants and benzoxazolinone in rye plants from precursors through enzymatic reactions and ensuing chemical changes [1], a glucoside $C_{14}H_{17}O_9N$ (m. p. 186–187°C) could be isolated from rye seedlings. From this glucoside the aglucone $C_8H_7O_4N$ (m. p. 152°C) is formed when the seedlings are crushed [2]. The aglucone is converted into benzoxazolinone $C_7H_5O_2N$ under liberation of formic acid [2]. In young wheat and maize plants the corresponding methoxy derivative, the glucoside $C_{15}H_{19}O_{10}N$ (m. p. 168–170°C) was found as an original compound from which the aglucone $C_9H_9O_5N$ (m. p. 156–157°C) was enzymatically formed [3]. 6-Methoxybenzoxazolinone $C_8H_7O_3N$ is a reaction product of the latter. The glucosides have only a very weak anti-*Fusarium* activity, whereas the aglucones inhibit the growth of *Fusarium nivale* in an oatmeal-glycerol nutrient medium already at a concentration of 0.1 % and benzoxazolinone at a concentration of 0.06 % [3].

Because the extract of rye seedlings, after the enzyme activity had been destroyed by boiling intact seedlings with 70 % ethanol, inhibits the growth of *Fusarium nivale* at concentrations corresponding to 1 to 1.5 g of fresh weight per ml substrate, the seedlings have to contain antimicrobial substances which as such are present in the seedlings and not formed only after the seedlings are crushed. The rye seedlings used in this work were of the varieties Pekka, Ensi, and Kungsråg. They were collected in November and December on the field.

On dividing the 70 % alcohol extract of the seedlings into ether-soluble neutral material, ether-soluble weakly acidic material (soluble in 1 N NaOH), ether-soluble acids (soluble in sodium bicarbonate), and water-soluble material (not extractable with ether from acidified water solution), about half of the total activity of the alcohol extract is found in the ether-soluble weakly acidic material, and the other half in the water-soluble material.

In the water-soluble material, part of the activity was extractable with *n*-butanol. On attempts to fractionate the water-soluble material by paper chromatography, the activity could not be recovered. Also on standing in the refrigerator for two months the activity was lost.

The ether-soluble weakly acidic material was shown to contain an active component moving with an R_F-value of 0.86 in *n*-propanol:ammonia (7:3) on Whatman No. 4 paper (desc.). This component was purified at first by cellulose-column chromatography in the *n*-propanol-ammonia solvent, and then by adsorption chromatography on a silica-gel column. The silica-gel was previously equilibrated against water-vapour of the pressure 5.6 mm Hg at 20°C, and the active material was eluted with ethanol-free chloroform containing rising amounts (2–50 %) of ether. Because the material was found to be subject to autoxidation the following steps were carried out in a CO_2 atmosphere, and illumination of the samples was avoided. The chloroform-ether fractions were evaporated to dryness *in vacuo* and dissolved in methanol. After addition of 20 % of water the solution was cooled and the inactive material crystallizing was filtered off. The filtrate was again evaporated to dryness *in vacuo* and the residue was chromatographed on a cellulose column in a solvent consisting of water-saturated benzene containing 0.2 % of methanol. The R_F-value of the active component in this solvent on Whatman No. 4 paper was 0.87 (asc.). The active fractions were evaporated to dryness *in vacuo*. The resulting slightly yellow-green oil was found to solidify at −14 to −15°C, and it melted at −12.5 to −11°C. The oil was crystallized from methanol at −40 to −50°C using an apparatus described by Kajanne [4]. After separation of the crystals they were allowed to melt, and the resulting oil was filtered at −10 to −5°C through a micro filter. After drying over P_2O_5 at +20°C and 0.01 mm Hg pressure, the oil solidified at −14°C and melted at −12 to −11°C. The yield was 68 mg oil from 255 g fresh weight of seedlings. Considering the amount of oil in the mother liquor after crystallization the total amount of oil in

the plants was calculated to be about 1.5 mg per g fresh weight.

Some of the properties of the substance are listed below:

Boiling point: At a pressure of 0.01 mm Hg signs of boiling were observed at about 170°C, with simultaneous darkening of the sample.

Empirical formula: Found: C 76.16; H 10.61; O 12.96. Calc. for $C_8H_{14}O$: C 76.2; H 11.1; O 12.7. Calc. for $C_8H_{13}O$: C 76.8; H 10.4; O 12.8.

Molecular formula: For the molecular weight, determined by the camphor method, the value 270–290 was found. Accordingly, the molecular formula is $C_{16}H_{28}O_2$ or $C_{16}H_{26}O_2$ with the calculated molecular weight of 252 or 250.

UV-absorption: In *cyclo*hexane the substance exhibits one maximum, at 236 mμ, $\varepsilon = 3020$.

Acidic properties: The substance dissolves in aqueous sodium carbonate solution and in sodium hydroxide, being precipitated on acidifying.

Hydroxyl content: The substance is esterified by acetylchloride. The product of catalytic hydrogenation reacts with one mole of acetyl chloride per mole substance.

Catalytic hydrogenation at room temperature with PtO_2: Two moles of hydrogen per mole substance are rapidly consumed.

Reducing properties: The substance decolorizes permanganate rapidly in sodium carbonate solution. One of the products from permanganate oxidation was identified as azelaic acid, $HOOC-(CH_2)_7-COOH$, by isolation and mixed melting point determination. The substance is also oxidized by periodic acid at room temperature within a few minutes. It exhibits a reducing action upon 2,6-dichlorophenolindophenol and reduces Hg(I)-nitrate and bromine slowly. It is autoxidized in the air, the reaction is light-catalyzed. The products of autoxidation seem to contain peroxides (liberation of iodine from iodide).

Other reactions: With silver ions the substance seems to form a complex. No colour formation with ferric chloride was observed. With diazotized *p*-nitroaniline a yellow colour appeared on making the solution alkaline. With 2,4-dinitrophenylhydrazine the substance is reacting, as shown by chromatography and spectrometry of the reaction mixtures, though no crystalline product could be obtained. No carboxylic acid-, lactone-, or ester-reaction could be obtained with the substance.

On the basis of the oxidation products the isolated substance contains a straight saturated chain of nine C-atoms. The other properties listed above suggest that the substance contains: two aliphatic double bonds, one C–OH group, and one C=O group.

The substance isolated inhibits the growth of *Fusarium nivale* to 50 % at a concentration of 0.5 to 0.7 mg per ml of an oat-meal-glycerol medium. On increasing the concentration to 2.4 mg/ml the inhibition increases to 85 %. Above this concentration the inhibition *vs.* concentration curve becomes parallel to the concentration axis, depending on the poor solubility of the substance in aqueous medium.

The support given by the *Rockefeller Foundation* is gratefully acknowledged.

1. Virtanen, A. I. and Wahlroos, Ö. *Suomen Kemistilehti* **B 31** (1959) 402.
2. Virtanen, A. I. and Hietala, P. K. *Suomen Kemistilehti* **B 32** (1959) 38, 138.
3. Wahlroos, Ö. and Virtanen, A. I. *Suomen Kemistilehti* **B 32** (1959) 139.
4. Kajanne, P. *Suomen Kemistilehti* **B 31** (1958) 188.

Received October 1, 1959.

Amino Acid Composition of the Fibrinopeptides A and B

JOHN SJÖQUIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

During the transformation of bovine fibrinogen to fibrin by bovine thrombin at least two peptides (A and B) are released [1,2]. The qualitative amino acid composition of the peptides has been given by Bettelheim [1] after isolation of the peptides by means of electrophoresis and reversed phase partition chromatography. We have published preliminary amino acid analyses, which were performed in connection with structural studies of the peptides [3]. Recently, Gladner *et al.*[4] have analyzed Peptides A and B for their amino acid composition. Differing from our preliminary report, they found Peptide B to contain threonine. However, the presence of threonine was later confirmed in our studies of the amino acid sequence of the peptides [5].

This report deals with the amino acid composition of highly purified fibrinopeptides isolated by the technique of Blombäck and Vestermark [6]. Samples of the peptides (0.5—1 mg) were hydrolyzed in 5.7 N HCl for 22 h at 110°C. The amino acids of the hydrolysates were converted to phenyl thiohydantoin derivatives [7] and quantitatively estimated after separation on paper chromatograms [8].

Table 1 gives the results of these analyses. The figures refer to air-dried samples. The data agree well with those of Bettelheim [1] and Gladner *et al.*[5] Our analyses on performic acid oxidized fibrinopeptides have revealed no cysteine or cystine. Measurements of the tryptophan absorbtion in ultra violet indicate that none is present. No analyses have been performed to determine the amide-N.

The minimum number of amino acid residues in Peptide A is nineteen and that of Peptide B is twentyone. Therefore, the calculated minimum molecular weights of Peptides A and B are 1 890 and 2 460, respectively.

This work was supported by grants from *Statens Medicinska Forskningsråd.*

Table 1.

Amino acid	Peptide A mmoles/100 g peptide			minimum number of residues	Peptide B mmoles/100 g peptide			minimum number of residues
	1st analysis	2nd analysis	mean		1st analysis	2nd analysis	mean	
Alanine	—	—	—	—	37.4	36.8	37.1	1
Arginine	44.5	42.0	43.3	1	75.1	76.0	75.6	2
Aspartic acid	128.2	126.0	127.1	3	142.1	148.0	145.1	4
Glutamic acid	92.5	90.8	91.7	2	105.2	107.0	106.1	3
Glycine	218.0	214.0	216.0	5	104.5	109.9	107.2	3
*Iso*leucine / Leucine	46.8	47.1	47.0	1	35.6	36.1	35.9	1
Lysine	—	—	—	—	33.6	34.4	34.0	1
Phenylalanine	46.0	47.0	46.5	1	35.8	34.5	35.2	1
Proline	92.8	94.3	93.6	2	68.2	70.0	69.1	2
Serine	72.3	70.2	71.3	2	—	—	—	—
Threonine	30.2	32.5	31.4	1	33.5	34.1	33.8	1
Tyrosine	—	—	—	—	34.8	35.0	34.9	1
Valine	47.3	47.1	47.2	1	39.1	38.6	38.9	1
Sum				19				21

1. Bettelheim, F. R. *Biochim. et Biophys. Acta* **19** (1956) 121.
2. Lorand, L. *Physiol. Revs.* **34** (1954) 742.
3. Blombäck, B. *Acta Physiol. Scand.* **43** (1958) Suppl. 148, p. 37.
4. Gladner, J. A., Folk, J. E., Laki, K. and Carroll, W. R. *J. Biol. Chem.* **234** (1959) 62.
5. Blombäck, B., Wallén, P. and Sjöquist, J. *Acta Chem. Scand.* **13** (1959) 819.
6. Blombäck, B. and Vestermark, A. *Arkiv Kemi* **12** (1958) 173.
7. Sjöquist, J. *Arkiv Kemi* **11** (1957) 129.
8. Sjöquist, J. *Submitted for publication.*

Received October 14, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1729–1732

Absorption Spectra of Some Vanadium(III) Fluorides

C. J. BALLHAUSEN and FLEMMING WINTHER

Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark

A number of vanadium(III) fluorides having the general formula $[VF_n(H_2O)_{6-n}]^{+(3-n)}$ were prepared and their reflection spectra measured. An identification of the various absorption bands observed is proposed based on a crystal field level scheme with spin-orbit interaction included. The presence of lower crystalline fields than O_h is deduced from the position of the first spin-forbidden band.

It is the purpose of this note briefly to study the application of the theoretical ($3d^2$) crystalline field level scheme recently given by Liehr and Ballhausen [1] to the absorption spectra of vanadium(III) complexes. To achieve this goal we have chosen the series $[VF_n(H_2O)_{6-n}]^{+(3-n)}$, the reason for our choice being the fact that going down this series from n = 6 to n = 3 some small "spin-forbidden" bands may be seen, some of which are not easily detected otherwise. It may of course be argued that the theory as outlined by Liehr and Ballhausen [1] is not at all applicable to non-cubic complexes. While this is perfectly true in a formal way, we must nevertheless remember that F^- and H_2O stand very close together in the spectro-chemical series. Thus we would not expect great deviations from "cubic" behaviour for these mixed complexes. Most important, we shall not find any kind of significant splittings of the "cubic" bands, due to the actual presence of lower symmetry. On the other hand we would expect the spin-forbidden bands to be easier to detect by comparing the spectra of the different complexes.

Furthermore, there are experimental and theoretical reasons to expect that all "cubic" V(III) complexes are distorted along the trigonal axis due to to a Jahn-Teller effect [2,3], and especially the excited states possessing E_g electronic states may be very strongly coupled to the various molecular vibrations. While this fact makes an *a priori* "exact" analysis nearly impossible, it seems nevertheless possible to understand the general features of the d^2 system assuming a pure cubic crystalline field [4–7]. For special systems it is further necessary to throw in a trigonal field component [8,9] in the Hamiltonian in order to account for specific features. This procedure was found to be necessary in the present work in order to explain the position of the first spin-forbidden band.

As the complexes in question are rather insoluble, the absorption spectra were recorded as reflection spectra taken upon the crystalline powder. This method gives quite accurate results in so far as the location of the various absorption bands are concerned, but it is completely out of the question to estimate band intensities in this way. We shall accordingly refrain from interpreting the oscillator strengths of the bands [10].

EXPERIMENTAL

The various complexes $[VF_3(H_2O)_3]$, $NH_4[VF_4(H_2O)_2]$, $(NH_4)_2[VF_5H_2O]$ and $(NH_4)_3[VF_6]$ were made according to Petersen [11,12]. $K_2[VF_5H_2O]$ was made according to Palmer [13]. An unsuccessful attempt was made to prepare the mixed complexes containing 1 and 2 fluorine atoms.

When pure all of the complexes mentioned above are quite stable in the atmosphere, the hexa- and pentafluorides being the most stable. Both the hexafluoride and the hexahydrate can be washed with alcohol without apparent structural changes, whereas this is not the case with the "mixed" complexes.

As all of the compounds mentioned have been well characterized and analyzed before [11,12], we have only analyzed the prepared complexes for the ratio between the vanadium and fluorine contents. The analyses were carried out in a semimicro scale.

The vanadium content was found by means of potentiometry using MnO_4^- as titrating agent. This was carried out in two steps: V(III) → V(IV) and V(IV) → V(V). As the titration took place in the presence of F^-, no glass apparatus was used.

The fluorine content was found after the method of Willard and Winter [14].

The reflection spectra were recorded on a Beckman DU spectrophotometer with MgO powder used as standard.

CONCLUSIONS

The spectra recorded all show broad maxima at ~15 000 cm^{-1} and ~23 000 cm^{-1}. These we attributed to the assignments [7]

15 000 cm^{-1} due to $^3T_{1g} \rightarrow {}^3T_{2g}$ and
23 000 cm^{-1} due to $^3T_{1g} \rightarrow {}^3T_{1g}$.

More exactly for $VF_6^{\equiv\equiv}$ we get $^3T_{1g} \rightarrow {}^3T_{2g}$ 14 800 cm^{-1}, from which we calculate [1,7] $Dq = 1\,585$ cm^{-1} taking $F_2 = 14\ F_4$ with $F_4 = 70$ cm^{-1} as in Liehr and Ballhausen [1].

Table 1. Calculated [1] and observed band maxima for V(III) complexes.

Calculated wavenumbers for d^2 cubic configuration $F_4 = 70$ cm^{-1} and $F_2 = 14\ F_4$ $Dq = 1\,585$ cm^{-1} and $\lambda = 65$ cm^{-1} Transitions from $^3T_{1g}$ to:		Observed wavenumbers			
		$VF_6^{\equiv\equiv}$	$VF_5(H_2O)^{=}$	$VF_4(H_2O)_2^{-}$	$VF_3(H_2O)_3$
^1E_g, $^1T_{2g}$	9 200 cm^{-1}	10 200	10 500	12 000	11 500
$^3T_{2g}$	14 800 cm^{-1}	14 800	15 500	16 000	16 200
$^1A_{1g}$	19 600 cm^{-1}				
$^3T_{1g}$	23 200 cm^{-1}	23 000	23 500	23 800	24 500
$^1T_{2g}$	25 200 cm^{-1}				
$^1T_{1g}$	27 300 cm^{-1}			28 200	
$^3A_{2g}$	31 200 cm^{-1}				

The calculated and the experimental absorption peaks are collected in Table 1.

The agreement between the calculated and observed absorption bands is seen to be fairly good with the single exception of the first "spin-forbidden" band $^3T_{1g} \rightarrow {}^1E_g, {}^1T_{2g}$. Assuming a pure cubic crystallinen field, the two levels ^1E_g and $^1T_{2g}$ are nearly degenerate. However, by superimposing a trigonal field upon the octahedral field the $^1T_{2g}$ level will split into a ^1E_g and $^1A_{1g}$ level, whereas ^1E_g is not split. Thus we would expect a strong repulsion to take place between the two ^1E_g levels by going down in symmetry, and this fact can explain why the "first" spin-forbidden band is pushed upwards about 1 000 cm^{-1}. For details see the appendix.

All in all it may thus be concluded that a slightly modified theoretical level scheme [1] is sufficient to explain the experimental facts.

Acknowledgement. We want to thank Dr. Claus E. Schäffer for valuable assistence in checking the results for the $VF_6^{\equiv}$ compound.

APPENDIX

The d-electrons in a cubic field are usually quantized along the four-fold axis, and are described as $t_{2g} = (xz)$, (yz) and (xy) and $e_g = (z^2)$ and (x^2-y^2). If instead we quantize along the three-fold axis of the octahedron, the following linear combinations of d-orbitals transform correctly as zero-order wavefunctions

$$t_{2g}^{+} = \sqrt{\tfrac{2}{3}}\,(x^2-y^2) - \sqrt{\tfrac{1}{3}}\,(xz)$$
$$t_{2g}^{-} = \sqrt{\tfrac{2}{3}}\,(xy) + \sqrt{\tfrac{1}{3}}\,(yz)$$
$$t_{2g}^{0} = (z^2) \qquad \text{and}$$
$$e_g^{+} = \sqrt{\tfrac{1}{3}}\,(x^2-y^2) + \sqrt{\tfrac{2}{3}}\,(xz)$$
$$e_g^{-} = \sqrt{\tfrac{1}{3}}\,(xy) - \sqrt{\tfrac{2}{3}}\,(yz)$$

Now choosing the most general trigonal perturbation as

$V_\tau = A_2^0 Y_2^0 + A_4^0 Y_4^0 + A_4^3 (Y_4^3 - Y_4^{-3})$, we obtain by taking a suitable reference energy

$$(t_{2g}^{\pm} \mid V_\tau \mid t_{2g}^{\pm}) = D\tau$$
$$(t_{2g}^{0} \mid V_\tau \mid t_{2g}^{0}) = -2\ D\tau.$$

By descending in symmetry from O_h to C_{3v} we get $^1T_{2g} \rightarrow {}^1E_g + {}^1A_{1g}$ and $^1E_g \rightarrow {}^1E_g$. Writing down zero-order determinental single-electronic wavefunctions which transform correctly both under O_h and C_{3v}, we obtain

$$^1E_g[^1E_g^{-}(t_{2g})^2] = -\sqrt{\tfrac{1}{6}}\,|\,\overset{+}{t_{2g}}\,\overset{-}{t_{2g}}\,| + \sqrt{\tfrac{1}{6}}\,|\,\overset{-}{t_{2g}}\,\overset{+}{t_{2g}}\,| + \sqrt{\tfrac{2}{6}}\,|\,\overset{+}{t_{2g}}\,\overset{-}{t_{2g}^{0}}\,| - \sqrt{\tfrac{2}{6}}\,|\,\overset{-}{t_{2g}}\,\overset{+}{t_{2g}^{0}}\,|$$
$$^1T_{2g}[^1E_g^{-}(t_{2g})^2] = \sqrt{\tfrac{2}{6}}\,|\,\overset{+}{t_{2g}}\,\overset{-}{t_{2g}}\,| - \sqrt{\tfrac{2}{6}}\,|\,\overset{-}{t_{2g}}\,\overset{+}{t_{2g}}\,| + \sqrt{\tfrac{1}{6}}\,|\,\overset{+}{t_{2g}}\,\overset{-}{t_{2g}^{0}}\,| - \sqrt{\tfrac{1}{6}}\,|\,\overset{-}{t_{2g}}\,\overset{+}{t_{2g}^{0}}\,|$$

where $|\cdots|$ designates an antisymmetrized, normalized wavefunction and the plus or minus sign above the single orbitals means α or β spin.

For the interaction of the two ^1E_g levels in C_{3v} we get:

$$\begin{vmatrix} -E & -\sqrt{2}\,D\tau \\ -\sqrt{2}\,D\tau & D\tau - E \end{vmatrix} = 0$$

with $E = 2D\tau$ and $-D\tau$.

Taking $D\tau$ to be 500 cm^{-1}, we find that the accidental degeneracy in O_h of ^1E_g and $^1T_{2g}$ at 9 200 cm^{-1} is done away with, and that instead we have three levels located at 10 200 cm^{-1}, 9 200 cm^{-1} and 8 700 cm^{-1}. This value of $D\tau$ is in close agreement with the results of Pryce and Runciman [9]. Due to experimental difficulties the two expected bands at $\sim$ 9 000 cm^{-1} have not yet been found.

REFERENCES

1. Liehr, A. D. and Ballhausen, C. J. *Ann. of Phys.* (N.Y.) **6** (1959) 134.
2. Öpik, U. and Pryce, M. H. L. *Proc. Roy. Soc. London* **A238** (1957) 425.
3. Liehr, A. D. and Ballhausen, C. J. *Ann. of Physics* (N.Y.) **3** (1958) 304.
4. Ilse, F. E. and Hartmann, H. *Z. Naturforsch.* **6a** (1951) 751.
5. Hartmann, H., Furlani, C. and Bürger, A. *Z. physik. Chem.* (N.F.) **9** (1956) 62.
6. Orgel, L. E. *J. Chem. Phys.* **23** (1955) 1004.
7. Ballhausen, C. J. *Z. physik. Chem.* (N.F.) **11** (1957) 205.
8. Ballhausen, C. J. *Z. physik. Chem.* (N.F.) **17** (1958) 246.
9. Pryce, M. H. L. and Runciman, W. A. *Discussions Faraday Soc.* **26** (1958) 34.
10. Ballhausen, C. J. and Liehr, A. D. *Mol. Physics* **2** (1959) 123.
11. Petersen, E. *Vanadinet og dets nærmeste Analoger.* Thesis. København 1888.
12. Petersen, E. *J. prakt. Chem.* **40** (1889) 44.
13. Palmer, W. G. *Experimental Inorganic Chemistry.* Cambridge University Press 1954.
14. Fresenius, W. and Jander, G. *Handbuch der analytischen Chemie.* 3. Vol. 7 p. 200 f.f. Springer 1950.

Received July 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1733–1736

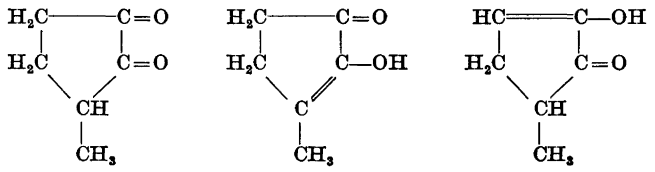
The Enol Structure of 3-Methyl*cyclo*pentane-1,2-dione

JOHAN B-SON BREDENBERG

Department of Chemistry, Institute of Technology, Helsingfors, Finland

Nuclear magnetic resonance investigations show that 3-methyl*cyclo*pentane-1,2-dione occurs as the enol 3-methyl*cyclo*pent-2-en-2-ol-1-one.

The α-diketone 3-methyl*cyclo*pentane-1,2-dione (I), a compound which has been found in soluble wood tar [1] and tall light oil [2] and which is formed by alkaline digestion of spruce wood or galactose [3], has been shown to occur as a monoenol in the crystalline form as well as in solution [4,5].



I II III

Two different enol structures (II, III) can be written for the compound. Both structures have been previously used [4,6,7]. Hesse and Breig [8] have recently tried to solve the problem of the direction of the enolisation by chemical methods. Halogenation and oxidation studies led them to advance structure III. The evidences are not, however, convincing, since the structures of the most important products were not unambiguously proven. In addition, it has to be remarked that substitution in the 5-ring has an influence that is not easily anticipated. The solution to the problem has therefore to be found in such physical methods as do not influence the tautomerism. An excellent method for this purpose is nuclear magnetic resonance spectrometry. The nmr spectrum of the compound in $CDCl_3$ with tetramethylsilane as an internal reference is given in Fig. 1 a. This spectrum shows conclusively that the compound has the structure II. The methyl resonance appears as a single peak at a τ value [9] of 7.97. The methylene groups are apparently nearly equivalent and give the resonance at τ 7.57. In view of the known influence of a keto

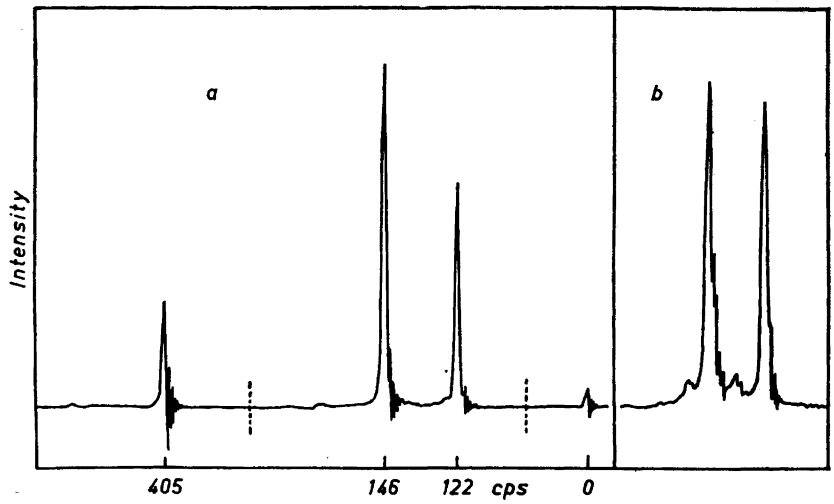


Fig. 1. Nuclear magnetic resonance spectra of 3-methyl-*cyclo*pent-2-en-2-ol-1-one in *a*. $CDCl_3$ solution, internal reference tetramethylsilane (parts of the base line are omitted at the broken lines), *b*. pyridine solution.

group and a double bond, respectively, in α-position to the methylene groups [10] the equivalence of the groups seems to be quite natural. The position at a τ value of 3.25 confirms the enol nature of the single proton peak. The areas appear to be in the ratio 3:4:1 which also tallies with structure II. Structure III would have given a much more complicated spectrum, *inter alia* a doublet for the methyl resonance. The nmr spectrum in pyridine did not reveal any shift in the enol equilibrium. However, the solvent effect had made the two methylene groups slightly non-equivalent and the spin-coupling between them was beginning to appear as a number of weak satellites (Fig. 1 b). The temperature was varied from the freezing to the boiling point of $CDCl_3$ with no evidence of anything but the enol form. These results, as well as infrared and ultraviolet investigations, show that the compound does not occur as an equilibrium mixture between the different tautomeric forms. Hesse and Breig [8] arrived at the same conclusion. Structure II finds further support from the absence of bands in the olefinic C-H stretching region of the infrared spectrum in CCl_4 (Fig. 2 b).

The enol structure II is the sterically favoured form since the C-methyl bond is staggered in relation to the C—H bonds on the adjacent atom in contrast to structure III where the C-methyl bond is opposed to one C—H bond. Hyperconjugation also favours structure II. The inductive effect of the methyl group which Hesse and Böckmann [7] thought to be operative in the tautomery can be observed in the ionisation of the enol. For the unsubstituted compound, *cyclo*pent-2-en-2-ol-1-one, the pK value is 9.14, and for compound II it is 9.60 [5]. This can be accounted for by structure II (compare phenol, pK 9.94, and *o*-cresol, pK 10.28 [11]). It is not possible, however, that the methyl group

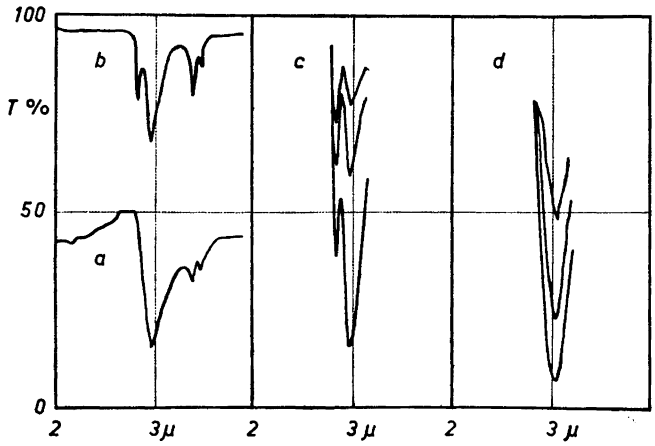


Fig. 2. Infrared spectra in the 2—4 μ region of 3-methyl-*cyclo*pent-2-en-2-ol-1-one, *a.* pellet, 2 mg in 300 mg KBr, *b.* saturated solution in carbon tetrachloride, *c.* solutions in chloroform, 1 g in 10, 30, and 50 ml, *d.* solutions in dioxan, 1 g in 10, 30, and 50 ml. All solutions in 0.1 mm cell.

in structure III should have had such a strong influence on the ionisation constant. A similar difference in the pK values is found in *cyclo*hex-2-en-2-ol-1-one and its 3-methyl homologue, the respective values being 10.30 and 11.23 [5]. This also confirms the enol structure for the last-mentioned compound advanced by Wallach [12] in connection with his investigations of the structure of diosphenol.

Methyl *cyclo*pentenolone is intermolecularly associated in the crystalline form, the OH stretching band absorbing at 3 350 cm^{-1} (Fig. 2 a). This band is also found in the carbon tetrachloride and chloroform solutions (Fig. 2 b, c). In these solutions there appears an additional band at 3 530 cm^{-1} which is strengthened upon dilution, the band at 3 350 cm^{-1} at the same time being weakened. The band at 3 530 cm^{-1} therefore corresponds to a chelate which, as a 5-ring hydrogen bridge, is rather weak. In dioxan the compound shows only one band at 3 280 cm^{-1} (Fig. 2 d).

EXPERIMENTAL

3-Methyl*cyclo*pent-2-en-2-ol-1-one, purum, Fluka A.-G., m. p. 106.5°, was used without further purification.

The nmr spectra were taken at Varian Associates, Palo Alto, California, with a 60 Mc High Resolution NMR Spectrometer. The infrared spectra were taken with a Beckman IR-5 spectrophotometer and the ultraviolet spectra with a Beckman DK-2 spectrophotometer.

Infrared maxima. Keto band, cm^{-1}, cryst. 1 710, in $CHCl_3$ 1 715, in CCl_4 1 715; double bond, cm^{-1}, cryst. 1 650, in $CHCl_3$ 1 667, in CCl_4 1 670.

Ultraviolet maxima. Water, λ_{max} 256 mμ (logε 4.14); ethanol, λ_{max} 258—259 mμ (logε 4.15); *cyclo*hexane, λ_{max} 251 mμ (logε 4.15).

The author wishes to express his thanks to Varian Associates, Palo Alto, California for taking the nmr spectra and to Dr. J. N. Shoolery for his comments on them.

REFERENCES

1. Meyerfeld, J. *Chemiker-Ztg.* **36** (1912) 549.
2. Bredenberg, J. B. *Soc. Sci. Fennica, Commentationes Phys.-Math.* **24** (1959) No. 1.
3. Enkvist, T. *Acta Chem. Scand.* **8** (1954) 51.
4. Rojahn, C. A. and Rühl, F. *Arch. Pharm.* **264** (1926) 211.
5. Schwarzenbach, G. and Wittwer, C. *Helv. Chim. Acta* **30** (1947) 663.
6. Staudinger, H., Ruzicka, L. and Reuss, E. *Ann. Acad. Sci. Fennicae, Ser. A.* **29** (1927) No. 17, 3.
7. Hesse, G. and Böckmann, K. W. F. *Ann.* **563** (1949) 37.
8. Hesse, G. and Breig, K. *Ann.* **592** (1955) 120.
9. Tiers, G. V. D. *J. Phys. Chem.* **62** (1958) 1151.
10. Chamberlain, N. F. *Anal. Chem.* **31** (1959) 56.
11. Sprengling, G. R. and Lewis, C. W. *J. Am. Chem. Soc.* **75** (1953) 5709.
12. Wallach, O. *Ann.* **437** (1924) 148.

Received July 16, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1737–1742

Electron Diffraction Conformational Analyses of *Trans*-1,4-Dichloro- and Dibromo*cyclo*hexane

V. A. ATKINSON and O. HASSEL

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

Electron diffraction studies have been carried out on two *trans*-1,4-dihalogen*cyclo*hexanes with chlorine and bromine as substituents. The results show that in the gas phase both compounds contain nearly equal amounts of the two possible conformations (*aa* and *ee*). The energy difference between the two conformations must be less than 170 cal/mole. Structural parameters of both conformations are given in Tables 1 and 2 for the dichloro and the dibromo compounds, respectively.

The fundamental principles of the stereochemistry of *cyclo*hexane and its derivatives have been set out several years ago [1]. It is now generally accepted that the two inter-convertable forms of a particular *cyclo*hexane derivative, both showing the "staircase" six-membered carbon ring, should in general possess different internal energies and therefore, according to statistical mechanics, be present in different concentrations in an equilibrium mixture. The energy difference will depend on the kind and number of substituents and on their positions in the molecule. The first attempt at a "conformational analysis" of a *cyclo*hexane derivative was made in 1943 [2] and the substance investigated was monochloro-*cyclo*hexane in the vapour state. The electron diffraction sector method was employed, but both the apparatus and the treatment of the experimental intensity curves used at that time were less satisfactory than those in use to-day in this laboratory. From differential radial distribution curves (*i.e.* the monochloroderivative minus *cyclo*hexane itself) the conclusion was drawn that the number of molecules in the equilibrium mixture with chlorine in the equatorial positions considerably larger than the number of molecules with axial chlorine atoms. A quantitative measurement of the equilibrium constant was not attempted at that time. Some attempts have more recently been undertaken both in this laboratory and elsewhere, to determine more quantitatively the conformational equilibrium of *cyclo*hexane derivatives using infrared spectroscopy or other experimental methods, but only in the liquid state or in the semi-liquid state of crystals containing "rotating" molecules [3]. The intermolecular forces can change the ratio of the con-

formations in condensed systems and we have therefore thought it worth while to try to determine equilibrium ratios in gaseous systems using modern electron diffraction technique. Considerable improvements have indeed been introduced since 1943 both in experimental technique and in the methods of treating the experimental data.

We have chosen *trans*-1,4-dichloro-*cyclo*hexane and the corresponding dibromo compound for our experiments. X-Ray crystallographic investigations have shown that these two substances are both diequatorial in the solid state. Infrared absorption spectra seem to indicate, however, that both conformations are present in liquid systems [4].

The measurements were carried out using the new Oslo electron diffraction apparatus [5] with distances of 19 and 48 cm between the nozzle and the photographic plate. The intensity data extended from $s = 1.25$ to $s = 45$. The data were treated in the manner described in a series of papers from this institute published after 1955. The radial distribution curves thus obtained are shown in Figs. 1(a) and 2(a).

RESULTS

Trans-1,4-dichlorocyclohexane

The radial distribution curve shown in Fig. 1(a) was obtained using a damping factor of 0.0015. The full arrows show where the C(3)-Cl, C(4)-Cl and Cl—Cl distances of the *ee* form would be expected and the broken arrows show where the same distances of the *aa* form would cause peaks. These distances are calculated on the basis of strictly tetrahedrally-directed carbon valences with bond distances: C—C = 1.53, C—Cl = 1.81 and C—H = 1.10 Å obtained from the positions of the first three peaks. The Cl—Cl peaks at 5.236 and 6.318 Å show that both conformations must be present in similar proportions.

Theoretical radial distribution curves were constructed from Gauss curves for both the *ee* and *aa* conformations. It then became apparent that structures based on the strictly tetrahedral carbon atom would not give a composite theoretical radial distribution curve which would agree satisfactorily with the experimental curve. At this time parallel work in our department [6] dealing with *cyclo*hexane itself showed that the carbon ring in the parent hydrocarbon is more "flat" than would be expected if the C—C—C angle were strictly tetrahedral (109° 28′). The value actually obtained from *cyclo*hexane (111.5°), was used to calculate revised theoretical radial distribution curves for the two conformations of *trans*-1-4-dichloro*cyclo*hexane.

In the case of the *ee* conformation the new curve appeared to agree with the experimental result, but a certain distortion of the *aa* model was necessary in order to obtain a close agreement with the experimental curve. This distortion was a tilting of the C—Cl bond away from the chief axis of the carbon ring. An angle of 6.3° between the C—Cl bonds and the axis turned out to give the best agreement with the experimentally observed maxima for which the *aa* conformation must be responsible. This tilting of the bonds of axial chlorine atoms agrees closely with earlier results from electron diffraction measurements of the *cyclo*hexane derivatives [7].

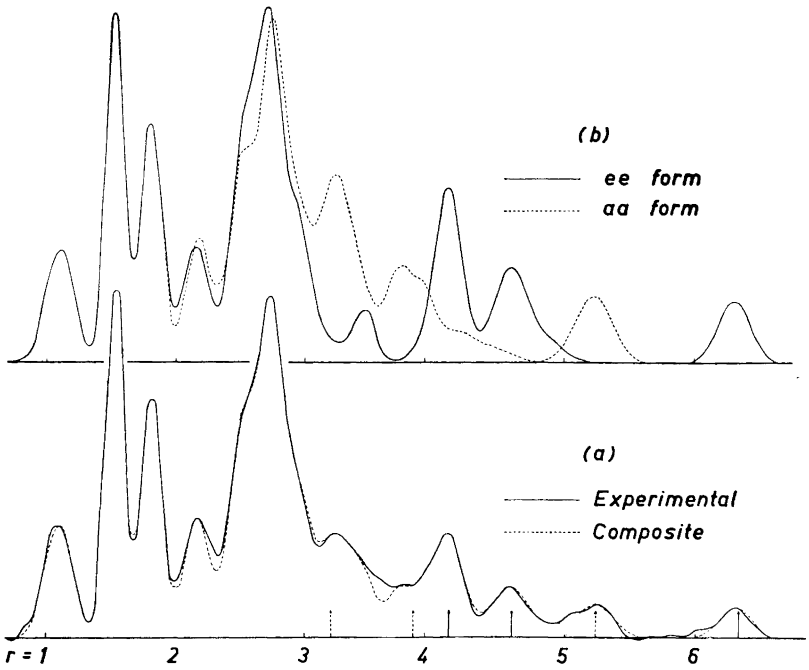


Fig. 1. Theoretical and experimental $\sigma(r)/r$ curves for *trans*-1,4-dichloro*cyclo*hexane.

Fig. 1(b) shows the theoretical radial distribution curves for both conformations based on the structural parameters in Table 1.

Least squares computations lead to the conclusion that the composite theoretical radial distribution curve giving best agreement with the experimental curve should be constructed on the basis of 49 % of the *ee* form and 51 % of the *aa* form. In Fig. 1(a) this composite radial distribution curve is compared with the experimental curve. The standard deviation is $\pm$ 6, *i.e.* 1.5 % of the vertical scale used.

Trans-1,4-dibromo*cyclo*hexane

Fig. 2(a) shows the experimental radial distribution curve obtained for this compound using a damping factor of 0.0036. The shape is similar to that of Fig. 1(a) showing that both conformations are again present in similar proportions.

The peak at 1.12 Å due to the C—H bond distance is lower than expected on theoretical grounds. This is probably due to some error in the selection of the experimental background to the intensity curve. However, this discrepancy, in a region where the *aa* and *ee* radial distribution curves are identical should not affect the conclusions, drawn from the other ends of the curves, about the proportions in which the two forms are present.

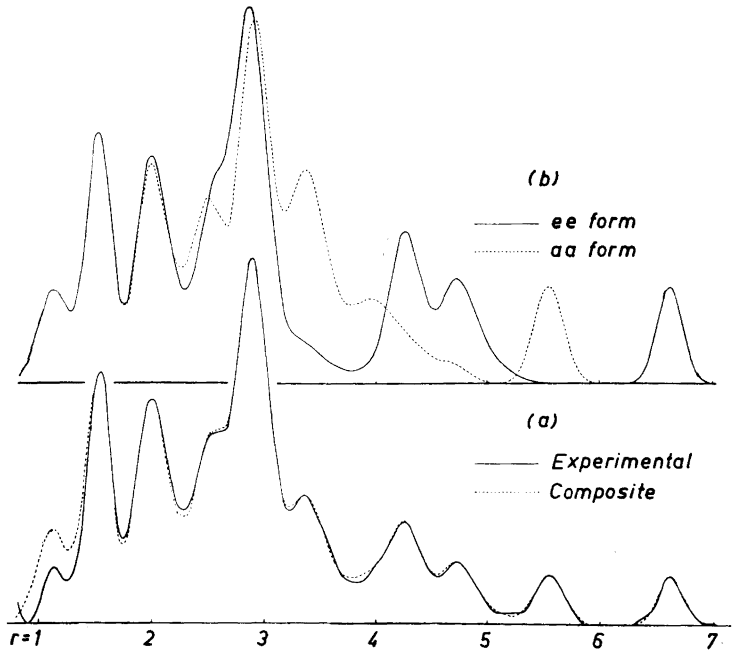


Fig. 2. Theoretical and experimental $\sigma(r)/r$ curves for *trans*-1,4-dibromo*cyclo*hexane.

Fig. 2(b) shows the theoretical radial distribution curves for the two conformations (*ee* and *aa*), based on the structural parameters given in Table 2. These parameters correspond to the same "flat" ring as was used for the chloro-compound. In the case of the *aa* form, there is again an extra distortion which tilts the bonds of the axial bromine atoms away from the main axis by an angle of 7.7°.

Least squares procedure showed that the composite radial distribution curve giving the best agreement with the experimental curve should have 51 % *ee* form and 49 % *aa* form. In Fig. 2(a) this composite theoretical curve is compared with the experimental curve. The standard deviation for the latter part of the curve (where $r > 1.5$) is $\pm$ 4.

DISCUSSION

The present conformational analyses clearly show that the amounts of the two forms present in the vapour state must be nearly equal. This result is contrary to that expected in the light of the old work on monochloro*cyclo*hexane [2].

The accuracy of the figures obtained clearly depends on the parameters chosen for the computation of the theoretical radial distribution curves for the *ee* and *aa* conformations. It appears very probable that small changes of the

Table 1. Structural parameters used for *trans*-1,4-dichloro*cyclo*hexane.

Bond distances used for both conformations		Bond angles	*ee* form	*aa* form
C—H	1.102 Å	C—C—C	111.5°	111.5°
C—C	1.530 Å	C—C—Cl = C—C—H	108.6°	110.6°
C—Cl	1.810 Å	H—C—Cl = H—C—H	111.5°	107.6°

Table 2. Structural parameters used for *trans*-1,4-dibromo*cyclo*hexane.

Bond distances used for both conformations		Bond angles	*ee* form	*aa* form
C—H	1.12 Å	C—C—C	111.5°	111.5°
C—C	1.530 Å	C—C—Br = C—C—H	108.6°	111.2°
C—Br	1.968 Å	H—C—Br = H—C—H	111.5°	100.1°

models would give slightly different theoretical radial distribution curves, which might have been combined in a slightly different proportion to give a composite curve which would have agreed equally well with the experimental curve. For this reason we assess our possible error in the ratio of the two conformations as high as ± 5 %. Even allowing for this error, the maximum energy difference between the two conformations obtained from Boltzmann's relation, is only 170 cal/mole.

The structural parameters employed when calculating the radial distribution curves are based on identical bond lengths in the two interconvertible conformations. There appears to be no reason to expect a difference in bond distances which could be of any significance for the present investigation. The distances recorded are taken from individual peaks whose positions can be assessed with an accuracy of ± 0.5 %. The value of the C—H bond distance for the bromine compound is associated with the major discrepancy between the theoretical and experimental curves and its error is estimated as ± 2 %.

The accuracy of the bond angles recorded in Tables 1 and 2 is more difficult to assess. The angles were chosen so that the theoretical and experimental radial distribution curves should show the best possible agreement. The influence of the bond distances involving hydrogen atoms on these curves is much less than that of the other distances. Therefore, only the C—C—C, C—C—Cl and C—C—Br angles recorded have real significance for our problem. Their accuracy is put at ± 0.5 %.

In both compounds it was necessary to assume that the axial bonds to halogen atoms were tilted away from the chief axis of the ring. The value of 6.3° for this deviation in the case of the dichloro compound agrees well with the 7° deviation assessed for 1*a*,2*a*,4*e*,5*e*-tetrachloro*cyclo*hexane [7], and for "*trans*"-1,2-dichloro*cyclo*hexane [8].

This tilting is ascribed to steric hindrance between the axial halogen and the axial hydrogens attached to C(3) and C(5). It is significant that, in both

trans-1,4-dihalogeno-*cyclo*hexanes, the effect of this deformation is to increase the H(3*a*)—Hal(*a*) and H(2*e*)—Hal(*a*) distances to approximately the same value. The actual distances were:

H(3*a*)—Cl(*a*) = 2.88 Å	H(2*e*)—Cl(*a*) = 2.86 Å
H(3*a*)—Br(*a*) = 3.00 Å	H(2*e*)—Br(*a*) = 3.05 Å

These are just within the limits expected from consideration of the van der Waal's radii (H = 1.2; Cl = 1.8 ; Br = 1.95 ± 0.1 Å)[9].

We wish to express our sincere gratitude to Mr. Arne Almenningen who was so kind as to take the electron diffraction pictures. We also wish to thank mag. scient. K. Lunde for letting us have pure samples of the compounds. Finally, thanks are due to *Norges Teknisk-Naturvitenskapelige Forskningsråd* for a postdoctorate fellowship (V.A.A.).

REFERENCES

1. Hassel, O. *Quart. Rev.* **7** (1953) 221.
2. Hassel, O. and Viervoll, H. *Tidsskr. Kjemi, Bergvesen, Metallurgi* **3** (1943) 35.
3. Yoshino, T. *J. Chem. Phys.* **23** (1955) 1974.
4. Lunde, K. *Unpublished work.*
5. Bastiansen, O., Hassel, O. and Risberg, E. *Acta Chem. Scand.* **9** (1955) 232.
6. Davis, M. *Unpublished work.*
7. Hassel, O. and Wang Lund, E. *Acta Cryst.* **2** (1949) 309.
8. Bastiansen, O., Hassel, O. and Munthe-Kaas, A. *Acta Chem. Scand.* **8** (1954) 872.
9. Pauling, L. *The Nature of the Chemical Bond*, Cornell University Press, N.Y. 1939.

Received July 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1743–1752

Studies in the Determination of Silica

II. The Separation of Phosphomolybdate from α-Silicomolybdate

LARS HENRIK ANDERSSON

Dept. of Analytical Chemistry, Institute of Chemistry, University of Uppsala, Uppsala, Sweden

Experiments described in this paper show that it is possible to separate quantitatively phosphomolybdate from α-silicomolybdate by extracting water phases with *n*-butanol or *n*-butanol-benzene mixtures under certain specified conditions.

When silica is determined spectrophotometrically as α-silicomolybdate, phosphorus in the form of orthophosphate ion interferes seriously. Among the methods used to separate the heteropolymolybdic acids of silicon and phosphorus, extraction of water phases with different organic solvents has repeatedly been investigated [1–4]. Probably because the authors cited have worked with solutions in which equilibrium between α- and β-silicomolybdates has not been reached, definite conclusions cannot always be drawn from their data.

King *et al.*[5] precipitated phosphate as ferric phosphate by the "basic ferric acetate method" prior to the determination of silica as silicomolybdate.

In this paper some experiments with the extraction of heteropolymolybdic acids of phosphorus and silicon are described. An elementary theoretical account of the extraction is also presented. Experimental data on the formation and some properties of phosphomolybdate are given as are also some experiments on the determination of phosphorus as phosphomolybdic acid extracted into organic phases.

1. EXPERIMENTAL DETAILS

For an account of some of the experimental methods and instruments the reader is referred to the first paper [6] in this series. The chemicals used were of analytical grade; the solvents, however, were also in general distilled once using a small Vigreux column.

pH measurements in solutions, in which the concentration of perchlorate ion is not low enough, can not be performed using saturated calomel electrodes with asbestos or sintered glass plugs, dipping into the solution to be measured, since $KClO_4$ will precipitate in the plug causing a drift of potential. Instead a calomel electrode with a bent-up

open tip at the end of the tube dipping into the solution was used in the case of perchloric acid solutions.

The extinction measurements were carried out in 1 cm cells of fused silica, fitted with ground in stoppers.

If not otherwise stated the experiments have been performed at 20.0°C.

2. SOME EXPERIMENTS ON THE FORMATION OF PHOSPHOMOLYBDATE IN AQUEOUS SOLUTIONS

The reaction between orthophosphate and molybdate ions to form phosphomolybdate seems to be too complex to be useful as a basis for a method for phosphorus determination. It has earlier been studied by, *e. g.*, Nydahl [7].

The following experiment shows the complex nature of the system orthophosphate-sodium molybdate-hydrochloric acid.

To two solutions, each containing 3×10^{-5} moles Na_2HPO_4, equal amounts of a Na_2MoO_4 solution was added followed by concentrated hydrochloric acid until the pH in one solution was 3.5 and in the other 0.5; they were then diluted to 50 ml in calibrated measuring flasks. By mixing suitable amounts of these two solutions a series of pH-values in the interval $0.5 \leqq pH \leqq 3.5$ could be obtained. Now varying the amount of Na_2MoO_4 taken and measuring the extinction of the solutions against suitable blanks at 400 mμ the extinction as a function of pH with Na_2MoO_4 as a variable was recorded.

The results are shown in Fig. 1, where the extinction is plotted against pH. Nowhere in the range of molybdate concentration studied is the extinction independent of that variable. Also pH plays an important role.

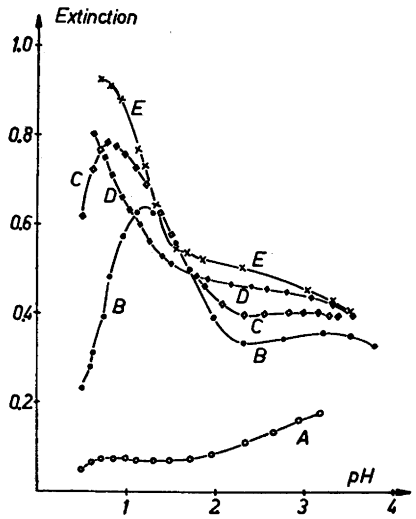


Fig. 1. Extinction of water solutions o f phosphomolybdate at 400 mμ.

curve A, $[Na_2MoO_4] = 6 \times 10^{-3}$ M
» B, » $= 1.2 \times 10^{-2}$ M
» C, » $= 3.0 \times 10^{-2}$ M
» D, » $= 1.5 \times 10^{-1}$ M
» E, » $= 0.3$ M.

3. A SIMPLIFIED THEORETICAL TREATMENT OF A CERTAIN TYPE OF EXTRACTION PROCESS

3.1. Notations and simplifying assumptions

The following notations will be used:

Concentrations in the water phase	[ ]
» » » organic »	[ ]'
The i:th acid dissociation constant of H_nA	k_i
Distribution constant for H_nA	γ

To begin with it will be assumed that the acid H_nA dissolves in the organic phase only as such (no polymerisation), dissociation taking place only in the water phase giving the ultimate product A^{-n} In this treatment also activity effects, mutual solubilities of the two phases and the influence of temperature are not taken into consideration.

3.2. The distribution of H_nA as a function of pH

By definition we have

$$k_i = \frac{[H^+]\,[H_{n-i}A^{-i}]}{[H_{n-i+1}A^{-i+1}]} \tag{1}$$

$$\gamma = \frac{[H_nA]'}{[H_nA]} \tag{2}$$

We also define an apparent distribution constant γ_a by

$$\gamma_a = \frac{[H_nA]'}{\sum_{i=0}^{n} [H_{n-i}A^{-i}]} \tag{3}$$

Putting $i = p$ in (1) and solving for $[H_{n-p}A^{-p}]$ we get, using (2)

$$[H_{n-p}A^{-p}] = \frac{1}{\gamma} \frac{\prod_{i=1}^{p} k_i}{[H^+]^p} [H_nA]' \tag{4}$$

Substituting eqn. (4) in (3) we arrive at the following expression for γ_a as a function of $[H^+]$:

$$\gamma_a = \frac{\gamma}{1 + \sum_{p=1}^{n} \frac{\prod_{i=1}^{p} k_i}{[H^+]^p}} \tag{5}$$

With fixed k_i, γ_a is therefore only a function of $[H^+]$. With the aid of eqn. (5) the function

$$\log \gamma_a = f(\mathrm{pH}) \tag{6}$$

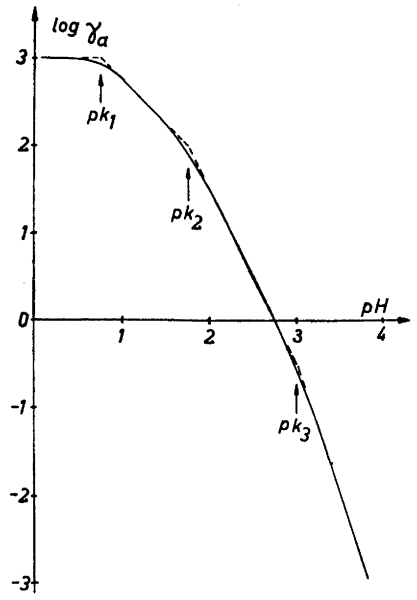


Fig. 2. Logarithm of apparent distribution constant as a function of pH.

can easily be constructed plotting log γ_a along the vertical and pH along the horizontal axis. Such a construction is shown in Fig. 2 for the hypothetical case H_3A with $pk_1 = 0.74$, $pk_2 = 1.75$, $pk_3 = 3$ and $\gamma = 10^3$. When pH$\rangle\rangle pk_3$ or when pH $\langle\langle$ pk_1 the curve approximates to straight lines; if the differences $pk_2 - pk_1$ and $pk_3 - pk_2$ are large enough, the form of the curve in the middle parts of the corresponding intervals will also be approximately straight lines with slopes -1 and $-1/2$, respectively. When pH $\rangle\rangle$ pk_3 the slope of the line is in the limit $-1/3$ and when pH $\langle\langle$ pk_1 the line is horizontal. These successive straight lines cut when pH = pk_1, pH = pk_2 and pH = pk_3, respectively.

3.3. Limits of applicability of foregoing treatment

The limits of applicability of the foregoing treatment can be summarised as follows:

a) the mass action law is strictly applicable only when activity is introduced instead of concentration.

b) the value of the distribution constant γ depends on the composition of the phases; this composition changes, *e. g.*, with the mutual solubilities of the two solvents, which depend on their salt contents.

c) polymerisation or other reactions can take place in one or both of the phases.

d) the "constants" k_i and γ are functions of temperature. In spite of these and other restrictions, experiments show that the treatment in 3.2 can to some extent be used to describe the behavior of extraction systems referred to later.

4. QUANTITATIVE EXTRACTION EXPERIMENTS

4.1. Description of experiments

The water phase containing α-silico- or phosphomolybdate, sodium molybdate, acid and when necessary additional salts was mixed in a beaker with the organic solvent using a magnetic stirrer to effect intimate phase contacts. A glass and a calomel electrode were dipped into the mixture being stirred; a reproducible pH-value could then after some stirring be read off the instrument. (pH-values measured in the way just described on solutions containing comparatively large amounts of organic compounds dissolved must only be regarded as relative, since the solvent is no longer water but a mixture of water and organic solvent.) If necessary concentrated acids were added to adjust pH to a desired value; when this was reached, the contents of the beaker were transferred to a graduated cylinder fitted with a ground glass stopper. This cylinder was placed in a thermostate and shaken now and then until equilibrium was reached. The volumes were read when the phases had separated completely; their contents of silicon and phosphorus were determined spectrophotometrically, silicon by the method described in the first paper in this series [6], phosphorus in the way described later in this paper (section 6). Finally the pH of the water phase was checked once more.

In the case of *n*-butanol-benzene mixtures a certain amount of care must be exercised if the original composition of the organic phase is to be maintained during the extraction process, since *n*-butanol and to a smaller extent benzene dissolve in the water phase. A large water phase is therefore first repeatedly shaken with new, small volumes of organic phase at a rather high pH (about = 4) until the water phase is in equilibrium with an organic phase, the composition of which is practically the same as the original organic mixture. The pH is then lowered to the desired value and the intended extractions carried out. This procedure is necessary when the water phase is more than about three times as large as the organic phase.

4.2. Extractions with *n*-butanol

4.2.1. *Preliminary notes.* The paper by Mellon and Wadelin [2] contains much information on the suitability of a number of organic solvents for the purpose of extracting heteropolymolybdic acids of phosphorus, arsenic and silicon. Some of these solvents have been tested and *n*-butanol was selected for more detailed experiments.

4.2.2. *Results of experiments.* The curves in Fig. 3 give the results obtained with water phases containing hydrochloric acid-sodium chloride. 15 ml water solution was extracted with 10 ml *n*-butanol. The water phase was 0.24×10^{-3} M in Si or P; curves B and D refer to water phases, which were also 0.6 M in NaCl. In Fig. 4 are given the extinctions of extracts from blank solutions as a function of pH.

The system nitric acid-sodium nitrate gives results which within experimental error are identical with those in Fig. 3. In the case of perchloric acid-sodium perchlorate corresponding curves are displaced about 0.2 pH units in

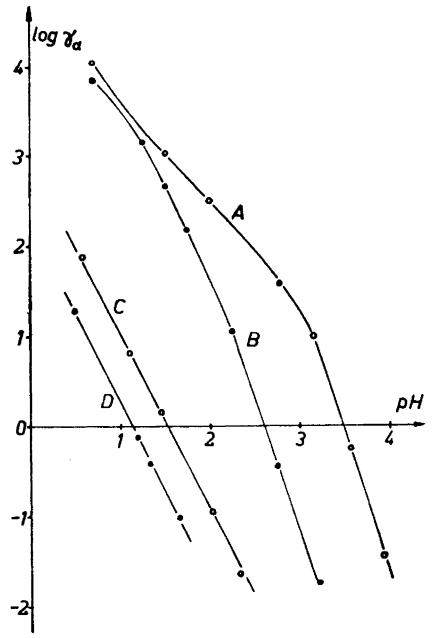


Fig. 3. Extractions with *n*-butanol from HCl—NaCl solutions.
curve A, phosphomolybdic acid,
» B, » » , water phases 0.6 M in NaCl.
curve C, silicomolybdic acid,
» D, » » , water phases 0.6 M in NaCl.

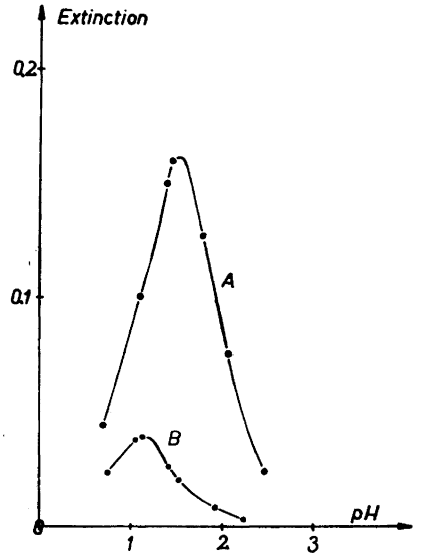


Fig. 4. Extinction as a function of wavelength for *n*-butanol extracts from blank HCl—NaCl solutions.
curve A, no NaCl added,
» B, water phase 0.6 M in NaCl.

the direction of decreasing pH. In all three cases the slopes of the lines are $-1/2$ in the case of silicomolybdic acid; phosphomolybdic acid seems to split off two protons of about the same strength in the neighbourhood of pH 3 if no extra salt has been added; below that pH-value only one proton seems to have been split off. However, all conclusions regarding the basicity of these acids, drawn from experiments such as these, must be regarded with some uncertainty.

The effect of salt additions is important. At high pH values the displacement of the phosphomolybdic acid line is greater than that of the silicomolybdic acid line for a given increase in salt concentration. This implies that when separation is to take place at say pH 2.5, it is necessary to keep the amounts of salts present to a minimum. On the other hand, when extractions are made at pH-values around 2, salt additions have about the same effect on both systems.

Another effect of adding salts is to lower the extinctions of extracts from blank solutions. This should be born in mind if phosphorus is to be determined in the organic phase. When silica is determined in the water phase the blanks

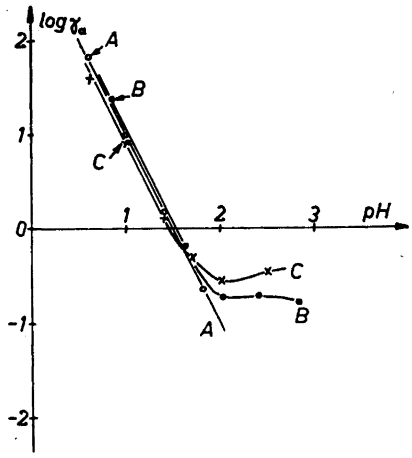


Fig. 5. Extractions with *n*-butanol of $H_2SO_4-Na_2SO_4$ water solutions.
curve A, no Na_2SO_4 added,
» B, $[Na_2SO_4]$ = 0.2 M,
» C, » = 0.4 M.

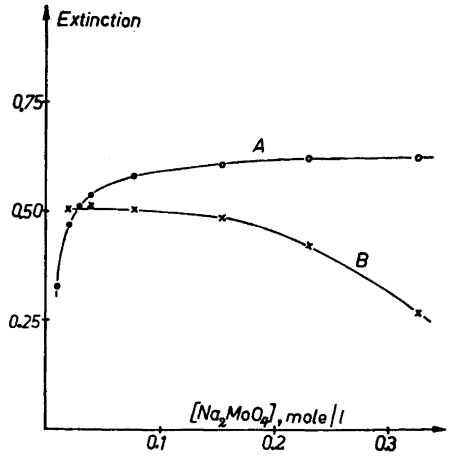


Fig. 6. Extinctions of water solutions (curve A) and extracts (curve B) of phosphomolybdic acid as functions of $[Na_2MoO_4]$.

are very low, being hardly affected if some molybdic acid has been extracted into the organic phase.

The system sulphuric acid-sodium sulphate exhibits a peculiar behaviour when silicomolybdic acid is extracted from these water phases, Fig. 5. The curves change direction when the pH has about the same value as the pK_s for HSO_4^- (2.0), the apparent distribution constants being much larger than expected. This implies that only small amounts of sulphate ions must be present if no silicomolybdic acid is to be extracted.

The results obtained in section 2 seem to show that the formation of phosphomolybdate in the experiments just described is not complete; in spite of this, however, straight lines are obtained in accordance with the simple theory in section 3. In this connection it must be pointed out that increasing the sodium molybdate concentration in the water phase has the effect of lowering the apparent distribution constant in the same way as when e.g. sodium chloride is added. This is demonstrated in the following experiment.

Water solutions, which were 0.8×10^{-3} M in Na_2HPO_4 and had a pH-value of 2.5, contained different amounts of Na_2MoO_4; 20 ml were extracted with 10 ml *n*-butanol in a beaker as described in section 4.1. The extinction of the extracts and of the original water solution were measured at 400 mμ. The results are shown in Fig. 6. Curve A demonstrates a rise in extinction, that could be predicted from the curves in Fig. 1, while on the other hand the decrease of extinction of the organic phase is evident from curve B.

4.3. Extractions with *n*-butanol — benzene mixtures

4.3.1. *Preliminaries.* To see if there was a composition of the organic mixture in question that was particularly suitable for extracting phosphomolybdic acid, the following experiment was performed.

15 ml water solution, 0.2×10^{-3} M in silicon or phosphorus, 0.02 M in Na_2MoO_4 and with the pH adjusted to 1.25 with HCl, was shaken with organic mixture (10 ml) until equilibrium was reached; by varying the composition of this organic mixture, organic phases with different extracting properties could be obtained. Extinctions of the extracts were measured at 400 mμ. Finally the pH of the water phase was checked once to see that it did not differ from the chosen value, 1.25.

In Fig. 7 the extinctions obtained are plotted along the vertical axis and the mole fraction of *n*-butanol in the organic mixture along the horisontal axis. A maximum extinction value is obtained, when the mole fraction is 0.5. The curves for silicomolybdic acid and for the blanks show that also at a mole fraction of 0.5 a separation between phosphomolybdic and silicomolybdic acids

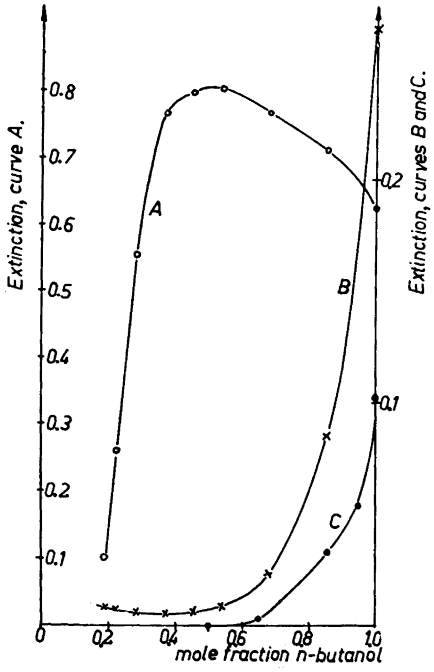


Fig. 7. Extinction of extracts as function of mole fraction *n*-butanol in the organic mixture.
curve A, phosphomolybdic acid extracted,
» B, blank solution extracted,
» C, silicomolybdic acid extracted.

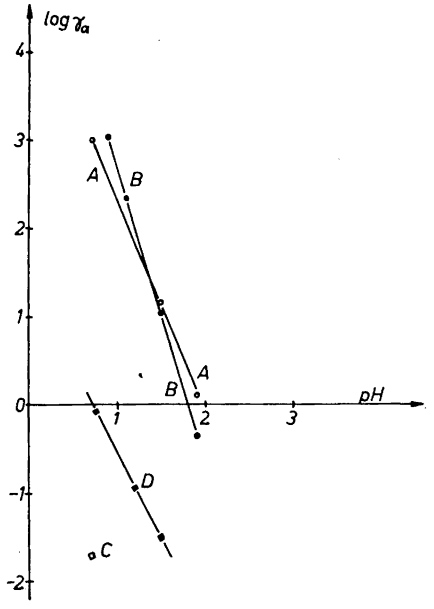


Fig. 8. Exyractions with 0.5 mole fraction *n*-butanol—benzene mixture from HCl—NaCl and H_2SO_4—Na_2SO_4 solutions.
curve A, phosphomolybdic acid,
» B, » » , water phase 0.3 M in NaCl.
point C, silicomolybdic acid,
curve D, » » , water phase 0.4 M in Na_2SO_4.

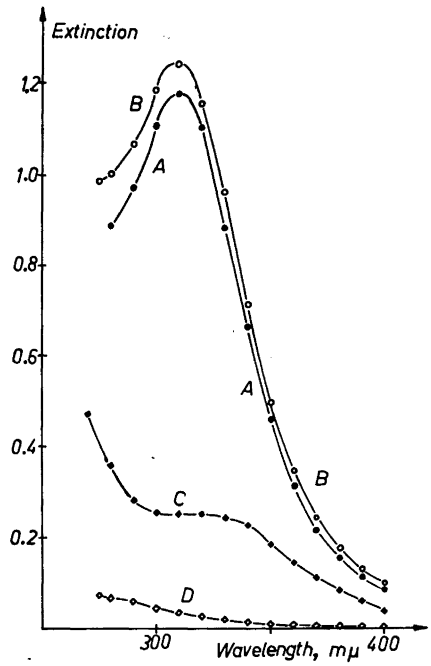


Fig. 9. Extinctions as a function of wavelength for phosphomolybdic acid in organic solvents.
curve A, extract No. 2 from Table 1, curve B, extract No. 6 from Table 1,
» C, corresponding blank, » D, corresponding blank.

is possible; at the same time a low blank is assured for the organic phase, if phosphorus is to be determined spectrophotometrically in that phase.

4.3.2. *Results of experiments.* In Fig. 8 the results of extraction experiments with water phases of the same composition as in 4.2.2 are shown. The systems with hydrochloric acid — sodium chloride and nitric acid — sodium nitrate behave in almost the same way. No extractions with perchloric acid — sodium perchlorate have been made, because at these low pH values and with organic phases containing only small amounts of water there are some risks of explosion. In the case of sulphuric acid — sodium sulphate, phosphomolybdic acid behaves in almost the same way as with hydrochloric acid — sodium chloride but silicomolybdic acid is more easily extracted: here too therefore only small amounts of sulphate should be present.

5. INFLUENCE OF TEMPERATURE

Extraction experiments with *n*-butanol and *n*-butanol — benzene mixtures showed that between 18° and 24°C only very small variations in apparent distribution constants with temperature were obtained, causing practically no changes in the curves of Figs. 3, 5 and 8.

Table 1.

No.	pH of water phase	Initial volume (ml) of water phase	org. phase	Final volume (ml) org phase	mmole P in initial 15 ml water phase	Extinction of final org. phase
			Extractions with *n*-butanol			
1	2.25	15	10	25	0.72×10^{-3}	0.050
2	2.25	15	10	25	1.20×10^{-3}	0.084
3	2.25	15	10	25	2.40×10^{-3}	0.170
4	2.25	15	10	25	3.60×10^{-3}	0.255
	Extractions with *n*-butanol-benzene mixture, mole fraction *n*-butanol = 0.5					
5	1.25	15	10	25	0.72×10^{-3}	0.060
6	1.25	15	10	25	1.20×10^{-3}	0.098
7	1.25	15	10	25	2.40×10^{-3}	0.196
8	1.25	15	10	25	3.60×10^{-3}	0.301

6. NOTE ON THE DETERMINATION OF PHOSPHORUS AS PHOSPHOMOLYBDIC ACID EXTRACTED INTO ORGANIC SOLVENTS

In connection with the determination of distribution constants for phosphomolybdic acid some experiments with the spectrophotometric determination of phosphorus were made. They are best discussed at this point.

The extractions were here carried out in separatory funnels after the pH-value of the water phase had been adjusted in the way described in section 4.1. When the phases had separated, the water phase (lower layer) was transferred to another funnel and the organic phase to a calibrated measuring flask; the water phase was once more extracted with a new portion of organic solvent. The combined extracts were diluted in the measuring flask to the mark with the corresponding solvent or solvent mixture. The extinctions were measured at 400 mμ; however, especially in the case of the 0.5 mole fraction mixture, a shorter wavelength (310 mμ, the maximum value of the extinction as a function of wavelength) can be chosen.

The results of these experiments can be found in Table 1; in Fig. 9 is also shown the extinctions as functions of wavelength for some typical extracts.

I wish to thank Dr. F. Nydahl, Head of the Department of Analytical Chemistry, for his continued interest in this investigation and for the facilities put at my disposal.

REFERENCES

1. Souchay, P. *J. Chim. Phys.* **42** (1945) 61.
2. Mellon, M. G. and Wadelin, C. *Anal. Chem.* **25** (1953) 1668.
3. Fischer, W., Rüdinger, P. and Abendroth, H. J. *Anal. Chim. Acta* **13** (1955) 38.
4. Ruf, E. *Z. anal. Chem.* **151** (1956) 169; **161** (1958) 1.
5. King, E. J., Stacy, B. D., Holt, P. F., Yates, D. M. and Pickles, D. *Analyst* **80** (1955) 441.
6. Andersson, L. H. *Acta Chem. Scand.* **12** (1958) 495.
7. Nydahl, F. *Kgl. Lantbruks-Högskol. Ann.* **10** (1942) 127–131.

Received July 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1753–1757

Addition Compounds of Phosphine Oxides

INGVAR LINDQVIST and GERD OLOFSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The formation of addition compounds with phosphine oxides as donor molecules has been studied. The following new compounds have been prepared: $SbCl_5 \cdot PO(C_6H_5)_3$, $SbCl_5 \cdot PO(CH_3)_3$, $SbCl_3 \cdot PO(CH_3)_3$, $SbCl_3 \cdot 2PO(CH_3)_3$, $AsCl_3 \cdot PO(CH_3)_3$, $AsCl_5 \cdot PO(CH_3)_3$, (stabilization of $AsCl_5$), $SeOCl_2 \cdot PO(CH_3)_3$, $HgCl_2 \cdot PO(C_6H_5)_3$, $HgCl_2 \cdot PO(CH_3)_3$, $5HgCl_2 \cdot 2PO(CH_3)_3$ and $SO_3 \cdot PO(C_6H_5)_3$.

The weak interaction between $HgCl_2$ and $PO(C_6H_5)_3$ has been revealed by a study of the infrared spectra of the compounds $HgCl_2 \cdot PO(C_6H_5)_3$ and $HgCl_2 \cdot PO(C_6H_5)_3$.

Addition compounds with phosphine oxides were described as early as 1861 by Hofmann [1] who prepared $ZnI_2 \cdot 2PO(C_2H_5)_3$. Sheldon and Tyree [2] have recently studied $SnCl_4 \cdot 2PO(C_6H_5)_3$ and $TiCl_4 \cdot 2PO(C_6H_5)_3$, and have shown that the phosphoryl stretching frequency in the infrared decreases 50—55 cm^{-1} as expected for adduct formation with the oxygen atom in $PO(C_6H_5)_3$ functioning as donor atom. The donor strength of $PO(CH_3)_3$ has been proved to be greater than that of $POCl_3$ with $SbCl_5$ as acceptor molecule [3]. In this connection we have found it interesting to investigate further the addition compounds of phosphine oxides in order to compare them with the $POCl_3$ adducts.

EXPERIMENTAL

Chemicals used. $PO(C_6H_5)_3$ was prepared according to Pickard and Kenyon [4]. Yield: 50 % calculated on $POCl_3$. Melting point +152—154°C.

$PO(CH_3)_3$ was prepared following McKee and Burg [5] but with use of $Mg(CH_3)Br$ instead of $Mg(CH_3)Cl$. Yield 75 % calculated on $POCl_3$. The colorless crystals melt at +137—139°C.

$SbCl_5$ and $SeOCl_2$ were purified by vacuum distillation.

$SbCl_3$ was sublimed directly onto a cool glass bulb placed 3 cm above the bulk of impure solid.

$AsCl_3$ and $POCl_3$ were purified by distillation and collected at +129° and 105°C.

$HgCl_2$, Merck *pro analysi*, was not further purified.

$Hg(C_6H_5)_2$ was prepared according to Pfeiffer and Truskier [6] and recrystallized from benzene. Yield 45 % calculated on $HgCl_2$. Melting point +124—125°C.

$Hg(C_6H_5)Cl$ was prepared following Nesmejanow [7]. Melting point +249—250°C.

Phase diagrams. The liquidus curves were studied by the same methods as described earlier [8]. In many cases the sublimation of $PO(CH_3)_3$ or the decomposition of the adduct compounds prevented the study of the liquidus curves in this way.

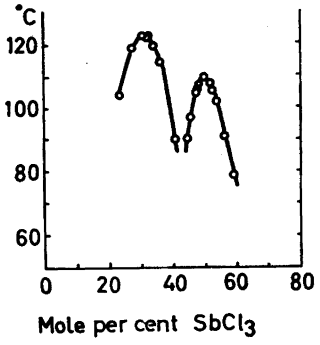


Fig. 1a. The system $SbCl_3$-$PO(CH_3)_3$.

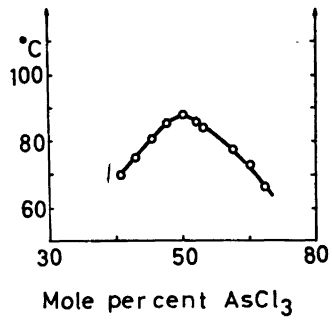


Fig. 1b. The system $AsCl_3$-$PO(CH_3)_3$.

Preparation of addition compounds. $SbCl_5 \cdot PO(C_6H_5)_3$ was precipitated from cool, dilute solutions of $SbCl_5$ and $PO(C_6H_5)_3$ in CCl_4 (mole ratio 1:1) and was recrystallized from $CHCl_3$ as almost colorless needle-shaped crystals. It is stable in air and can be melted carefully without decomposition at +196—197°C. The crystals are soluble in benzene (colorless solution), ether, chloroform or ethylene chloride. Preparation directly from $SbCl_5$ and $PO(C_6H_5)_3$ gives HCl evolution and darkly colored decomposition products (Found: Cl 31.0. Calc. 30.7.)

$SbCl_5 \cdot PO(CH_3)_3$ was precipitated as colorless crystals from a supersaturated solution in ethylene chloride. It is stable in air, melts without decomposition at +176—177°C, and can be dissolved in benzene (no color change). The thermal decomposition with evolution of HCl starts at +260°C (Found: Cl 44.7. Calc. 45.3.)

$SbCl_3 \cdot PO(CH_3)_3$ and $SbCl_3 \cdot 2PO(CH_3)_3$ were found in the phase diagram (Fig. 1a). The melting points are +110° and +124°C. Supercooling prevented the study of the system near the eutectic point. Crystals of both compounds prepared directly from the components are stable in air but we could not prepare them from CCl_4 solutions; only oil drops dispersed in the solution were obtained.

$AsCl_3 \cdot PO(CH_3)_3$ was found in the phase diagram (Fig. 1b). The melting point is +88°C. The system cannot be studied at higher phosphine oxide contents because of sublimation. The crystals can also be prepared from CCl_4 solutions. They are hygroscopic and decompose in air within a few minutes.

$AsCl_5 \cdot PO(CH_3)_3$ is formed when dry chlorine gas is led at 25°C through a liquid mixture of $AsCl_3$ and $PO(CH_3)_3$ (mole ratio 3:1). The color of the solution changes to orange, and orange crystals form upon cooling. They decompose near +50°C with loss of color. They also decompose in air with color disappearance. A rapid analysis of four different samples gave an average Cl content of 51.1 % (Calc. 51.5 %).

$SeOCl_2 \cdot PO(CH_3)_3$ was found in the phase diagram (Fig. 2a). Its melting point is +90°C.

$HgCl_2 \cdot 2PO(C_6H_5)_3$ was found in the phase diagram (Fig. 2b). The existence of the $HgCl_2 \cdot PO(C_6H_5)_3$ 1:1 compound was confirmed by powder photographs. The 1:2 compound can be precipitated from ethylene chloride solution and has been described earlier [4]. Both compounds are stable in air. The melting points are +110° and +126°C. The latter was measured with precipitated 1:2 crystals; the value +128°C found in the liquidus curve is less accurate.

$HgCl_2 \cdot PO(CH_3)_3$ and $5HgCl_2 \cdot 2PO(CH_3)_3$ were precipitated from acetone or alcohol solutions with excess of $PO(CH_3)_3$ and $HgCl_2$, respectively. They can be recrystallized with unchanged compositions. Their melting points are +137°C and +187°C. Phase diagrams cannot be studied because of the sublimation of $PO(CH_3)_3$. (Found: Hg 55.2. Calc. for $HgCl_2 \cdot PO(CH_3)_3$: 55.1.) (Found: Hg 64.9. Calc. for $5HgCl_2 \cdot 2PO(CH_3)_3$: 65.0.)

$HgCl_2$ does not give any addition compounds with $POCl_3$ as checked by us in a series of preparations.

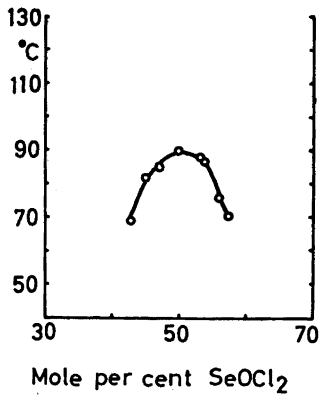


Fig. 2a. The system $SeOCl_2$-$PO(CH_3)_3$.

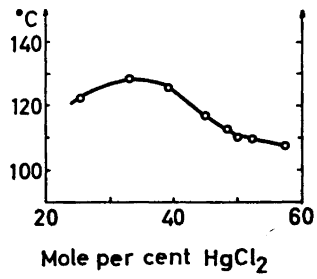


Fig. 2b. The system $HgCl_2$-$PO(C_6H_5)_3$.

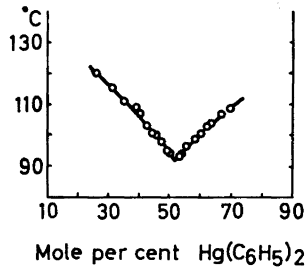


Fig. 2c. The system $Hg(C_6H_5)_2$-$PO(CH_3)$.

$Hg(C_6H_5)_2$ and $Hg(C_6H_5)Cl$ do not give any addition compounds as shown by the phase diagram for $Hg(C_6H_5)_2$ (Fig. 2c) and by repeated melting and precipitation experiments for $Hg(C_6H_5)Cl$.

$SO_3 \cdot PO(C_6H_5)_3$ was prepared by leading SO_3 through a U-tube filled with $PO(C_6H_5)_3$ dissolved in ethylene chloride. A white precipitate formed which dissolved in an excess of SO_3. It melts with decomposition at +102°C. (Found: S 8.63. Calc. 8.94.)

$SO_3 \cdot PO(CH_3)_3$ which has earlier been described by McKee and Burg [5] was also prepared and found to dissolve in an excess of SO_3.

Infrared spectra. The infrared spectra of $HgCl_2 \cdot PO(C_6H_5)_3$ and $HgCl_2 \cdot 2PO(C_6H_5)_3$ were recorded on a Perkin-Elmer Model 21 Spectrophotometer equipped with NaCl and CsBr prisms (in the Institute of Biochemistry). The crystals were dispersed in Nujol and studied between KBr and TlBr-TlI plates. The phosphoryl stretching frequency was found at 1 149 and 1 161 cm^{-1} and the antisymmetrical Hg-Cl bond stretching frequency at 352 and 345 cm^{-1} respectively. The corresponding values for pure $PO(C_6H_5)_3$ and $HgCl_2$ were 1 186 and 371 cm^{-1}.

DISCUSSION

1. In a paper on the addition compounds of sulfoxides and sulfones [9], a qualitative discussion of the cohesion energies in the solid state was attempted. The results were successful in so far as the principal predictions about the structures of the adduct molecules between $SbCl_5$ and sulfoxides or sulfones have later been confirmed by X-ray crystallography [10]. Spectroscopic investiga-

tions [11] indicate, however, that the conditions of interaction are quite complicated and that the reasonable results obtained in the paper on the sulfoxides and sulfones are rather fortuitous. This suggestion is strongly supported by the results presented here. The melting points of the compounds $SbCl_5 \cdot POCl_3$, $SbCl_5 \cdot PO(CH_3)_3$, and $SbCl_5 \cdot PO(C_6H_5)_3$ are 115, 40, and 43°C higher than the melting points of the pure donor molecules $POCl_3$, $PO(CH_3)_3$, and $PO(C_6H_5)_3$. The same argument as the one applied to $SbCl_5 \cdot SOCl_2$, $SbCl_5 \cdot SO(CH_3)_2$, *etc.* would here lead to the conclusion that the structures of $SbCl_5 \cdot POCl_3$ and $SbCl_5 \cdot PO(CH_3)_3$ have quite different bond angles at the donor atom oxygen (*cf.* Fig. 3 of Ref.[9]). We have, however, been able to show [12] that the structure of $SbCl_5 \cdot PO(CH_3)_3$ is very similar to the structure of $SbCl_5 \cdot POCl_3$[13]. The discussions of the resulting dipole moments and the melting point relations which were proposed [9] tentatively should therefore now be considered as too crude simplifications to permit any safe predictions.

2. It is difficult to express any opinion about the structures of $SbCl_3$ and $AsCl_3$ adducts. Structure determinations would be of great interest because of the labelling of the similar reactions of $SbCl_3$ or $AsCl_3$ with $POCl_3$ as weak electrostatic interaction, on the basis of the spectroscopic data [11].

3. The stabilization of $AsCl_5$ by complex formation with the strong donor molecule $PO(CH_3)_3$ was successful and can probably be achieved with other similar donors. The non-existence of free $AsCl_5$ thus seems to depend upon unfavorable energy conditions in the trigonal bipyramid $AsCl_5$, while the octahedral co-ordination is more favorable. The same difference is found with the very strong Lewis acid $SbCl_5$ but in that case the free $SbCl_5$ is also stable.

4. The importance of 1:2 adducts of $SbCl_3$ is confirmed by the existence of the compound $SbCl_3 \cdot 2PO(CH_3)_3$ in analogy with $SbCl_3 \cdot 2POCl_3$ and $SbCl_3 \cdot 2SO(CH_3)_2$[9].

5. $SeOCl_2$ on the other hand is here exceptional as an acceptor molecule in giving a 1:1 compound $SeOCl_2 \cdot PO(CH_3)_3$. The adducts with $POCl_3$ and sulfoxides cannot be prepared because of metathetical processes [8] but many 1:2 compounds are known, and the crystal structure of $SeOCl_2 \cdot 2NC_5H_5$ has recently been determined [14]. A comparative study of $SeOCl_2 \cdot PO(CH_3)_3$ would be of great interest.

6. The differences between the reactions of $PO(CH_3)_3$, $PO(C_6H_5)_3$, and $POCl_3$ with $HgCl_2$ are obvious. We have without success tried to isolate a 1:2 compound with $PO(CH_3)_3$ and a 5:2 compound with $PO(C_6H_5)_3$.

7. The interaction between $HgCl_2$ and $PO(C_6H_5)_3$ is of the same weak type as between $AsCl_3$ and $POCl_3$[11]. The spectra of $HgCl_2$ and $PO(C_6H_5)_3$ are both preserved and only slightly perturbed in the spectra of the adduct molecules. The band shifts are in the predicted direction and magnitude. The P-O bonds would thus be most perturbed for the highest mole ratio of $HgCl_2$, and the 1:1 adduct actually shows the largest decrease in the phosphoryl band position. The Hg-Cl bond on the other hand must be most perturbed in the 1:2 compound which has the highest $PO(CH_3)_3$ content, and the corresponding stretching frequency is accordingly shifted furthest in that compound.

8. The structure of $HgCl_2 \cdot 2PO(C_6H_5)$ can thus probably not be characterized by simple tetrahedral co-ordination around Hg but rather by a distorted square

co-ordination of the same type as in $HgCl_2 \cdot C_4H_8O_2$[15]. The structures of the 1:1 and 5:2 adducts are difficult to predict. They will be investigated by Mr. Brändén of this Institute as part of an extensive study on the crystal chemistry of $HgCl_2$ adduct molecules.

9. The negative results of the experiments with $Hg(C_6H_5)_2$ and $Hg(C_6H_5)Cl$ confirm earlier suggestions about the negligible or very weak acceptor properties of these compounds [16]. Strohmeier has, however, proved that $Hg(C_2H_5)_2$ has some acceptor character [17].

This work has been sponsored by the *Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Forces*, through its European Office under contract No. AF 61(052)-43. This support is gratefully acknowledged. The Hg analyses were made by Dr. Lilly Gustafsson in the Analytical Department and the infrared spectrograph was put at our disposal by Professor Tiselius. We wish to thank them and Professor Hägg, Head of the Department of Inorganic Chemistry, for friendly help.

REFERENCES

1. Hofmann, A. W. *Ann.* **1861**, Suppl. I. 2.
2. Sheldon, J. C. and Tyree, S. Y. *J. Am. Chem. Soc.* **80** (1958) 4775.
3. Kinell, P. O., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **13** (1959) 190.
4. Pickard, R. H. and Kenyon, J. *J. Chem. Soc.* **1906** 262.
5. McKee, W. and Burg, A. *J. Am. Chem. Soc.* **73** (1951) 4590.
6. Pfeiffer, P. and Truskier, P. *Ber.* **37** (1904) 1127.
7. Nesmejanow, A. N. *Ber.* **62** (1929) 1010.
8. Agerman, M., Andersson, L. H., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **12** (1958) 477.
9. Lindqvist, I. and Einarsson, P. *Acta Chem. Scand.* **13** (1959) 420.
10. Hansson, A. and Claesson, M. *To be published.*
11. Kinell, P. O., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **13** (1959) 1159.
12. Brändén, C. I. and Lindqvist, I. *To be published.*
13. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134; *Acta Cryst.* **12** (1959) 642.
14. Lindqvist, I. and Nahringbauer, G. *Acta Cryst.* **12** (1959) 638.
15. Hassel, O. and Hvoslef, J. *Acta Chem. Scand.* **8** (1954) 1953.
16. Coates, G. E. *Organo-Metallic Compounds*, Methuen, London 1956.
17. Strohmeier, W. and Hümpfner, K. H. *Z. Elektrochem.* **60** (1956) 1111.

Received July 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1758–1760

Addition Compounds of Diphosphorus Trioxide Tetrachloride

INGVAR LINDQVIST, MARGARETA ZACKRISSON and SIGVARD ERIKSSON

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

The formation of addition compounds with diphosphorus trioxide tetrachloride as donor molecule has been studied. The following new compounds have been prepared: $2SbCl_5 \cdot P_2O_3Cl_4$ and $SnCl_4 \cdot P_2O_3Cl_4$, and their infrared spectra have been recorded.

It has been established by a series of X-ray crystallographic structure determinations [1,2] that in phosphoryl chloride $POCl_3$, the oxygen atom functions as the donor in adduct formation. We have been interested in finding out how the related diphosphorus trioxide tetrachloride, $P_2O_3Cl_4$, reacts with typical acceptor molecules (Lewis acids) such as $SbCl_5$ and $SnCl_4$. The molecular structure of $P_2O_3Cl_4$ is not known, but it can safely be predicted to contain an oxygen bridge between the two phosphorus atoms. The bridge oxygen cannot be expected to function as a donor atom as efficiently as the oxygen atoms of the two phosphoryl groups. A study of the compositions of the addition compounds formed was supposed to give some information about this problem.

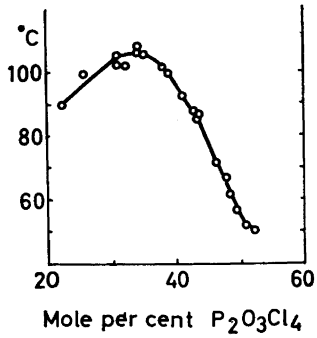


Fig. 1a. The system $SbCl_5-P_2O_3Cl_4$.

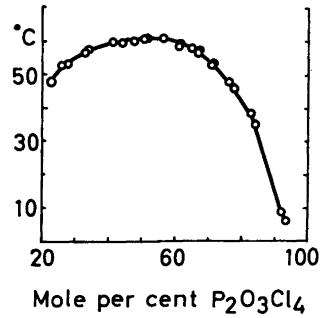


Fig. 1b. The system $SnCl_4-P_2O_3Cl_4$.

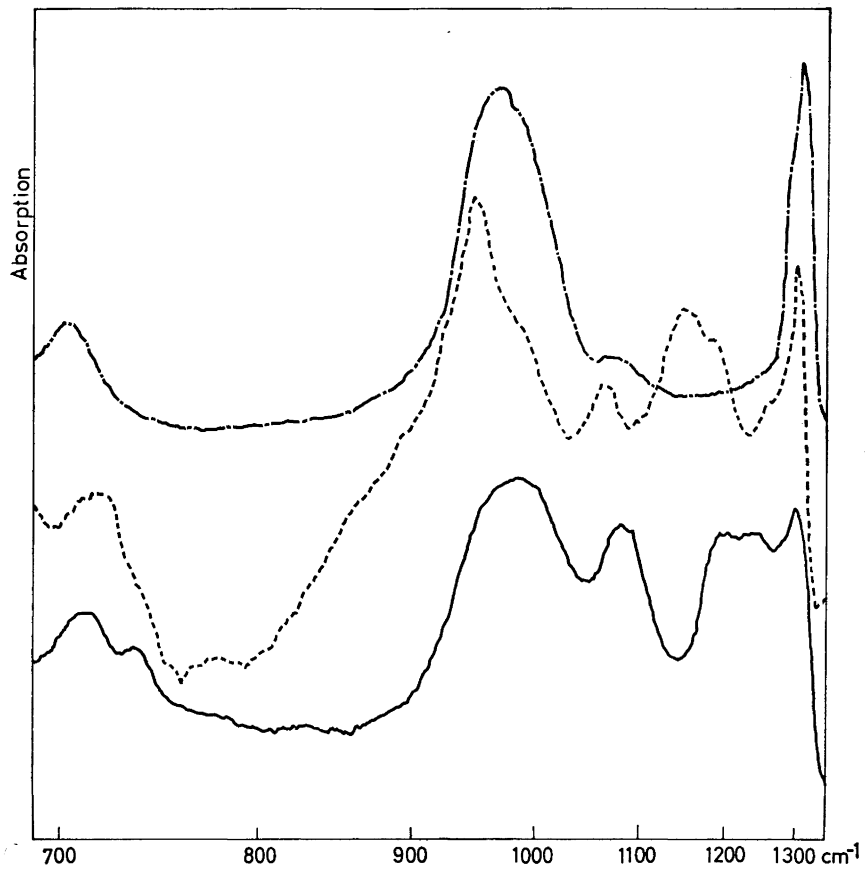


Fig. 2. The IR spectra of $P_2O_3Cl_4$ (broken line), $2SbCl_5 \cdot P_2O_3Cl_4$ (dashed line) and $SnCl_4 \cdot P_2O_3Cl_4$ (full line). In order to obtain a clearer picture the spectra have been arbitrarily displaced along the absorption axis.

EXPERIMENTAL

$P_2O_3Cl_4$ was prepared according to Grunze [3]. $SbCl_5$ and $SnCl_4$ were purified by vacuum distillation. Liquidus curves were studied by a method described earlier [4]. Infrared spectra were recorded on a Perkin-Elmer Model 21 Spectrophotometer equipped with a NaCl prism (in the Institute of Biochemistry). The spectrum of $P_2O_3Cl_4$ was run with the liquid in a thin layer between two NaCl plates. The spectra of the addition compounds were run in Nujol between NaCl plates.

RESULTS

1. The liquidus curve of the system $SbCl_5$-$P_2O_3Cl_4$ (Fig. 1*a*) shows a maximum at the composition $2SbCl_5 \cdot P_2O_3Cl_4$ where the entire liquid phase solidifies. The melting point is $+107-109°C$. At higher mole ratios of $P_2O_3Cl_4$ the study is prevented by strong super-cooling.

2. The liquidus curve of the system $SnCl_4$-$P_2O_3Cl_4$ (Fig. 1*b*) has one rather flat maximum at the composition $SnCl_4 \cdot P_2O_3Cl_4$. The melting point is +72°C.

3. The infrared spectra (in the region 7.5—14.5 μ) are shown in Fig. 2.

DISCUSSION

1. The existence of the compounds $2SbCl_5 \cdot P_2O_3Cl_4$ (*cf.* $SbCl_5 \cdot POCl_3$) and $SnCl_4 \cdot P_2O_3Cl_4$ (*cf.* $SnCl_4 \cdot 2POCl_3$) clearly indicates that in $P_2O_3Cl_4$ the oxygen atoms of both phosphoryl groups but not the bridge oxygen can function as donor atoms. In $2SbCl_5 \cdot P_2O_3Cl_4$ two molecules of $SbCl_5$ would thus be attached to the same molecule of $P_2O_3Cl_4$. In $SnCl_4 \cdot P_2O_3Cl_4$ two alternatives are possible. One possibility is that the two oxygen donor atoms in $P_2O_3Cl_4$ are bonded to the same molecule of $SnCl_4$ in *cis*-position (*cf.* the *cis*-structure of $SnCl_4 \cdot 2POCl_3$[2]). Another possibility is that two different $SnCl_4$ molecules interact with the same molecule of $P_2O_3Cl_4$. The structure would then not contain discrete molecular units of the addition compound.

2. The analysis of the spectrum of $P_2O_3Cl_4$ is very complicated, but we had hoped to be able to identify at least the phosphoryl bond stretching frequency by comparison with the spectra of the addition compounds. We do not feel competent, however, to attempt a correlation. Splitting and decrease of the phosphoryl frequency have probably both taken place as earlier found with $POCl_3$[5], but in this case the complex nature of the pure $P_2O_3Cl_4$ spectrum makes the interpretation more difficult.

This work has been sponsored by the *Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Forces,* through its European Office under contract No. AF 61(052)-43. This support is gratefully acknowledged. The infrared spectrograph was put at our disposal by Professor Tiselius. We wish to thank him and Professor Hägg, Head of the Department of Inorganic Chemistry for friendly help.

REFERENCES

1. Lindqvist, I. and Brändén, C. I. *Acta Chem. Scand.* **12** (1958) 134; *Acta Cryst.* **12** (1959) 642.
2. Brändén, C. I. and Lindqvist, I. *Two papers to be published.*
3. Grunze, H. *Z. anorg. allgem. Chem.* **298** (1959) 152.
4. Agerman, M., Andersson, L.-H., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **12** (1958) 477.
5. Kinell, P. O., Lindqvist, I. and Zackrisson, M. *Acta Chem. Scand.* **13** (1959) 1159.

Received July 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1761–1766

On the Mechanism of the Formation of Hyodeoxycholic Acid in the Pig

Bile Acids and Steroids 89

SUNE BERGSTRÖM, ÅKE GÖRANSSON and BENGT SAMUELSSON

Department of Chemistry, Karolinska Institutet, Stockholm, and Djursjukhuset, Hälsingborg, Sweden

The mechanism of the formation of hyodeoxycholic acid has been studied in the pig with chenodeoxycholic acid-7β-^{3}H, 24-^{14}C. This acid was injected intraperitoneally into pigs and a bile fistula was prepared three days later. By this procedure the administered acid is hydroxylated in the liver to hyocholic acid (3α,6α,7α-trihydroxycholanic acid), which is then dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) by the microorganisms in the intestine. Determination of the ^{3}H and ^{14}C content of chenodeoxycholic, hyocholic and hyodeoxycholic acids, isolated from the bile, showed that the ^{3}H-label is retained during the hyodeoxycholic acid formation. The mechanism of the elimination of the 7α-hydroxyl group is discussed.

The main bile acids in the normal pig bile are chenodeoxycholic, hyocholic and hyodeoxycholic acids, which occur conjugated mainly with glycine but also to a small extent with taurine [1]. The metabolism of the above mentioned bile acids in the pig has recently been investigated in our laboratories [2]. It was found that chenodeoxycholic acid (3α,7α-dihydroxycholanic acid) is 6α-hydroxylated to hyocholic acid (3α,6α,7α-trihydroxycholanic acid) in the liver, and the latter acid is then dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) during the enterohepatic circulation. Hyodeoxycholic acid is not rehydroxylated in the liver. Immediately after the preparation of a bile fistula all three acids are present in the bile. However, hyodeoxycholic acid gradually vanishes and after two days only chenodeoxycholic and hyocholic acids are present [2]. Furthermore, after administration of cholesterol-4-^{14}C to a bile fistula pig the only labelled acidic products excreted in the bile are chenodeoxycholic and hyocholic acids [3]. All these experiments demonstrate

that hyodeoxycholic acid is formed secondarily from hyocholic acid in the intestine and exclude that it may be synthesized primarily in the liver.

The aim of the present investigation was to study the mechanism of hyodeoxycholic acid formation with the aid of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C.

MATERIAL AND METHODS

Labelled acids: Chenodeoxycholic acid 7β-^{3}H was prepared by reducing 7-ketolithocholic acid with tritium-labelled sodium borohydride as described previously [4]. Specific activity: 0.5 μC/mg.

Chenodeoxycholic acid-24-^{14}C was prepared according to the method of Bergström *et al.*[5] Specific activity: 10 μC/mg.

A stock solution of chenodeoxycholic acid-7-β-^{3}H, 24-^{14}C was prepared by dissolving 21.0 mg of chenodeoxycholic acid-7-β-^{3}H and 0.5 mg of chenodeoxycholic acid-24-^{14}C in acetone. The ratio between tritium and ^{14}C of this acid was 1.13, when determined by the gas phase counting procedure.

Animal experiments. 10 mg of doubly labelled chenodeoxycholic acid was injected intraperitoneally as the sodium salt in 0.9 % aqueous sodium chloride into each of two young female pigs weighing about 20 kg. The bile fistulas were prepared three days after the injection of the labelled bile acid, according to the technique described earlier [3], and bile collection in ethanol immediately started.

Fractionation of the bile. The bile from 5 h collection periods was filtered, evaporated to dryness and hydrolyzed with 150 ml of 2 N KOH at 120° for 6 h in a sealed flask. The free bile acids were extracted from the acidified solution with ether.

Chromatographic separations. Reversed phase partition chromatography as described by Bergström, Norman and Sjövall [6,7] was used for separation of the free bile acids. The following solvent systems were used:

System	Moving phase	ml	Stationary phase	ml
C[6]	Methanol:water	150:150	Chloroform:*iso*octanol	15:15
F[7]	Methanol:water	165:135	Chloroform:heptane	45:5

3 ml of the stationary phase was supported on 4.5 g of Hostalene (Farbwerke Hoechst G.m.b., West Germany) [8]. Hostalene was purified before use by extraction with 95 % ethanol for 48 h in a Soxhlet extractor and then dried at 75°. All the chromatograms were run at a constant temperature of +23°.

Isotope analyses. The administered and isolated bile acids were combusted to CO_2 and water. The water was converted to butane with butyl magnesium bromide and the ^{14}C and ^{3}H of the CO_2 and butane was determined by gas phase counting as described by Glascock [6]. Two determinations were made on each bile acid sample.

RESULTS

Chenodeoxycholic acid 7β-^{3}H, 24-^{14}C was injected intraperitoneally into two pigs and bile fistulas were prepared three days after the injection. It has previously been demonstrated that chenodeoxycholic acid is hydroxylated at the 6α-position to hyocholic acid (3α,6α,7α-trihydroxycholanic acid) in the liver. The latter acid is dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) by the intestinal microorganisms during the enterohepatic circulation. By the above-mentioned procedure it was thus expected that the injected doubly labelled chenodeoxycholic acid would be converted into both hyocholic and hyodeoxycholic acids.

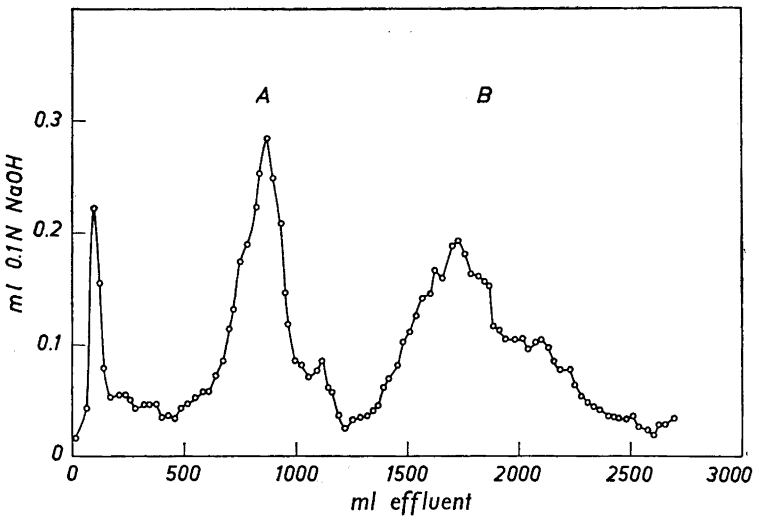


Fig. 1. Chromatographic separation of acids from hydrolyzed pig bile, excreted during 5h following the preparation of the bile fistula. Chenodeoxycholic acid-7β-^{3}H,24-^{14}C was administered 3 days before the operation. Column: 45 g Hostalene. Solvent system: Type C.

The hydrolyzed bile was chromatographed with solvent system C. In Fig. 1 is shown a chromatogram of the bile acids excreted during 5 h immediately following the operation (Pig I).

The material in the fractions of the peak (A. 650—1 000 ml effluent) which has the elution volume characteristic of hyocholic acid, was crystallized from aqueous acetic acid and aqueous acetone. Yield: 141.6 mg of hyocholic acid, m. p. 184—185°. (Lit.[10] m. p. 188—189°.) The second peak of the chromatogram shown in Fig. 1 (B. 1 350—2 400 ml effluent) was rechromatographed with solvent system F (Fig. 2), which is suitable for separation of dihydroxycholanic acids. Two major peaks appear, of which the first one (150—290 ml effluent) is eluted with the elution volume of hyodeoxycholic acid and the second one (300—410 ml effluent) with that of chenodeoxycholic acid. The material in these peaks was crystallized from ethyl acetate and ethylacetate/light petroleum, respectively, yielding 135.7 mg of hyodeoxycholic acid m. p. 198—199°, (Lit.[10] m. p. 197—198°) and 95.8 mg of chenodeoxycholic acid, m. p. 140—141° (Lit.[11] m. p. 140—142°).

The ^{3}H and ^{14}C content of the administered chenodeoxycholic acid 7β-^{3}H, 24-^{14}C and the isolated chenodeoxycholic, hyocholic and hyodeoxycholic acids from two pigs was determined. From the results of these determinations (Table 1) it is obvious that the original ^{3}H-labelling in chenodeoxycholic acid is retained both in hyocholic acid and in hyodeoxycholic acid.

Hyodeoxycholic acid, isolated from the bile, was oxidized with chromic acid in aqueous acetic acid at 2—3° to 3α-hydroxy-6-ketocholanic acid (m.p. 156—160°) [12] which had the same ^{3}H content as the starting material (Table 1).

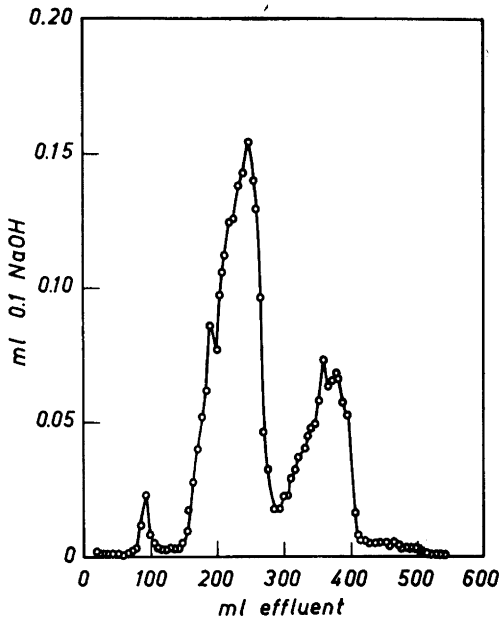


Fig. 2. Chromatographic separation of peak B of the chromatogram, shown in Fig. 1. Column: 22.5 g Hostalene. Solvent system: Type F.

Table 1.

Compound	^3H, c.p. m/mg	^3H, c.p. m/mg mean	^{14}C, c.p. m/mg	^{14}C, c.p. m. mean	$^3H^{14}/C$	Per cent ^3H retained
Administered chenodeoxy-	220		192			
cholic acid-7α-^3H, 24-^{14}C	215	218	194	193	1.13	
Chenodeoxycholic acid, Pig I	29		25			
» » Pig I	25	27	25	25	1.08	96
» » Pig II	19		17			
» » Pig II	20	20	17	17	1.18	104
Hyocholic acid, Pig I	75		76			
» » Pig I	83	79	73	75	1.05	93
» » Pig II	93		78			
» » Pig II	87	90	79	79	1.14	101
Hyodeoxycholic acid, Pig I	58		53			
» » Pig I	56	57	58	56	1.02	90
» » Pig II	74		73			
» » Pig II	76	75	74	74	1.01	89
3α-Hydroxy-6-keto	72		69			
cholanic acid	71	72	70	70	1.03	91
3α-Hydroxy-6-keto allo-	5		70			
cholanic acid	2	4	73	72	0.06	5

The keto acid (200 mg) was dissolved in 15 ml 2 N KOH and refluxed for 24 h. The product was crystallized from ethylacetate yielding 112 mg of 3α-hydroxy-6-keto allocholanic acid (m. p. 187—188°) [12]. This acid contained only 5 % of the original ^3H labelling.

DISCUSSION

The formation of hyodeoxycholic acid in the pig involves elimination of the 7α-hydroxyl group from hyocholic acid, which is formed in the liver through 6α-hydroxylation of the primarily synthesized chenodeoxycholic acid.

This type of elimination reaction was originally demonstrated in the rabbit [13], in which the main bile acid, deoxycholic acid, is formed by dehydroxylation of cholic acid during the enterohepatic circulation. The same reaction has also been found in man [14] and in the rat [15]. The elimination of the hydroxyl group in hyocholic acid as well as in cholic acid is carried out by the microorganisms in the intestine. The mechanism of the elimination of the 7α-hydroxyl group has been studied in the rabbit with the aid of 7β-tritio-cholic acid [16]. The fact that the tritium label was retained in the deoxycholic acid excluded that an intermediate ketone formation occurs, although 7-keto-deoxycholic acid was rapidly converted into deoxycholic acid. This makes it probable that the dehydroxylation consists of a dehydration-reduction reaction sequence or a direct elimination. As 7α-hydroxylation of the formed deoxycholic acid in the rat liver resulted in complete elimination of the tritium label it appears probable that it had been moved into the 7α-position. In the present investigation the formation of hyodeoxycholic acid through an analogous reaction was studied with chenodeoxycholic acid-7β-^3H, 24-^{14}C. Determination of ^3H and ^{14}C in the administered and isolated chenodeoxycholic acid and isolated hyocholic and hyodeoxycholic acids showed that the ^3H-label is retained in the molecule during these transformations, *i. e.* hydroxylation at C6 and dehydroxylation at C7. The ^3H-label was still present in 3α-hydroxy-6-ketocholanic acid, formed by chromic acid oxidation of hyodeoxycholic acid, but could be eliminated by a base-catalyzed exchange of the keto-derivative, indicating that the ^3H-label was retained at C7 in hyodeoxycholic acid. The available data do not indicate if the label was in the 7α- or 7β-position (*cf.* above).

The most probable mechanisms for the elimination reaction, which are consistent with the isotopic experiments, are summarized below.

1) Direct elimination of the hydroxyl group at C7.
2) a) Dehydration with the formation of a Δ^6 or Δ^7-double bond.
   b) Reduction of this double bond.
3) a) Dehydration to the Δ^6-unsaturated derivative.
   b) Enol-ketone-rearrangement to 3α-hydroxy-6-ketocholanic acid.
   c) Reduction of the 6-ketoderivative to hyodeoxycholic acid.

The first two mechanisms have been discussed previously for the elimination of the 7α-hydroxyl group from cholic acid. Nor is it possible to differentiate between these two possibilities by the experiments reported in this paper.

In the hyodeoxycholic acid formation, however, a third mechanism cannot be excluded. Dehydration of hyocholic acid to the Δ^6-unsaturated derivative entails the formation of a compound with an enol configuration that might very easily rearrange to the ketone. Further work is in progress to clarify the above-mentioned questions.

Acknowledgements. We are very grateful to Dr. Fritz Sevelius, Djursjukhuset, Hälsingborg, for the facilities and help placed at our disposal. The technical assistance of Miss B. Turedotter, Miss B. Holmberg and Mr. S. Jönsson is gratefully acknowledged.

This work is part of investigations supported by *Knut och Alice Wallenbergs Stiftelse*, Sweden, and the *National Heart Institute, National Institutes of Health*, Bethesda, Maryland, USA (H 2842).

REFERENCES

1. Haslewood, G. A. D. and Ogan, A. U. *Biochem. J.* **67** (1957) 30 P.
2. Bergström, S., Danielsson, H. and Göransson, Å. *Acta Chem. Scand.* **13** (1959) 776.
3. Samuelsson, B. *J. Biol. Chem.* **234** (1959) 2852.
4. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 976.
5. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
6. Bergström, S. and Norman, A. *Proc. Soc. Exptl. Biol. Med.* **83** (1953) 71.
7. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
8. Wiss, O. and Gloor, U. *Z. physiol. Chem. Hoppe-Seyler* **310** (1958) 260.
9. Glascock, R. *Isotopic Gas Analysis for Biochemists.* Acad. Press Inc. N.Y. 1954.
10. Haslewood, G. A. D. *Biochem. J.* **62** (1956) 637.
11. Fieser, L. F. and Rajagopalan, S. *J. Am. Chem. Soc.* **72** (1950) 5530.
12. Wieland, H. and Dane, E. *Z. physiol. Chem. Hoppe-Seyler* **212** (1932) 41.
13. Lindstedt, S. and Sjövall, J. *Acta Chem. Scand.* **11** (1957) 421.
14. Lindstedt, S. *Arkiv Kemi* **11** (1957) 145.
15. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
16. Bergström, S., Lindstedt, S. and Samuelsson, B. *J. Biol. Chem.* **234** (1959) 2022.

Received July 8, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1767–1774

High Temperature X-Ray Study of the Thermal Expansion of PtS_2, $PtSe_2$, $PtTe_2$ and $PdTe_2$

ARNE KJEKSHUS and FREDRIK GRØNVOLD

Kjemisk Institutt A, Universitetet i Oslo, Blindern, Norway

PtS_2, $PtSe_2$, $PtTe_2$ and $PdTe_2$, all with structures of the $Cd(OH)_2$-type and $c/a < 1.633$, have been studied by the X-ray powder method at temperatures in the range from 20 to 950°C. The thermal expansion is considerably anisotropic for all four compounds. For the a-axis it is almost linear over the whole temperature range, with linear thermal expansion coefficient, β_a, equal to 2.3×10^{-6} °C^{-1} for PtS_2 (20–562°C), 5.7×10^{-6} °C^{-1} for $PtSe_2$ (20–946°C), 5.8×10^{-6} °C^{-1} for $PtTe_2$ (20–870°C) and 12.0×10^{-6} °C^{-1} for $PdTe_2$ (20–574°C). For the c-axis the thermal expansion is approximately linear at lower temperatures only, and the expansion coefficient, β_c, equal to 37×10^{-6} °C^{-1} for PtS_2 (20–250°C), 20×10^{-6} °C^{-1} for $PtSe_2$ (20–283°C), 27×10^{-6} °C^{-1} for $PtTe_2$ (20–591°C) and 30×10^{-6} °C^{-1} for $PdTe_2$ (20–284°C). At higher temperatures the c-axis of PtS_2, $PtSe_2$ and $PtTe_2$ increases more slowly than at lower temperatures, while the c-axis of $PdTe_2$ increases more rapidly. For all compounds the volume expansion has a temperature dependence similar to that of the c-axis. The average thermal volume expansion coefficient, α, is 40×10^{-6} °C^{-1} for PtS_2 (20–250°C), 31×10^{-6} °C^{-1} for $PtSe_2$ (20–283°C), 39×10^{-6} °C^{-1} for $PtTe_2$ (20–591°C) and 57×10^{-6} °C^{-1} for $PdTe_2$ (20–284°C). The interatomic distances between metal and metalloid atoms, and between the metalloid atoms, have been calculated for the various temperatures.

Although detailed investigations are necessary for a complete description of the thermal movement of the atoms or molecules forming a crystal, some information might, in simple cases, be obtained from the thermal expansion of the crystal. Because of anharmonic vibrations in the crystals the expansion is generally not isotropic [1]. For layer structures the anisotropic expansion in favor of the direction normal to the layers is explainable on the basis of weak bonds between the atoms in different layers.

The present paper concerns the thermal expansion of PtS_2, $PtSe_2$ and $PtTe_2$[2] and $PdTe_2$[3], all of which have structures of the $Cd(OH)_2$-type. A drawing of four unit cells of the $Cd(OH)_2$-type structure is shown in Fig. 1. Filled circles represent metal atoms in (0 0 0), while open circles represent metalloid atoms

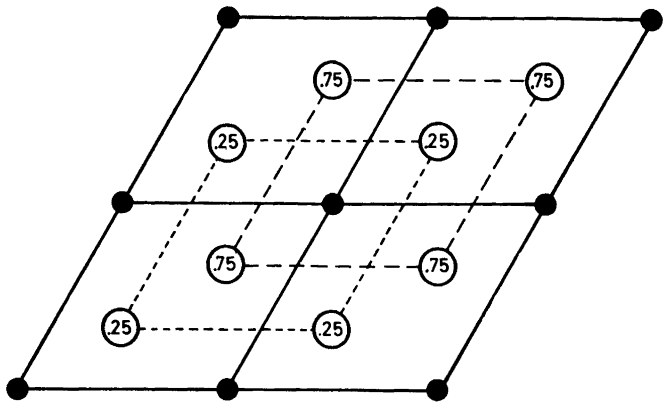


Fig. 1. Projection of four unit cells of the $Cd(OH)_2$-type structure on (001).

in ($\frac{2}{3}$ $\frac{1}{3}$ z) and ($\frac{1}{3}$ $\frac{2}{3}$ $\bar{z}$), with $z = 0.250$. From Fig. 1 it is seen that both metal and metalloid atoms form hexagonal nets, but at different heights. In the projection they are repeated at every 1/3 face diagonal.

Only a few compounds with this type of structure have been examined at temperatures other than room temperature. Megaw [4] studied the thermal expansion of single crystals of $Ca(OH)_2$ and $Mg(OH)_2$ by X-ray methods from 0 to 100°C. For these compounds the coefficient of expansion normal to the hexagonal layers was found to be 3 to 4 times larger than parallel to the layers.

Bredig [5] examined CdI_2, which forms polytypic structures based upon the $Cd(OH)_2$-structure [6], and found that this compound also exhibits relatively large thermal expansion normal to the hexagonal layers. (From 25 to 325°C β_c is approximately 4×10^{-5} °C^{-1}, while β_a is 1×10^{-5} °C^{-1}).

$NiTe_2$, with $Cd(OH)_2$-type structure, has been studied at several temperatures up to 800°C by Schneider and Imhagen [7]. They also determined the lattice constants of different samples in the composition range from NiTe (NiAs-type structure) to $NiTe_2$ as function of temperature.

Other compounds with layer structures, *e.g.* graphite [8], and boron nitride [9], show contraction in the hexagonal layers with increasing temperature, and large expansion perpendicular to the layers.

EXPERIMENTAL

The platinum chalcogenides were prepared from stoichiometric amounts of the elements heated in evacuated, sealed silica tubes at 825°C and slowly cooled to room temperature. The palladium ditelluride was prepared at 600°C.

Information about the purity of the elements is given elsewhere [2,3].

X-Ray powder photographs of the compounds were taken in a 190 mm Unicam high-temperature camera, with the samples sealed in thin-walled quartz capillaries. The samples were studied at temperatures between 20 and 950°C. PtS_2 and $PdTe_2$ could not, however, be examined at the very highest temperatures, PtS_2 because of its great dissociation pressure [10] which made the thin-walled quartz capillaries burst, and $PdTe_2$ because of its relatively low melting point of about 700°C.

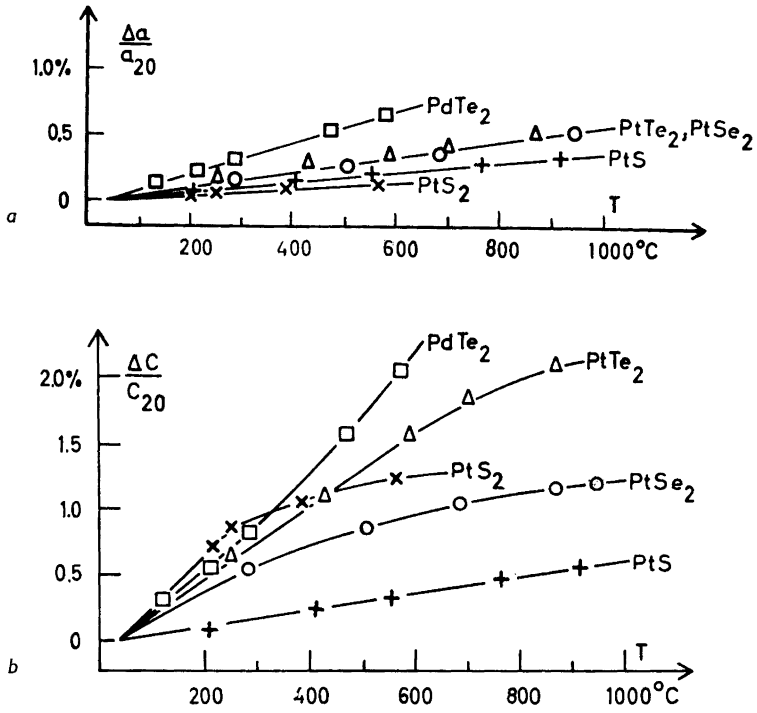
By means of a voltage regulator, the registered temperature of the furnace surrounding the specimen was kept constant within $\pm 5°C$. The Pt/Pt-Rh thermocouples of the furnace had been calibrated with a standard couple located at the position of the specimen. Lattice constants are given in Ångström units ($\lambda CuK_{\alpha_1} = 1.54050$ Å). Only the high-angle reflections of the diffraction pattern were used in the calculation of the lattice constants. The probable error in the lattice constant determinations at temperatures above room temperature is estimated to be about 0.02 %. This includes a possible error due to variation in the composition of the sample with temperature.

RESULTS

The results of the lattice constant measurements are listed in Table 1. Here is also listed the c/a ratio, the unit cell volumes, the shortest interatomic distances between metal and metalloid atoms [(000)(2/3 1/3 0.250)] and between the metalloid atoms [(2/3 1/3 0.250) (1/3 2/3 $\overline{0.250}$)]. For every measured or

Table 1. Lattice constants, unit cell volumes and interatomic distances for PtS_2, $PtSe_2$, $PtTe_2$ and $PdTe_2$ at different temperatures.

Sample	T(°C)	a(Å)	c(Å)	c/a	V(Å^3)	Metal-metalloid distance (Å)	Metalloid-metalloid distance (Å)
PtS_2	20	3.5431	5.0389	1.422	54.782	2.402	3.245
	215	3.5442	5.0745	1.432	55.203	2.408	3.260
	250	3.5449	5.0822	1.434	55.309	2.409	3.261
	385	3.5463	5.0911	1.436	55.449	2.411	3.267
	562	3.5474	5.1000	1.438	55.580	2.413	3.271
$PtSe_2$	20	3.7278	5.0813	1.363	61.152	2.499	3.330
	283	3.7337	5.1089	1.368	61.679	2.506	3.343
	503	3.7377	5.1235	1.371	61.988	2.510	3.350
	683	3.7420	5.1325	1.372	62.240	2.513	3.355
	865	3.7463	5.1384	1.372	62.455	2.516	3.358
	946	3.7482	5.1405	1.372	62.544	2.517	3.360
$PtTe_2$	20	4.0259	5.2209	1.297	73.283	2.666	3.495
	256	4.0326	5.2531	1.303	73.981	2.673	3.510
	431	4.0357	5.2777	1.308	74.442	2.678	3.520
	591	4.0386	5.3026	1.313	74.900	2.682	3.531
	707	4.0419	5.3152	1.315	75.201	2.685	3.537
	870	4.0452	5.3279	1.317	75.504	2.689	3.543
$PdTe_2$	20	4.0365	5.1262	1.270	72.333	2.660	3.464
	120	4.0420	5.1422	1.272	72.757	2.664	3.472
	210	4.0446	5.1538	1.274	73.015	2.667	3.478
	284	4.0495	5.1685	1.276	73.401	2.671	3.485
	470	4.0577	5.2061	1.283	74.233	2.680	3.502
	574	4.0632	5.2303	1.287	74.782	2.686	3.513



Figs. 2a, b. Relative lattice constant variations as function of temperature.

calculated property x_T, the ratio $\frac{x_T - x_{20}}{x_{20}} = \frac{\Delta x}{x_{20}}$ is calculated and plotted as $\frac{\Delta x}{x_{20}}$ *versus* T. The calculations of the expansion coefficients were based upon these data.

The change in length of the cell edges with temperature is rather different along the different axes. As shown in Fig. 2a, the a-axis of all four compounds increases linearily with increasing temperature. The expansion coefficient β_a is equal to 2.3×10^{-6} °C^{-1} for PtS_2 (20—562°C), 5.7×10^{-6} °C^{-1} for $PtSe_2$ (20—946°C), 5.8×10^{-6} °C^{-1} for $PtTe_2$ (20-870°C) and 12.0×10^{-6} °C^{-1} for $PdTe_2$ (20—574°C). The corresponding curves for the c-axis are shown in Fig. 2b. Those for PtS_2, $PtSe_2$ and $PtTe_2$ are of similar shape, while that for $PdTe_2$ is different. At lower temperatures the thermal expansion of the c-axis is linear, with expansion coefficient β_c equal to 37×10^{-6} °C^{-1} for PtS_2 (20—250°C), 20×10^{-6} °C^{-1} for $PtSe_2$ (20—283°C), 27×10^{-6} °C^{-1} for $PtTe_2$ (20—591°C) and 30×10^{-6} °C^{-1} for $PdTe_2$ (20—284°C). At higher temperatures the c-axis of PtS_2, $PtSe_2$ and $PtTe_2$ increases more slowly than at lower temperatures. The curve for the c-axis of $PdTe_2$ has the opposite curvature, and rises more sharply than at lower temperatures. In the temperature region just

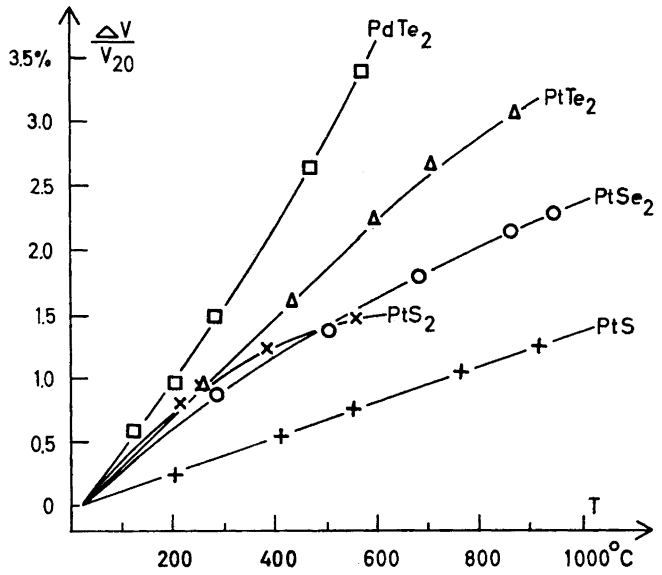


Fig. 3. Relative volume expansions as function of temperature.

below melting a fairly large increase in the coefficient of thermal expansion for the *c*-axis of $PdTe_2$ seems to occur.

The volume expansion is plotted as function of temperature in Fig. 3. For all compounds the volume expansion has a similar temperature dependence as the *c*-axis. The first part of the curves is approximately linear. The average thermal volume expansion coefficient α is 40×10^{-6} °C^{-1} for PtS_2 (20—250°C), 31×10^{-6} °C^{-1} for $PtSe_2$ (20—283°C), 39×10^{-6} °C^{-1} for $PtTe_2$ (20—591°C) and 57×10^{-6} °C^{-1} for $PdTe_2$ (20—284°C).

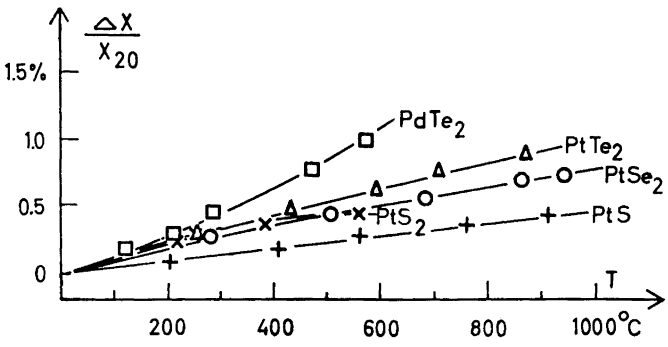


Fig. 4. Relative changes in interatomic bond distance between metal and metalloid atoms with temperature.

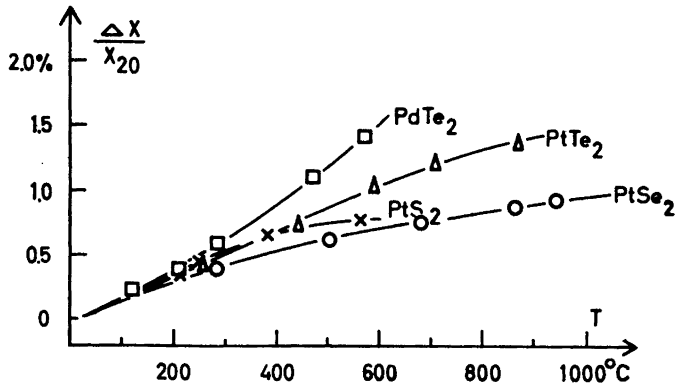


Fig. 5. Relative changes in the shortest metalloid-metalloid distance with temperature.

As seen from Fig. 4, the changes in bond distance are of the same order of magnitude as the average linear expansion. The expansion curves have a linear region with expansion coefficients 11×10^{-6} °C^{-1} for PtS_2 (20—250°C), 10×10^{-6} °C^{-1} for $PtSe_2$ (20—283°C), 11×10^{-6} °C^{-1} for $PtTe_2$ (20—591°C), and 16×10^{-6} °C^{-1} for $PdTe_2$ (20—284°C). The shortest metalloid-metalloid distances (*cf.* Fig. 5) change similarly, while the shortest metal-metal distances vary as the *a*-axis. The expansion coefficient over the linear region in Fig. 5 is 17×10^{-6} °C^{-1} for PtS_2 (20—250°C), 15×10^{-6} °C^{-1} for $PtSe_2$ (20—283°C), 17×10^{-6} °C^{-1} for $PtTe_2$ (20—591°C), and 23×10^{-6} °C^{-1} for $PdTe_2$ (20—284°C). The changes in the metal-metalloid and the metalloid-metalloid distances are calculated on the assumption that the parameter z has the same value $z = 0.250$ at all temperatures.

DISCUSSION

The present results show agreement with the expansion measurements [4] of $Mg(OH)_2$ and $Ca(OH)_2$ with regard to linearity and strong anisotropy of the expansion coefficients in the lower temperature range. The measurements were, however, only carried to about 100°C and deviations from linearity of the expansion coefficient might well take place at higher temperatures. From the results of Schneider and Imhagen [7] on $NiTe_2$ the curvature of the lattice constant variation could not be obtained with certainty.

Comparison with results from thermal expansion of compounds with other than $Cd(OH)_2$-type structures will here be limited to compounds with supposedly similar chemical bond type. Results for MnTe [11] and CrSb [12], both with NiAs-type structure, are similar in the temperature range below the antiferromagnetic transition temperature to those found for $PdTe_2$. In the range from −100 to + 50°C the thermal expansion coefficient of the *a*-axis of MnTe is almost zero, while for the *c*-axis it increases with temperature. In the range from 0 to 450°C the *a*-axis of CrSb increases linearly, while the *c*-axis increases more rapidly than at lower temperatures. FeS and $FeS_{1.09}$ also with NiAs-like

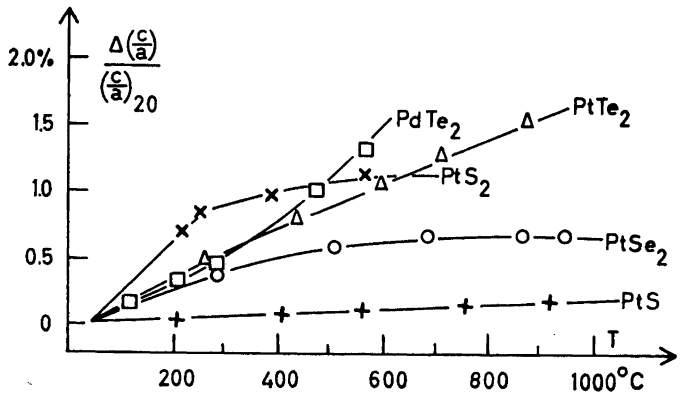


Fig. 6. Relative changes in the c/a ratio with temperature.

structures have been examined [13] in the temperature range from 20 to 385°C. Both a-axis and c-axis are found to increase with temperature. The expansion in the hexagonal plane is larger than perpendicular to it. For FeS $\beta_a \approx 1.5 \times 10^{-5}$ °C^{-1}, $\beta_c \approx 0$ (20 — 138°C) and $\beta_a \approx 1.7 \times 10^{-4}$ °C^{-1}, $\beta_c \approx 4 \times 10^{-5}$ °C^{-1} (138 — 325°C), and for $FeS_{1.09}$ $\beta_a \approx 5 \times 10^{-5}$ °C^{-1}, $\beta_c \approx 8 \times 10^{-6}$ °C^{-1} (75 — 325°C).

The present authors have examined the thermal expansion of PtS with B17-type structure. This compound has linear expansion both in the a- and c-directions, with $\beta_a \approx 3.9 \times 10^{-6}$ °C^{-1} (20—915°C) and $\beta_c \approx 5.8 \times 10^{-6}$ °C^{-1} (20—915°C) (*cf.* Figs. 2a, b). As apparent from Fig. 6 the expansion is approximately isotropic. The volume expansion (*cf.* Fig. 3) is linear with coefficient (α) 13.7×10^{-6} °C^{-1} in the temperature range studied. The platinum-sulfur bond distance shows a linear change in the examined temperature range with expansion coefficient 4.7×10^{-6} °C^{-1}, (*cf.* Fig. 4). The shortest metal-metal distance is also here equal to the length of the a-axis and shows the same variation with temperature (*cf.* Fig. 2a). There are two different short sulfur-sulfur distances which have the same relative variation as the a-axis and c-axis, respectively (*cf.* Figs. 2a, b).

Starting from absolute zero, the first part of a thermal expansion curve should be of sigmoidal type similar to the heat capacity curve. The reasons for the increase in volume at higher temperatures are not fully explained by the existing theories for the perfect lattice. On approaching melting, the curve is expected to show a rapid increase caused by an increasing number of lattice defects [14]. Fletcher [15] has attempted to show that the theories might account for the enhanced expansion without assuming lattice defects.

Of the compounds studied here only $PdTe_2$ shows an increase of the relative volume expansion at higher temperatures. This compound also has the lowest melting point. The relative expansions of PtS_2, $PtSe_2$ and $PtTe_2$ are still on the upper part of the sigmoidal curve.

Fajans [16] has found that in chemical compounds there is usually an increase in the thermal expansion with decreasing electronegativity difference between the elements. This rule applies to the two compounds $PtSe_2$ and $PtTe_2$ with α equal to $31 \times 10^{-6}\,°C^{-1}$ and $39 \times 10^{-6}\,°C^{-1}$, respectively, but not to PtS_2 and $PtSe_2$ or $PtTe_2$ since the expansion coefficient of PtS_2, $40 \times 10^{-6}\,°C^{-1}$, is greater than for the two others. The possibility that this might have been caused by PtS_2 loosing sulfur due to dissociation at higher temperatures cannot be excluded. If one, however, considers the a-axes, the expansion coefficient β_a increases from the sulfide to the telluride.

Acknowledgement. The authors wish to thank Professor Haakon Haraldsen for his interest in this study and for placing laboratory facilities at their disposal.

REFERENCES

1. Grüneisen, E. and Goens, E. *Z. Physik* **29** (1924) 141.
2. Grønvold, F., Haraldsen, H. and Kjekshus, A. *To be published.*
3. Grønvold, F. and Røst, E. *Acta Chem. Scand.* **10** (1956) 1620.
4. Megaw, Helen D. *Proc. Roy. Soc. (London)* **142** (1933) 198.
5. Bredig, M. A. *J. Chem. Phys.* **24** (1956) 1037.
6. Mitchell, R. S. *Z. Krist.* **108** (1956) 296, 341.
7. Schneider, A. and Imhagen, K. H. *Naturwiss.* **44** (1957) 324.
8. Nelson, B. and Riley, D. P. *Proc. Phys. Soc. (London)* **57** (1945) 477.
9. Pease, R. S. *Acta Cryst.* **5** (1952) 356.
10. Biltz, W. and Juza, R. *Z. anorg. u. allgem. Chem.* **190** (1930) 161; see also Richardson, F. D. and Jeffes, J. H. F. *J. Iron Steel Inst. (London)* **171** (1952) 165.
11. Greenwald, Selma *Acta Cryst.* **6** (1953) 396.
12. Snow, A. I. *Revs. Modern Phys.* **25** (1953) 127.
13. Haraldsen, H. *Z. anorg. u. allgem. Chem.* **246.** (1941) 195.
14. Lawson, A. W. *Phys. Rev.* **78** (1950) 185.
15. Fletcher, C. C. *Phil. Mag.* **2** (1957) 639.
16. Fajans, K. *Phys. Rev.* **61** (1942) 543.

Received June 21, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1775–1780

Crystal Structure of the Addition Compound 1,4-Dioxan-Chlorine

O. HASSEL and KNUT O. STRØMME

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

An X-ray analysis of the 1:1 compound 1,4-dioxan chlorine carried out at temperatures between −30° and −90°C shows that the crystals are isomorphous with those of the corresponding bromine compound [1]. The monoclinic unit cell has the dimension a = 9.36 Å, b = 8.83 Å, c = 4.13 Å and β = 91.5°. The observed O—Cl and Cl—Cl distances associated with the chlorine molecule bridges are 2.67 and 2.02 Å, respectively.

During the last years a series of addition compounds formed by molecules containing a donor atom (A) and halogen molecules have been studied in the solid state by X-ray crystallographic methods. One of the most striking features observed in these compounds is the existence of linear atomic arrangements A-halogen-halogen. So far, the halogen molecules chosen have been those of iodine or bromine, in some cases also iodo monochloride. In no case have addition compounds in which chlorine is directly attached to the donor atom been studied. From our earlier experience it would in fact seem probable that a charge transfer bond between, *e.g.*, an oxygen atom and a chlorine atom should be even weaker than the bond to a bromine atom which is in turn certainly weaker than an oxygen-iodine bond.

Results, recently obtained, tend to confirm this view but show that a series of donor molecules actually form addition compounds with chlorine which are isomorphous with the bromine and even the iodine compounds. This might perhaps appear a little surprising when we consider the difference in atomic radii of the different halogen atoms. The A-halogen bond distance, however, is determined both by the size of the halogen atom and by the strength of the bond established by electron transfer between the two atoms. Although the smaller radius of the chlorine atom will tend to shorten this bond distance a certain compensation results from the fact that the bond is weaker in the case of chlorine than in the case of bromine. The analogy between crystals containing a donor molecule and chlorine and those containing the same donor molecule and bromine, even suggests the formation of »chlorine molecule bridges» in chlorine addition compounds.

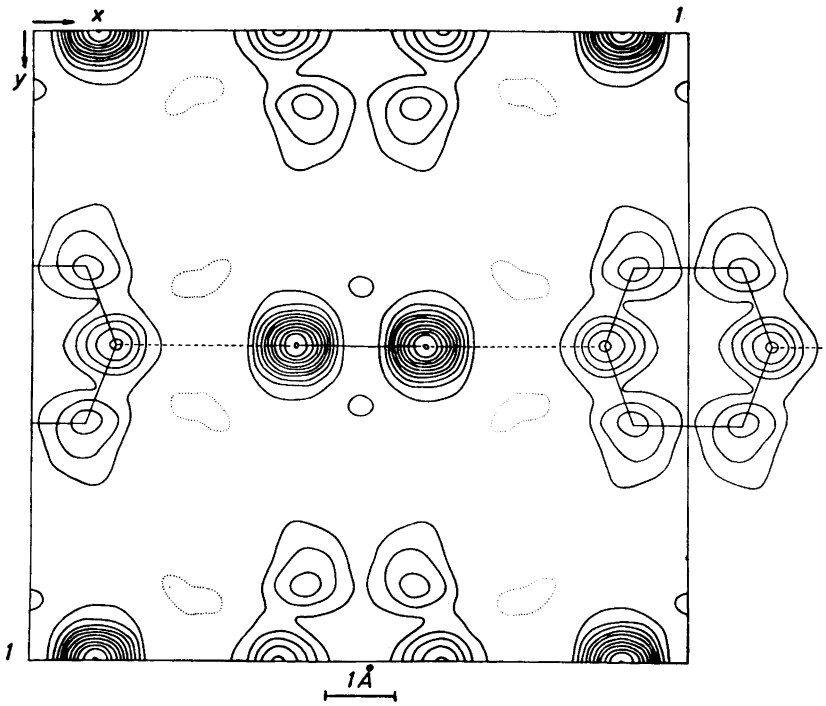


Fig. 1. Projection along [001]. Contour intervals $2\sum e$. Å^{-2}. Zero contour dotted.

Choosing first the 1:1 compound between 1,4-dioxan and chlorine, a report on the determination of the crystal structure of this compound will be given here.

Single crystals were obtained by cooling an equimolecular mixture of the two substances in a capillary tube mounted in a Weissenberg camera equipped with a cooling system. The melting point of the addition compound is approximately −5°C. Zero layer line Weissenberg photographs were taken at temperatures below −30°C, mostly at −90°C, and with rotation about the directions [001], [110] and [101] of the monoclinic cell.

The lattice parameters obtained from the diagrams are:

$$a = 9.36 \text{ Å} \qquad b = 8.83 \text{ Å} \qquad c = 4.13 \text{ Å} \qquad \beta = 91.5°$$

and the number of molecules in the unit cell *two* (density about 1.55). These figures and the following analysis confirmed the suggestion that the compound is actually isomorphous with the corresponding bromine compound and belongs to the space group $C2/m$. It was found difficult, however, to obtain good single crystals, particularily if the crystals had their c-axis approximately parallel to the axis of the capillary tube. It was observed that the crystals

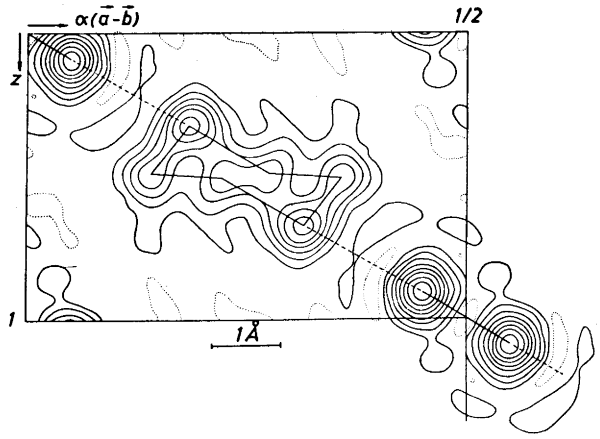


Fig. 2. Projection along [110]. Contours: 0, 2, 4, 8, 12, 16, — for chlorine and 0, 2, 4, 6, 8, 10 — for light atoms. Zero contour dotted.

have a marked tendency to grow along directions which are approximately parallel to the directions of the endless molecular chains of which the entire structure is found to be built up.

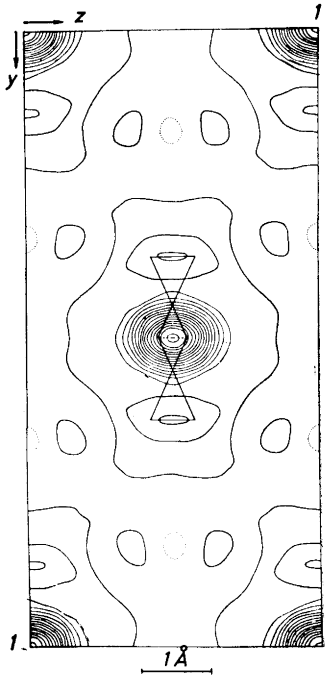


Fig. 3. Projection along [101]. Contours: 0, 2, 6, 10, 14 —. Zero contour dotted.

Table 1. Atomic coordinates in fractions of the cell-edges. Origin at a center of symmetry.

	x	*y*	*z*
O	0.371	0.000	0.322
Cl	0.101	0.000	0.094
C	0.419	0.134	0.492

Table 2. Bond distances and bond angles in the dioxan molecule.

C—C	1.52 Å (1.529);	C—O	1.44 Å (1.421)
∠ C—O—C	111° (112.5);	∠C—C—O	109° (109.0)

The suggestion that the dioxan-chlorine structure is very similar to the dioxan-bromine structure [1] made it appear reasonable to carry out the first Fourier synthesis along [001] inserting chlorine parameters almost identical with the bromine parameters found for the dioxan-bromine compound. The intensity material obtained from zero layer line photographs with rotation about the *c*-axis appeared, however, to be less satisfactory than intensity data obtained from other diagrams. For this reason the intensity material was partly supplemented by intensity data obtained from diagrams with rotation about other crystallographic directions.

The final electron density maps are reproduced in Figs. 1—3. The atomic coordinates computed using these maps are given in Table 1.

In the projection along [101] serious overlapping of atoms occurs. This is not the case in the two other projections, but the intensity material on which the [110] projection is based was considered more reliable than that leading to the [001] projection. Table 1 is mainly based on the coordinates obtained from this projection.

From the coordinates given in Table 1 the bond distances and bond angles within the dioxan molecule listed in Table 2 have been computed.

The values in parantheses are those recently obtained in this laboratory by Michael Davis from an electron diffraction study of dioxan vapour.

The Cl—Cl bond distance is 2.02 Å and thus only slightly longer than the spectroscopic value for the free molecule (1.988). The Cl—O distance obtained is 2.67 Å. The standard deviation in the Cl—Cl distance is 0.013 Å, in the *Cl*—O distance 0.026 Å and in the C—C distance 0.045 Å. It appears probable that the conclusion is legitimate that the chlorine-oxygen distance in the dioxan chlorine compound is actually slightly smaller than the bromine-oxygen distance in the corresponding bromine compound. The angle O—Cl—Cl is found equal to 178°. It appears doubtful whether or not the deviation from 180° is real. The deviation of the O—Cl bond from the direction of an equatorial bond is about 19° and in the same direction as that obtained in the bromine and iodine compounds.

The relatively small values of the temperature factor *B* ranging from 2.0 for exposures at the lowest temperature to 2.8 for the high temperature, indicate a relatively rigid structure.

Table 3. Observed and calculated structure factors. The figures represent one half of the absolute values.

h k l	*F*obs	*F*calc	*h k l*	*F*obs	*F*calc
	(*h h l*) zone				
1 1 4	11.9	−13.1			
1 1 0	1.4	1.7	2 2 4	5.4	− 5.2
2 2 0	6.1	5.1	3 3 4	6.2	− 5.9
3 3 0	1.0	1.2	4 4 4	3.4	3.6
4 4 0	15.7	−15.5	5 5 4	3.7	4.2
5 5 0	12.7	−11.9	1 1 $\bar{4}$	<1.4	− 0.3
6 6 0	10.1	− 9.0	2 2 $\bar{4}$	<1.4	1.0
7 7 0	<1.3	− 1.0	3 3 $\bar{4}$	7.6	9.3
8 8 0	2.2	1.5	4 4 $\bar{4}$	7.3	8.9
0 0 1	3.9	− 2.1	5 5 $\bar{4}$	1.8	1.6
1 1 1	16.1	13.6	0 0 5	8.0	−11.1
2 2 1	4.0	4.4	1 1 5	2.0	− 2.4
3 3 1	22.0	−20.3			
4 4 1	16.1	−15.5	2 2 5	2.6	− 3.8
5 5 1	6.2	− 5.2	1 1 5	2.5	− 2.3
6 6 1	5.9	− 4.4	2 2 5	0.8	0.8
7 7 1	<1.2	1.4	3 3 5	0.5	0.5
8 8 1	4.6	4.9			
1 1 $\bar{1}$	44.3	47.3		(*h k* $\bar{h}$) zone	
2 2 $\bar{1}$	10.2	7.6	0 2 0	37.1	38.1
3 3 $\bar{1}$	6.6	5.5	0 4 0	19.1	16.4
4 4 $\bar{1}$	4.8	− 5.1	0 6 0	23.3	19.5
5 5 $\bar{1}$	12.6	−12.1	0 8 0	15.2	15.1
6 6 $\bar{1}$	7.0	− 5.5	0 10 0	6.1	5.5
7 7 $\bar{1}$	9.5	− 9.3	1 1 $\bar{1}$	41.9	42.1
8 8 $\bar{1}$	<0.7	− 0.7	1 3 $\bar{1}$	19.3	19.1
0 0 2	10.9	12.8	1 5 $\bar{1}$	17.9	16.0
1 1 2	1.3	− 1.1	1 7 $\bar{1}$	17.1	17.5
2 2 2	17.3	−18.1	1 9 $\bar{1}$	10.0	8.9
3 3 2	14.2	−13.9	1 11 $\bar{1}$	2.8	3.3
4 4 2	6.2	− 3.8	2 0 $\bar{2}$	25.8	28.3
5 5 2	10.6	− 9.0	2 2 $\bar{2}$	18.3	19.9
6 6 2	3.1	2.7	2 4 $\bar{2}$	12.4	12.4
7 7 $\bar{2}$	7.9	9.0	2 6 $\bar{2}$	11.9	12.6
1 1 $\bar{2}$	6.7	7.6	2 8 $\bar{2}$	9.7	9.6
2 2 $\bar{2}$	18.9	21.5	2 10 $\bar{2}$	3.4	4.3
3 3 $\bar{2}$	6.2	5.7	3 1 $\bar{3}$	12.0	13.3
4 4 $\bar{2}$	6.1	7.2	3 3 $\bar{3}$	8.6	10.4
5 5 $\bar{2}$	5.0	− 5.2	3 5 $\bar{3}$	8.1	8.9
6 6 $\bar{2}$	11.2	−11.2	3 7 $\bar{3}$	6.7	6.9
7 7 $\bar{2}$	3.3	− 2.0	4 0 $\bar{4}$	3.7	6.2
0 0 3	3.0	− 4.2	4 2 4	5.3	6.7
1 1 3	9.8	− 8.6	4 4 4	4.6	6.3
2 2 3	12.6	−12.8	4 6 4	<1.9	4.2
3 3 3	5.9	− 5.5			
4 4 3	9.7	− 9.2		(*h k* 0) zone	
5 5 3	4.4	4.6	2 0 0	17.8	17.7
6 6 $\bar{3}$	6.9	6.9	4 0 0	31.4	−31.4
1 1 $\bar{3}$	5.3	4.7	6 0 0	16.0	−16.1
2 2 $\bar{3}$	11.1	11.9	8 0 0	4.1	3.6
3 3 $\bar{3}$	10.9	12.5	1 1 0	1.2	1.6
4 4 $\bar{3}$	2.2	2.4	3 1 0	<1.8	0.6
5 5 $\bar{3}$	5.0	4.9	5 1 0	6.2	− 4.0
6 6 $\bar{3}$	3.9	− 2.2	7 1 0	3.4	− 2.9
0 0 4	3.7	− 3.1	0 2 0	38.2	38.7

h k l	*F*obs (*h k* 0) zone	*F*calc	*h k l*	*F*obs	*F*calc
			4 6 0	10.5	−11.6
2 2 0	5.3	5.5	6 6 0	9.0	− 6.9
4 2 0	19.6	−21.7	8 6 0	<2.9	2.9
6 2 0	7.9	− 8.0	1 7 0	<3.1	1.5
8 2 0	4.1	5.9	3 7 0	<3.2	− 0.6
1 3 0	21.7	19.8	5 7 0	3.5	− 3.1
3 3 0	0.9	1.1	7 7 0	<2.8	− 0.6
5 3 0	14.9	−15.0	0 8 0	12.8	14.0
7 3 0	7.9	− 8.5	2 8 0	3.8	3.8
0 4 0	16.1	15.8	4 8 0	7.8	− 8.6
2 4 0	<2.3	− 0.6	6 8 0	6.7	− 6.4
4 4 0	13.9	−13.5	8 8 0	1.9	1.3
6 4 0	3.1	− 2.4	1 9 0	<3.1	2.7
8 4 0	4.4	6.5	3 9 0	<2.8	− 0.7
1 5 0	10.6	11.1	5 9 0	<2.5	− 3.4
3 5 0	<2.8	0.2	7 9 0	<1.5	− 1.2
5 5 0	11.2	− 9.8	0 10 0	5.1	5.0
7 5 0	4.5	− 5.8	2 10 0	<2.5	0.7
0 6 0	19.7	18.8	4 10 0	3.7	− 4.2
2 6 0	3.0	3.9			

The reliability factors of the final projections are as follows: [001], R=8.0%; [110], R = 11.6 %; [101], R = 9.0 %.

In Table 3 calculated and observed structure factors are listed. The number of reflexions available for the [001], [110] and [101] projections are 34, 64, and 24, respectively (maximum with CuK-radiation 60, 69, and 26). f-Values given by Berghuis *et al.*[2] were used for carbon and oxygen, for chlorine the values given in *Int. Tab.*[3].

This investigation has been sponsored by the *United States Air Force Development Command* under contract AF 61 (052)-71.

REFERENCES

1. Hassel, O. and Hvoslef, J. *Acta Chem. Scand.* **8** (1954) 873.
2. Berghuis, J., Haanappel, I. M., Potters, M., Loopstra, B. O., Mac Gillavry, C. H. and Veenendaal, A. L. *Acta Cryst.* **8** (1955) 478.
3. *Internationale Tabellen zur Bestimmung von Kristallstrukturen*, Gebrüder Borntraeger, Berlin 1935.

Received July 1, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1781–1786

Crystal Structure of the Addition Compound Benzene-Chlorine (1:1)

O. HASSEL and KNUT O. STRØMME

Universitetets Kjemiske Institutt, Blindern-Oslo, Norway

The crystal structure of an equimolecular addition compound benzene-chlorine has been determined using single crystal Weissenberg technique and working at low temperatures, mainly about −90°C. The crystals are *monoclinic* (space group $C2/m$) and isomorphous with those of the corresponding benzene-bromine compound [2]. Chains of alternating benzene and chlorine molecules are present in the crystal, the Cl—Cl distance is 1.99 Å and the distance from one particular chlorine atom to the nearest benzene plane (3.28 Å), a little shorter than the corresponding distance in the bromine compound.

A considerable amount of experimental material has been gathered during the last few years concerning crystal structures of charge transfer compounds in which molecules of the heavier halogens and iodo monochloride are the electron acceptors. The most conspicuous results of these investigations are the facts that in all compounds so far investigated the arrangement, donor atom-halogen-halogen, is *linear*, and that both atoms of a particular halogen molecule may be capable of forming bonds to donor atoms.

Recently X-ray work has been started on addition compounds formed by *chlorine*. The first crystal structure to be studied was that of the 1:1 compound formed by 1,4-dioxan and chlorine [1]. It could be shown that this compound is isomorphous with the corresponding bromine compound and a proof of the existence of chlorine molecule bridges between oxygen atoms was thereby given. The next step in our research programme was to look for a possible solid compound between benzene and chlorine. We actually succeeded in growing crystals of a 1:1 compound and in determining its crystal structure.

The procedure followed was very similar to that briefly described for the benzene-bromine compound [2]. By cooling an equimolecular mixture of benzene and chlorine in a capillary tube to temperatures below −50°C (m.p. −40°C) single crystals of the 1:1 compound could be grown which were suitable for X-ray analyses in a Weissenberg camera. Photographs with CuK-radiation

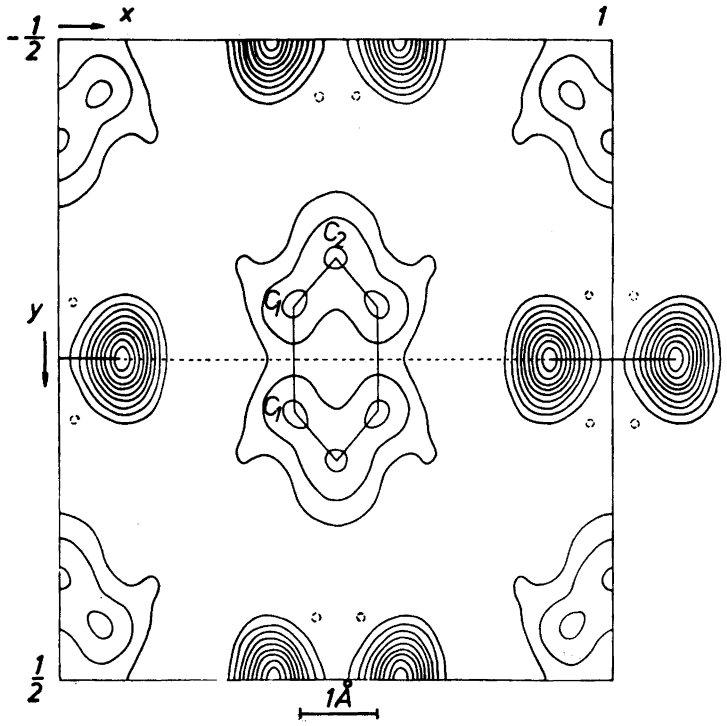


Fig. 1. Fourier along [001]. Contour intervals 2 e . Å^{-2}. Zero contour broken.

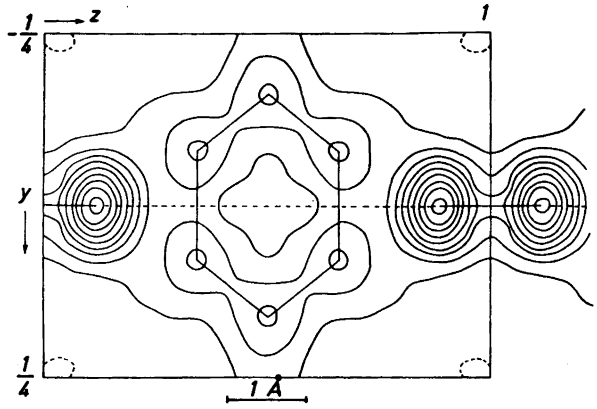


Fig. 2. Fourier projection along [100]. Contours as in Fig. 1.

Table 1. Atomic coordinates in fractions of the cell-edges. Origin at a center of symmetry.

Atom	x	y	z
Cl	0.116_5	0.000	0.116_5
C_1	0.575	0.080	0.342
C_2	0.500	0.161	0.500

and rotation about three different crystallographic directions were obtained at temperatures mainly at about −90°C.

The crystals turned out to be monoclinic, space group $C2/m$ with lattice parameters: $a = 7.41$ Å, $b = 8.61$ Å, $c = 5.65$ Å, $\beta = 99.5°$. With these parameters and assuming the unit cell to contain two molecules of either kind a density of 1.40 g/cm^3 is computed. This conforms to the expected density of a compound containing equal numbers of the two kinds of molecules. These findings make it appear extremely probable that the crystalline chlorine-benzene compound is isomorphous with the bromine-benzene compound previously investigated.

From zero layer line Weissenberg diagrams with rotation about [001], [100] and [10$\bar{1}$] intensity data were derived which could be used for the computation of electron density projections. It was assumed that the structure is indeed closely similar to that of the benzene-bromine compound [2]. The final Fourier maps with projections along [001], [100], and [10$\bar{1}$] are reproduced in Figs. 1—3. The number of reflexions observed in the ($hk0$) zone is 28 (theor. 48), in the ($0kl$) zone 26 (theor. 39) and in the (hkh) zone 19 (theor. 27). The atom form factors were taken from McWeeney [3] (hydrogen), Berghuis *et al.*[4] (carbon) and International Tables [5] (chlorine).

In Table 1 the atomic coordinates derived from the Fourier projections are listed. On computing the reliability factors it was found that the agreement between observed and calculated F-values improved on including the contribution from the hydrogen atoms, the coordinates of which were taken from a model. The R-values obtained were:

[001] 11.0 %; [100] 7.3 %; [10$\bar{1}$] 11.3 %

When calculationg the R-values a few strong reflexions which are suffering from extinction effects were omitted. Calculated and observed F-values are listed in Table 2. The following (anisotropic) temperature factors were introduced:

$$B_{100} = 3.2 \; ; \quad B_{010} = 7.8 \; ; \quad B_{001} = 5.2$$

The experimental results thus indicate anisotropic vibrations of the chlorine atoms, the vibrations being more pronounced perpendicular to [101] (the Cl—Cl direction) than along this direction. It appears justified, however, to assume that the thermal vibrations are independent of the direction within planes perpendicular to [101] (parallel to the benzene rings). This assumption actually conforms very well to the experimentally observed damping of the X-ray reflections.

Table 2. Observed and calculated structure factors. The figures represent one half of the absolute values. F—'s suffering from extinction are marked with an asterisk.

h k l	Fobs	Fcalc
	(h k 0) zone	
2 0 0	20.6	22.1
4 0 0	15.7	−17.0
6 0 0	5.2	− 5.2
8 0 0	4.8	4.7
1 1 0	<0.8	1.1
3 1 0	14.9	−16.0
5 1 0	8,5	− 8.0
7 1 0	4.2	5.4
9 1 0	2.7	4.1
0 2 0	25.0 *	29.9
2 2 0	3.3	3.2
4 2 0	18.1	−16.0
6 2 0	6.2	− 5.7
8 2 0	3.6	3.0
1 3 0	20.1	19.0
3 3 0	6.1	− 4.4
5 3 0	5.7	− 5.4
7 3 0	4.1	4.1
9 3 0	<1.8	3.8
0 4 0	8.6	7.5
2 4 0	<2.0	− 2.4
4 4 0	8.6	− 9.4
6 4 0	2.2	− 1.6
8 4 0	2.4	3.2
1 5 0	11.0	10.1
3 5 0	4.9	− 4.8
5 5 0	6.4	− 6.9
0 6 0	6.2	3.0
2 6 0	<2.0	0.4
4 6 0	2.2	− 2.8
6 6 0	2.0	1.2
1 7 0	3.8	4.4
3 7 0	<2.0	− 2.9
5 7 0	2.5	− 3.7
7 7 0	<1.2	− 0.26
0 8 0	2.1	1.9
2 8 0	<2.2	− 0.36
4 8 0	<1.9	− 2.00
1 9 0	<2.0	2.00
010 0	<2.0	1.0
	(0 k l) zone	
0 2 0	31.2	30.0
0 4 0	6.9	7.4
0 6 0	5.1	4.8
0 8 0	1.7	1.7
010 0	<1.4	0.9
0 0 1	1.0	− 0.7
0 2 1	17.1	17.7
0 4 1	14.0	13.6
0 6 1	4.9	5.0
0 8 1	1.9	2.5
010 1	<1.1	0.8
0 0 2	2.1	3.1
0 2 2	3.6	− 3.0
0 4 2	1.8	0.5
0 6 2	3.1	3.0
0 8 2	<1.0	0.4
0 0 3	5.1	− 4.3
0 2 3	2.2	− 2.4
0 4 3	5.2	− 4.9
0 6 3	5.2	− 5.4
0 8 3	1.5	− 1.4
0 0 4	8.6	− 9.4
0 2 4	9.9	−10.0
0 4 4	5.3	− 5.2
0 6 4	<1.5	− 1.2
0 8 4	<1.2	− 1.2
0 0 5	6.3	− 6.8
0 2 5	3.8	− 4.0
0 4 5	2.7	− 2.3
0 6 5	1.4	− 1.8
0 0 6	1.4	1.2
0 2 6	<1.4	− 0.5
0 4 6	<1.2	− 1.2
0 0 7	<0.9	0.2
	(h k h) zone	
2 0 2	8.2	− 6.4
4 0 4	5.7	6.2
1 1 1	20.6	20.0
3 1 3	2.1	− 2.0
5 1 5	<1.2	1.0
0 2 0	25.0 *	30.8
2 2 2	15.3	−13.8
4 2 4	3.7	4.1
1 3 1	3.2	− 2.8
3 3 3	4.2	− 4.6
0 4 0	7.3	7.6
2 4 2	11.9	−12.0
4 4 4	2.9	3.7
1 5 1	2.1	− 1.7
3 5 3	1.5	− 1.2
0 6 0	5.2	3.8
2 6 2	4.3	− 5.1
4 6 4	2.0	3.3
1 7 1	<2.1	− 0.5
3 7 3	<2.0	− 0.4
0 8 0	1.7	− 2.5
2 8 2	1.7	− 1.7
1 9 1	<2.1	− 0.5
010 0	<1.9	0.6

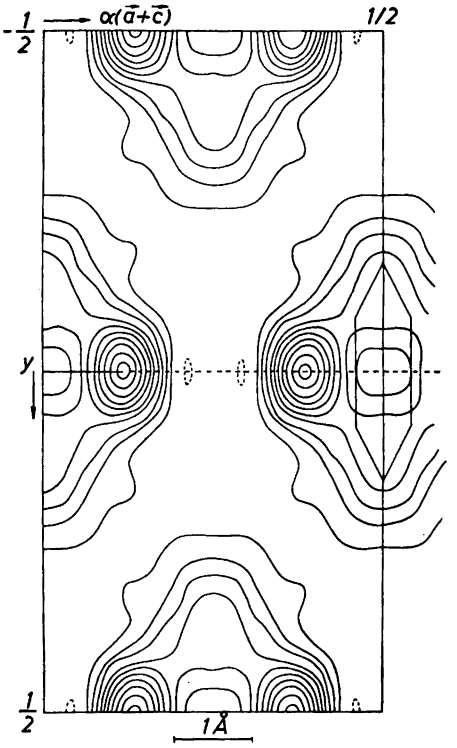


Fig. 3. Fourier projection along [10$\bar{1}$]. Contours as in Fig. 1.

All our findings thus confirm the expected close analogy between the structures of the benzene compounds of chlorine and bromine. In both cases the thermal movement of the atoms takes place preferably parallel to the benzene planes. The Cl—Cl bond direction is at least very nearly perpendicular to the benzene rings. The distance from a particular chlorine atom to the nearest benzene ring is 3.28 Å and thus 0.08 Å shorter than the corresponding distance in the bromine compound [2]. The observed Cl—Cl distance is 1.99 Å and thus identical with the spectroscopical value for the free molecule. In Table 3 the angles and interatomic distances in the benzene ring have been listed. The standard deviations of the reported distances are the following:

Cl—Cl 0.013 Å; C—Cl 0.026 Å; C—C 0.034 Å.

Table 3. Interatomic distances and angles within the benzene ring.

Bond	Bond length (Å)	Angle	
C_1-C_1	1.38	$C_1-C_1-C_2$	120.7°
C_1-C_2	1.37	$C_1-C_2-C_1$	118.6°

The present structure thus affords a second example of a chlorine molecule bridge in an addition compound, and of isomorphism between addition compounds in which chlorine and bromine are the electron acceptors. It is to be expected that many new examples will be found in the future. The reason for this isomorphism is no doubt, that the lengthening of the bond between donor molecule and chlorine is compensated by the smaller atomic radius of the chlorine atom.

This investigation has been sponsored by the *United States Air Force Development Command* under contract AF 61 (052)—71.

REFERENCES

1. Hassel, O. and Strømme, Knut O. *Acta Chem. Scand.* **13** (1959) 1775.
2. Hassel, O. and Strømme, Knut O. *Acta Chem. Scand.* **12** (1958) 1146.
3. McWeeney, R. *Acta Cryst.* **4** (1951) 513.
4. Berghuis, J., Haanappel, I. M., Potters, M., Loopstra, B. O., MacGillavry, C. H. and Veenendaal, A. L. *Acta Cryst.* **8** (1955) 478.
5. *Internationale Tabellen zur Bestimmung von Kristallstrukturen*, Gebrüder Borntraeger, Berlin 1935.

Received July 1, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1787–1790

Separation of some O-Phosphorylated Amino Acids and Peptides on Anion Exchange Resin

LARS STRID

Department of Medical Biochemistry, University of Göteborg, Göteborg, Sweden

A chromatographic procedure for the separation of O-phosphorylated amino acids and peptides on the anion exchange resin Dowex 1-X2, is described. The following synthetic compounds have been separated: SerP *, ThrP, Gly-SerP-Gly, Gly-SerP, SerP-Gly, Leu-SerP, SerP-Leu, SerP-Asp, SerP-Glu and SerP-SerP.

Investigations of phosphoproteins in this laboratory [1-4] involve degradation of such proteins by enzymes and by acid, whereby a mixture of phosphorylated amino acids and phosphopeptides are obtained. In the synthesis of phosphopeptides [5-7], using diphenylphosphoryl chloride as the phosphorylating reagent, a mixture of phosphopeptide and by-products are obtained. Consequently, it was found necessary to work out a simple preparative separation method.

Ion exchange chromatography of phosphopeptides from partial acid hydrolysed phosphoproteins has earlier been used by several researchers. Most of them have used cation exchange resin [8,9] and in some cases rechromatography on anion exchange resin [10-13].

Using a cation exchange resin Österberg [3] obtained a pure phosphopeptide, containing three phosphate groups, from an enzymic digest of casein.

With anion exchange resin Kennedy and Smith [14] reported complete separation of the phosphates of the following amino acids: hydroxyproline, threonine, serine and tyrosine.

EXPERIMENTAL

Materials

All reagents used were of analytical grade.

2 M sodium formate was prepared by mixing equal volumes of 4 M sodium hydroxide and 4 M formic acid. The pH was adjusted to 5.5 with 4 M formic acid.

* The following abbreviations are used in this paper: SerP is O-phosphorylserine and ThrP is O-phosphorylthreonin. Peptides are abbreviated as commonly done in literature; thus Gly-SerP-Gly equals glycyl-(O-phosphoryl)-serylglycine (*cf.* also Table 1).

0.1 M formic acid.

2 M pyridine formate. 511 g 90 % formic acid and 790 g pyridine were mixed and diluted with water to 5 l. Resulting pH was 4.5. From this stock solution, 0.25, 0.50, and 1.00 M solutions were obtained by dilution with water.

Model compounds used in the chromatography are listed in Table 1.

Dowex 1-X2 (Cl^-) 200—400 mesh. 500 ml resin was washed on a Büchner funnel with 2 litres of 2 N HCl, followed by 2 litres of water. The resin was suspended in water and after the resin had settled for 10 min, the supernatant was decanted to remove the finest particles. Coarse particles were removed by wet screening through a 50 DIN sieve. The resin was then poured into a column and converted to the formate form by allowing 2 M sodium formate pH 5.5 to flow slowly through the column, until the effluent gave a negativ test for chloride. It was then washed with distilled water on a Büchner funnel and stored in moist form.

Method

Column. About 150 ml of the resin was washed with 700 ml 0.1 M formic acid and then suspended in 200 ml of the same acid and freed from air by evacuation. The suspended resin was poured into a column 1.6 × 50 cm to a height of 43 cm at a pressure of 15 cm Hg. The top of the column was connected to a mixing chamber of 550 ml, furnished with a magnetic stirrer. The mixing chamber was connected to a separatory funnel. The top of the column, mixing chamber and separatory funnel were all filled with 0.1 M formic acid, and after 100 ml acid had passed, the column was ready for use.

Chromatography. 0.5—2 mg of each component (Table 1) in 0.1—0.2 ml H_2O or 0.1 M formic acid was applied to the column and washed into it by washing twice with 0.2 ml of 0.1 M formic acid. The gradient system was connected, and elution started. The following elution schedule was used with the gradient given as limit concentration:

I 0.1 M formic acid (165 ml)
II Gradient to 0.25 M pyridine formate, pH 4.5 (725 ml)
III Gradient to 0.50 M pyridine formate, pH 4.5 (310 ml)
IV Gradient to 1.00 M pyridine formate, pH 4.5 (320 ml)

Fractions of 3.5 ml were collected. For the sake of convenience the flow rate in the experiment described here was 20 ml/h but can be increased considerably. The chromatographic run was performed at room temperature.

Analysis and identification of the eluate. 1 ml of each fraction was mixed with 1 ml of 0.2 M citrate buffer pH 5.0. 1 ml of ninhydrin solution was added [16] and the sample heated for 15 min in boiling water. After dilution with 10 ml of ethanol:water (1:1), the optical density was read at 570 mμ.

Table 1. Model compounds.

Compound *	Abbreviation
O-Phosphorylserine	SerP
Glycyl-(O-phosphoryl)-serine	Gly-SerP
O-Phosphorylserylglycine	SerP-Gly
Glycyl-(O-phosphoryl)-serylglycine	Gly-SerP-Gly
Leucyl-(O-phosphoryl)-serine	Leu-SerP
O-Phosphorylseryl-leucine	SerP-Leu
O-Phosphorylserylglutamic acid	SerP-Glu
O-Phosphorylserylaspartic acid	SerP-Asp
O-Phosphorylseryl-(O-phosphoryl)-serine	SerP-SerP
O-Phosphorylthreonine	ThrP
Aspartic acid	Asp
Serine	Ser

* The three last mentioned compounds were obtained from Mann Research Lab., N.Y. All other compounds were synthesized in this laboratory [5,6,15].

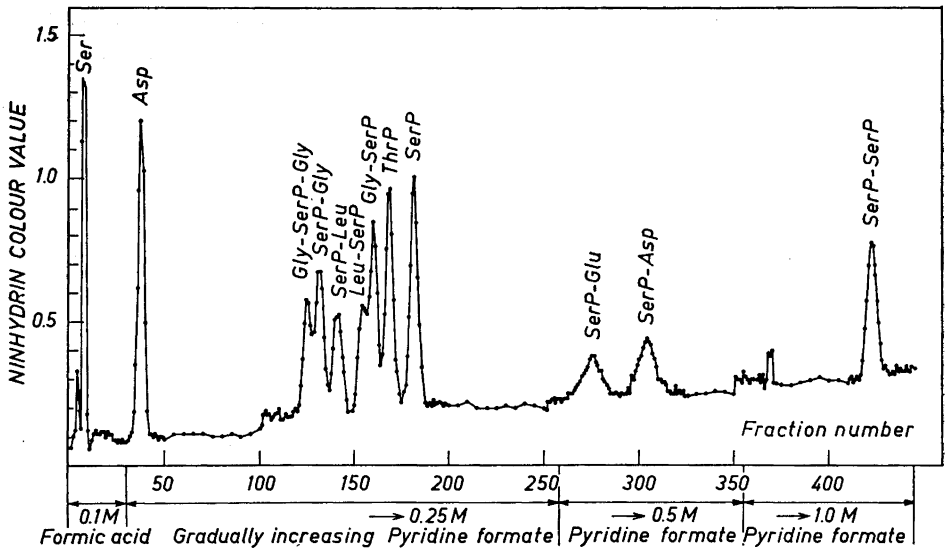


Fig. 1. Chromatogram obtained by separation of a synthetic mixture of phosphorylated amino acids and peptides. Dimensions of the column 1.6 × 43 cm. Volume of each fraction 3.5 ml. 1 ml of each fraction was used for the colour reaction.

The fractions from each peak were combined. Wherever overlapping occurred the intermediate fractions were discarded. The solutions were evaporated to dryness at 40° *in vacuo*. A small amount of water was added and the evaporation repeated until no smell of pyridine could be detected. As it was sometimes difficult to free the product from pyridine [6], particularly the slower fractions in the chromatography, it was found convenient to remove the pyridine on a small Dowex 50(H^+) column and to elute the peptide with water or weak acid.

Identification of the peaks was made from runnings where certain compounds were omitted, and by comparison between the applied and recovered ninhydrin colour.

The peaks were also identified by paper chromatography with the solvent system ButOH:HAc:H_2O(4:1:5). Each fraction gave only one spot. R_F-values and colours were compared with references (Table 1).

RESULTS AND DISCUSSION

It is evident that chromatography on anion exchange resin is useful for the separation of acidic substances such as phosphorylated peptides. These compounds are often so acidic that they are difficult to adsorb on cation exchange resins. On strong anion exchange resins on the other hand they are strongly retained. The resin Dowex 1-X2, being a strong anion exchanger, was used in this investigation. The low cross-linking was chosen to allow separation also of larger peptides.

Concerning elution medium the requirements were firstly that it should be easy to evaporate at temperatures where phosphopeptides are stable, and secondly that it should not produce colour reaction with ninhydrin. Pyridine formate meets these requirements and was therefore selected.

Synthetic phosphorylated amino acids and peptides [5,6,15] were used for working out the method. Serine and aspartic acid have been included to show where a neutral and acidic amino acid is eluted.

Fig. 1 shows a typical chromatogram. It can be seen that the more acidic the compounds, the more difficult they are to elute from the column. SerP-SerP, containing two phosphate groups per two amino acid residues, is the most acidic of the applied compounds, and is not eluted until the concentration of pyridine formate in the solvent is about 0.8 M. The phosphopeptides containing acidic amino acids are somewhat less acidic. They also show a strong binding to the column and are eluted in the order expected.

Phosphorylated amino acids and phosphopeptides containing neutral amino acids are eluted within a rather narrow region. Gly-SerP-Gly contains one phosphate group per three amino acid residues and is, as expected, eluted first. The phosphorylated derivatives of threonine and serine, having one phosphate group per amino acid residue, are eluted after the phosphopeptides containing neutral amino acids. The order of elution between the compounds agrees fairly well with the order of the pK values for their carboxyl groups, reported [17] by Fölsch and Österberg as being: Gly-SerP-Gly 3.29, SerP-Gly 3.13, SerP-Leu 3.11, Gly-SerP 2.90 and SerP 2.07.

As the method has been worked out for preparative use, no exact determinations of the recovery have been performed. The ninhydrin colour of each compound indicates a yield of between 85—107 %.

With slight modifications the present method has been used for isolation of phosphorylated peptides from products obtained by hydrogenation of O-diphenylphosphorylated peptide derivatives [6,7].

Acknowledgement. The author wishes to express sincere thanks to Professor Olof Mellander for his support in this work.

The work was supported by grants from the *Swedish Medical Research Council* to Professor Olof Mellander.

REFERENCES

1. Mellander, O. *Upsala Läkarfören. Förh.* **52** (1947) 107.
2. Mellander, O. and de Verdier, C.-H. *Acta Soc. Med. Upsaliensis* **57** (1952) 218.
3. Österberg, R. *Arkiv Kemi* **13** (1959) 409.
4. Bennich, H., Johansson, B. and Österberg, R. *Acta Chem. Scand.* **13** (1959) 1171.
5. Fölsch, G. *Acta Chem. Scand.* **12** (1958) 561.
6. Fölsch, G. *Acta Chem. Scand.* **13** (1959) 1407.
7. Theodoropoulos, D., Bennich, H., Fölsch, G. and Mellander, O. *Nature.* **184** (1959) 187.
8. Schaffer, N. K., May, S. C., Jr. and Summerson, W. H. *J. Biol. Chem.* **202** (1953) 67.
9. Schaffer, N. K., Simet, L., Harshman, S., Engle, R. R. and Drisko, R. W. *J .Biol. Chem.* **225** (1957) 197.
10. Flavin, M. *J. Biol. Chem.* **210** (1954) 771.
11. Hipp, N. J., Groves, M. L. and McMeekin, T. L. *J. Am. Chem. Soc.* **79** (1957) 2559.
12. de Verdier, C.-H. *Acta Chem. Scand.* **7** (1953) 196.
13. de Verdier, C.-H. *Acta Chem. Scand.* **8** (1954) 1302.
14. Kennedy, E. P. and Smith, S. W. *J. Biol. Chem.* **207** (1954) 153.
15. Fölsch, G. and Mellander, O. *Acta Chem. Scand.* **11** (1957) 1232.
16. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
17. Fölsch, G. and Österberg, R. *J. Biol. Chem.* **234** (1959) 2298.

Received July 27, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1791–1808

Studies on the Hydrolysis of Metal Ions

23. The Hydrolysis of the Ion $Bi_6(OH)_{12}^{6+}$ in Perchlorate Medium

ÅKE OLIN

Department of Inorganic Chemistry, The Royal Institute of Technology, Stockholm 70, Sweden

The hydrolysis of the ion $Bi_6(OH)_{12}^{6+}$ has been studied at 25°C in 0.1 M sodium perchlorate medium by means of the cells

$$\begin{array}{l} - \text{ Bi—Hg / S / ref. } + \\ - \text{ glass \quad / S / ref. } + \end{array}$$

where S had the general composition

B M Bi(III), H M H^+, $(0.1-3B-H)$ M Na^+, 0.1 M ClO_4^-.

The small solubility of the hydrolysis products necessitated the low concentration of the neutral salt. To keep the activity factors constant, the total metal ion concentration had to be diminished accordingly. B was varied between 4 and 0.5 mM. The data may be explained by the equilibria

$$\begin{array}{lll} 1.5\ Bi_6(OH)_{12}^{6+} + 2H_2O \rightleftharpoons Bi_9(OH)_{20}^{7+} + 2H^+ & \log K = -3.5 & \pm 0.1 \\ Bi_9(OH)_{20}^{7+} + H_2O \rightleftharpoons Bi_9(OH)_{21}^{6+} + H^+ & \log K_1 = -3.2 & \pm 0.2 \\ Bi_9(OH)_{21}^{6+} + H_2O \rightleftharpoons Bi_9(OH)_{22}^{5+} + H^+ & \log K_2 = -2.6 & \pm 0.2 \end{array}$$

The hydrolysis of Bi(III) in perchlorate medium can, for ease in description, be arbitrarily divided into two parts. The first part may be called the hydrolysis of the bismuth ion (Bi^{3+}) and the second part the hydrolysis of the $Bi_6(OH)_{12}^{6+}$ ion.

The hydrolysis of the bismuth ion has been studied a number of times [1,2]. The explanations of the experimental data, though divergent in details, give as main hydrolysis products polynuclear complexes with the ratio Bi:O equal or close to one. Recent studies [2,2a] strongly indicate $Bi_6(OH)_{12}^{6+}$ (or $Bi_6O_6^{6+}$ *etc.*) as the predominating species.

Holmqvist [3] found that solutions of the analytical composition $BiOClO_4$ are acid, which shows that the hydrolysis proceeds beyond the "bismuthyl" stage. He suggested the formation of the complex $Bi_2(OH)_5^+$. In 1954 Faucherre [4] published log h measurements on bismuth perchlorate solutions which had a composition varying between $BiOClO_4$ and $Bi_2(OH)_5ClO_4$. From the

measurements he concluded that the complex $Bi_8(OH)_{20}^{4+}$ is formed in this region. Faucherre states, however, that the result is quite uncertain. The present investigation is an extension to lower log h values of a previous study by the present author [2] on the hydrolysis of bismuth perchlorate solutions using electrometric methods.

Some of the symbols frequently encountered in the text are defined below:

B	total bismuth(III) concentration
b	concentration of Bi^{3+}
C	total bismuth(III) concentration, calculated as $Bi_6(OH)_{12}^{6+}$; $C = B/6$
c	concentration of $Bi_6(OH)_{12}^{6+}$
H	analytical hydrogen ion concentration
h	actual hydrogen ion concentration
Z	average number of hydrogen ions split off by one Bi^{3+}
Z'	average number of hydrogen ions split off by one $Bi_6(OH)_{12}^{6+}$. $Z' = 6(Z-2)$
$\eta =$	log B/b
$\eta' =$	η + (unknown) constant defined by eqn. (7)
$\delta =$	concentration of basic impurity in $NaClO_4$
$\varkappa_{P,Q}$	formation constant for $Bi_Q(OH)_P$ from $Bi_6(OH)_{12}^{6+}$ and H_2O
$y(x)_v$	y as a function of x at constant v

Other symbols used are defined when they first appear. All concentrations in the tables and figures are expressed in M (mole/l) and equilibrium constants are given on the M scale. In the text, however, the more convenient unit mM will be used frequently.

Method of investigation and choice of experimental conditions

The hydrogen ion concentration and the variation of free $[Bi^{3+}]$ with log h have been measured electrometrically for a large number of bismuth perchlorate solutions at equilibrium. As usual the measurements were carried out as potentiometric titrations keeping the total bismuth concentration constant in each run and varying log h by adding acid or base.

Because of the fairly low solubility [11] (*ca.* 25 mmoles/l) of the salt described as $Bi_2(OH)_3OClO_4$ it was found necessary to use a total ClO_4^- concentration as low as 0.1 M in order to keep the bismuth in solution in the log h range between -3 and -5. To minimize variations in the activity factors, the metal ion concentration has to be diminished accordingly. In 3 M $(Na)ClO_4$ it appears possible to use solutions containing at least 0.1 M of the metal ion investigated without causing serious variations in the activity factors; since little is known about these factors in solutions of low neutal salt concentration, the highest bismuth(III) concentration was now chosen by proportionality as 4 mM. In this way one is likely to be on the safe side [5].

The solutions measured had the general composition

$$[Bi(III)] = B \text{ M}; \quad [H^+] = H \text{ M}; \quad [Na^+] = (0.1-3B-H) \text{ M}; \quad [ClO_4^-] = 0.1 \text{ M}$$

where B denotes the total bismuth(III) concentration, and H the analytical hydrogen ion concentration defined by

$$H = [ClO_4^-] - 3B$$

In the present study H is always a negative number.

The following cells were used to measure the hydrogen ion concentration, h, and unhydrolysed Bi^{3+}, b.

$$- \text{ glass } /H^+/ \text{ ref } + \qquad \text{(I)}$$

$$E_g = E_g^\circ - 59.15 \log h - E_j \qquad (1)$$

$$- \text{ Bi—Hg } /Bi^{3+}/ \text{ ref } + \qquad \text{(II)}$$

$$E_{am} = E_{am}^\circ - 19.72 \log b - E_j \qquad (2)$$

As reference half-cell

$$0.1 \text{ M } NaClO_4/0.1 \text{ M } ClO_4^-, 0.095 \text{ M } Na^+, 0.005 \text{ M } Ag^+/AgCl, Ag \qquad \text{(III)}$$

was used, and the salt bridge was of the "Wilhelm" type [6].

To study the effect of a change in the nature of the neutral salt a few titrations were carried out in 0.1 M$(Ba)ClO_4$. In addition the direction of titration was reversed and $Ba(OH)_2$ was added to an acid solution. The use of this base ensures the absence of interfering carbonate. In general, acid was added to a hydrolysed solution. The experiments were carried out at 25°C.

Determination of the liquid junction potential in 0.1 M $NaClO_4$ and free acid or base in neutral salt solutions

The liquid junction potential in 0.1 M $(Na)ClO_4$ was determined between 0 and 15 mM H^+ by means of the cell

$$Ag, AgCl/0.005 \text{ M } Ag^+, H \text{ M } H^+, (0.095 - H) \text{ M } Na^+, 0.1 \ ClO_4^-/\text{ref.} \qquad \text{(IV)}$$

$$E = E^\circ - 59.15 \log[Ag^+] - E_j = E^{\circ\prime} - E_j \qquad (3)$$

since $[Ag^+]$ was kept constant. The liquid junction potential so defined contains any variation in the activity factors. The procedure to obtain E_j from the measurements has been described by Biedermann and Sillén [7] The above-mentioned cell has the advantage, over those containing log h sensitive electrodes, that small errors in the hydrogen ion concentration do not influence the result at low concentrations. Moreover the extrapolation involved in the determination can be carried out with greater confidence since the extrapolated value ($E^{\circ\prime}$), *i.e.* the potential of the cell with no acid added, can also be measured as a check.

The results fit the simple Henderson equation [8] with

$$E_j = -59.15 \log(1 + 26.7\ h) \qquad 0 < h < 0.015 \text{ M} \qquad (4)$$

The formula reproduces the experimental data to better than 0.05 mV.

When using the cell

$$\log h \text{ sensitive electrode } / h \text{ M } H^+, I - h \text{ M } Na^+, I \text{ M } ClO_4^-/ \text{ ref.} \qquad \text{(V)}$$

$$E = E^\circ - 59.15 \log h - E_j \qquad (5)$$

to determine E_j, it is often found that the points lying close to the origin ($H'<3$ mM) are off the straight line $E + 59.15 \log H'$ (H'). This is due to small acidic or basic impurities in the neutral salt, which cause the calculated hydrogen ion concentration, H', and actual hydrogen ion concentration, h, to be different.

The amount of impurity, $\delta = H'-h$, can be found by the following procedure. Let us assume, to be specific, that we want to know the concentration of small amounts of acid or alkali suspected to be present in a I M $NaClO_4$ solution at 25°C. To a known volume of this solution is added gradually a standardized $HClO_4$ solution, also containing $NaClO_4$ to make $[ClO_4^-] = I$ M, and the emf of the above cell (V) is measured. $E + 59.15 \log H'$ is then plotted as a function of H', where H' is the hydrogen ion concentration calculated from the volume of acid added. At higher acidities, where the impurity is small compared to H', the plot is linear and can be extrapolated to $H' = 0$ to give $E°$. Once $E°$ is known, h can be found for the first few points. The difference between h and H' for each point is then used to find the concentration of the impurity. It seems possible to determine this impurity to $\pm$ 0.01 mM.

The liquid junction potentials determined with the quinhydrone electrode agreed within $\pm$ 0.1 mV with those obtained with the silver-silverchloride electrode, which is to be expected if the activity factors stay constant (or change in the same manner with log h), since the basicity of quinone [9] does not show up if h <15 mM.

MATERIALS AND ANALYSIS

Sodium perchlorate was prepared from recrystallized Na_2CO_3 (Merck p.a.) and $HClO_4$ (Kebo p.a.). The product was recrystallized once from distilled water. The stock solution of $NaClO_4$ was analysed by evaporating a known volume to dryness and weighing the residue as $NaClO_4$. The determination of acidic or basic impurities was made as described above. After a preliminary determination of this impurity, a calculated amount of acid or base was added to make the solution neutral. The final product was reanalysed and found to be neutral to within $\pm$ 0.01 mM H^+ or OH^-.

Barium perchlorate was prepared from $Ba(OH)_2$ (Merck p.a.) and $HClO_4$. The product was recrystallized twice to reduce the chloride content to the order of 10^{-6} M in a 0.05 M solution. The stock solution was analyzed by evaporating a known volume to dryness and weighing the residue as $Ba(ClO_4)_2$. $Ba(ClO_4)_2$ is known to be a very hygroscopic substance, therefore the result was checked by passing known volumes through a cation-exchange resin and titrating with standard base. The two methods gave results identical to 0.1 %.

Perchloric acid solutions were prepared by diluting $HClO_4$ (Kebo p.a. 70—72 %) and were standardized against $KHCO_3$.

Barium hydroxide solutions were prepared by dilution of a filtered stock solution of $Ba(OH)_2$. All solutions containing this base were handled under nitrogen.

Bismuth (III) perchlorate solutions were prepared from Bi_2O_3 and $HClO_4$. Two stock solutions were made. The first solution, which had the analytical composition $BiOClO_4$, was prepared from known amounts of the oxide and the acid. As a check it was analysed for bismuth according to Winkler [10] and for perchlorate by cation exchange. Calculated and found values agreed to 0.1 %. The second solution was prepared by dissolving the oxide in hot acid but now choosing the molar proportion Bi_2O_3 : $HClO_4$ = 1:1.2. On cooling the solution crystals separated. These crystals gave the same X-ray powder photographs as crystals prepared by adding a concentrated $NaClO_4$ solution to $BiOClO_4$, or NaOH to mixtures of $NaClO_4$ and $Bi(ClO_4)_3$ solutions as described by Holmqvist[3] and Prytz and Nagel [11]. These authors found from the analysis a salt of composition $Bi_2(OH)_5OClO_4$. In addition to this salt Prytz and Nagel found a yellow salt, which precipitated when a large amount of $NaClO_4$ was added to a bismuthyl perchlorate solution. Its composition was given as $Bi_3(OH)_3O_2(ClO_4)_2$. This salt was not obtained in the present investigation.

The crystals were dissolved by adding water. On standing overnight, however, the solution became turbid. After filtration and standing the turbidity developed again. Since no impurities were found in the distilled water used, carbon dioxide from the air was suspected as interfering. This was confirmed by passing in CO_2. The formation of the insoluble carbonate is a slow process, and a pronounced turbidity appears only after one

or two h. The final stock solution was filtered through a bacteria filter (Jena G 5), and some acid added. The solution was analyzed and thereafter stored and handled under nitrogen. When stored under nitrogen the solution remained clear.

The Bi_2O_3 used was obtained by igniting twice recrystallized $Bi(NO_3)_3$ (Baker p.a.).

Bismuth amalgam was prepared and handled as described previously [2]. The bismuth concentration in the amalgam was 0.5 % (weight).

The emf measurements

The log h measurements were made with a Radiometer glass electrode, which had been checked against the quinhydrone electrode in an acetate buffer also containing 0.1 M $NaClO_4$. The potential between the two electrodes remained constant throughout the log h range -2 to -6. Thus the glass electrode behaved as a perfect hydrogen electrode. The bismuth amalgam electrode consisted of a pool on the bottom of the titration vessel.

Since E°_{am} could not be determined we could find only relative concentrations of unhydrolysed bismuth from the potentials. In order to compare titrations made at different times, a certain titration was repeated at regular intervals, and the average potential change (at most 0.4 mV) from the first of these standard titrations, for identical points, was taken as the shift in E°_{am} and used as a correction to the measured E.

All potentials were recorded with a Radiometer valve potentiometer (PHM 3) with the exception of the liquid junction potential measurements, where a Cambridge potentiometer was used.

The equilibria were rapidly attained. No time effects were present. This was established by comparing titrations for the same total bismuth concentration but starting from different analytical hydrogen ion concentrations. The Z and η' curves for the two titrations, in the log h region where they overlapped, were identical within the limits of the experimental error.

The glass electrode measurements showed, between identical titrations, a reproducibility which in general lay within the accuracy of the instrument (± 0.2 mV). The agreement between titrations on solutions made up from the two stock solutions was better than 0.01 Z units in most of the range studied. In the most acidic region, the spread in the glass electrode measurements, however, caused a somewhat larger uncertainty in Z.

The behavior of the amalgam electrode changed remarkably during the titration. As log h decreased the electrode became more and more sensitive to shakings and vibrations. The time required to attain a stable potential increased and finally no constant potentials were obtained. The last few potentials recorded were uncertain to at least 0.5 mV since the potential fluctuated by this amount. A swirling motion of the amalgam changed the potential by more than 20 mV and it regained the previous value after 1—2 h. This may be contrasted with the behavior at log $h = -2$ and higher. In this region the cell attained the equilibrium value immediately and shaking did not affect the potential at all.

RESULTS AND CALCULATIONS

Owing to the pronounced hydrolysis of Bi^{3+} it is not possible, when working with 0.1 M $(Na)ClO_4$ medium, to add enough acid to repress this reaction. Thus E° values could not be obtained by inserting analytical concentrations in eqns. (1) and (2). Instead, E°_g had to be determined in $HClO_4$—$NaClO_4$ mixtures, whereas E°_{am} remains unknown. Therefore only changes in the concentration of Bi^{3+} can be determined and not absolute values.

From eqn. (2) we find $\log b = \frac{E^\circ_{am}}{19.72} - \frac{(E_{am} + E_j)}{19.72}$ and from the definition of η;

$$\eta = \log B - \log b = \log B + \frac{(E_{am} + E_j)}{19.72} - \frac{E^\circ_{am}}{19.72} \qquad (6)$$

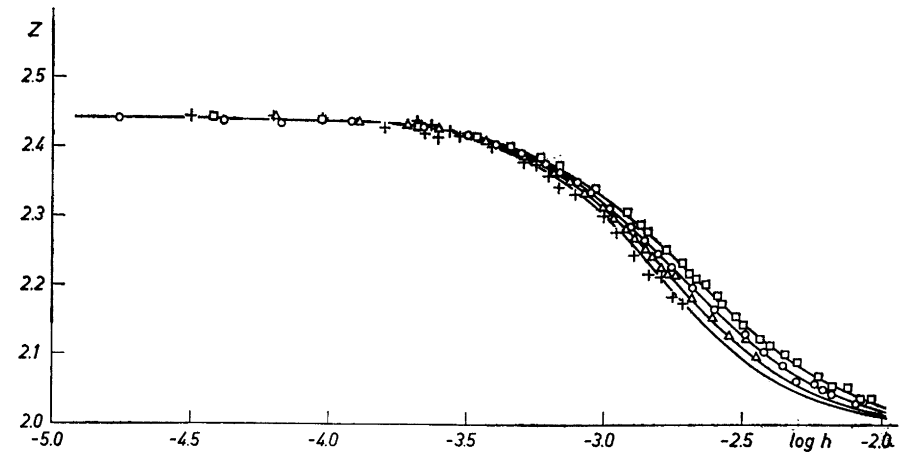


Fig. 1. Average number, Z, of protons split off by one Bi(III) as a function of log h. The lines are calculated with log $\varkappa_{20,9} = -3.5$, log $K_1 = -3.2$ and log $K_2 = -2.6$. □ 4 mM Bi(III); ○ 2 mM Bi(III); △ 1 mM Bi(III); + 0.5 mM Bi(III); $\delta = 0.01$ mM OH^-.

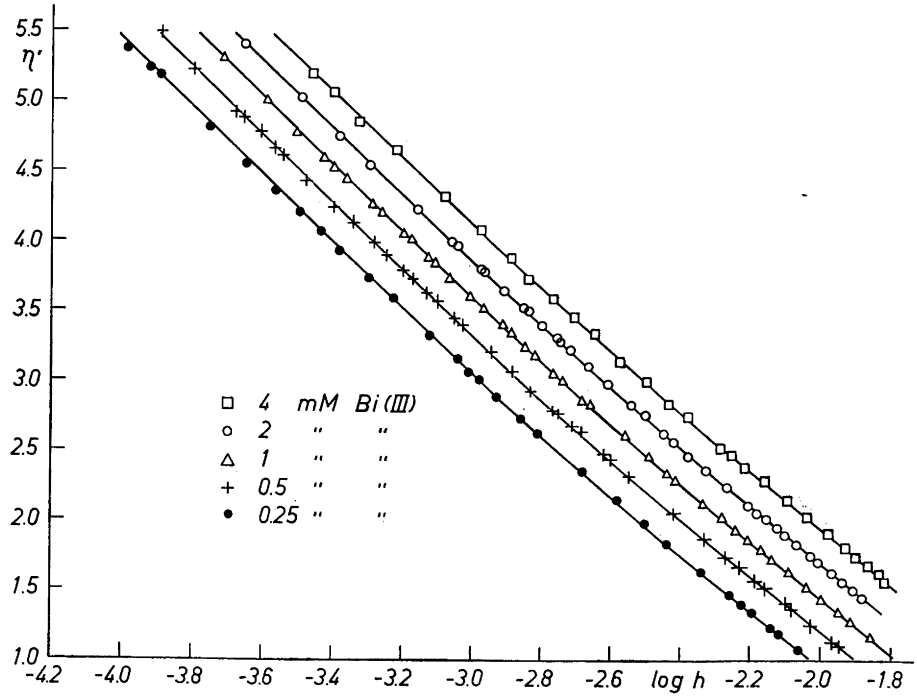


Fig. 2. $\eta' = \log \dfrac{[\mathrm{Bi(III)}]_{tot}}{[\mathrm{Bi}^{3+}]} + \nabla$ as a function of log h. The lines are calculated with log $\varkappa_{20,9} =$ 3.5, log $K_1 = -3.2$ and log $K_2 = -2.6$.

Table. 1. Corresponding values of log h, Z and η'. In general only points where both Z and η' were measured simultaneously are given.

Bi(III) = 4 mM

$-\log h$	Z	η'	$-\log h$	Z	η'
4.427	2.442	in 0,1 M $(Ba)ClO_4$	2.662	2.206	3.339
4.035	2.437		2.576	2.169	3.142
3.681	2.424		2.500	2.138	2.990
3.460	2.410	5.200	2.437	2.122	2.837
3.326	2.394	4.866	2.383	2.102	2.721
3.225	2.380	4.653	2.290	2.082	2.528
3.085	2.350	4.318	2.255	2.076	2.482
2.976	2.321	4.080	2.219	2.051	2.381
2.913	2.303	3.902	2.158	2.043	2.254
2.890	2.294	3.882	2.097	2.040	2.148
2.838	2.277	3.750	2.040	2.020	2.026
2.770	2.249	3.588	1.981	2.028	1.904
2.713	2.230	3.451	1.881	—	1.702
			1.835	—	1.620

Bi(III) = 2 mM

$-\log h$	Z	η'	$-\log h$	Z	η'
4.758	2.441	in 0,1 M $(Ba)ClO_4$	2.611	2.162	2.988
4.379	2.437		2.544	2.130	2.836
4.038	2.435		2.449	2.110	2.633
3.916	2.433	—	2.380	2.085	2.470
3.654	2.422	5.407	2.317	2.060	2.349
3.495	2.410	5.021	2.270	2.054	2.252
3.384	2.398	4.742	2.207	2.046	2.121
3.298	2.386	4.539	2.156	2.034	2.019
3.227	2.373	4.357	2.104	2.032	1.902
3.161	2.359	4.230	2.060	—	1.816
3.059	2.333	3.992	2.008	—	1.705
2.976	2.309	3.804	1.971	—	1.629
2.900	2.285	3.637	1.940	—	1.563
2.839	2.250	3.510	1.911	—	1.507
2.757	2.222	3.312	1.884	—	1.451
2.665	2.185	3.109			

Bi(III) = 1 mM

$-\log h$	Z	η'	$-\log h$	Z	η'
4.195	2.436	—	2.822	2.232	3.189
3.891	2.429	5.724	2.768	2.205	3.062
3.718	2.421	5.303	2.740	2.204	3.016
3.595	2.414	5.009	2.659	2.172	2.824
3.505	2.404	4.786	2.577	2.115	2.646
3.430	2.393	4.598	2.491	2.112	2.463
3.365	2.384	4.446	2.441	2.089	2.347
3.290	2.375	4.259	2.391	—	2.251
3.261	2.366	4.198	2.351	—	2.159
3.200	2.357	4.050	2.285	—	2.027
3.178	2.350	4.015	2.209	—	1.860
3.127	2.339	3.888	2.143	—	1.728
3.066	2.321	3.741	2.094	—	1.632
3.003	2.299	3.604	2.040	—	1.515
2.968	2.285	3.518	1.996	—	1.429
2.918	2.271	3.407	1.952	—	1.343
2.889	2.256	3.336	1.916	—	1.267
2.850	2.241	3.249	1.856	—	1.145

Table 1. Continued

Bi(III) = 0,5 mM

$-\log h$	Z	η'	$-\log h$	Z	η'
4.535	2.430	—	2.765	2.162	2.796
4.204	2.426	—	2.711	2.154	2.680
4.019	2.422	5.763	2.689	2.096	2.640
3.889	2.418	5.489	2.620	2.104	2.482
3.798	2.408	5.225	2.600	2.072	2.440
3.653	2.400	4.881	2.557	2.082	—
3.619	2.412	4.774	2.545	2.094	2.320
3.566	2.404	4.657	2.530	2.042	—
3.546	2.388	4.612	2.415	—	2.030
3.478	2.394	4.429	2.339	—	1.879
3.400	2.380	4.247	2.270	—	1.747
3.341	2.376	4.130	2.233	—	1.670
3.284	2.344	3.988	2.187	—	1.564
3.247	2.350	3.902	2.158	—	1.524
3 200	2.338	3.790	2.104	—	1.407
3.167	2.322	3.729	2.030	—	1.260
3.132	2.308	3.638	1.967	—	1.133
3.048	2.294	3.450	1.949	—	1.103
3.027	2.280	3.400	1.903	—	1.011
2.944	2.262	3.207	1.849	—	0.905
2.883	2.214	3.070			

Bi(III) = 0.25 mM

$-\log h$	η'	$-\log h$	η'
3.894	5.193	2.679	2.363
3.754	4.828	2.581	2.150
3.651	4.554	2.503	1.988
3.565	4.356	2.440	1.841
3.495	4.209	2.339	1.638
3.434	4.072	2.258	1.481
3.382	3.930	2.224	1.405
3.298	3.738	2.195	1.344
3.228	3.591	2.141	1.233
3.122	3.332	2.118	1.192
3.041	3.165	2.060	1.075
3.009	3.068	1.996	0.949
2.927	2.891	1.933	0.837
2.858	2.734	1.884	0.731
2.809	2.627	1.813	0.599

The measured E_{am} ranged between 500 and 400 mV. In the following calculations E°_{am} was arbitrarily replaced by 360.6 mV, which gave a convenient quantity η' defined as (∇ = unknown constant)

$$\eta' = \log B + \frac{(E_{am} + E_j)}{19.72} - \frac{360.6}{19.72} = \eta + \frac{(E^{\circ}_{am} - 360.6)}{19.72} = \eta + \nabla \qquad (7)$$

In order to be able to find possible reactions giving rise to Z values above 2 it is necessary to make some assumptions as to what species are formed

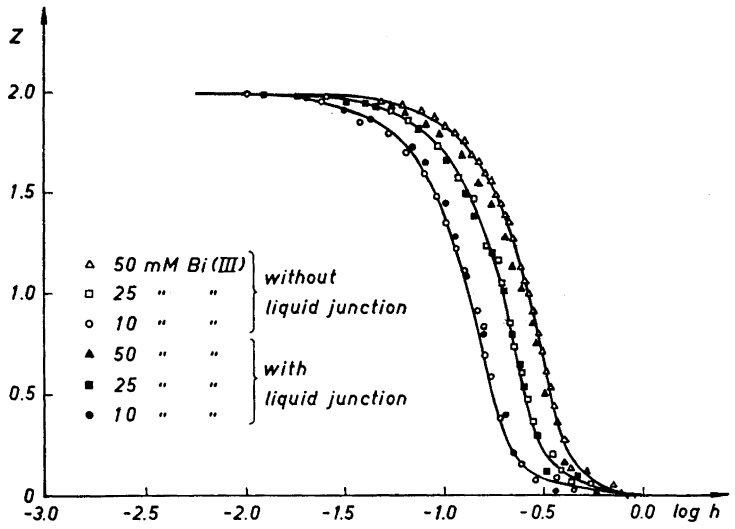


Fig. 3. Average number, Z, of protons split off by one Bi^{3+} in 3 M $(Na)ClO_4$ as a function of log h. The lines are calculated with $\log \beta_{12,6} = 0.33$ and $\log \beta_{11} = -1.58$.

in earlier stages of the hydrolysis. Previous measurents of η with 3 M$(Na)ClO_4$ medium[2] have indicated that the main complex formed is $Bi_6(OH)_{12}^{6+}$, and that in addition some $BiOH^{2+}$ is formed. Assuming only these two complexes it was possible to explain quantitatively the data over a wide range of concentrations. This hypothesis has been the basis for the present calculations.

The experimental results are shown graphically in Figs. 1 and 2 and numerically in Table 1.

With 0.05 M $Ba(ClO_4)_2$ — to be strict, 0.1 M $(Ba)ClO_4$ — as medium only log h measurements with $[Bi(III)_{tot}] = 4$ and 2 mM were made. The data obtained in general agreed to 0.005 Z units with those found with 0.1 M $(Na)ClO_4$ as medium. Therefore only a few data in the low log h region not covered by the measurements with the latter medium are given.

The η' values in the most acid region are characterized by parallel curves for the various B values, a slope $(\Delta\eta'/\Delta \log h)_B$ close to -2, and $(\Delta \log B/\Delta \log h)_{\eta'} = 2.4$ to 2.5. Thus the curves have the same properties as found with 3 M $(Na)ClO_4$, which makes it likely that the same complex, $Bi_6(OH)_{12}^{6+}$, predominates also at lower ionic strengths. With decreasing log h, $(\Delta\eta'/\Delta \log h)_B$ decreases, and the curves tend to become closer together, as seen along the log h axis.

The Z curves clearly indicate that $Bi_6(OH)_{12}^{6+}$ is in equilibrium with *larger* complexes, since the curves are separate and do not cross. The curves do not show a pronounced shelf at $Z = 2$ but turn fairly sharply downwards after having passed this value. Unfortunately it is not possible to follow the hydrolysis in 0.1 M $(Na)ClO_4$ for much smaller values than $Z = 2$, for the experimental

accuracy declines rapidly as the acidity increases. That there is a short separation between the reactions occuring above and below $Z = 2$ is evident from Fig. 3, which shows some Z curves from measurements with 3 $M(Na)ClO_4$. They exhibit a pronounced bending off towards $Z = 2$. These measurements are described in an appendix to the present paper. Holmberg, Kraus and Johnson [12] also found $Z_{lim} = 2.0$ from log h measurements.

An upper limit seems to exist for Z around 2.44. The solutions had a very low buffer capacity in this region. A small change in analytical composition led to a large log h change and finally precipitation started. It is remarkable that this limiting value is different from 2.5 which is the ratio OH:Bi in the solid phase [3,11]. The divergence of the limiting value from 2.5 is not likely to be due to an experimental error, since it was obtained with two independent stock solutions both in $NaClO_4$ and $Ba(ClO_4)_2$. Moreover protons were set free when the precipitate was formed, as is expected if the ratio OH:Bi = 2.5 in the solid phase and < 2.5 in the solution.

Elucidation of possible hydrolysis products. The formulas of the products formed by the hydrolysis of the $Bi_6(OH)_{12}^{6+}$ ion are difficult to find with certainty mainly for two reasons. Firstly, only low total metal ion concentrations could be measured, which causes the Z values in general to be uncertain to 0.01 unit. When we have polynuclear complexes present the spacing between the Z curves is very important in finding possible species to explain the data. With a small concentration range considerable uncertainty is introduced. Secondly high powers are present in the expression for the law of mass action for the complexes formed. This leads to a scatter of the experimental points used in the calculations.

It is convenient to consider the complexes as built up from $Bi_6(OH)_{12}^{6+}$ (C) and $OH^-(-H^+)$ and not from Bi^{3+} and OH^-. The complex $Bi_q(OH)_p$ is therefore written as

$$Bi_q(OH)_p = C_q(OH)_{p'} \text{ with } q = 6q' \text{ and } p = 12q' + p' \tag{8}$$

where p' and $6q'$ are whole numbers.

$\varkappa_{p',q'}$ denotes the equilibrium constant for the reaction

$$q'C + p'H_2O \rightleftharpoons C_{q'}(OH)_{p'} + p'H^+ \tag{9}$$

The expression (9) is so general that it includes all conceivable species, also Bi^{3+} ($q' = 1/6$, $p' = -2$) and $BiOH^{2+}$ ($q' = 1/6$, $p' = -1$) known to exist in small amounts.

The law of mass action gives

$$C = c + \Sigma q' \varkappa_{p'q'} c^{q'} h^{-p'} \tag{10}$$

$$CZ' = \Sigma p' \varkappa_{p'q'} c^{q'} h^{-p'} \tag{11}$$

The concentration of $Bi_6(OH)_{12}^{6+}$, c, is found from the amalgam data. We start with the equation

$$\frac{[Bi_6(OH)_{12}^{6+}] \cdot h^{12}}{[Bi^{3+}]^6} = \beta_{12,6} \text{ or } \log c = \log \beta_{12,6} + 6 \log b - 12 \log h \tag{12}$$

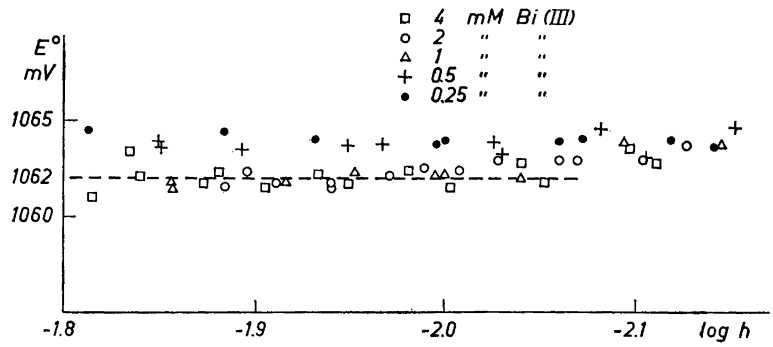


Fig. 4. The values of E° found from eqn. (13) as a function of log h.

Inserting log b and log h from eqns. (1) and (2) we get

$$19.72 \log c = 19.72 \log \beta_{12,6} + 6E^{\circ}_{am} - 4E^{\circ}_{g} + 4E_{g} - 6E_{am} - 2E_{j} = \\ = E^{\circ} + 4E_{g} - 6E_{am} - 2E_{j} \quad (13)$$

In this equation both $\beta_{12,6}$ and E°_{am} are unknown but by inserting $B/6$ for c, $E^{\circ} = 19.72 \log \beta_{12,6} + 6E^{\circ}_{am} - 4E^{\circ}_{g}$ can be found from the potentials in the most acid region where $Bi_6(OH)_{12}^{6+}$ is the predominating species. Since the amalgam data are presented as η', an equation from which c can be calculated using η' will be given.

$$\eta' = \log B - \log b + \nabla \text{ or } \log b = \log B - \eta' + \nabla \quad (14)$$

Inserting this expression for log b in (12)

$$\log c = \log \beta_{12,6} + 6\nabla + 6 \log B - 6\eta' - 12 \log h \quad (15)$$

is obtained. Log $\beta_{12,6} + 6\nabla$ is found by inserting $B/6$ for c as indicated above.

Fig. 4 shows the values of E° obtained from eqn. (13) in the region where $Bi_6(OH)_{12}^{6+}$ may be expected to predominate. It is seen that for the three highest concentrations E° comes out quite constant $= 1\,062 \pm 0.5$ mV, which is almost better than could be expected, considering the factors 4 and 6 in eqn. (13). The lower concentrations yield an E° *ca.* 2 mV higher. This is not unexpected since $Bi_6(OH)_{12}^{6+}$ is certainly not the sole species present. If for example the equilibrium constants were the same as given in Ref.[2] for 3 M $(Na)ClO_4$, we may estimate that for $B = 0.25$ mM, 5—7 % of the bismuth(III) is present as Bi^{3+} and $BiOH^{2+}$ at log $h = -2$. For the highest values of B we may also have disturbances from species more hydrolysed than $Bi_6(OH)_{12}^{6+}$ as indicated by the $(\Delta\eta'/\Delta \log h)_B$ values which are smaller than -2. In the calculations on the 0.5 mM titrations we have used the value of E° found for the higher concentrations, but only used the data in the low log h region where an error in E° is not so serious. No calculations have been performed on the 0.25 mM titrations and the reason for this is, that beside the uncertainty

in $E°$, these data must be subject to large errors since $(\Delta\eta'/\Delta \log h)_B$ does not decrease regularly with decreasing $\log h$ values. This contradicts the law of mass action.

When we know c, the quantity $CZ'/(C-c)$, which is the average number of OH^- per $Bi_6(OH)_{12}^{6+}$ unit in the complexes, can be calculated. The $CZ'/(C-c)$ ($\log h$) curves for the 4 and 2 mM titrations coincided or very nearly so, whereas the 1 and 0.5 mM titrations were slightly off the curve. However, if the $NaClO_4$ contained a basic impurity, δ, of the order of 0.01 mM, the $CZ'/(C-c)$ ($\log h$) curves for the different B coincide.

If only one complex had been formed, $CZ'/(C-c)$ should have come out as a constant equal to p'/q'. The hypothesis that only one complex is formed is ruled out, since $CZ'/(C-c)$ is found to be variable.

The same conclusion is reached from consideration of Z data alone [13]. Eqns. (10) and (11) give

$$C = c + q'\varkappa_{p'q'}c^{q'}h^{-p'} \tag{10a}$$

$$CZ' = p'\varkappa_{p'q'}c^{q'}h^{-p'} \tag{11a}$$

and after elimination of c

$$\log X = \log h - \frac{1}{p'}\log(p'\varkappa_{p'q'}C^{q'-1}) = \frac{1}{p'}\log\frac{(p'-Z'q')^{q'}}{p'^{q'}Z'} \tag{16}$$

Comparison with $Z'(\log X)$ curves calculated for different q' and choosing p' to give a $Z_{\lim} \approx 2.4$, showed no fit. The theoretical curves are steeper than the experimental ones, which means that intermediate complexes must be present.

The fact that the $CZ'/(C-c)$ ($\log h$) curves are independent of C, suggests that the complexes formed are homonuclear. The hydrolysis can then be represented by the reactions

$$Q'C + P'H_2O \rightleftharpoons C_{Q'}(OH)_{P'} + P'H^+ \tag{17}$$

$$C_{Q'}(OH)_{P'} + n\ H_2O \rightleftharpoons C_{Q'}(OH)_{P'+n} + n\ H^+ \quad (n = 1,2,3,\ ---N) \tag{18}$$

where Q', P' and N are unknown. With $\varkappa_{\overline{P',\ Q'}} = \varkappa$ and $\varkappa_{P'_i+n,\ Q'} = \alpha_n\varkappa$ the law of mass action gives

$$C = c + Q'\varkappa c^{Q'}h^{-P'}(1 + \alpha_1h^{-1} + \alpha_2h^{-2} + - - -) \tag{19}$$

$$CZ' = \varkappa c^{Q'}h^{-P'}(P' + (P'+1)\alpha_1h^{-1} + (P'+2)\alpha_2h^{-2} + - - -) \tag{20}$$

For the complex of highest Z, $C_{Q'}(OH)_{P'+N}$ or $Bi_Q(OH)_{P+N}$, the following relations must be fulfilled

$$(P+N)/Q = 2.44 \pm 0.02 \text{ or } (P'+N)/6Q' = 0.44 \pm 0.02;\ Q > 6;\ P,Q,N,P',$$

and $6Q'$ whole numbers.

A tentative value for Q' is found from eqn. (19). If homonuclear complexes are formed and the correct value of Q' is chosen $\log(C-c) - Q'\log c = f(h)$ when plotted as a function of $\log h$ should give one curve independent of C. Different Q' values were tested starting from $Q' = 7/6$. This test excluded all values of Q' except 7/6 and 9/6. $Q' = 8/6$ is ruled out since there is no corre-

sponding P' that gives a Z_{lim} in agreement with experiment. For $Q' > 9/6$ the experimental data fall less and less satisfactorily on a single curve.

With the help of the Q' values 7/6 and 9/6 a tentative value for N can be found. The experimental $CZ'/(C-c)$ (log h) curve ranges between 2.7 $\pm$0.1 and 1.3 $\pm$0.2, with these limits as asymptotes. These limiting values should be equal to $(P' + N)/Q'$ and P'/Q'. For $Q' = 7/6$ we should have $P' = 1.5 \pm 0.2$ and $P' + N = 3.15 \pm 0.1$. For $Q' = 9/6$ we find $P' = 1.95 \pm 0.3$ and $P' + N = 4.05 \pm 0.15$. We may thus conclude $N = 2$.

Determination of α_1 and α_2. Eqns. (19, 20) with $N = 2$ give after elimination of c

$$Q'\left(\frac{CZ'}{C-c}\right) - P' = \frac{\alpha_1 h^{-1} + 2\alpha_2 h^{-2}}{1 + \alpha_1 h^{-1} + \alpha_2 h^{-2}} = n \qquad (22)$$

Experimental $\bar{n}$(log h) were compared with normalized curves for the right hand side of the equation according to Sillén [14]. The sets tested were $P' = 1$, $Q' = 7/6$ (or $P = 15$, $Q = 7$) and $P' = 2$, $Q' = 9/6$ (or $P = 20$, $Q = 9$). Both combinations reasonably fitted the normalized curves, and α_1 and α_2 were determined for each set.

Determination of $\varkappa$. To determine $\varkappa$ and to make possible a choice of best values for P and Q the following equations were developed. Replacing $1 + \alpha_1 h^{-1} + \alpha_2 h^{-2}$ by φ (h) in eqn. (19) and $P' + (P' + 1)\alpha_1 h^{-1} + (P' + 2)\alpha_2 h^{-2}$ by $\omega(h)$ in eqn. (20) for simplification, eliminating c and introducing the new variables y and x defined by

$$y = Z' \frac{\varphi(h)}{\omega(h)}, \quad x = \log h - \frac{1}{P'} \log \varphi(h) - \frac{(Q'-1)}{P'} \log C \qquad (23, 24)$$

we get

$$X = x - \frac{1}{P'} \log \varkappa = \frac{1}{P'} \log \frac{(1-Q'\,y)^{Q'}}{y} \qquad (25)$$

Experimental $y(x)$ curves calculated with eqns (23, 24) and the previously determined constants α_1 and α_2 may be compared with normalized $y(X)$ curves from eqn. (25). If a fit is obtained, $P'^{-1} \log \varkappa$ is the difference between the coordinates on the x and X axes in this position. This comparison ruled out the set $P' = 1$, $Q' = 7/6$ but gave a good fit for the set $P' = 2$, $Q' = 9/6$ if we allow for a $\delta = 0.01$ mM OH^-.

The same calculations were carried out for complexes $Bi_{11}(OH)_{25+n}$ for $N = 2$. A fairly good fit was obtained if δ is assumed to be 0.02 mM OH^-, which is, however, larger than the estimated error in the experimental value for $\delta = 0.00 \pm 0.01$ mM OH^- or H^+.

Sillén's "core and links" approach [15,16] to the explanation of polynuclear complex formation will be briefly discussed since it has been successfully applied to the hydrolysis of many metal ions. If this assumption is valid it should be possible to represent the complexes by the formula $Bi_6(OH)_{12}[Bi_6(OH)_{12}(OH)_t]_n$, where t is a constant and n varies. nt and $6n$ must, of course, be whole numbers. This hypothesis requires that the $Z(\log h)_B$ curves should be parallel, and that $(\Delta \log B/\Delta \log h)_Z = t$ is constant. These characteristics

are not shown by the present data especially at high Z values. It may still be instructive to apply this hypothesis to the data, since the curves are sufficiently close together that a fairly small experimental error can distort the curves. In these calculations $t = 6$ was used in order to conform with a limiting value 2.5 for Z, and n arbitrarily assumed to take the values 0, 1/6, 2/6...1. The t value used is greater than the experimental average value which is closer to 5. The calculations indicated that the complexes with the smallest n predominate, but no satisfactory explanation of the data was afforded by this approach using simple assumptions about how the equilibrium constants vary with n. It is, however, not unlikely that a number of complexes with different values of q, as for instance in a "core + links" series, could also be made to fit the data, if no relation is assumed to exist between the equilibrium constants.

The determination of the equilibrium constants for the final choice of complexes, $Bi_9(OH)_{20}^{7+}$, $Bi_9(OH)_{21}^{6+}$, and $Bi_9(OH)_{22}^{5+}$, will be given in some detail using the methods indicated in the general discussion. We start with the determination of α_1 and α_2 which are the equilibrium constants for the reactions

$$Bi_9(OH)_{20}^{7+} + H_2O \rightleftharpoons Bi_9(OH)_{21}^{6+} + H^+$$
$$Bi_9(OH)_{20}^{7+} + 2H_2O \rightleftharpoons Bi_9(OH)_{22}^{5+} + 2H^+$$

This was accomplished by a combination of the η' and Z' data. We plotted the left hand side of eqn. (22), $1.5\,CZ'/(C-c)$-2, as a function of log h and compared the plot with normalized curves for the right hand side of the same equation. The normalization is brought about by the following substitutions

$$v^2 = \alpha_2 h^{-2} \text{ and } k = \alpha_1 \alpha_2^{-1/2} \qquad (26, 27)$$

which give

$$\bar{n} = \frac{\alpha_1 h^{-1} + 2\alpha_2 h^{-2}}{1 + \alpha_1 h^{-1} + \alpha_2 h^{-2}} = \frac{kv + 2v^2}{1 + kv + v^2} \qquad (28)$$

Curves $\bar{n}$ (—log v) were calculated for different values of k to find the value which gave the best agreement between the experimental and normalized curve. When this value was found, log α_2 was determined as twice the difference between the coordinates on the log h and —log v axes when the two curves overlapped and log α_1 was determined from eqn. (27). The best value of k was found to be 0.5. This value is, however, not very accurate owing to the scatter among the experimental points (Fig. 5).

The values found for log α_1 and log α_2 were

$$\log \alpha_1 = -3.2 \pm 0.2$$
$$\log \alpha_2 = -5.8 \pm 0.1$$

Knowing α_1 and α_2 we proceeded to determine $\varkappa_{20,9}$ from eqns. (23, 24 and 25). For each experimental point $\varphi(h)$ and $w(h)$ were first computed and then y and x. The plot $y(x)$ was compared with the theoretical $y(X)$ curve calculated from eqn. (25) with $Q' = 9/6$ and $P' = 2$, and examined for a fit. In this position twice the difference between the x and X coordinates gives log $\varkappa_{20,9}$ (Fig. 6).

$$\log \varkappa_{20,9} = -3.5 \pm 0.1$$

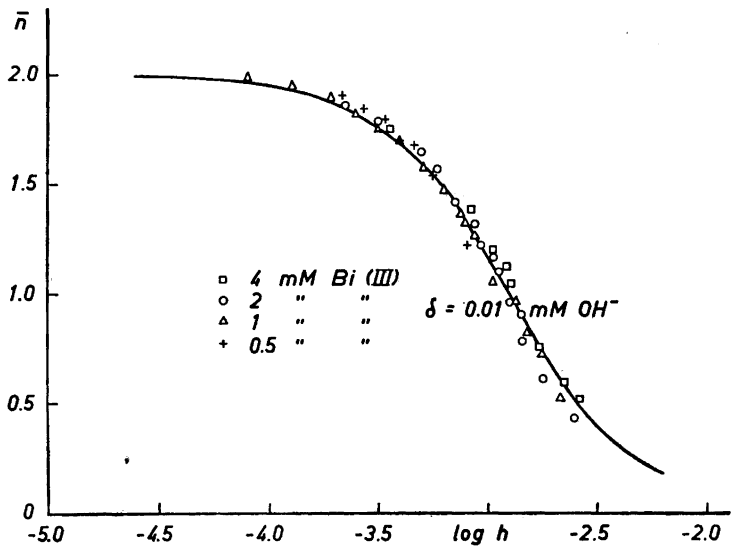


Fig. 5. Determination of α_1 and α_2 by curve fitting according to eqn. (28). The line represents the normalized curve in position of best fit.

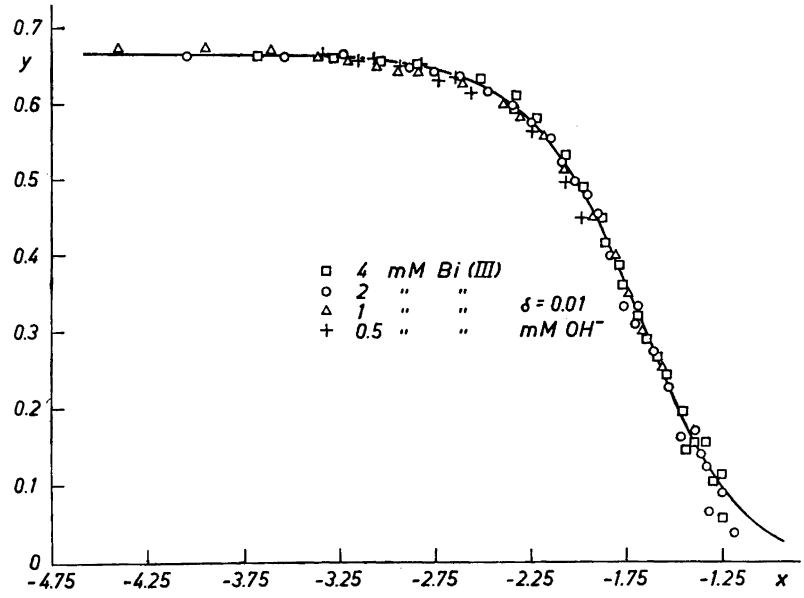


Fig. 6. Determination of $\varkappa_{20,9}$ by curve fitting according to eqn. (25). The line represents the normalized curve in position of best fit.

Log $\varkappa_{20,9}$ was also calculated from the amalgam data using eqn. (19) from which we find

$$\log \varkappa_{20,9} = \log(C-c) - 1.5 \log c + 2 \log h - \log \varphi(h) - \log 1.5 \qquad (19a).$$

$\varphi(h)$ was computed with the values for α_1 and α_2 already determined. The mean value obtained for log $\varkappa_{20,9}$ was

$$\log \varkappa_{20,9} = -3.45 \pm 0.1$$

When taking the mean value a few values from the most hydrolysed solutions were excluded. It is not suprising that the log $\varkappa_{20,9}$ values calculated from these points are erroneous. The estimated uncertainty in the amalgam potentials of at least 0.5 mV leads to an uncertainty in log $\varkappa_{20,9}$ of 0.23.

The data may also be represented by log $\varkappa_{20,9}$ and the two acidity constants (K_1 and K_2) of $Bi_9(OH)_{20}^{7+}$. We find that log K_1 = log α_1 and log K_2 = log α_2 − log α_1.

The numerical values are

$$\log K_1 = -3.2 \pm 0.2$$
$$\log K_2 = -2.6 \pm 0.2$$

Finally the experimental $Z(\log h)_B$ and $\eta'(\log h)_B$ curves were compared with the theoretical ones calculated with the equilibrium constants given (Figs 1 and 2).

Comparison with Faucherre's measurements

The only previous work on the hydrolysis of the "bismuthyl ion", which contains enough data to warrant a calculation of the species formed, is that by Faucherre. With some hesitation he gives $Bi_8(OH)_{20}^{4+}$ as a possible complex. To make a comparison with the present data easier, Faucherre's data were converted to give Z as a function of log h. This cannot be accomplished very accurately, since he presents only a small graph showing log h as a function of the analytical composition of the solutions. The most notable difference between the two sets of data is the disagreement between the limiting values for Z. Whereas in the present work Z values larger than 2.44 to 2.45 were not obtained and the form of the curves indicates that this is a limiting value, he found Z values at least larger than 2.46 before precipitation started. In this region his curves are still bending quite steeply upwards so that the limiting value 2.5 for Z proposed by him is reasonable. Some doubt may be raised concerning the complex $Bi_8(OH)_{20}^{4+}$. If we accept $Bi_4O_4^{4+}$ as the "mother complex", as was done by Faucherre, and calculate a theoretical curve for the combination $Bi_4O_4^{4+}$ and $Bi_8O_{10}^{4+}$, it is found that the agreement is poor between Faucherre's experimental data and the calculated curve even if we consider the previously mentioned uncertainty in the Z values. The theoretical curve is too steep for this explanation of the data. This was the situation met with when the present data were tested for the hypothesis that only one complex is formed.

DISCUSSION

It is not easy to discuss the plausibility of the proposed hydrolysis mechanism. On hydrolysis, the metal ions seem to be quite individualistic in their behavior so we have little or no help from analogies with other cations. The mechanism proposed shows some parallelism with that found for the VO_2^+ ion by Rossotti and Rossotti [17] but is widely different from what has been found for other ions. The VO_2^+ ion hydrolyses to the decavanadate $H_2V_{10}O_{28}^{4-}$, which splits off two hydrogen ions.

One intriguing feature of the mechanism proposed is the discrepancy between the number of OH^- groups bound per Bi(III) in the aqueous complexes and in the solid phase. However, the relationship between the solid phase described as $Bi_2(OH)_3OClO_4$ and the complexes in solution may not be simple, since protons were set free when the salt started to precipitate. It would be desirable to know the crystal structure of this salt. It may contain structural units that could give hints as to what is happening in the solution.

APPENDIX

In this appendix some log h measurements on bismuth(III) in 3 M $(Na)ClO_4$ will be described briefly. They were carried out with the cell — glass/0.01 M Ag^+, B M Bi(III), H M H^+,(2.99—3B—H)M Na^+, 3 M ClO_4^-/Ag, AgCl +

$$E_g = E_g^\circ - 59.15 \log h + \varepsilon$$

where ε is a correction term for the deviation of the glass electrode from ideality. The electrode used had the slope 59.15 up to H^+ = 350 mM. After 350 mM H^+, deviations from this slope became apparent. At 750 mM H^+, which was the highest acidity used, ε reached —0.4 mV.

The above cell was used in an effort to reduce the uncertainty in the measured potentials by elimination of the liquid junction. Since Bi^{3+} starts to hydrolyse in fairly acid solutions small differences in the measured emf cause large differences in Z.

The results are shown in Fig. 3. When the new data were compared with older ones, obtained from cells with liquid junctions, discrepancies were observed. These discrepancies were found to be caused by the E_g° chosen. When 59.15 log h was plotted as a function of H it was found that the plots for the two cells were perfectly superposable but slightly displaced from each other. The difference was 0.2 mV for the 10 and 25 mM Bi(III) and 0.5 mV for 50 mM. The rather large difference in the latter case merely reflects the difficulty of obtaining good E_g° values in very acid solutions where the liquid junction potential is large and corrections have to be applied to the glass electrode.

In Fig. 3 the drawn curves have been calculated with the equilibrium constants previously found from η data [2]

$$6Bi^{3+} + 12H_2O \rightleftharpoons Bi_6(OH)_{12}^{6+} + 12H^+ \quad \log \beta_{12,6} = 0.33$$
$$Bi^{3+} + H_2O \rightleftharpoons BiOH^{2+} + H^+ \quad \log \beta_{1,1} = -1.58$$

we thus find that the number of protons set free by the hydrolysis of Bi(III) in 3 M $(Na)ClO_4$ can be satisfactorily accounted for by the formation of $Bi_6(OH)_{12}^{6+}$ and $BiOH^{2+}$.

Note added in proof. After the completion of this work I was told by Dr. R.S. Tobias (private communication; results to be published in *J.Am.Chem.Soc.*) that from log h measurements in 1 M $(Na)ClO_4$ he had suggested the formation of $Bi_6O_6^{6+}$ and $Bi_6O_6(OH)_3^{3+}$. The agreement is good on $Bi_6O_6^{6+}$ (which is equivalent to $Bi_6(OH)_{12}^{6+}$ but the present data cannot be explained on the assumption that $Bi_6O_6(OH)_3^{3+}$ is formed as a main species in the latter stages of the hydrolysis of the Bi^{3+} ion.

Acknowledgements. The author wishes to thank sincerely Professor Lars Gunnar Sillén for his continual interest in this work. The author also wishes to thank Professor Robert E. Connick, Univ. of Calif., for helpful comments on the manuscript and Dr. George Biedermann for discussions during the work. Dr. Helen S. Dunsmore and Mr. David L. Martin have corrected the English of this paper. Mr. Frans Ericson has been helpful in constructing and maintaining the apparatus. This work has been supported by *Statens Tekniska Forskningsråd (Swedish Technical Research Council)*.

REFERENCES

1. Granér, F. and Sillén, L. G. *Acta Chem. Scand.* **1** (1947) 631.
2. Olin, Å. *Acta Chem. Scand.* **11** (1957) 1445, and references therein.
2a. Granér, F., Olin, Å. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 476.
3. Holmqvist, A. *Svensk Kem. Tidskr.* **48** (1936) 106.
4. Faucherre, J. *Bull. soc. chim. France* **1954** 253.
5. Bates, S. J. and Urmston, J. W. *J. Am. Chem. Soc.* **55** (1933) 4068.
6. Forsling, W., Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **6** (1952) 901.
7. Biedermann, G. and Sillén, L. G. *Arkiv Kemi* **5** (1953) 425.
8. Sillén, L. G. and Ekedahl, E. *Arkiv Kemi* **22A** (1946) No. 16.
9. Biedermann, G. *Acta Chem. Scand.* **10** (1956) 1340.
10. Winkler, L. W. *Die chemische Analyse* Bd. 29, Ferdinand Enke, Stuttgart 1931.
11. Prytz, M. and Nagel, P. *Z. anorg. allgem. Chem.* **227** (1936) 65.
12. Holmberg, R. W., Kraus, K. A. and Johnson, J. S. *J. Am. Chem. Soc.* **78** (1956) 5506.
13. Method suggested by L. G. Sillén (not published). First used in Ref.[17].
14. Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 186.
15. Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 299.
16. Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 318.
17. Rossotti, F. J. C. and Rossotti, H. *Acta Chem. Scand.* **10** (1956) 957.

Received June 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1809 – 1813

Vibrational Mean-Square Amplitude Matrices

VI. Planar Symmetrical XY_3 Molecules

S. J. CYVIN

Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The mean-square amplitude quantities for the planar symmetrical XY_3 molecular model are treated theoretically. The L matrix elements ($S = LQ$), and the symmetrized mean-square amplitude matrix elements at the temperatures $T = 0$ and 298 °K, are given numerically for the four boron trihalides.

The vibrations of planar symmetrical XY_3 molecules have been subjected to several investigations. For publications containing theoretical considerations on this problem, and involving force constant determinations, references are made to [1–7]. For the computations of mean amplitudes of vibration from spectroscopic data, it is referred to [7–9]. To the knowledge of the author, no determinations of mean amplitudes of vibration have so far been performed from electron-diffraction, for any molecule of the structure here considered. In the present paper, the mean-square amplitude quantities of planar symmetrical XY_3 molecules are treated, and numerical values for boron trihalides are reported.

THEORETICAL TREATMENT

For the in-plane vibrations of the considered molecular model there exist four independent harmonic vibrational constants. The following set of constants consists of mean-square amplitude quantities, based on a set of valence force coordinates.

$$\sigma = \langle r_1^2 \rangle = \langle r_2^2 \rangle = \langle r_3^2 \rangle \quad (1)$$

$$\sigma' = \langle r_1 r_2 \rangle = \langle r_2 r_3 \rangle = \langle r_3 r_1 \rangle \quad (2)$$

$$\tau = R^2\langle \alpha_1^2 \rangle = R^2\langle \alpha_2^2 \rangle = R^2\langle \alpha_3^2 \rangle \quad (3)$$

$$\tau' = R^2\langle \alpha_1\alpha_2 \rangle = R^2\langle \alpha_2\alpha_3 \rangle = R^2\langle \alpha_3\alpha_1 \rangle \quad (4)$$

$$\varrho = R\langle r_1\alpha_1 \rangle = R\langle r_2\alpha_2 \rangle = R\langle r_3\alpha_3 \rangle \quad (5)$$

$$\varrho' = R\langle r_1\alpha_2 \rangle = R\langle r_1\alpha_3 \rangle = R\langle r_2\alpha_3 \rangle = R\langle r_2\alpha_1 \rangle = R\langle r_3\alpha_1 \rangle = R\langle r_3\alpha_2 \rangle \quad (6)$$

In these equations, R is the equilibrium X—Y bond length, r representing the deviations of the interatomic X—Y distances, and α the inter-bond angle deviations, from their equilibrium values. The numbering of the coordinates is the same as that used in previous papers [7,9]. From the redundant condition $\alpha_1 + \alpha_2 + \alpha_3 = 0$ it is found

$$\tau + 2\tau' = 0, \qquad \varrho + 2\varrho' = 0 \tag{7}$$

for the quantities defined by eqns (3)—(6).

The interatomic distance deviations between the non-bonded atom pairs may be taken into account instead of the angle displacements, and designated r_1^*, r_2^* and r_3^*. Then the following mean-square amplitude quantities are to be introduced.

$$\tau^* = \langle (r_1^*)^2 \rangle = \langle (r_2^*)^2 \rangle = \langle (r_3^*)^2 \rangle \tag{8}$$

$$\varrho^* = \langle r_1 r_1^* \rangle = \langle r_2 r_2^* \rangle = \langle r_3 r_3^* \rangle \tag{9}$$

$$\varrho'^* = \langle r_1 r_2^* \rangle = \langle r_1 r_3^* \rangle = \langle r_2 r_3^* \rangle = \langle r_2 r_1^* \rangle = \langle r_3 r_1^* \rangle = \langle r_3 r_2^* \rangle \tag{10}$$

These quantities may be expressed in terms of the quantities from eqns. (1)—(6) by the following linear combinations.

$$\tau^* = \frac{3}{2}(\sigma + \sigma') + \frac{1}{6}(\tau - \tau') - 3^{-\frac{1}{2}}(\varrho - \varrho') \tag{11}$$

$$\varrho^* = 3^{\frac{1}{2}}\,\sigma' + \frac{1}{3}(\varrho - \varrho') \tag{12}$$

$$\varrho'^* = \frac{1}{2}\,3^{\frac{1}{2}}\,(\sigma + \sigma') - \frac{1}{6}(\varrho - \varrho') \tag{13}$$

The vibrational analysis is facilitated by introducing symmetry coordinates. The elements of the symmetrized mean-square amplitude matrix are given by

$$\Sigma_1 = \langle S_1^2 \rangle = \sigma + 2\sigma' \tag{14}$$

$$\Sigma_3 = \langle S_{3a}^2 \rangle = \langle S_{3b}^2 \rangle = \sigma - \sigma' \tag{15}$$

$$\Sigma_4 = \langle S_{4a}^2 \rangle = \langle S_{4b}^2 \rangle = \tau - \tau' \tag{16}$$

$$\Sigma_{34} = \langle S_{3a} S_{4a} \rangle = \langle S_{3b} S_{4b} \rangle = \varrho - \varrho' \tag{17}$$

Table 1. Symmetry coordinates for the planar symmetrical XY_3 molecular model.

Species	No.	Coordinate
A_1'	1	$3^{-\frac{1}{2}}\,(r_1 + r_2 + r_3)$
E'_a	3a	$6^{-\frac{1}{2}}\,(2r_1 - r_2 - r_3)$
	4a	$6^{-\frac{1}{2}}\,R\,(2\alpha_1 - \alpha_2 - \alpha_3)$
E'_b	3b	$2^{-\frac{1}{2}}\,(r_2 - r_3)$
	4b	$2^{-\frac{1}{2}}\,R(\alpha_2 - \alpha_3)$

The symmetry coordinates (S) are formed as normalized linear combinations of the valence force coordinates, and specified in Table 1. For the mean-square amplitude quantities of eqns. (14)—(17) one has the relations

$$\Delta_1 = \Sigma_1 \mu_Y^{-1} \tag{18}$$

$$\Delta_3 + \Delta_4 = [\tfrac{1}{2}(\Sigma_3 + \tfrac{1}{3}\Sigma_4)(3\mu_X + 2\mu_Y) - 3^{\frac{1}{2}}\,\Sigma_{34}\mu_X]\,(3\mu_X + \mu_Y)^{-1}\mu_Y^{-1} \tag{19}$$

$$\Delta_3\Delta_4 = \tfrac{1}{3}(\Sigma_3\Sigma_4 - \Sigma_{34}^2)(3\mu_X + \mu_Y)^{-1}\mu_Y^{-1} \tag{20}$$

Here μ_X and μ_Y denote the inverse masses of the X and Y atoms, respectively, and the values of Δ are connected with the vibrational normal frequencies (ν) through

$$\Delta_k = (h/8\pi^2\nu_k)\,\coth(h\nu_k/2kT) \tag{21}$$

where h is Planck's constant, k Boltzmann's constant, and T the absolute temperature. Another set of relations, involving the force constants, is given below.

$$\varepsilon_1 = K_1\Sigma_1 \tag{22}$$

$$\varepsilon_3 + \varepsilon_4 = K_2\Sigma_3 + \Phi\Sigma_4 + 2\Gamma\Sigma_{34} \tag{23}$$

$$\varepsilon_3\varepsilon_4 = (K_2\Phi - \Gamma^2)(\Sigma_3\Sigma_4 - \Sigma_{34}^2) \tag{24}$$

The force constants, *viz.* K_1, K_2, Φ and Γ, are based on the chosen symmetry coordinates (Table 1), and given in terms of usual valence force coordinates in Ref.[9] The values of ε are given by

$$\varepsilon_k = \tfrac{1}{2}h\nu_k\,\coth\,(h\nu_k/2kT) \tag{25}$$

If the coefficients of the transformation connecting the symmetry coordinates (S) with the normal coordinates (Q), *viz.*

$$S_i = \sum_k L_{ik}\,Q_k \tag{26}$$

are known, the symmetrized mean-square amplitude matrix elements are simply obtained by the following expressions.

$$\Sigma_1 = L_{11}^2\,\Delta_1 \tag{27}$$

$$\Sigma_3 = L_{33}^2\Delta_3 + L_{34}^2\Delta_4 \tag{28}$$

$$\Sigma_4 = L_{43}^2\Delta_3 + L_{44}^2\Delta_4 \tag{29}$$

$$\Sigma_{34} = L_{33}L_{43}\Delta_3 + L_{34}L_{44}\Delta_{44} \tag{30}$$

The most important ones of the mean-square amplitude quantities here concerned, are the two designated σ and τ^*, and defined by eqns. (1) and (8), respectively. They are given in terms of the symmetrized mean-square amplitude matrix elements by the expressions

$$\sigma = \tfrac{1}{3}(\Sigma_1 + 2\Sigma_3) \tag{31}$$

$$\tau^* = \Sigma_1 + \tfrac{1}{2}(\Sigma_3 + \tfrac{1}{3}\Sigma_4) - 3^{-\frac{1}{2}}\Sigma_{34} \tag{32}$$

Table 2. Transformation coefficients L_{ik} in (atomic weight units)-$\frac{1}{2}$ for boron trihalides.

$^{11}BF_3$	Q_1	Q_3	Q_4
S_1	0.2294	...	...
S_3	...	0.4346	0.001331
S_4	...	0.5413	0.5230
$^{11}BCl_3$	Q_1	Q_3	Q_4
S_1	0.1679	...	...
S_3	...	0.4051	−0.01749
S_4	...	0.5981	0.3681
$^{11}BBr_3$	Q_1	Q_3	Q_4
S_1	0.1119	...	...
S_3	...	0.3856	0.006125
S_4	...	0.6074	0.2779
$^{11}BI_3$	Q_1	Q_3	Q_4
S_1	0.08876	...	...
S_3	...	0.3789	−0.02293
S_4	...	0.6333	0.1765

These quantities represent the mean-square amplitudes of vibration for the bonded and non-bonded interatomic distances, respectively, and would also be obtainable from electron-diffraction. See, *e.g.* Refs.[10–15]

NUMERICAL COMPUTATIONS

In the numerical computations reported in the following, the three vibrational frequencies for $^{11}BY_3$ (Y = F,Cl,Br,I) together with ν_3 for $^{10}BY_3$ were used to determine the vibrational constants. The experimental data for BF_3 and BCl_3 have been taken from Ref.[4], those for BBr_3 and BI_3 from Ref.[5], and are quoted in a previous paper [7]. This paper [7] also contains the calculated force constants.

The coefficients L_{ik} defined by eqn. (26), for the trihalides of the ^{11}B isotope, are given numerically in Table 2. The resulting mean-square amplitude quantities Σ_1, Σ_3, Σ_4 and Σ_{34} at the absolute zero point and the temperature 298 °K, are given in Table 3.

Table 3. Mean-square amplitude quantities in Å² units for boron trihalides.

Molecule	Symbol	Mean-square amplitude quantity $T = 0$	298°K
$^{11}BF_3$	Σ_1	0.000999	0.001027
	Σ_3	0.002191	0.002195
	Σ_4	0.013000	0.015103
	Σ_{34}	0.002753	0.002763
$^{11}BCl_3$	Σ_1	0.001010	0.001242
	Σ_3	0.002921	0.002999
	Σ_4	0.015723	0.024279
	Σ_{34}	0.003835	0.003521
$^{11}BBr_3$	Σ_1	0.000759	0.001297
	Σ_3	0.003063	0.003190
	Σ_4	0.016386	0.033563
	Σ_{34}	0.005011	0.005572
$^{11}BI_3$	Σ_1	0.000699	0.001631
	Σ_3	0.003527	0.004051
	Σ_4	0.014858	0.032465
	Σ_{34}	0.005064	0.003261

The mean amplitudes of vibration are in the present notation given by

$$u_{XY} = \sigma\tfrac{1}{2}, \qquad u_{YY} = (\tau^*)\tfrac{1}{2} \tag{33}$$

and are obtainable according to eqns. (31) and (32). Their numerical values for the molecules here considered, have been given in Table 2 of Ref.[7]

REFERENCES

1. Heath, D. F. and Linnett, J. W. *Trans. Faraday Soc.* **44** (1948) 873.
2. Heslop, W. R. and Linnett, J. W. *Trans. Faraday Soc.* **49** (1953) 1262.
3. Venkateswarlu, K. and Sundaram, S. *J. Chem. Phys.* **23** (1955) 2368.
4. Lindeman, L. P. and Wilson, M. K. *J. Chem. Phys.* **24** (1956) 242.
5. Wentink, T. Jr. and Tiensuu, V. H. *J. Chem. Phys.* **28** (1958) 826.
6. Pistorius, C. W. F. T. *J. Chem. Phys.* **29** (1958) 1174.
7. Cyvin, S. J. *Acta Chem. Scand.* **13** (1959) 334.
8. Bakken, J. *Acta Chem. Scand.* **12** (1958) 594.
9. Cyvin, S. J. *Spectrochim. Acta* **15** (1959) 341.
10. Debye, P. *J. Chem. Phys.* **9** (1941) 55.
11. Karle, I. L. and Karle, J. *J. Chem. Phys.* **17** (1949) 1052.
12. Bartell, L. S., Brockway, L. O. and Schwendeman, R. H. *J. Chem. Phys.* **23** (1955) 1854.
13. Morino, Y. and Hirota, E. *J. Chem. Phys.* **23** (1955) 737.
14. Bastiansen, O. and Cyvin, S. J. *Nature* **180** (1957) 980.
15. Cyvin, S. J. *Kgl. Norske Videnskab. Selskabs Skrifter. In press.*

Received July 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1814–1816

The Distribution of some Dialkylfluorophosphates in the Liquid Systems Carbon Tetrachloride/Water and Oil/Water

SVERRE ÖKSNE

Norwegian Defence Research Establishment, Toxicological Group, Lilleström, Norway

The distribution of four dialkylfluorophosphates (I–IV) has been investigated in the systems carbon tetrachloride/water and whale-oil/water, and that of III also in the system peanut oil/water. In the systems whale-oil/water and carbon tetrachloride/water the following sequence for the distribution coefficient (K) was found: I < III < II < IV.

In connection with investigations on the inhibition of cholinesterase, it was desirable to determine the distribution of some dialkylfluorophosphates (I–IV) between an organic solvent and water, and an oil and water. The compounds investigated had the formula $(RO)_2P(O)F$. R = ethyl (I), R = propyl (II), R = *iso*propyl (III), R = butyl (IV).

The distribution of III between peanut oil and water has been investigated by Augustinsson [1] who used the cholinesterase inhibiting effect of the water phase as a measure of the amount of III present.

In the work described herein two different methods were used for the quantitative determination of the dialkylfluorophosphates. The one is a *o*-tolidine perborate method for colorimetric estimation of dialkylfluorophosphates. The other is a method based upon analysis of fluoride. The values of the distribution coefficients found all refer to room temperature (20–24°C).

The results of a typical experiment, *viz.* the determination of the distribution coefficient (K) of III in the liquid systems whale-oil/water and carbon tetrachloride/water are given in Table 1.

A summary of the distribution coefficients (K) obtained is given in Table 2, which also includes the number of experiments made for each determination and the standard deviation.

The values of K vary widely with the length of the alkyl group. In the systems carbon tetrachloride/water and whale-oil/water the value of K varies in the following way with the alkyl group, ethyl < *iso*propyl < propyl < butyl.

Table 1. Data for the determination of the distribution of III between carbon tetrachloride and water and between whale-oil and water.

Solvent	Expt.	Total III mg	Vol.ratio solvent / water	mg of dialkylfluorophosphate recovered		Recovery %	*K*
				Solvent	Water		
Carbon tetrachloride	1	18.80	1 : 5	17.44	2.23	104.6	39.2
	2	18.37	1 : 3	17.41	1.39	102.1	37.5
	3	13.78	1 : 7	11.86	2.17	101.8	38.2
Whale-oil	1	111.6	1 : 8.87	55.8 [a]	55.8		8.87
	2	55.9	1 : 8.30	27.9 [a]	28.0		8.26
	3	45.0	1 : 19.10	14.0 [a]	31.0		8.64
	4	65.0	1 : 13.90	26.5 [a]	38.5		9.57
	5	54.3	1 : 8.41	26.3 [a]	28.0		7.90

[a] Calculated by difference.

EXPERIMENTAL

Syntheses of dialkylfluorophosphates. The dialkylfluorophosphates were prepared by a one stage method [2]. Benzene was used as solvent. The products were redistilled until constant refractive index was obtained. No other test for purity was made. The refractive index was measured with a Hilger-Abbe refractometer, and the following values for n_D^{20} and boiling point respectively were found: I: 1.3722, 57°/11 mm; II: 1.3891, 80°/11.5 mm; III: 1.3817, 71°/14 mm; IV: 1.4013, 54°/0.02 mm.

Distribution coefficients of I—IV between carbon tetrachloride and water. The method used is a modification of the *o*-tolidine perborate method for estimation of phosphonofluoridates [3,4]. The following reagents and organic solvents were used:

Acetone *pro analysi*, purified with potassium permanganate and distilled.

Carbon tetrachloride *pro analysi* (Merck).

o-Tolidine reagent. *o*-Tolidine dihydrochloride (B.D.H.) was purified by treatment with stannous chloride and active carbon until a concentrated aqueous solution was colourless. A 1 % solution in water was used (prepared daily).

Phosphate buffer 0.05 M, pH = 11.5, prepared by dissolving 8.7 g of K_2HPO_4 in 1 litre of distilled water, and adjusted to pH 11.5 with saturated NaOH solution.

Table 2. Distribution coefficients of dialkylfluorophosphates in the liquid systems carbon tetrachloride/water, whale-oil/water and peanut oil/water.

Dialkylfluorophosphate	Distribution carbon tetrachloride/water			Distribution whale-oil/water			Distribution peanut oil/water	
	No. of expts.	*K*	Stand. dev.	No. of expts.	*K*	Stand. dev.	No. of expts.	*K*
I	5	3.46	0.16	5	2.7	1.11		
II	4	60.2	1.02	6	15.0	2.04		
III	3	38.3	0.86	5	8.6	0.63	4	7 6
IV	3	102	10.74	9	62	7.52		

Sodium perborate reagent. 1.25 % aqueous solution of sodium perborate (prepared daily).

For estimation of the calibration curve a known solution of dialkylfluorophosphate in carbon tetrachloride was prepared, and the reagents mixed in the following quantities and sequence. Acetone (20 ml), phosphate buffer (2.0 ml), sodium perborate reagent (1.5 ml), *o*-tolidine reagent (1.0 ml), water saturated with carbon tetrachloride (6.5 ml), carbon tetrachloride saturated with water (1—n) ml and a solution of dialkylfluorophosphate in carbon tetrachloride (n ml).

The intensity of colour was measured after 20 min in a Fisher Electrophotometer (model AC) with a blue filter, and the colorimetric readings, corrected for blanks, were plotted against mg of dialkylfluorophosphate.

For the determination of the distribution coefficient a measured volume of a known solution of dialkylfluorophosphate in carbon tetrachloride was shaken vigorously for 1 min with a known volume of distilled water. The phases were allowed to separate and samples of both of them were with-drawn and analyzed for dialkylfluorophosphate as described for the calibration. In analyzing the water phase (6.5—n) ml of water, n ml of water phase and 1 ml of carbon tetrachloride were used. The concentration in each phase was determined by comparison of the net colorimetric readings with the calibration curve.

Distribution coefficients of I—IV between whale-oil and water. The amount of dialkylfluorophosphate in the water phase was determined by colorimetric estimation of fluoride by interaction with a thorium lake [5]. The following reagents were used:

Refined whale-oil from De-No-Fa, Fredrikstad, Norway.

Thorium reagent, 0.001 M with respect to thorium nitrate and 1.0 M with respect to sodium sulphate, formic acid and sodium formate.

Alizarin indicator 0.00025 M. 0.0855 g of alizarin monosodium sulphonate was dissolved in 1 litre of distilled water.

For estimation of the calibration curve 20—40 mg of dialkylfluorophosphate was dissolved in 100 ml of distilled water. Samples of not more than 25 ml were boiled with reflux for 90 min with 10 ml of 0.1 M NaOH solution. The samples were filtered through paper filter and quantitatively transfered to 100 ml flasks. 20 ml of alizarin indicator was added, and the samples were titrated with 0.6 M nitric acid until a pure yellow colour was obtained. Then 20 ml of thorium reagent was added and the solution diluted to 100 ml with distilled water. The intensity of colour was measured after 30 min in a Fisher Electrophotometer (model AC) with a green filter, and the colorimetric readings, corrected for blanks, were plotted against mg of dialkylfluorophosphate.

For estimation of the distribution coefficient the density of a 3 % solution of dialkylfluorophosphate in oil was determined. A calculated volume (about 5 ml) of a known solution of dialkylfluorophosphate, about 3 % in oil, was shaken cautiously with 50 ml of distilled water for 5 min. The two phases were allowed to separate and samples of 5 ml of the water phase were withdrawn for analyses. The analyses were performed as described for the calibration, and the concentration of dialkylfluorophosphate in the water phase was determined by comparison of the net colorimetric readings with the calibration curve.

We did not succeed in analyzing the oil phase. The amount of dialkylfluorophosphate in the oil phase therefore was calculated by difference.

Distribution coefficient of III between peanut oil and water. The first part of the procedure was as described for the system whale-oil/water. The second part, the analyzing of the water phase, was performed by the *o*-tolidine perborate method as described before.

REFERENCES

1. Augustinsson, K. B. *Arkiv Kemi* **6** (1954) 331.
2. Monard, C. *Bull. soc. chim. France* **1952** 544.
3. Epstein, J. and Bauer, V. E. *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy.* February 27—March 2, 1956.
4. Rosenthal, Robert W., Proper, R. and Epstein, J. *J. Phys. Chem.* **60** (1956) 1596.
5. Milton, R. F. and Waters, W. A. *Methods of quantitative micro-analysis*, London 1955, p. 324.

Received July 21, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1817–1827

The Separation of Intestinal Invertase and Three Different Intestinal Maltases on TEAE-cellulose by Gradient Elution, Frontal Analysis, and Mutual Displacement Chromatography

ARNE DAHLQVIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The separation of intestinal invertase and maltase activities on ion exchange columns was further studied by use of a modified ion exchange cellulose, TEAE-cellulose.

The gradient elution, frontal analysis, and mutual displacement techniques were applied to the separation of intestinal invertase and maltase activities on these columns.

The results with these separation methods supported the theory that intestinal maltase activity consists of three components, only one of which is associated with invertase activity.

The intestinal invertase activity behaved as one single enzyme. The homogeneity of intestinal invertase activity also appears from the K_s values for the invertase activity in different fractions of a chromatogram.

The method for the separation of intestinal maltase activities by partial heat inactivation was improved.

By combining the methods of heat inactivation and ion exchange chromatography, the three components of intestinal maltase activity were purified. The three maltase fractions had different K_s values, which supports the theory that they are different enzymes.

In a previous paper the separation of intestinal maltase activity in one heat-stable component (amounting to 1/3 of the total maltase activity) and one heat-labile component, was described [1]. The heat-stable maltase was without invertase activity. The heat-labile maltase, too, was partially separated from the invertase activity. In a preparation containing heat-labile maltase and invertase activity with a maltase/invertase activity quotient of 0.6, these activities could not, however, be further separated. In this preparation the invertase and maltase activities may therefore possibly be caused by the same enzyme.

As a working hypothesis, the possible existence of three different maltases in intestinal mucosa was therefore assumed. For the further study of these enzymes, however, improved separation methods were required.

In the present paper further separation experiments with intestinal invertase and maltase activities are described. Use was made of a modified ion exchange cellulose (TEAE-cellulose) and, in addition to *gradient elution*, two other techniques for chromatography, namely *frontal analysis* and *mutual displacement chromatography*. The separation by partial heat inactivation was improved, too. The enzyme fractions obtained were characterized by their K_s values.

MATERIALS AND METHODS

Solubilized glycosidases were prepared from pig intestinal mucosa as previously described [2].

Substrates. Sucrose of analytical grade purity was obtained from J. T. Baker Co. Maltose of analytical grade purity was obtained from Merck A. G. (Germany) and Pfanstiehl Chemical Co. The maltose preparations were found to contain oligosaccharides with a lower mobility than maltose on the paper chromatogram. For maltase determinations the preparations were used without further purification, but for K_s determinations chromatographically pure maltose was used, which was obtained by carbon column chromatography [3].

Determination of invertase and maltase activity was performed under the same conditions as used earlier, *i. e.* at 0.1388 M substrate concentration, in 0.05 M maleate buffer pH 6.5 and with toluene as preservative [1,2]. One unit of glycosidase activity produces 1 mg of monohexoses (*i. e.* 1 % of hydrolysis in 2.0 ml of reaction mixture) in 1 h at 37°C under these conditions. When the degree of hydrolysis did not exceed 10 %, the amount of monohexoses produced was proportional to the amount of enzyme present.

Determination of invertase activity. 1.0 ml of substrate solution (9.5 % sucrose *, 0.1 M maleate buffer pH 6.5 and a few ml of toluene per lit.) and 1.0 ml of enzyme solution with 0.5 – 3 units of invertase were mixed in a test-tube in a water-bath at 37°C. After 60 min the reaction was interrupted by the addition of 2.0 ml of the 3,5-dinitrosalicylic acid reagent of Sumner [4]. A blank was prepared with the same composition, in which, however, the 3,5-dinitrosalicylic acid reagent was added *before* the enzyme solution. The samples were heated in a boiling water-bath for 10 min, chilled with tap water, and diluted with 20.0 ml of distilled water. After mixing, the red colour was determined with a Beckman B spectrophotometer at 530 mμ. The colour was stable for at least 20 h. The amount of monohexoses produced was calculated from a standard curve with known amounts of glucose. Glucose and fructose have the same extinction coefficient with the 3,5-dinitrosalicylic acid reagent.

Determination of total maltase activity. Equal volumes of substrate solution (10.0 % maltose monohydrate, 0.1 M maleate buffer pH 6.5 and a few ml of toluene per lit.) and enzyme solution with 5 – 10 units of maltase per ml were mixed in a test-tube in a water-bath at 37°C. At zero time and after 60 min, 0.10 ml samples were withdrawn from the reaction mixture and immediately precipitated [5] with 1.0 ml of 5 % $ZnSO_4$ and 1.0 ml of 0.3 N $Ba(OH)_2$, diluted with water to 4.0 ml and centrifugated. 2.0 ml of the supernatant was transferred to a Folin's tube for sugar determination, 2.0 ml of the acid copper reagent of Tauber and Kleiner [6] was added, and the tube was boiled for 8 min in a water-bath. After chilling with tap water for 2 min, 2.0 ml of Benedicts molybdate reagent [6] was added, and after mixing the solution was diluted to the 25 ml mark with distilled water. After 3 – 30 min the blue colour was determined with a Beckman B spectrophotometer at 600 mμ. With each set of determinations, a series of tubes were boiled containing known amounts of maltose and glucose in mixture, corresponding to 0, 5, 10, and 15 % hydrolysis.

* Per cent means g of solute per 100 ml of solution.

Determination of heat-stable maltase activity. To the enzyme solution was added water or phosphate buffer (depending on the buffer content of the enzyme solution) until reaching a final concentration of 0.01 M phosphate buffer pH 6.0. The solution was then heated in a water-bath at 60°C for 45 min, which completely inactivated the heat-labile maltase activity, while the heat-stable maltase was unaffected [1]. The heat-stable maltase activity was determined with the procedure described above.

The heat-labile maltase activity was calculated as the difference between the total maltase activity and the heat-stable maltase activity.

K_s *for invertase activity* was determined as described earlier [7].

K_s *for maltase activity* was determined with 0.030—0.002 M maltose solutions. The degree of hydrolysis was not allowed to exceed 20 %. With these dilute substrate solutions it was necessary to use a larger volume of the reaction mixture for the quantitative determination of the glucose produced. Since amounts greater than 5 μmoles of the maleate buffer interfered with the reagents of Tauber and Kleiner, the modified reagents described by Caputto, Leloir and Trucco [8] were used for these determinations. Amounts as large as 50 μmoles of maleate buffer pH 6.5 did not interfere with these reagents.

Protein was determined with the method of Lowry *et al.*[9] by use of human serum albumin (kindly supplied by A.B. Kabi) as a standard.

Conductivity measurements (for determining the concentration of phosphate buffer in chromatography fractions) were made with a Philips conductometer, type PR 9500.

Diethylaminoethyl cellulose (DEAE-cellulose) was prepared from Solkafloc cellulose powder SW 40 A with the method of Peterson and Sober [10]. The product contained 0.75—0.85 mequiv. of titrable hydroxyl groups per g dry weight. The titrations were performed in 0.5 M NaCl [10].

Triethylaminoethyl cellulose (TEAE-cellulose) was prepared from DEAE-cellulose by ethylation as described by Porath [11]. The ethylation process increased the pK value of the ion exchanger from 9.5 (DEAE-cellulose) to 10.0 (TEAE-cellulose). Since the free base of TEAE-cellulose was found unstable even at room temperature, TEAE-cellulose was stored and used in its bromide form.

Before use, the TEAE-cellulose was stirred for 30—60 min with the buffer to be used as starting buffer in the chromatography. After packing the column, it was washed with several column volumes of the same buffer.

RESULTS AND DISCUSSION

Gradient elution from TEAE-cellulose. During gradient elution chromatography on DEAE-cellulose, intestinal maltase and invertase activties formed one single peak, which, however, was not homogeneous [2]. Porath [11] has reported the preparation of a modified anion exchange cellulose, TEAE-cellulose, with somewhat different adsorption properties. The chromatography of the intestinal glycosidases on TEAE-cellulose was therefore studied.

To a 1 × 11 cm column of TEAE-cellulose, which had been equilibrated with 0.010 M phosphate buffer pH 6.0, a solubilized intestinal glycosidase preparation containing 12.2 mg of protein, 180 units of invertase and 360 units of maltase in 0.010 M phosphate buffer pH 6.0 was applied. The enzymes were eluted with phosphate buffer pH 6.0 with continuously increasing concentration (Fig. 1). The experiment was performed at room temperature with a flow rate of 0.5 ml/min. Each fraction collected had a volume of 3.0 ml.

Also with TEAE-cellulose the invertase and maltase activities formed one common peak. The resolution within this peak seemed better, however, than that with DEAE-cellulose (*cf.* Fig. 1 in this publication with Fig. 3 in Ref.[2]). The first half of the invertase activity peak had a low maltase/invertase activity quotient (0.6) and is apparently identical with the fraction obtained earlier [1], which had the same maltase/invertase activity quotient, and in which the invertase

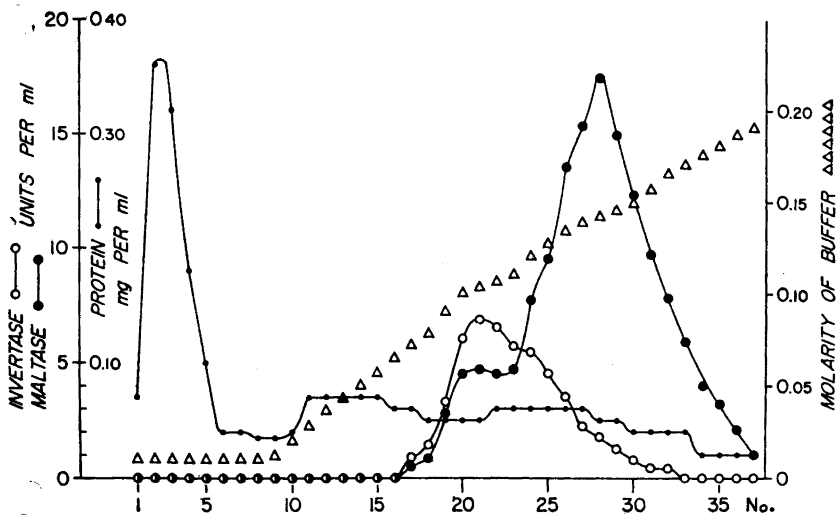


Fig. 1. Gradient elution of intestinal glycosidases on TEAE-cellulose at pH 6.0. For experimental conditions, see text. The molarity of the buffer in the effluent was determined by conductometry.

and maltase activities could not be further separated by rechromatography or by heat inactivation at varying pH. With TEAE-cellulose, too, the heat-stable maltase activity was the main component of the descending branch of the maltase peak.

Attempts to improve the separation by use of longer columns, lower flow rate, or step-wise elution, were not successful.

Frontal analysis. In frontal analysis the column is first washed with pure solvent. Then a relatively large volume of a solution of the substance to be analyzed is filtered through the column. Since the substance is adsorbed to the column, the filtrate is at first pure solvent. While more solution is filtered through the column, however, an increasing part of the column becomes saturated with the substance, and at last the substance appears in the effluent. If the flow rate is not too high, the concentration of the substance in the effluent rises rapidly from zero to the same concentration as in the influent. If there are more than one substance present in the solution, these substances may from separate fronts, depending on their concentration and the capacity of the column for each substance, and also on the competition of the different substances for the adsorbent (mutual displacement).

The frontal analysis principle was introduced by Tiselius [12], and its application to the separation of sugars and fatty acids on charcoal columns has been thoroughly studied by Claesson [13]. The frontal analysis principle has also been applied to the separation of inorganic ions on ion exchange column, which has been studied by Samuelsson [14]. As far as the present author is aware, however, frontal analysis has not been applied to the separation of proteins.

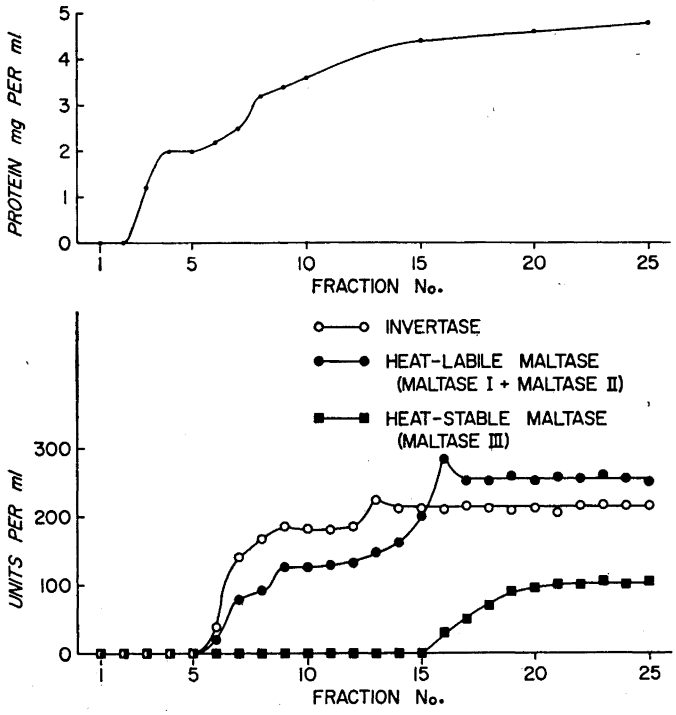


Fig. 2. Frontal analysis of intestinal glycosidases on TEAE-cellulose. 0.070 M phosphate buffer pH 6.0 was used as solvent. For experimental conditions, see text.

The frontal analysis of a solubilized intestinal glycosidase preparation on TEAE-cellulose was studied by use of phosphate buffer pH 6.0, and with varying molarity, as solvent. The experiments were performed on 1 × 6 cm columns containing 0.5 g (dry weight) of TEAE-cellulose, previously equilibrated with the same buffer as was used as solvent for the glycosidase preparation.

It was found necessary to use a relatively low flow rate, 2—3 drops (< 0.2 ml) per min, because otherwise very flat fronts were obtained. The experiments were always performed at room temperature (at lower temperature, a still lower flow rate would have been required).

The buffer concentration was found essential for the results. At low buffer concentration (0.010 M) all the glycosidases appeared as one common front. At higher concentrations (0.060—0.080 M) separate fronts were obtained. At still higher buffer concentration the glycosidases were not adsorbed to the column, but appeared in the effluent together with the solvent front. Since an important factor in frontal analysis seems to be the mutual displacement effect, these observations on the importance of the buffer concentration are in accordance with the findings of Boman [15,16].

A frontal analysis experiment is demonstrated in Fig. 2. The enzyme solution used contained 220 units/ml of invertase, 360 units/ml of maltase

and 4.8 mg/ml of protein in 0.070 M phosphate buffer pH 6.0. The flow rate was 3 drops/min and each fraction collected had a volume of 1.8 ml. The first glycosidase front was formed by invertase activity, accompanied by a heat-labile maltase activity with maltase/invertase activity quotient 0.6. This front corresponds to the maltase fraction I (invertase fraction) postulated earlier [1]. The appearance of these activities as one single, homogeneous front, strongly supports the theory that these activities are caused by one single enzyme. The fact that a second front of heat-labile maltase later appeared, is also in favour of the theory that two different heat-labile intestinal maltases exist.

The second front of heat-labile maltase activity (maltase II [1]) and the front of the heat-stable maltase activity (maltase III [1]) appeared in the same tubes. This front is, however, not homogeneous, since the increase in heat-labile maltase activity is more rapid than the increase in heat-stable maltase activity. This indicates an incomplete mutual displacement of these two activities, which was also found in mutual displacement technique chromatography.

Mutual displacement chromatography. In mutual displacement cromatography the substances to be separated are adsorbed to the upper end of an adsorbent column. The substances are then eluted by the addition of another substance to the influent, which is adsorbed to the column with greater force than any of the substances to be separated. The developer should have a sufficiently high concentration to elute the substances to be separated completely. These substances therefore travel down the column as one band, just in front of the developer. During the passage down the column, the different components of the band compete with each other for the adsorbent, which makes them form separate zones of increasing adsorbability. If sufficiently small fractions are collected, the components may therefore be obtained in different fractions.

This principle for chromatography was introduced by Tiselius [17], and was first applied to the separation of sugars on charcoal columns. It has also been applied to the separation of proteins, but only in a few cases and not always under clear-cut experimental conditions [16]. That one protein may displace another from an ion exchange column has been demonstrated by Boman [15,16]. He found, however, that protein-protein displacement is ineffective at low buffer concentration, *i.e.* when the protein to be displaced is strongly adsorbed.

The mutual displacement chromatography of intestinal glycosidases was performed on TEAE-cellulose columns, which had been equilibrated with phosphate buffer pH 6.0 with varying molarity. After adsorption of the glycosidase solution to the column, this was first eluted with the starting buffer to remove some inactive protein which was not adsorbed to the column [2]. The molarity of the influent buffer then at once was increased to 0.200 M, which eluted all the glycosidases. The enzymes appeared in the effluent as one single protein peak, which was, however, composed of zones with different enzymatic activity (Fig. 3).

The experiments were always performed at room temperature. To obtain good separation, it was necessary to use so low a flow rate as 1 drop/min (0.04 ml). In accordance with the experiences of Boman, the molarity of the starting buffer was of great importance for the separation. With 0.010 M

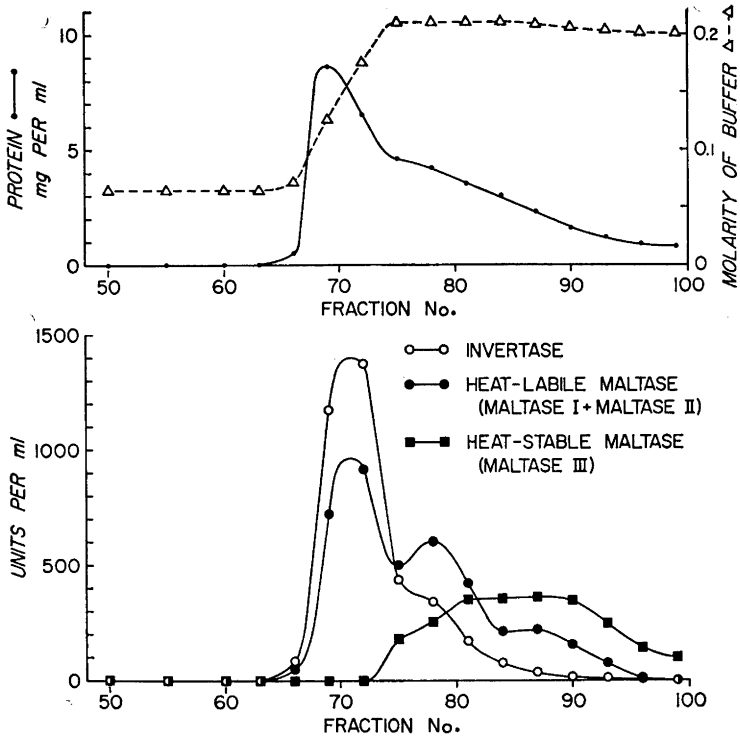


Fig. 3. Mutual displacement chromatography of intestinal glycosidases on TEAE-cellulose with 0.065 M phosphate buffer pH 6.0 as starting buffer. For experimental conditions, see text.

buffer practically no separation was obtained. The most effective separation was found with 0.065—0.080 M buffer. With 0.090 M buffer the separation was again poor, although the glycosidases were adsorbed to the column with this buffer.

The length of the column also had an influence on the separation, since better separation was obtained with a 1 × 100 cm column than with a 1 × 50 cm column. This is in contrast to the gradient elution technique, in which the length of the column was found to be of practically no importance.

These facts seem to prove that the separation obtained is really caused by mutual displacement, and that the experiments cannot be described as a gradient elution with a very steep gradient [16].

One mutual displacement chromatography of intestinal glycosidases is demonstrated in Fig. 3. A 1 × 100 cm column containing 9 g (dry weight) of TEAE-cellulose was equilibrated with 0.065 M phosphate buffer pH 6.0. 48.5 ml of an intestinal glycosidase preparation containing 313 units/ml of invertase, 430 units/ml of maltase, and 5.75 mg/ml of protein in 0.065 M phosphate buffer pH 6.0 was applied to the column with a flow rate of 3 drops

(0.12 ml)/min. The column was first eluted with 230 ml of 0.065 M phosphate buffer pH 6.0, which eluted 37 % of the protein applied to the column, but no invertase or maltase activity. The influent buffer was then shifted to 0.200 M phosphate pH 6.0, and at the same time the flow rate was lowered to 1 drop (0.04 ml)/min. Each fraction collected contained 20 drops (0.8 ml). The number of the fractions in Fig. 3 refers to the number of fractions collected from the moment the influent buffer was shifted to 0.200 M.

As is seen from the figure, the enzymes appear in the effluent as one single protein peak. The first part of this peak (fractions Nos. 66—72) contains the main part of the invertase activity and has a maltase/invertase activity quotient of 0.6. This part of the peak apparently corresponds to maltase fraction I (invertase fraction) described earlier [1]. The tailing of the invertase activity peak was reduced by the mutual displacement technique (*cf.* Fig. 1) but it was not completely abolished. K_s determinations for the invertase activity in fraction Nos. 69 and 85 gave the same values, 2.6×10^{-2}, in good accordance with the value obtained for a crude intestinal glycosidase preparation [7]. This shows that the intestinal invertase activity is caused by one single enzyme. As was found in the frontal analysis experiments, the mutual displacement between maltase II (invertase-free heat-labile maltase activity)[1] and maltase III (heat-stable maltase activity) [1] was much less effective, although a certain mutual displacement took place between these two enzymes also. The heat-stable maltase activity may be purified simply by heating the solution at 70°C [1], but the invertase-free heat-labile maltase fraction seems more difficult to separate. Rechromatography of fractions Nos. 75—84 did not appreciably increase the separation of this activity.

Heat inactivation of intestinal glycosidases. The frontal analysis experiments supported the theory that the heat-labile intestinal maltase is made up of two components. These two activities are rapidly inactivated at 60°C in 0.01 M phosphate buffer pH 6.0. At 50°C in the same buffer, the invertase activity was inactivated by 90 % in one hour. During the same time, however, the maltase activity was diminished only by 30 % (see Fig. 2 in Ref.[1]). If intestinal invertase and maltase I are supposed to be identical, this must mean that maltase II is not measurably inactivated under these conditions. Consequently, there must be a small difference in heat lability between maltases I and II as well.

At pH 7.0 this difference was found to be increased. A solubilized glycosidase preparation was used, which had been dialyzed against distilled water. The solution had a conductivity corresponding to about 0.005 M phosphate and contained 167 units/ml of invertase, 280 units/ml of maltase and 3.6 mg/ml of protein. The pH of the solution was 6.95, as measured with a glass electrode. When this solution was heated at 50°C, the invertase and maltase I activities were inactivated in 30 min, while the maltase II and III activities were not affected even after heating at 50°C for 60 min (Fig. 4). That the difference was caused by the shift in pH, was found by heating another sample of the solution in the presence of 0.010 M phosphate buffer pH 7.0 with the same results.

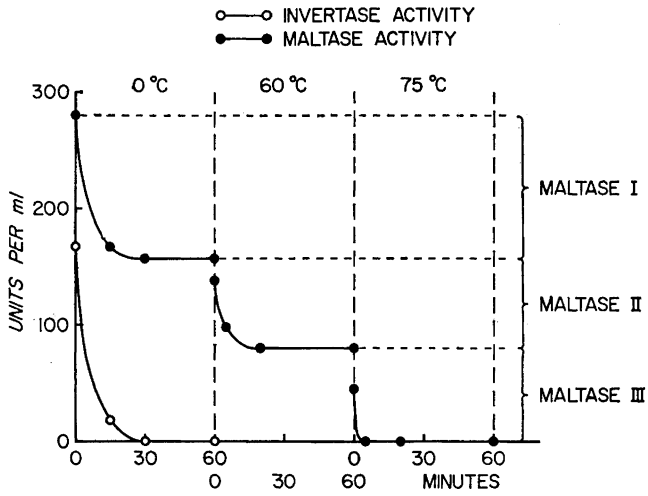


Fig. 4. Heat inactivation of intestinal maltase activity in three steps. Inactivation at 50°C was performed without buffer present (pH of the solution was 6.95). Inactivation at 60°C and 75°C was performed in 0.010 M phosphate buffer pH 6.0. The volume change by addition of the buffer is corrected for in the figure. Preheating was performed as described earlier [1]. The enzyme solution contained 167 units/ml of invertase, 280 units/ml of (total) maltase, and 3.6 mg/ml of protein.

When phosphate buffer pH 6.0 was added to a final concentration of 0.010 M (the change of volume has been corrected for in the figure) and the temperature increased to 60°C, the maltase II was rapidly inactivated while the maltase III was unaffected (Fig. 4).

In this way it was found possible to inactivate the invertase and maltase I activities with preservation of the maltase II and III activities. An attempt

Table 1. Change in adsorbability of the protein denaturated at 50°C at pH 7.0. The table shows the distribution of the protein in two corresponding mutual displacement chromatograms on TEAE-cellulose of the same enzyme solution, before and after heating to 50°C at pH 7.0 for 1 h. The amount of protein *not* eluted from the column was calculated from the amount of protein applied to the column.

	Protein as per cent of total	
	Enzyme solution not heated	Enzyme solution heated at 50°C at pH 7.0 for 1 h
Eluted with 0.080 M phosphate buffer pH 6.0	38	39
Eluted with 0.200 M phosphate buffer pH 6.0	32	18
Not eluted from the column	30	43

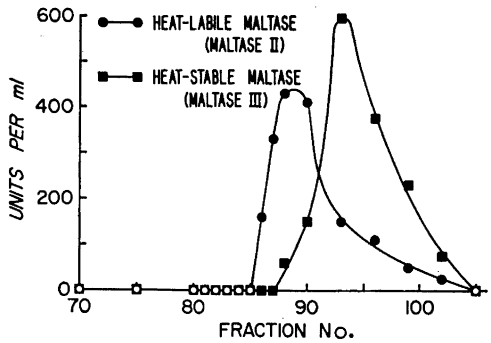


Fig. 5. Mutual displacement chromatography of intestinal maltase activities II and III on TEAE-cellulose with 0.080 M phosphate buffer pH 6.0 as starting buffer. For experimental conditions, see text.

was made to remove the inactivated protein by centrifugation, but it could not be sedimented even at 50 000 *g* (in a Spinco preparative ultracentrifuge). The adsorption properties of the inactivated protein were, however, found to be changed. The distribution of the protein in two corresponding chromatograms of the same glycosidase solution, one before and the other after heating at 50°C for 1 h, is shown in Table 1. It is seen that the heat-inactivated protein is not eluted from TEAE-cellulose by 0.200 M phosphate pH 6.0.

Mutual displacement chromatography of maltase II and III. A 1 × 100 cm column of TEAE-cellulose was equilibrated with 0.080 M phosphate buffer pH 6.0. 50 ml of an intestinal glycosidase preparation, in which the invertase and maltase I activities had been inactivated by heating for 1 h at 50°C, was used for the experiment. The solution contained 160 units/ml of maltase (II + III) and 3.6 mg/ml of protein. After addition of phosphate buffer pH 6.0 to a final concentration of 0.080 M, the solution was applied to the column, which was first eluted with 100 ml of 0.080 M phosphate buffer pH 6.0, and then with 0.200 M phosphate buffer of the same pH. The flow rate was 1 drop (0.04 ml)/min and each fraction collected contained 20 drops (0.8 ml).

The results of the chromatography is seen in Fig. 5. The mutual displacement between maltase II and maltase III was very incomplete. Only the two first fractions (Nos. 86 and 87) of the enzyme peak were free from heat-stable maltase activity.

Table 2. K_s values for three different intestinal maltases.

	K_s
Maltase I (invertase fraction)	1.1×10^{-2}
Maltase II (invertase-free heat-labile maltase activity)	2.2×10^{-3}
Maltase III (heat-stable maltase activity)	4.2×10^{-3}

K_s-values for the different maltase activities. For K_s determinations the following fractions were used:

Maltase I: Heat-labile maltase activity with maltase/invertase activity quotient 0.6, obtained by mutual displacement chromatography (fractions Nos. 66—72 of the chromatogram shown in Fig. 3).

Maltase II: Heat-labile maltase activity without invertase activity, obtained by mutual displacement chromatography of enzyme solutions which has been heated at 50°C at pH 7.0 for 1 h (fractions Nos. 86—87 of the chromatogram shown in Fig. 5 and corresponding fractions from similar chromatograms).

Maltase III: Heat-stable maltase activity, obtained from fractions Nos. 86—99 of the chromatogram shown in Fig. 3, fractions Nos. 93—102 of the chromatogram shown in Fig. 5, and the corresponding fractions from similar chromatograms. Traces of heat-labile maltase and invertase activity were inactivated by heating at 60°C for 1 h.

The K_s values for the different maltase activities are shown in Table 2. The figures reported are the mean of several separate determinations, with good agreement between them. The divergent K_s values demonstrate that the different maltase fractions are really different enzymes.

Acknowledgement. The author's thanks are due to Professor Bengt Borgström for his interest in my work and for valuable discussions.

This work has been supported by grants from the *Medical Faculty of the University of Lund.*

The skilful technical assistance of Mrs. K. Hagebris is gratefully acknowledged.

REFERENCES

1. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 945.
2. Borgström, B. and Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 1997.
3. Whistler, R. L. and Durso, D. F. *J. Am. Chem. Soc.* **72** (1950) 677.
4. Sumner, J. B. and Graham, V. A. *J. Biol. Chem.* **47** (1921) 5.
5. Somogyi, M. *J. Biol. Chem.* **160** (1945) 69.
6. Tauber, H. and Kleiner, I. S. *J. Biol. Chem.* **99** (1932) 249.
7. Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 2012.
8. Caputto, R., Leloir, F. and Trucco, R. E. *Enzymologia* **12** (1947) 350.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
10. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
11. Porath, J. *Arkiv Kemi* **11** (1957) 97.
12. Tiselius, A. *Arkiv Kemi, Mineral. Geol.* **14B** (1940) No. 22.
13. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **23A** (1946) No. 1.
14. Samuelsson, O. *Ion Exchangers in Analytical Chemistry.* Uppsala 1952.
15. Boman, H. G. *Ion Exchange Chromatography of Proteins and Some Applications to the Study of Different Phosphoesterases.* Diss. Uppsala 1958.
16. Boman, H. G. *Mutual Displacement in Protein Chromatography,* in Neuberger, A. *Symposium on Protein Structure,* New York 1958.
17. Tiselius, A. *Arkiv Kemi, Mineral. Geol.* **16A** (1943) No. 18.

Received July 11, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1828–1838

Studies on the Hydrolysis of Metal Ions

24. Hydrolysis of the Uranyl Ion, UO_2^{2+}, in Perchlorate Self-Medium

SIRKKA HIETANEN and LARS GUNNAR SILLÉN

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

The hydrolysis of UO_2^{2+} has been studied in perchlorate self-medium at 25°C, using quinhydrone or glass electrodes, which gave concordant results. The data indicate the two main reactions: $2UO_2^{2+} + H_2O \rightleftharpoons (UO_2)_2OH^{3+} + H^+$ (equilibrium constant β_{12}) and $2UO_2^{2+} + 2H_2O \rightleftharpoons (UO_2)_2(OH)_2^{2+} + 2\,H^+$ (equilibrium constant β_{22}). Small deviations at low acidities could be explained assuming the reaction $3UO_2^{2+} + 4H_2O \rightleftharpoons (UO_2)_3(OH)_4^{2+} + 4H^+$ (equilibrium constant β_{43}). The values obtained were: in the medium 0.4 UO_2^{2+}, 1.0 $(Na)ClO_4$, $\log \beta_{12} = -3.66 \pm 0.20$, $\log \beta_{22} = -6.02 \pm 0.03$. In 1.4 UO_2^{2+}, 3.0 $(Na)ClO_4$, $\log \beta_{12} = -3.68 \pm 0.20$, $\log \beta_{22} = -6.31 \pm 0.03$, $\log \beta_{43} \approx -12.6$ (quinh.), -12.9? (glass). The values for β_{22} and β_{43} are of the same order as obtained in an inert medium. The concentration of $(UO_2)_2OH^{3+}$ is appreciable only at high UO_2^{2+} concentration.

In an earlier paper [1] in this series, it was suggested that added information on the complex formation between two reagents, A and B, can be gained by equilibrium studies in what was termed as "self-medium". This means that one of the reagents, say B, which is charged, is kept at a high and constant total concentration and forms the major part of the ions of this sign of charge; the inert ions of opposite charge are kept at constant concentration. Self-medium measurements should especially give information on complexes A_pB_q with a higher ratio q/p than may be studied with the usual "inert medium" method.

The principle was applied to the hydrolysis of the thorium ion, Th^{4+}, in chloride self-medium. The data could be explained assuming the two complexes $Th_2(OH)_2^{6+}$ and Th_2OH^{7+}; for the first, a formation constant was found which agreed well with inert-medium data, the second complex was discovered by the self-medium work [1].

In the present work, the self-medium method has been applied to the hydrolysis of the uranyl ion in perchlorate self-medium. Denoting as usual the total concentration of B by B, and the "analytical" excess concentration of H^+ by H, which was negative in the majority of our solutions, the equilibrium solutions were composed as follows:

Medium	B	H	$[Na^+]$	$[ClO_4^-]=I$
0.4 UO_2^{2+}, 1.0 (Na)ClO_4	0.4	H	$0.2 - H$	1.0
1.4 UO_2^{2+}, 3.0 (Na)ClO_4	1.4	H	$0.2 - H$	3.0

The experiments were performed as titrations, starting with a certain solution S and adding from a buret measured amounts of another solution T. The compositions of the various solutions S and T followed the scheme above; Table 1 gives the values for H in S, T, and the final solution in the various experiments.

Table 1. Survey of titrations.

B	I	H S	H T	Final H quinh.	Final H glass	Corr mV quinh.	Corr mV glass	Symbol quinh.	Symbol glass
0.4	1.0	0.20	−0.30	0.08	−0.06	—	—	+	○
		−0.14	0.20	0.09	0.09	0.5	—	×	□
1.4	3.0	−0.20	0.20	0.05	0.05	0.5	−1.8	+	□
		0.20	−0.20	0.04	0.04	0.5	−1.8	+	□
		0.22	−0.18	0.02	0.02	—	—	×	●
		−0.18	0.22	0.02	0.02	−0.7	—	×	●
		0.20	−0.20		0.00		+50		○
		−0.20	0.20		0.04		+49.5		○

Rounded values are given for H. The two last titrations were made with a different type of glass electrode. In each solution, $[UO_2]_{total} = B$, $[ClO_4^-] = I$, $[Na^+] = 0.2 - H$.

After each addition of T, the hydrogen ion concentration, h, was measured with one of the cells

$$- \text{ glass electrode}|\text{equilibrium solution}||\text{SE}+ \quad \text{(I)}$$
$$-\text{Pt}|\text{Q,QH}_2\text{, equilibrium solution}||\text{SE}+ \quad \text{(II)}$$

Separate experiments were made with cells I and II. The reference electrode was

$$\text{SE} = B \text{ M } UO_2^{2+},\ 0.20 \text{ M } Na^+,\ I \text{ M } ClO_4^-|B \text{ M } UO_2^{2+},\ 0.01 \text{ M } Ag^+,\ 0.19 \text{ M } Na^+,\ I \text{ M } ClO_4^-|Ag$$

It so happens that the predominating species for UO_2^{2+} seem to have the same types of formula as for Th^{4+}; for the calculations one may then take over a number of equations which were derived and applied in the previous paper [1].

Symbols

A, B = reactants, here OH^- ($-H^+$) and UO_2^{2+} ; a = concentration of free A, here $a = h^{-1}$; b = concentration of free B ; B = total concentration of B ; E = electromotive force of cell (I) or (II) ; E_0 = constant in eqn. (1) ; $f = 2u^2 + 2ku$, eqn (6) ; $F(h) = BZ + \Delta$, eqn (2) ; $h = [H^+]$; H = analytical excess of H^+, often negative ; $I = [ClO_4^-]$ in medium ; j = constant in term for liquid junction potential, eqn (1) ; k = parameter defined by eqn (6) ; p,q = coefficients in formula of complex A_pB_q ; u = normalized variable, corresponding to a, eqn (6) ; Z = average number of A (here OH^-) bound per B (here UO_2^{2+}) ; β_{pq} = equilibrium constant for formation of A_pB_q, eqn (3) ; Δ = analytical error in H, eqn (2).

In the equilibrium constants, and figures, the unit M (mole/liter) is used throughout.

REAGENTS

Uranyl perchlorate. In our first experiments, with $B = 0.4$, we started from ammonium uranyl hydroxide. This substance had long ago been precipitated from uranyl nitrate, which had been purified by ether extraction. The hydroxide was ignited, and the U_3O_8 formed was dissolved in HNO_3. The solution was evaporated repeatedly with $HClO_4$ under an infrared lamp, until no trace of nitrate ion could be detected.

For the experiments with $B = 1.4$, we started with spectroscopically pure UO_3, obtained from AB Atomenergi. A possible impurity of NH_3 was removed by igniting the oxide to U_3O_8, which was then dissolved in 6 M $HClO_4$, 30 % H_2O_2 being added. The excess of acid was removed by evaporation under an infrared lamp.

The total uranium concentration was determined by precipitating with oxine or NH_3 at pH = 7, igniting the precipitate, and weighing as U_3O_8. The results obtained with oxine or NH_3 agreed within 0.1 %.

Total perchlorate was determined by passing a known amount of solution through a H^+-saturated ion exchanger (Wofatit KPS-200), and titrating the eluate with NaOH.

The hydrogen ion excess, H, in the stock solutions was determined by emf titration with NaOH, after diluting to 0.02, 0.04, or 0.08 M UO_2^{2+}, using diagrams according to Gran [2]. The values for H from experiments of different dilutions were consistent, and the estimated uncertainty in H will correspond to an error in Z of at most 0.004, probably only 0.002. The H so determined came out slightly higher than $[ClO_4^-] - 2B$, which is the difference between two much larger numbers and cannot be determined with sufficient accuracy.

Sodium perchlorate was prepared from sodium carbonate p.a. and perchloric acid p.a. *Perchloric acid* was standardized against solid $KHCO_3$ and standard NaOH. *Sodium hydroxide* solution was prepared as described earlier [3].

Apparatus. The Ag, AgCl electrodes were prepared according to Brown [4]. In the quinhydrone half-cell, a bright Pt foil was used. The glass electrodes were Radiometer "red" electrodes G 202A (for $B = 0.4$), G 202 BT57, and G 202 B38 (for $B = 1.4$). The electrode vessel was of the usual "Wilhelm" type [5], and the cells were kept in a paraffin oil thermostat at 25.0 ± 0.1°C in a thermostated room. The emfs were measured with a Vernier compensator, or with a Radiometer potentiometer PHM 3.

With the medium 0.4 UO_2^{2+}, 1.0 (Na)ClO_4, E became constant within 5 min; in general, the reading was taken 10 min after each addition. In the medium 1.4 UO_2^{2+}, 3.0 (Na)ClO_4, the emfs stabilized somewhat more slowly, especially with the glass electrodes, so one had to wait 15–30 min for a constant reading.

TREATMENT OF DATA

The primary data given by each titration were sets of values (E,H), where E is the emf of cell (I) or (II), and H is the analytical excess of hydrogen ions calculated for each point; (in the range with appreciable hydrolysis, H was negative). The fundamental equations are

$$E = E_0 - 59.15 \log h + jh \quad (1)$$
$$H = h - BZ - \Delta = h - F(h) \quad (2)$$

Here, E_0 and j are constants; jh is the liquid junction potential, which is expected to be linear with h. BZ is the amount of OH^- bound by B per liter of solution, and may be expressed as a function of h applying the law of mass action, eqns (4) and (5) below; Δ is the analytical error in H.

The $E(H)$ plots for various titrations did not coincide exactly, which might be ascribed either to variations in E_0, or to variations in Δ. In the same way as described in our previous paper [1], it was checked graphically that the spread is in E_0 and not in Δ. Indeed, there was no reason to assume an appreciable

Table 2. Medium 0.4 UO_2^{2+}, 1 (Na)ClO_4. Experimental data H and E (corrected) for four titrations. Every other point given, log h calculated from E with $E_0 + 3 \cdot 59.15 = 164.9$ mV for quinhydrone titrations, and $E_0 + 3 \cdot 59.15 = 493.7$ for glass electrode titrations, $j = 51.0$ mV M^{-1}.

quinhydrone electrode

H	E	log h	Z	H	E	log h	Z
symbol +				symbol ×			
0.2020	38.6	−0.691	−0.006	−0.1450	165.2	−3.005	0.360
0.1671	41.9	−0.777	0.000	−0.1136	159.2	−2.904	0.282
0.1275	47.0	−0.897	−0.002	−0.0760	150.8	−2.760	0.189
0.0866	54.8	−1.064	−0.001	−0.0421	140.2	−2.580	0.107
0.0529	65.3	−1.270	0.001	−0.0191	128.0	−2.372	0.053
0.0246	82.0	−1.578	0.005	0.0029	104.5	−1.970	0.015
0.0005	110.4	−2.079	0.020	0.0275	78.1	−1.506	0.004
−0.0202	129.9	−2.408	0.060	0.0561	63.3	−1.232	0.001
−0.0383	139.0	−2.562	0.103	0.0919	53.2	−1.030	−0.001
−0.0627	147.3	−2.702	0.162				

glass electrode

H	E	log h	Z	H	E	log h	Z
symbol ○				symbol □			
0.2020	367.4	−0.691	0.004	−0.1430	494.8	−3.017	0.360
0.1671	370.7	−0.777	0.000	−0.1116	488.8	−2.917	0.282
0.1275	375.8	−0.897	−0.002	−0.0740	480.1	−2.770	0.189
0.0866	383.4	−1.061	0.001	−0.0401	469.3	−2.587	0.107
0.0529	394.2	−1.272	0.002	−0.0171	457.2	−2.383	0.053
0.0246	411.1	−1.582	0.004	0.0049	433.4	−1.977	0.014
0.0005	440.1	−2.094	0.019	0.0295	407.0	−1.509	0.004
−0.0202	459.7	−2.425	0.060	0.0581	392.0	−1.230	0.002
−0.0383	468.8	−2.579	0.102	0.0939	381.9	−1.029	−0.001
−0.0599	476.1	−2.702	0.155				

Table 3. Medium 1.4 UO_2^{2+}, 3 (Na)ClO_4. Experimental data H and E for six titrations. Every other point given, log h calculated from E with $E_0 + 3 \cdot 59.15 = 157.2$, $j = 8.0$ mV M^{-1} for quinhydrone titrations, and $E_0 + 3 \cdot 59.15 = 260.7$, $j = 8.8$ mV M^{-1} for glass electrode titrations.

quinhydrone electrode

H	E	log h	Z	H	E	log h	Z
symbol +				symbol ×			
−0.1960	125.6	−2.466	0.142	0.2220	19.8	−0.647	0.002
−0.1710	122.9	−2.420	0.125	0.2091	21.2	−0.672	0.002
−0.1490	120.2	−2.374	0.109	0.1856	24.2	−0.726	0.001
−0.1204	116.1	−2.305	0.089	0.1649	26.9	−0.775	0.002
−0.0819	109.1	−2.187	0.063	0.1297	32.7	−0.878	0.002
−0.0489	100.4	−2.040	0.041	0.0887	41.4	−1.030	0.003
−0.0145	86.3	−1.801	0.022	0.0620	49.2	−1.166	0.004
0.0320	61.3	−1.375	0 007	0.0220	66.4	−1.462	0.009
0.2034	22.1	−0.689	0.000	−0.1780	123.5	−2.430	0.131
0.1905	23.7	−0.718	0.000	−0.1651	122.1	−2.407	0.121
0.1670	26.8	−0.773	0.001	−0.1416	119.5	−2.363	0.105
0.1463	29.8	−0.826	0.002	−0.1208	116.4	−2.310	0.090
0.1111	36.2	−0.943	0.002	−0.0857	109.9	−2.200	0.066
0.0701	46.5	−1.118	0.004	−0.0446	98.8	−2.013	0.039
0.0434	56.1	−1.286	0.006	−0.0180	87.7	−1.825	0.024
				0.0183	67.9	−1.489	0.010

glass electrode

H	E	log h	Z	H	E	log h	Z
symbol □				symbol ● contd.			
−0.1960	230.2	−2.484	0.142	−0.1781	229.5	−2.473	0.130
−0.1710	227.3	−2.435	0.125	−0.1502	225.9	−2.412	0.111
−0.1490	224.4	−2.386	0.109	−0.1185	221.0	−2.329	0.088
−0.1204	220.1	−2.314	0.090	−0.0798	213.2	−2.197	0.062
−0.0819	213.0	−2.194	0.063	−0.0539	206.2	−2.079	0.045
−0.0489	203.7	−2.036	0.041	−0.0280	196.8	−1.919	0.029
−0.0145	189.9	−1.801	0.022	−0.0003	182.6	−1.678	0.015
0.0320	164.8	−1.374	0.007	0.0220	170.0	−1.463	0.009
0.2034	125.9	−0.691	0.000	symbol ○			
0.1905	127.3	−0.716	0.001	0.2040	125.9	−0.691	0.000
0.1670	130.3	−0.770	0.002	0.1790	128.8	−0.743	0.001
0.1463	133.3	−0.824	0.003	0.1468	133.7	−0.831	0.001
0.1111	139.6	−0.936	0.003	0.1117	139.8	−0.939	0.002
0.0701	150.2	−1.122	0.004	0.0767	148.0	−1.083	0.004
0.0434	160.0	−1.291	0.006	0.0440	159.2	−1.277	0.006
				0.0040	179.8	−1.632	0.014
symbol ●							
0.2215	123.5	−0.648	0.004	−0.1960	230.2	−2.484	0.142
0.2122	124.6	−0.667	0.002	−0.1710	227.4	−2.437	0.125
0.1856	127.8	−0.726	0.002	−0.1489	224.6	−2.390	0.109
0.1553	131.9	−0.799	0.002	−0.1203	220.4	−2.319	0.089
0.1297	136.0	−0.873	0.003	−0.0817	213.0	−2.194	0.063
0.1027	141.7	−0.975	0.002	−0.0513	204.8	−2.055	0.043
0.0800	147.2	−1.071	0.003	−0.0183	192.0	−1.837	0.023
0.0573	154.5	−1.196	0.005	0.0040	180.0	−1.632	0.014
0.0353	163.2	−1.347	0.007	0.0316	164.9	−1.375	0.007
0.0220	169.9	−1.462	0.009				

error Δ. Since UO_2^{2+} hydrolyses at lower acidities than Th^{4+}, one may determine H accurately by acid-base titration and Gran plot, which was not possible for Th^{4+}. In the following, $\Delta = 0$ was assumed.

The quinhydrone and glass electrode measurements for $B = 0.4$ were made to coincide by applying shifts along the E axis, whereas for $B = 1.4$, data with glass and quinhydrone were treated separately. Thus three separate curves $E(H)$ were obtained for the calculations. These corrected data are given in Tables 2 ($B = 0.4$) and 3 ($B = 1.4$).

Just as in the case of thorium, a preliminary treatment showed that the data for the lowest Z agreed well with a mechanism with a complex containing one OH group, whereas for higher Z, they indicated a complex with 2 OH. Data up to about $Z = 0.15$ were used in the calculations.

It was concluded that the main products are the complexes $AB_2 = (UO_2)_2OH^{3+}$ and $A_2B_2 = (UO_2)_2(OH)_2^{2+}$. The assumption of 2 B per complex will be justified in the discussion below. Later on also the complex A_4B_3 had to be included to account for certain deviations.

We denote by β_{pq} the equilibrium constant for the formation of A_pB_q:

$$p\ H_2O + q\ UO_2^{2+} \rightleftharpoons (UO_2)_q(OH)_p^{(2q-p)+} + p\ H^+;$$

$$[A_pB_q] = \beta_{pq}[H^+]^{-p}[UO_2^{2+}]^q = \beta_{pq}a^pb^q \qquad (3)$$

The law of mass action, and the definitions, then give

$$B = b + 2\ \beta_{12}ab^2 + 2\ \beta_{22}a^2b^2\ (+ 3\ \beta_{43}a^4b^3) \qquad (4)$$

$$BZ = \beta_{12}ab^2 + 2\beta_{22}a^2b^2\ (+ 4\beta_{43}a^4b^3) \qquad (5)$$

In the calculations, eqns (1) and (2)' were used alternately for successive approximations, as described in the thorium paper [1] until a theoretical function $F(h)$, and values for E_0 and j, had been found which generated each other on application of (1) and (2) to the experimental data (E,H).

In the calculations, the auxiliary normalized variable u, the parameter k, and the abbreviation $f(u)$ were used [1]:

$$u^2 = 2\beta_{22}a^2B\ ;\ ku = 2\beta_{12}aB\ ;\ f = 2u^2 + 2ku \qquad (6)$$

One may then derive an expression (Ref [1], eqn 13) for Z as a function of u, and construct plots of $Z(\log u)_k$ for a number of values for k, which may be compared with experimental curves $Z(\log a)$. From the value of k, and the difference of coordinates ($\log u - \log a$), at the position of the best fit, $\log \beta_{22}$ and $\log \beta_{12}$ could be obtained, using (6) above or Ref.[1], eqns 14 and 15.

When this method was applied, it was seen that for the very lowest acidities there was an unavoidable bend in the plot ($E + 59.15 \log h$) *versus* h, indicating the existence of at least one more complex. Now, the data in inert medium [6,7] had indicated the existence of a series of complexes, $B(A_2B)_n$. The first member, A_2B_2, had already been found with self-medium. It was then reasonable

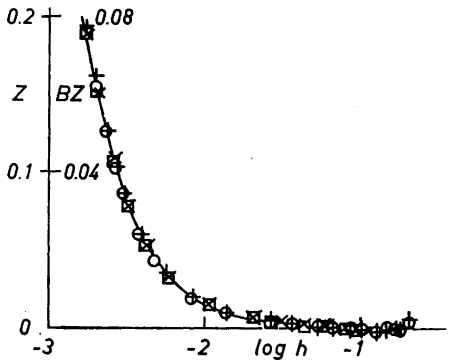


Fig. 1. Hydrolysis of UO_2^{2+}, medium 0.4 UO_2^{2+}, 1.0 (Na)ClO_4. *Z versus* log *h*. Points: calculated from experimental data *E*(*H*), Table 2, using E_0 and *j* from Table 4. Curve: calculated *Z*, assuming values for β_{22} and β_{12} from Table 4.

to assume that the deviations are due to the second member, A_4B_3, and on this assumption, β_{43} was calculated using (5), which gives

$$BZ-\beta_{12}ab^2-2\beta_{22}a^2b^2 = 4\beta_{43}a^4b^3 \qquad (7)$$

The value for *b* in each point was obtained from *B*, *a*, and eqn (4). In this way, log β_{43} was estimated for the stronger medium ($B = 1.4$); if this correction was applied to *BZ* in *F*(*h*), the E_0 plots were found to be more nearly linear (Fig. 4, Fig. 5). Because of the greater spread of the glass electrode data, the value for β_{43} derived from them is especially uncertain. For the weaker medium ($B = 0.4$), the deviation is smaller, and no attempt was made to estimate β_{43}.

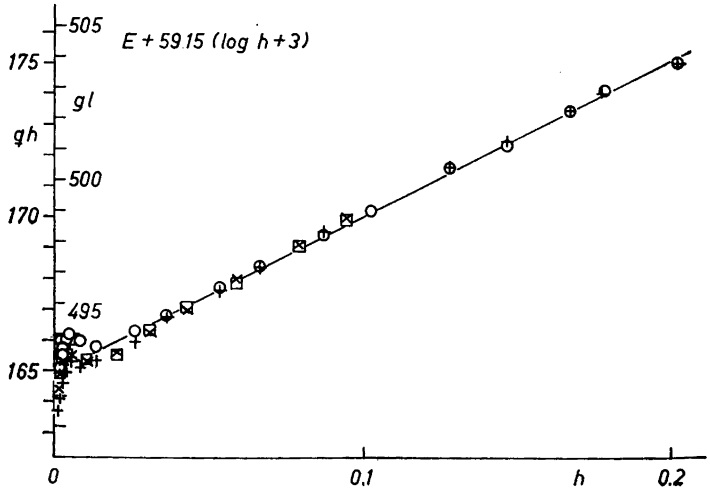


Fig. 2. Medium 0.4 UO_2^{2+}, 1.0 (Na)ClO_4. Abscissa: log *h*, calculated from *H* and *Z*, using values for β_{12} and β_{22} in Table 4. Ordinate: $E + 59.15$ (log h + 3). Straight line: given by values of *j* and E_0 in Table 4.

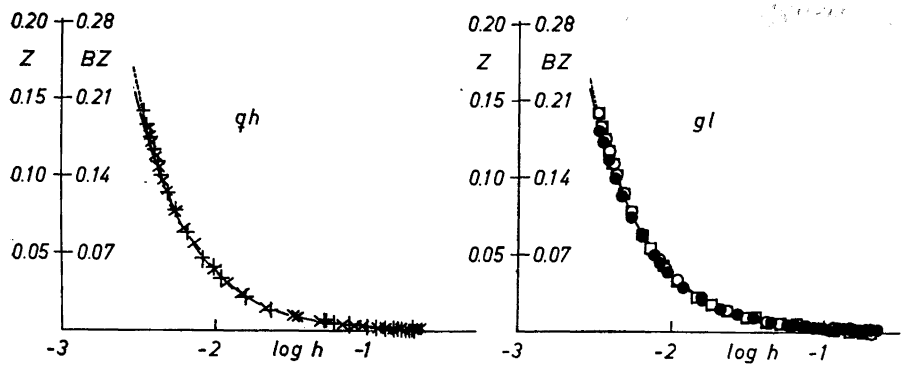


Fig. 3. Hydrolysis of UO_2^{2+}, medium 1.4 UO_2^{2+}, 3.0 (Na)ClO_4. Experimental points and full-drawn curve obtained as in Fig. 1. Left: quinhydrone electrode data, right: glass electrode data. The dotted lines show the correction obtained by assuming the complex A_4B_3.

It has been pointed out by Professor Robert Connick that if the liquid junction potential is calculated from the Henderson equation, a plot like Fig. 2 would show an upward bend of (E + 59.15 log h) at the lowest values for h, because in this range Na^+ ions replace a considerable part of the UO_2^{2+} ions. Rough estimates of the ionic mobilities, using unpublished data of Dr George Biedermann, indicate that this effect would be of the order of a few 0.1 mV thus much less than the observed effect, which we have ascribed to β_{43}. Although this estimate is too uncertain to justify any correction, it shows that one should be careful not to press the data too much in this region.

The values finally obtained for equilibrium constants, E_0, and j, are given in Table 4. The calculated values for h and Z are given in Tables 2 and 3

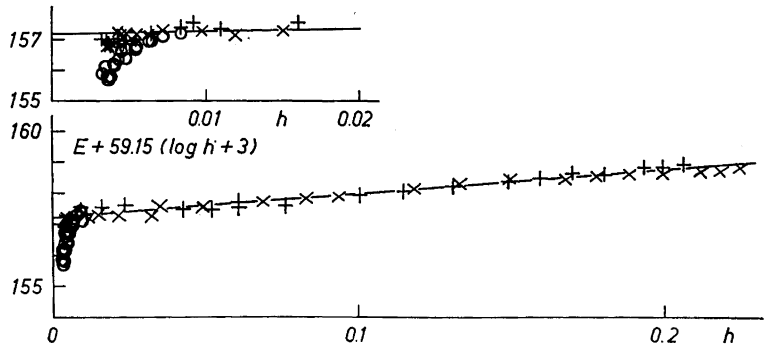


Fig. 4. Medium 1.4 UO_2^{2+}, 3.0 (Na)ClO_4, quinhydrone electrode. Plots for E_0 and j, calculated as in Fig. 2. Crosses: corrected for β_{43}. Circles: not corrected for β_{43}, shown only for $h < 10$ mM; for higher h, the difference is negligible.

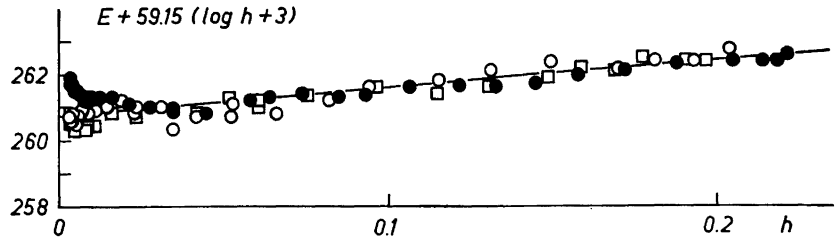


Fig. 5. Medium: 1.4 UO_2^{2+}, 3.0 (Na)ClO_4, glass electrode. Plot of $E + 59.15$ (log $h + 3$) *versus* h, calculated as in Fig. 2, assuming log $\beta_{43} = -12.9$. Difference between series indicates spread in glass electrode data.

(every other point given), and the corresponding diagrams are given in Figs. 1, 2 ($B = 0.4$), 3, 4, and 5 ($B = 1.4$).

It has been asked, whether the assumption of AB_2 is necessary and whether the whole effect could not be explained by A_2B_2, and some deviation of the liquid junction potential from linear behavior. To answer this question, it was assumed that A_2B_2 is the only complex present; assuming various values for β_{22}, eqn (2) makes it possible to calculate h directly from H; inserting this in (1), one might plot $E + 59.15$ log h *versus* h; the results for $B = 1.4$ M are shown in Fig. 6. Deviations from linearity of the order of 10—15 mV cannot be avoided, whatever value for β_{22} is chosen, and so it does not seem possible to dispense with AB_2 in the explanation.

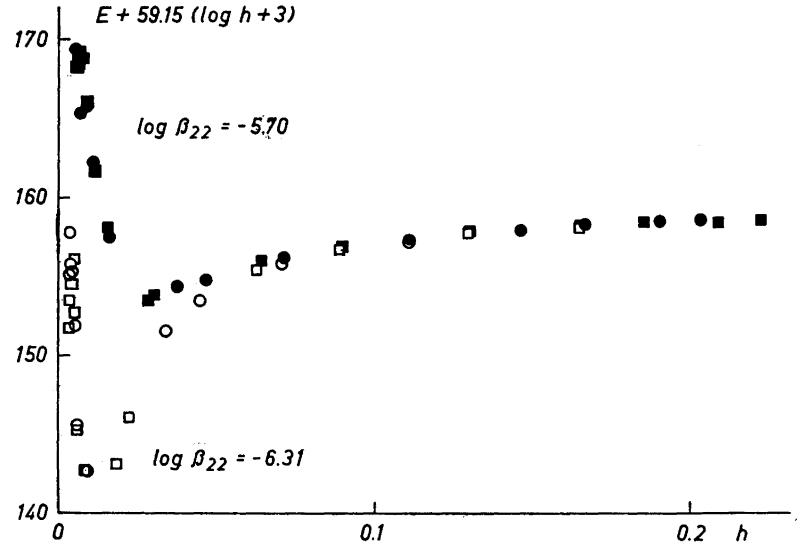


Fig. 6. Medium: 1.4 UO_2^{2+}, 3.0 (Na)ClO_4. Plots for E_0 and j, calculated neglecting AB_2 and assuming log $\beta_{22} = -5.70$ (black symbols) and -6.31 (open symbols). Quinhydrone electrode; circles correspond to + in Fig. 4, squares to ×. Non-linearity of plot indicates that the data cannot be explained by A_2B_2 alone.

Table 4. Equilibrium constants etc obtained by successive approximations from the experimental data $E(H)$. Emfs in mV, concentrations in M.

Quantity	0.4 UO_2^{2+}, 1.0 (Na)ClO_4	1.4 UO_2^{2+}, 3.0 (Na)ClO_4 quinh	1.4 UO_2^{2+}, 3.0 (Na)ClO_4 glass
log k	−0.7	−0.3	−0.3
log u − log a	−3.06	−2.93	−2.94
log β_{22}	−6.02	−6.31	−6.32
log β_{12}	−3.66	−3.68	−3.69
log β_{43}	(not est.)	−12.6	(−12.9)
j	51.0	8.0	8.8
E_0 + 3 . 59.15	164.9 (qh), 493.7 (gl)	157.2	260.7

Estimated uncertainty: approximately ± 0.2 in log k and log β_{12}, ±0.03 in log β_{22}. Different type of glass electrode was used in the two media. Of the j values 8.0 and 8.8, that for "quinh" was more accurately determined. The difference is within the limits of error of the "glass" values.

DISCUSSION

When equilibria A—B are studied in a self-medium with a high and constant concentration of B, the data allow a rather certain conclusion on the values of p in the predominating complexes A_pB_q, whereas the assignment of q cannot be made with certainty from self-medium data alone.

From the data presented in this paper one may conclude that there are species with 1 OH, 2 OH, and probably 4 OH. The formulas ascribed above have been AB_2, A_2B_2, and A_4B_3, thus $(UO_2)_2OH^{3+}$, $(UO_2)_2(OH)_2^{2+}$, and $(UO_2)_3(OH)_4^{2+}$, as usual with unknown amounts of H_2O and ClO_4^-.

For the uranyl ion, just as in the case of thorium [1], the predominating complexes cannot reasonably be mononuclear. If they had been, the measurements at lower B in an inert medium [6,7] would have come out much differently from what is actually observed: hydrolysis would have set in at higher acidities, and moreover, one would have obtained a single curve ("mononuclear wall"). instead of a family of parallel curves.

The assignment of the formula A_2B_2 rather than, say, A_2B_3, or A_2B_4, is made probable by the fact that the log β_{22} so obtained is of the same order of magnitude as the log β_{22} obtained for other ionic media: 1 (Na)ClO_4, −6.05 to −6.10 [7,8], 0.1 ClO_4^- −5.94 [9], and similar values in other media studied in this laboratory.

The value for log β_{43}, which appears only as a correction term, also is of the same order as obtained in earlier work, −12.4 to −12.5 *etc.*[7,8].

It seems reasonable for structural reasons that the complex with one A is AB_2. Had it been, say, AB_3, one might also have expected "log β_{12}" to be about 0.5 units higher in 1.4 than in 0.4 M uranyl medium.

Two of the ions inferred from the self-medium measurements are the same as those observed in inert medium, whereas AB_2 is new. It is understandable that it has not been observed in the earlier inert medium measurements, if it is noticed that the equilibrium constant for

$$(UO_2)_2(OH)_2^{2+} + H^+ \rightleftharpoons (UO_2)_2OH^{3+} + H_2O$$

will give

$$[(UO_2)_2OH^{3+}][(UO_2)_2(OH)_2^{2+}]^{-1} = \beta_{12}\beta_{22}^{-1}h \approx 10^{2.5}h$$

Thus, $[AB_2] > [A_2B_2]$ only for $h > \beta_{22}\beta_{12}^{-1} \approx 10^{-2.5}$. Hydrolysis at such a high acidity is appreciable only at rather high concentrations of uranyl. For instance, at $h = \beta_{22}\beta_{12}^{-2}$, $Z \approx 3\ B\beta_{12}^{2}\beta_{22}^{-1} \approx 0.2\ B$. Even at $B = 0.1$ M, a high value for inert medium measurements, Z is then only 0.02.

Acknowledgements. This work has been supported by *Atomkommittén (Swedish Atomic Energy Commission)* and *Statens Naturvetenskapliga Forskningsråd (Swedish Natural Science Research Council)*. We wish to thank Professor Robert Connick for discussing and correcting the manuscript, and Mr. Frans Ericson for practical help with the apparatus.

REFERENCES

1. Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **13** (1959) 533, Part 22.
2. Gran, G. *Analyst* **77** (1952) 661.
3. Ingri, N., Lagerström, G., Frydman, M. and Sillén, L. G. *Acta Chem. Scand.* **11** (1957) 1034.
4. Brown, A. S. *J. Am. Chem. Soc.* **56** (1934) 646.
5. Forsling, W., Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **6** (1952) 901.
6. Ahrland, S. *Acta Chem. Scand.* **3** (1949) 374.
7. Ahrland, S., Hietanen, S. and Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 1907.
8. Rossotti, F. J. C., Rossotti, H. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 203.
9. Sutton, J. *J. Chem. Soc.* **1949**, S 275.

Received June 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1839–1850

An Electrophoretic Investigation of the Effect of pH on the Degradation of α-Casein by Crystalline Rennin

BÖRJE LINDQVIST and TORSTEN STORGÅRDS

The Central Laboratory of Mjölkcentralen, Stockholm, Sweden

The enzymatic degradation of electrophoretically homogeneous α-casein by crystalline rennin breaks down the casein into a few high molecular weight components. These are degraded further in various ways depending on the pH of the solution. In both the primary and most of the later degradation reactions the parent substance is split into two components with electrophoretic mobilities above and below, respectively, that of the original substance. In addition to the simple fission of α-casein and its primary degradation products there was also observed a slow loss of low molecular weight substances (NPN). The pH optima for the fission and for the NPN formation do not coincide. These results provide a simple explanation of the earlier contradictory properties ascribed to the different components of α-casein and "para-α-casein". Most of these components can now be described as enzymatic cleavage products of the α-casein and of the κ-casein and λ-casein which also contribute to the α-peak.

Although a number of electrophoretic investigations of the enzymatic degradation of α-casein, β-casein and whole casein have been carried out [1–8], only a few of these have dealt with the action of the rennet enzyme (rennin or chymosin) as a proteolytic enzyme in casein hydrolysis [2,4,5]. From these reports it is not possible to draw more than limited conclusions about the groups in the casein that can be split off and about the factors that govern the enzymatic processes. Particularly, little work has been done on the effect of pH on the degradation of α-casein and β-casein by pure rennet enzyme. Several extensive investigations have, however, been carried out without the use of electrophoresis. Nitschmann and coworkers, for instance, have made a detailed study of the primary reaction in the attack of rennin on the casein [9–11] and Ågren [12] and others have separated and identified several components of enzymatic hydrolysates of casein.

In the present investigation it has been found that the enzymatic hydrolysis of casein can proceed in a number of different ways depending on the pH. This explains the somewhat problematic nature of the results of earlier investigations on casein degradation.

METHODS

Preparation of α-casein. α-Casein was prepared by a method essentially the same as the urea method developed by Hipp and coworkers [13]. Great importance was attached to carrying out the preparation rapidly and under mild conditions. In the preparation of the acid casein, only two hours passed between precipitation and the dissolution of the casein in the urea solution. To make this possible the washing and the reprecipitation of the casein was done once only. If a longer time is taken for the preparation there may be enzymatic destruction of the casein and there is a risk of oxidation of sensitive groups in the protein. Three reprecipitations from the urea solution were usually sufficient to give an electrophoretically pure preparation. The casein was obtained in an enzyme-free state by dissolving in dilute alkali and heating at 80° for 30 min. To prepare a suitable form for storage the casein was precipitated with acid, washed and freeze dried. When a very pure preparation was required the casein solution was freed from fat and colloidal material by sedimentation in a Spinco model L ultracentrifuge at 30 000 rpm for 1 hour. 0.5 % thioglycol was added to all the solutions used in the preparation in order to reduce oxidation by atmospheric oxygen, and toluene was added as a bacteriostatic agent.

Preparation of rennin. Crystalline rennin was prepared from "Chr. Hansens Standardlöpe" in powder form by the method of Berridge [14]. The preparation was followed by microscopic examination and by electrophoresis and only preparations with pure, typical crystals and a well defined single peak in the electrophoresis curve were used. It was usually necessary to recrystallise from a magnesium acetate buffer to get crystals that dissolved to give a colorless, clear solution.

The experimental work was done in sets of three parallel runs about 0.2 pH units apart which took between two and three weeks depending on the speed of the degradation. The following conditions are typical of those used for a three week run:

α-Casein (72 g), which was checked electrophoretically immediately before use to ensure that it had not been affected enzymatically during storage, was dispersed in distilled water (*ca.* 1 500 ml) and the pH was adjusted to between 7 and 9 by dropwise addition of 10 % sodium hydroxide solution with vigorous stirring. After a few hours when the casein had dissolved, the pH was adjusted to 9.0 with 1 N NaOH using a Beckman Automatic Titrator and the solution was made up to 1 800 ml. The 4 % casein solution obtained was then heated to 80° for 10 min to destroy any micro-organisms and any enzymes remaining and then cooled after adding 1 % toluene.

Three buffered rennin solutions were prepared containing:

16 g NaCl
8.4 g NaH_2PO_4
x ml 1 N HCl or NaOH (0—30 ml)
y ml rennin solution (*ca.* 1 ml with 300—400 Ru/ml *)
distilled water to 200 ml.

These quantities were chosen so that a 3:1 mixture of casein solution and buffer solution would have the following concentrations:

NaCl	2 %
NaH_2PO_4	0.0675 M
Rennin	400 Ru/ml

The following volumes of HCl or NaOH solution were added in making up the rennin buffer solutions.

The 4 % casein solution was measured out in 15 ml portions into three sets of twelve 25 ml test-tubes with glass stoppers and in 60 ml portions into three sets of six 100 ml conical flasks with glass stoppers. Buffer solution (5 and 20 ml, respectively) was added to each of these casein solutions and the test-tubes were immediately shaken strongly to give a mixture as homogeneous as possible. Toluene (0.2 and 0.8 ml, respectively) was added to each mixture and they were again shaken.

* Ru indicates rennin activity units according to Berridge[14]

Table 1. Volumes of acid or alkali solution added to the buffer solution and corresponding pH values in the substrate (see text).

1 N HCl or NaOH, ml	pH of casein-buffer solution
17.5 NaOH	7.0
12.5 »	6.7
7.5 »	6.4
0 »	5.89
1.5 HCl	5.81
3 »	5.75
5 »	5.45
10 »	5.21
15 »	4.85
20 »	4.79
25 »	4.48
30 »	4.21

A sticky precipitate was formed when the solutions were mixed, occluding part of the buffer salts and enzyme. Full equilibrium inside and outside this gel was not attained immediately but was gradually reached in about a day. Flasks from each mixture were withdrawn for analysis of the contents every Monday, Wednesday and Friday.

Analyses. To determine the distribution of nitrogen the flasks containing 80 ml of mixture were shaken lightly, trichloroacetic acid (TCA, 5 ml, 25 %) was added and the flasks were shaken again vigorously and then centrifuged for 15 min at 3 500 rpm. The supernatant was then decanted through a filter and a nitrogen determination was made on a 20 ml sample of the filtrate by the macro-Kjeldahl method. The amount of nitrogen soluble in 2 % TCA as found by this method was corrected for the amount of soluble nitrogen in the original casein before the rennin was added. The $NPN_{2\%\ TCA}$ (non-protein nitrogen soluble in 2 % TCA) was then expressed as a percentage of the total nitrogen in the original mixture.

The electrophoreses were run on the mixture in the 25 ml tubes. The contents of each tube were first treated with EDTA (0.4 g) to bind any traces of calcium remaining in the casein and with 0.2 ml of 30 % NaOH to raise the pH. After rapid mixing by shaking the pH of the mixture had risen to about 9. This destroyed the rennin and stopped the enzyme reaction. At the same time the casein began to dissolve and after shaking for 2 h the casein was completely dissolved. The protein solution was then dialysed against two changes of the electrophoresis buffer in a rotary dialyser, first for 3 h and then for 16 h. The ratio of sample to buffer during the dialysis was about 1:100.

After dialysis the protein solution was slightly opaque due to the presence of globules of fat and toluene and particles of other material in suspension. After sedimentation in a Spinco model L ultracentrifuge * at 30 000 rpm for 1 h the solution gave a very small bottom layer (*ca.* 0.1 ml), a clear middle layer, and an opaque top layer. A 15 ml sample of the clear layer was taken for the electrophoresis. Centrifuging gave optically clear solutions much more rapidly and conveniently than any of the methods used previously and gave much better reproducibility.

The electrophoreses were run in a Spince model H instrument at 1°C using a phosphate buffer, μ 0.1, pH 7.3, containing 0.02 % merthiolate as preservative. Standard conditions of 120 min at 12 mA were used throughout the investigation.

The sensitivity of the optical recording device and the protein concentration in the sample were kept constant and the electrophoresis curves were therefore directly comparable. The pH of the electrophoresis samples was measured before the addition of EDTA and sodium hydroxide. The variations between tubes were usually about 0.03—0.04 pH units; the values given below are means of the values found for each series.

The conductivity of the buffer and of the electrophoresis sample were measured with an LKB conductivity bridge with the electrodes immersed in the electrophoresis bath.

* Centrifuge purchased with grants from the SMR fund for research in dairy science.

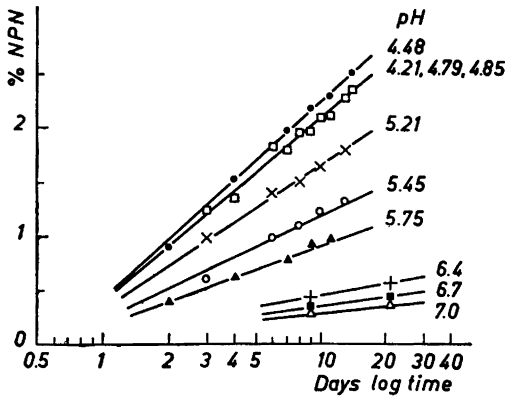


Fig. 1. The release of NPN from α-casein by rennin.

RESULTS

A. Formation of soluble nitrogen

The soluble nitrogen values obtained, when plotted against a logarithmic time scale, gave a linear set of curves with a common origin at 0 % NPN. This point lies rather high on the logarithmic time scale — at approximately 0.6 days (Fig. 1). The slope of the curves which is of course a measure of the rate of the enzymatic process, has a pH optimum at 4.48. At pH 7.0 the reaction rate is very low.

Plotting the NPN value at 15 days as a function of the pH gives curves of the type shown in Fig. 2. To decide whether the curve is symmetrical around the pH 4.5 ordinate would require measurements further down the pH scale

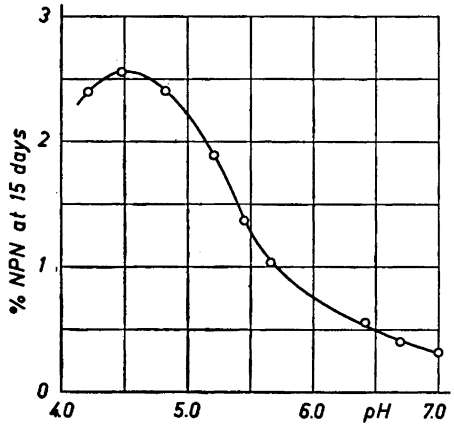


Fig. 2. NPN liberated from α-casein in 15 days.

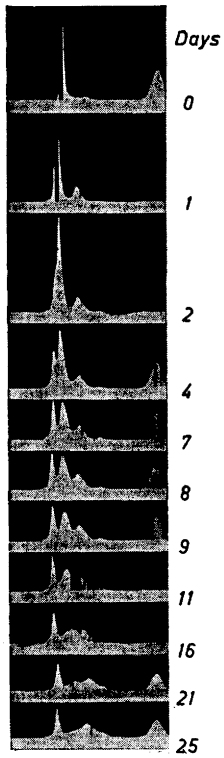


Fig. 3. Electrophoresis curves showing the course of degradation of α-casein by rennin at pH 5.81.

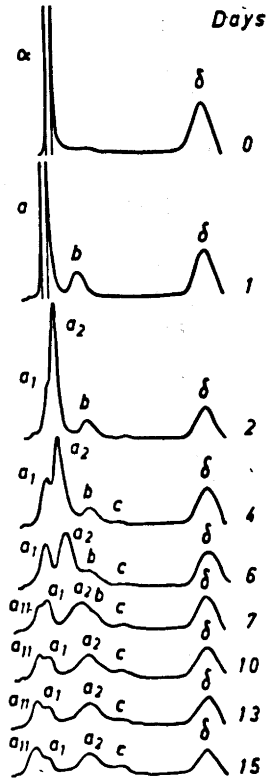


Fig. 4. Electrophoresis curves showing the course of degradation of α-casein by rennin at pH 5.21. Tracings from the photographic curves.

B. Course of the degradation

More than 200 electrophoreses were run in order to elucidate the course of the degradation and approximately 75 of these would be essential to show the phenomena observed completely. For practical reasons only a few will be given here. The curves selected have been supplemented by diagrams showing the course of the more important phenomena occurring during the degradation.

Fig. 3, for example, shows the degradation of α-casein at pH 5.81. The sequence of changes shown is typical of that occurring at pH values between 5 and 7. It shows clearly the successive breakdown of the α-casein into a few components which then undergo further degradation at different rates.

Fig. 4 shows the degradation at pH 5.21. By using only tracings of the curves it has been possible to get the curves close enough to each other to show successive changes more clearly. In this case the degradation is more compli-

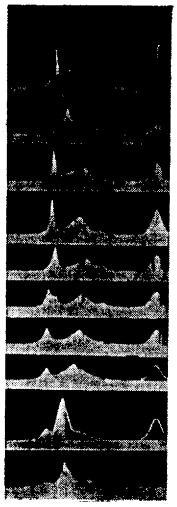


Fig. 5. Typical electrophoresis curves at an intermediate stage of the degradation for different pH values.

cated than at pH 5.81 and some of the components formed first are split again into two components before the subsequent degradation.

Fig. 5 shows a cross section of the electrophoresis curves through the whole pH scale. These have been selected to show the typical sequence at eacn pH. It is apparent that at a pH greater than 6 or less than 4.5 the electrophcresis curves are much simpler than in the interval between. The appearance and degradation of some of these main components will be discussed below.

The two components first formed are designated here as a and b. Component a splits into a_1 and a_2 and then a_1 is further degraded to a_{11}. Component a_2 is degraded more or less continuously without forming any definite degradation product. In some cases where indications of degradation products of a_2 were observed they have been numbered 1, 2 and 3. No special designation has been given to minor substances that could not be traced back to any parent substance except in the case of a component labelled c.

General description of the degradation. At all pH values between 4 and 7 the α-casein is split immediately after adding the enzyme (within a few hours) into two components, a main component (a) with a slightly higher migration rate than the α-casein and a component present in smaller amount (b) with a lower migration rate. The course of the subsequent degradation varies considerably depending on the pH. The b component appears to break down comparatively slowly at a rate independent of the pH and its migration rate changes little once the component has been formed. The formation of the final form of the b component proceeds more slowly the higher the pH and at a pH above 6.5 it takes more than 25 days under the conditions used here. At a pH lower than this it reaches a stable migration rate in two to three days.

Fig. 6 which illustrates the changes in the migration rates of all components shows that there is a strong reduction in the mobility of the b component after 7 to 10 days at pH 5.8—6.4. This may, however, be only an artifact due

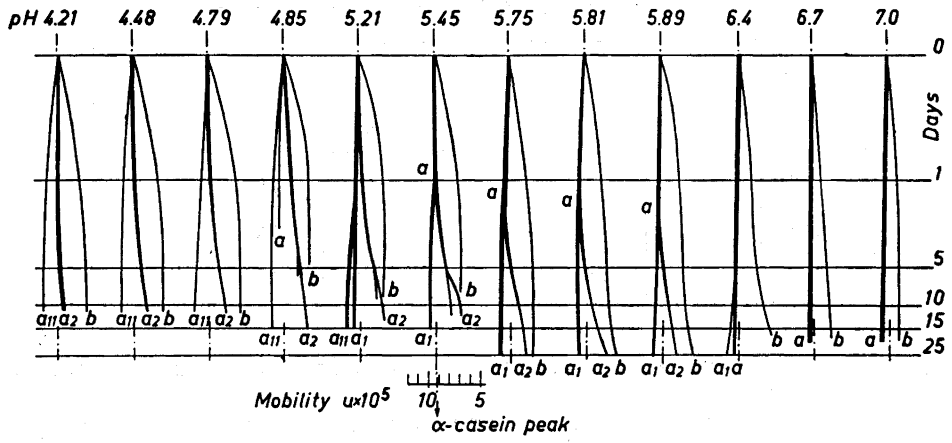


Fig. 6. Changes in electrophoretic mobility during degradation at different pH values. The dotted line shows the position of the α-casein peak at the start of the experiment. The full lines show the changes of mobility of those peaks that can be followed. The thickness of the lines gives a rough indication of the amount of the component present in the mixture.
The electrophoretic migration is from right to left; mobilities can be read from the scale under the pH 5.45 curve.

to the movement through the b component peak of peaks due to other components as their speed changes in the course of the degradation. This would either displace the apparent position or the b peak or form an asymmetric peak with a displaced centre of gravity.

The a-component. The migration rate of the a component is only slightly higher than that of the α-casein (9.3×10^{-5} as aginst 9.1×10^{-5}). The degradation of the a-casein becomes apparent quite soon after it is formed but the rate of degradation depends strongly on the pH. At pH 6.4 it can be observed after 10 days and at pH 5.2—5.3 the degradation is so fast that the a-casein is completely changed after only a day. At pH values lower than this the a-casein is not observed and only its degradation products, a_1 and a_2, appear.

Component a_2. The a_1 and a_2 caseins have mobilities higher and lower, respectively, than that of the parent a-casein in the same way as the a and b caseins have higher and lower migrations rates, respectively, than that of the α-casein. This simultaneous formation of fission products with migration rates higher and lower than that of the parent substance appears to be a general rule in this type of casein degradation.

The mobility of the a_2 component gradually falls in the same way as that of the b-casein. The final product with a stable migration rate is reached most rapidly at a pH of about 5.2. Both at lower and at higher pH values the change is much slower. The final migration rate coincides at optimal pH values with that of the b component but at other pH values is usually higher.

In the pH range 4.8—5.5 there is a tendency for a_2 to split up in the intermediate stages into several components. Later, however, these are largely

Table 2. Composition (%) of the casein degradation product.

Preparation		a		b	c
pH	days	a_1	a_2		
5.75	2	83.2		15.0	1.7
5.45	1	83.3		15.0	1.7
5.75	4	25.5	53.2	19.3	2.0

broken down and only one or two remain. The final migration speed of a_2 coincides even in this case more or less with that of the b-component but varies somewhat depending on the amount and type of these intermediate components left. The degradation of the a_2 subcomponents apparently does not stop but continues as long as there is any material left in the sample.

Component a_1. The a_1 component like the a component is broken down very rapidly at certain pH values. However, within the pH range 4.85—6.4 the ratio of the rate of formation to the rate of degradation is such that it can be observed during relatively long period. At a pH of 5.21 or lower it changes to a form with a slightly higher migration rate as shown in Figs 7 and 8; this has been designated here as a_{11}. At a pH of 4.8 or less a_1 is broken down so rapidly that a appears to be transformed directly into a_{11} and a_2. Like other components a_{11} gradually attains a fairly stable migration rate.

Quantitative measurements. The electrophoresis curves obtained were suitable for quantitative estimates only during the first part of the degradation. The rates of breakdown of a into a and b and the rate of formation of the component c, which is discussed below, can be measured fairly readily. In some cases it was also possible to measure the splitting of a into a_1 and a_2 but the subsequent breakdown of a_2 is rapid and gives components which coincide with b so that the measurements are then much less certain. Table 2 gives estimates of the proportions of the different degradation products.

DISCUSSION

Even though the α-casein and the crystalline rennin used in these experiments were electrophoretically pure they cannot be regarded as homogeneous. Waugh and von Hippel [15] have reported the presence in α-casein of up to 12 % of a component which they named $\varkappa$-casein and Long, Winkle and Gould [16] have isolated from electrophoretically homogeneous α-casein an additional substance, which they named λ-casein, that forms about 3 % of the α-casein. The degradation products observed here cannot therefore be referred immediately to any particular one of the three substances in the mixture formerly regarded as α-casein.

Crystalline, electrophoretically pure rennin has been found by Schwander *et al.*[17] to contain a small amount of a substance which, according to the

results of sedimentation in an ultracentrifuge and of diffusion measurements, is of higher molecular weight. Ernstrom [18] has found, moreover, by electrophoretic examination of crystalline rennin, that the single rennin peak obtained under normal conditions is split into several components at low ionic strengths. It cannot be excluded that one or more of these components may have proteolytic properties different from those of the main component of the rennin.

It is thus apparent that the present investigation, no more than any other hitherto published on the enzymatic degradation of casein, cannot satisfy the requirements set out by Ogston for a defined biochemical process [19]. Nevertheless, while awaiting the development of methods for the preparation of absolutely homogeneous casein fractions and chemically pure rennin, the results obtained give some perspective to the investigations that have been made on the structure and properties of the phosphopeptones and polypeptides obtained from casein. The results of the present investigation show more directly that it is possible to find experimental conditions under which the degradation products have simpler electrophoretic properties than are found under other conditions. It now seems possible on the basis of the present observations to give a fairly simple explanation of some older observations on "paracasein".

Enzymatic processes in a heterogeneous mixture of the type concerned here do not lend themselves to rate determinations nearly as readily as when they occur in a homogeneous phase. In the present case there was strong gel formation and precipitation of α-casein as soon as the pH came below 5.3. However, by adapting the technique used to these conditions it was found possible to obtain more or less reproducible results, as is apparent from Figs. 1 and 2. At pH 5.45 and higher pH values where the α-casein is in solution the curves obtained had approximately the same origin on the time scale as when the pH was less than 5.45 and the casein insoluble. This shows that the rate and the course of the processes occurring were more or less the same in both cases. The delay of 0.6 days that was observed in all these experiments may be due to the presence of an inhibitor of the type mentioned by Dixon [20]. The results of Amundstad's investigations [21], when plotted on a semi-logarithmic scale as in Fig. 1 show a similar delay. In this case the delay varied rather more than in the present investigation but was of approximately the same order. Amundstad worked with total casein and technical rennet and the chances of some poisoning effect occurring are rather different from the present case. An alternative explanation of the delay could be that the NPN is not liberated in the first stages of the degradation but only after certain key links are broken by the rennin.

Some support for this hypothesis is obtained from a comparison of the changes in the electrophoresis curve with the change in the amount of NPN. At a given degree of degradation, for instance 1 % NPN, the electrophoresis curves are different for different pH values (Fig. 7). The primary degradation thus cannot have any direct effect on the formation of NPN. This supposition was supported by the very slow formation of NPN at pH 7 and the simultaneous relatively rapid fission of the α-casein at this pH into a and b components (Figs. 5 and 7). Another criterion supporting this is that the optimum pH

Fig. 7. Electrophoresis curves at NPN 1 %. The absence of any relationship between the NPN content and the appearance of the curve is an indication that these concern two independent processes.

for NPN formation is 4.5, as shown in Fig. 2, but is 5.2—5.5 for the degradation of a_2 as is visible in Figs. 6 and 7.

These phenomena are parallelled by the rapidity with which a glyco-macropeptide is split off during the primary reaction observed by Nitschmann *et al.*[10] in the coagulation of casein with rennet and the slowness of the subsequent, general proteolytic action noted by Nitschmann[9] in the tertiary stage. There is as yet no proof that any of our components is identical with Nitschmann's glyco-macropeptide nor, on the other hand, is there any contradictory evidence.

Nitschmann and Lehmann[2] found that the paracasein which is formed when casein is coagulated with rennet gave two peaks in place of the α-casein peak. They called these rennet α_1- and rennet α_2- caseins and recorded mobilities in phosphate buffer at pH 7.35, μ 0.077, 2°C of

$$\alpha_1 : 9.55 \times 10^{-5} \quad \text{and} \quad \alpha_2 : 8.83 \times 10^{-5}$$

The α-casein had a mobility of 9.30×10^{-5}. This is in good agreement with our observations where the α-casein had a mobility in phosphate buffer at pH 7.3, μ 0.1, 1°C of 9.09×10^{-5}. The mobilities of the a and a_1 components fall within the range $9.2—10.8 \times 10^{-5}$ while a_2 has a mobility between 9.1 and 7.0 depending on the extent of the degradation of the casein.

It seems quite possible that Nitschmann's rennet α_1-and rennet α_2-caseins are identical with the a or a_1 components and with a_2 respectively, with Nitschmann's material representing forms of an intermediate degree of degradation.

More difficult to explain is the observation of Cherbuliez[22] that casein that has not been treated with rennet also gives two α peaks which he called

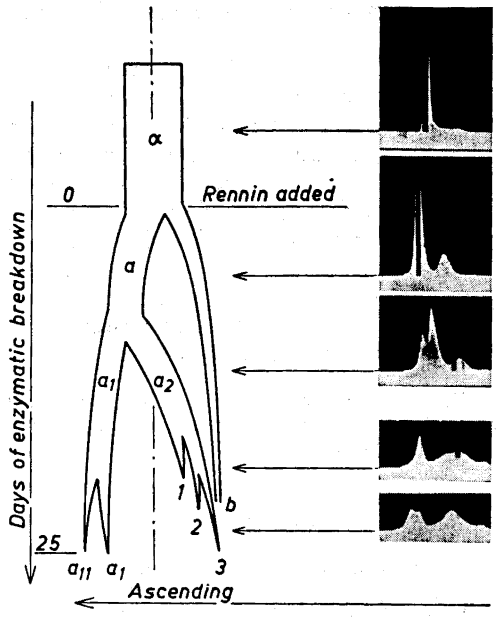


Fig. 8. The curves in Fig. 7 slightly idealized to cover the pH range 5.0—5.5 and to illustrate the major changes taking place during the α-casein decomposition.

α_I and α_{II}. However, he used a rather time-consuming method to obtain ash-free and fat-free preparations and it is quite possible that proteolytic enzymes originally present in the milk, which would accompany the α-casein in the preparation, may easily have given rise to a degradation similar to that produced by the rennet. The components later found by Cherbuliez [23] in para α-casein may very well have been rather degraded products formed according to the scheme described here for the further degradation of a_1 and a_2. In this case it would not be possible to compare the mobilities since the experimental conditions would be quite different.

Fig. 6 summarises all the electrophoresis results obtained as branched curves showing how the mobilities of the different degradation products are related to the mobilities of the parent substance, which is marked with a dashed line. As is shown in the figure the rates of the secondary, tertiary and further degradation reactions, that is the branching points of the curves, are very strongly dependent on the pH. A diagram showing the full course of the degradation for the pH range 5.0—5.5 is given in Fig. 8. The course of the degradation at pH values outside this range can be seen from a comparison of Figs. 7 and 8. The most striking point about these diagrams is that components such as a_{11} and a_1 and b are either extremely stable to rennin or else their migration rates do not change at all on further degradation while a_2 on the other hand is rapidly broken down forming substances of lower mobility.

This must of course be dependent on the amino-acid composition of the fission products.

Since the a and b produced in the first step of the degradation of the α-casein have a higher and a lower mobility, respectively, than the parent substance it is probable that a contains relatively more serine-threonine bound phosphoric acid and more glutamic acid and aspartic acid than the α-casein, while b must contain more basic amino acids than the α-casein or a. The large changes in the mobility of a_2 during the degradation must be ascribed to the presence of relatively greater amounts of easily removed acidic amino-acids and of difficultly removable basic amino-acids. The distribution of the amino-acids in a_1 and a_{11} on the other hand must be such that the neutral amino-acids predominate or that any acidic and basic amino-acids present neutralise each other and, moreover, are not too readily accessible to proteolysis.

Acknowledgement. The authors wish to express their gratitude to the Scientific Council of Swedish Dairies' Association wich made the investigation possible by placing the ultracentrifuge at their disposal.

REFERENCES

1. Mellander, O. *Upsala Läkareförens. Förh.* **52** (1947) 107.
2. Nitschmann, H. and Lehmann, W. *Experientia* **3** (1947) 153.
3. Perlmann, G. E. *Discussions Faraday Soc.* **1953** 67.
4. Hagberg, E. C. and Sullivan, R. A. *J. Dairy Sci.* **36** (1953) 569.
5. Tsugo, T. and Yamauchi, K. *XIV International Dairy Congr. Rome 1956. Proc.* Vol II, part II, 588.
6. Lillevik, H. A. and Anwar, R. A. *J. Dairy Sci.* **40** (1957) 597.
7. Petersson, R. F., Naumann, L. W. and Mc. Meekin, T. L. *J. Am. Chem. Soc.* **80** (1958) 95.
8. Groves, M. L., Hipp, N. J. and Mc Meekin, T. L. *J. Am. Chem. Soc.* **80** (1958) 716.
9. Nitschmann, H. and Keller, W. *Helv. Chim. Acta* **38** (1955) 942.
10. Nitschmann, H., Wissmann, H. and Henzi, R. *Chimia* **11** (1957) 76.
11. Wissmann, H. and Nitschmann, H. *Helv. Chim. Acta* **40** (1957) 356.
12. Ågren, G. and Glomset, J. *Acta Chem. Scand.* **7** (1953) 1071.
13. Hipp, N. J., Groves, M. L., Custer, J. H. and Mc Meekin, T. L. *J. Dairy Sci.* **35** (1952) 272.
14. Berridge, N. J. and Woodward, C. *J. Dairy Research* **20** (1953) 255.
15. Waugh, D. F. and von Hippel, P. H. *J. Am. Chem. Soc.* **78** (1956) 4576.
16. Long, J., van Winkle, Q. and Gould, J. A. *J. Dairy Sci.* **41** (1958) 317.
17. Schwander, H., Zahler, P. and Nitschmann, H. *Helv. Chim. Acta* **35** (1952) 553.
18. Ernstrom, C. A. *J. Dairy Sci.* **41** (1958) 1663.
19. Ogston, A. G. *Ann. Rev. Biochem.* **24** (1955) 181.
20. Dixon, M. *Enzymes*, London 1958, p. 94.
21. Amundstad, O. *Statens Mejeriförsök, Alnarp 1950 (Sweden)*, Medd. No. 28.
22. Cherbuliez, E. and Baudet, P. *Helv. Chim. Acta* **33** (1950) 398.
23. Cherbuliez, E. and Baudet, P. *Helv. Chim. Acta* **33** (1950) 1673.

Received May 28, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1851–1854

Further Investigations on Displacement Potentials

F. CARLSEN, W. DANSGAARD and C. E. JENSEN †

Universitetets biofysiske laboratorium, and Universitetets fysisk-kemiske institut, Copenhagen, Denmark

By the aid of a vibrating reed electrometer it is shown that displacement potentials of the order of 100—150 mV can be generated when very high molecular hyaluronate is used. The phenomenon exhibits effects of fatigue and hysteresis.

It was shown by Jensen, Koefoed and Vilstrup [1] that if a solution of high molecular hyaluronate is displaced in a glass capillary an electric potential is generated which is characterized by the fact that the potential difference persists as long as the solution is strained. The same phenomenon was observed with regard to genuine endolymph from the piked dogfish *(Acanthias vulgaris)*, and Vilstrup, Jensen and Koefoed [2] suggested that these potentials might be of the greatest importance for the function of the labyrinth. Very recently also Dohlman [3] has accepted the conclusion that the transformation of mechanical movements to electrical charges is what we have reason to believe to be the fundamental potential, influencing the generation of excitatory reactions in the nerve endings at the crista. The presence of acid mucopolysaccharides in the gelatinous substance in the internal ear (Jensen and Vilstrup [4], Dohlman, Ormerod and Mc Lay [5], and Poulsen [6]) suggests reversible electrical changes due to displacement potentials which might be collected by the hairs, acting as electro-sounds for the hair cells of the sound-perceiving organs of the inner ear (suggested in an oral discussion, 1955, by J. A. Christiansen).

In the first published experiments the electrical effect seemed to be relatively small, varying from fractions of mV to a few mV, but recently it was found (Jensen, Christiansen and Vilstrup [7]) that the potentials resulting from displacements of very high molecular polyelectrolytes in a glass capillary and measured potentiometrically amounted to about 100 mV.

In the present work this observation has been confirmed by using a vibration reed electrometer, and further it is shown that the relation between displacement and potential can be described by hysteresis loops, and that the phenomenon exhibits an effect of fatigue.

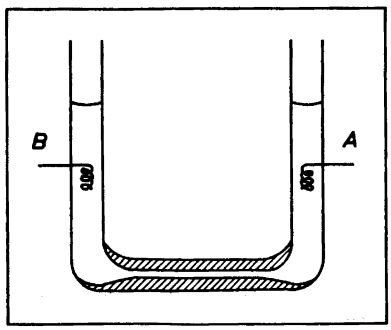


Fig. 1. U-Tube in which palladium electrodes are inserted.

MATERIALS

The sample of alginate used was a commercial preparation ("Protanal HF", manufactured and kindly supplied to us by A/S Protan, Drammen, Norway). The number average molecular weight was determined to be about 1×10^5 after the osmotic method of Christiansen and Jensen [8]. By fractional precipitation with alcohol we obtained a sample with a molecular weight of about 2×10^5.

The sample of potassium hyaluronate employed was prepared from human umbilical cords according to Jensen [9]. The molecular weight of hyaluronate obtained by this method is about 5×10^5 (determined at a concentration of 1 %). By adding alcohol to an 0.1 % aqueous solution of this precipitation until the solution was about 35 % in respect of alcohol we obtained a preparation which had a molecular weight greater than 1.5×10^6 as judged from osmotic measurements. In all experiments care was taken to avoid bacterial contamination. All measurements of the potentials were performed at room temperature.

EXPERIMENTS AND RESULTS

The employed glass apparatus with palladium electrodes is shown in Fig. 1. The deflection of a highly sensitive galvanometer (aperiodically damped; resistance 455 ohms) connected to the electrodes indicated their potential difference.

With distilled water in the apparatus no significant potentials were observed. However, when a 0.05 % solution of alginate was introduced into the apparatus through the branch called A in Fig. 1 the electrode in branch B became positive. The magnitude of the potential difference (called V_0 below) depended upon the concentration of the solute, the method of filling and the dimensions of the apparatus.

Taking V_0 as a zero point, displacement of the solution towards A rendered this branch negative relative to B as long as the solution was moving, but positive when the solution came at rest. By displacing the solution towards B the temporary as well as the final potential differences changed their signs. Repeating of this procedure decreased the potential differences (effect of fatigue, shown in Fig. 2). After some days, however, the effect regenerated.

The potentials appeared to be a function not only of the actual displacement. They were also influenced by the magnitude and direction of former

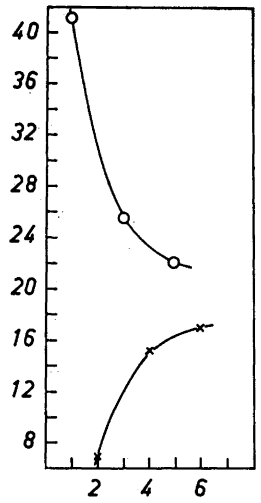


Fig. 2. Curves showing the phenomenon of fatigue. Ordinates: Arbitrary galvanometer readings. Abscissae: Sequence of displacements. Circles represent the potentials following displacements to the right, crosses to the left.

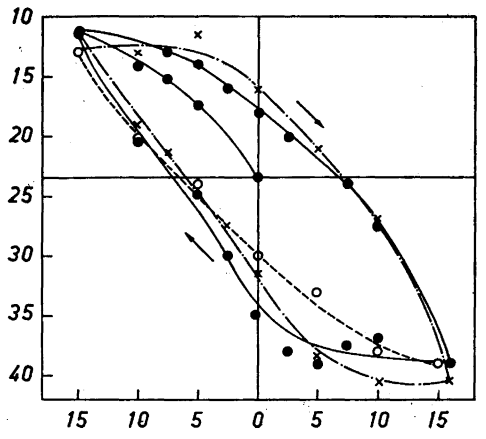


Fig. 3. Hysteresis loops. Ordinates: Arbitrary galvanometer readings. Abscissae: Displacements in mm to the right and to the left.

displacements (hysteresis, shown in Fig. 3). Increased potentials were observed upon insertion of thin glass rods in the capillary tube.

By recent application of a vibrating reed electrometer the electric current through the apparatus became negligible. Under these circumstances values of V_0 up to 140 mV were registered after filling the apparatus with a 0.25 % solution of hyaluronate. Furthermore potentials of the order of 10 mV occurred as a result of displacements of the order of 1 cm. V_0 was not quite constant in time, but generally the drift was less than 20 % during several days.

DISCUSSION

The experiments reported here show that under laboratory conditions the displacement potential may exceed 100 mV, and although such high voltages may not be present in the sensulae of the inner ear, an even much smaller voltage may be considered sufficient for adequate stimulation of the hair cells, *i. e.* for the modulation of the spontaneous cell action.

As shown above the displacement potential displays the phenomenon of fatigue; in this connection it may be mentioned that the phenomenon of fatigue is also well-known in the labyrinth.

We wish to thank Professor J. A. Christiansen, Head of the Physico-chemical Institute of the University of Copenhagen, for many valuable discussions and suggestions. One of us (C. E. J.) gratefully acknowledge financial support from *Philips Fond of 1958.*

REFERENCES

1. Jensen, C. E., Koefoed, J. and Vilstrup, Th. *Nature* **174** (1954) 1101.
2. Vilstrup, Th., Jensen, C. E. and Koefoed, J. *Ann. Otol. Rhinol. Laryngol.* **64** (1955) 406.
3. Dohlman, G. *J. Laryngol. Otol.* **73** (1959) 154.
4. Jensen, C. E. and Vilstrup, Th. *Acta Oto-laryngol. In press.*
5. Dohlman, G., Ormerod, F. C. and Mc Lay, K. *Acta Oto-laryngol.* **50** (1959) 243.
6. Poulsen, H. *Myxoedematøse bindevævsforandringers indflydelse på labyrinthens funktion.* (With a summary in English). Munksgaard, Copenhagen 1959.
7. Jensen, C. E., Christiansen, J. A. and Vilstrup, Th. *Symposium über Makromoleküle in Wiesbaden 1959.*
8. Christiansen, J. A. and Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 1247.
9. Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 603.

Received July 22, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1855–1862

Studies on the Chemistry of Lichens

XIII.* The Structure of Pannaric Acid

B. ÅKERMARK, H. ERDTMAN and C. A. WACHTMEISTER

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Pannaric acid, $C_{16}H_{12}O_7$, a phenolic acid from *Crocynea membranacea* (Dicks.) Zahlbr., has been shown to contain two carboxyl groups, two phenolic hydroxyl groups and two C-methyl groups. On decarboxylation, it forms a phenol $C_{14}H_{12}O_3$, identified as 3,9-dihydroxy-1,7-dimethyldibenzofuran, which was synthesised. From biogenetic considerations, the most probable structure would be (V). This structure has been confirmed by permanganate oxidation of di-O-methylpannaric acid to 1,2,6,7-tetracarboxy-3,9-dimethoxydibenzofuran, previously obtained from porphyrilic acid (I). Structure (V) is also in harmony with colour reactions and the infra-red absorption spectra of pannaric acid and its derivatives.

One of the main aims of these investigations has been the isolation of compounds illustrating the role of oxidative coupling of phenols in biosynthesis. The importance of this reaction in lichen chemistry, in the lignan field and also to an understanding of the biosynthesis of thyroxin [1] was discussed by one of us in a lecture to the Chemical Society, Stockholm, in December 1933 shortly after the structural elucidation of the depsidone salazinic acid by Asahina [2]. The depsidones were considered to be coupling products of phenol oxygen radicals and mesomeric carbon radicals.

Examples of the pairing of carbon radicals were expected and have since been found: usnic acid [3], the "depsone" picrolichenic acid [4,5] and the dibenzofurans strepsilin [6], didymic acid [7] and porphyrilic acid [8,9]. Usnic acid and picrolichenic acid are particularly interesting because one of the nuclei has not returned to the aromatic state.

The biosynthetic aspects of phenol dehydrogenation in general have been recently discussed by Barton and Cohen [10] and by Erdtman and Wachtmeister [11] and with particular reference to lichen chemistry by Wachtmeister [12].

---

* Part XI *Acta Chem. Scand.* **12** (1958) 147.
Part XII *Svensk Kem. Tidskr.* **70** (1958) 117.

In this search for new oxidative coupling products attention has been focussed on lichen acids which cannot be cleaved hydrolytically into two or more components; these can easily be found by paper chromatography [13].

Hesse's pannaric acid * from *Crocynea membranacea* (Dicks.) Zahlbr. (= *Pannaria lanuginosa* Ach.) has such properties. According to Hesse [14], pannaric acid has the composition $C_9H_8O_4$ but the high melting point (Hesse reports 224°) indicates a higher molecular weight.

Exhaustive extraction of the lichen with ether afforded a crude crystalline product from which roccellic acid could be removed by extraction with hot petroleum ether. The residual crude pannaric acid (about 1.5 % of the dry lichen) was conveniently recrystallised from diacetone alcohol and further purified by filtration through alumina. The purity of the acid was checked chromatographically on phosphate-impregnated paper [13].

Pannaric acid exhibits a bluish-white fluorescence in ultra-violet light. It is readily soluble in the lower alcohols and in acetone containing about 2 % of water but only sparingly soluble in dry acetone, ether, benzene and chloroform. It dissolves with effervescence in aqueous sodium hydrogen carbonate to give a colourless solution. Like porphyrilic acid [9], pannaric acid gives a blue colour with iron(III) chloride and a blue colour with Gibb's reagent [15]; with bleaching powder it gives the green colour characteristic of all known lichen acids belonging to the dibenzofuran group.

Pannaric acid crystallises from aqueous acetone as colourless needles containing solvent of crystallisation. It melts with decomposition at 243—245° and after drying *in vacuo* gave analytical results in agreement with the composition $C_{16}H_{12}O_7$. Pannaric acid possesses two strongly acidic groups and carbon dioxide is evolved on thermal decomposition. Kuhn-Roth determination indicated the presence of two C-methyl groups.

Methylation of pannaric acid with a large excess of diazomethane in the presence of some methanol yielded dimethyl di-O-methylpannarate, $C_{12}H_2O(CH_3)_2(OCH_3)_2(COOCH_3)_2$ which on hydrolysis gave di-O-methyl pannaric acid, $C_{12}H_2O(CH_3)_2(OCH_3)_2(COOH)_2$. Treatment of pannaric acid at −10° with a slight excess of diazomethane for 1—2 min afforded dimethyl pannarate, $C_{12}H_2O(CH_3)_2(OH)_2(COOCH_3)_2$, which was readily acetylated to a diacetate.

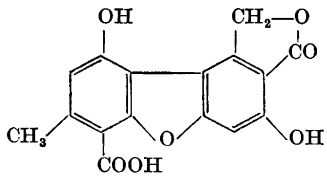
Pannaric acid itself yielded a diacetate $C_{12}H_2O(CH_3)_2(OCOCH_3)_2(COOH)_2$ which decomposed above 240° without melting.

On prolonged boiling with formic acid, pannaric acid was decarboxylated with the formation of pannarol, $C_{12}H_4O(CH_3)_2(OH)_2$, m.p. 183—184°, which gave a diacetate and a dimethyl ether. As described by Hesse, pannarol was also formed on thermal decomposition of the dry acid.

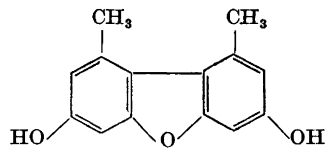
The ultra-violet absorption curves of pannarol and of its diacetate were very similar to those of 3,7-dihydroxy-1,9-dimethyldibenzofuran [7] (II) and its diacetate, respectively, but pannarol was not identical with compound II.

---

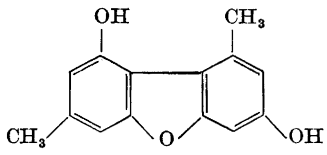
* The name pannaric acid, unfortunately, has also been given to an oxidation product obtained from pannarin, a chlorine-containing depsidone isolated from the Japanese *Pannaria lanuginosa* and some other *Pannaria* species by Yosioka. Compare Asahina, Y. and Shibata, S. *Chemistry of Lichen Substances*, Tokyo 1954.



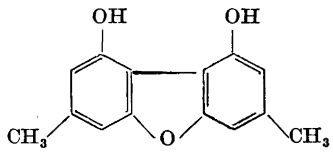
I



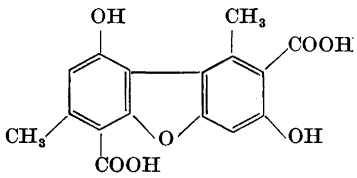
II



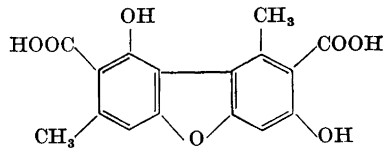
III



IV



V



VI

For biogenetic reasons, pannarol was considered to be either 3,9-dihydroxy-1,7-dimethyldibenzofuran (III) or 1,9-dihydroxy-3,7-dimethyldibenzofuran (IV). These phenols were therefore synthesised.

Dibenzofurans of these types are readily available by Ullmann coupling of the appropriate iodides and cyclisation of the tetramethoxydiphenyl obtained. Although 2-iodo-3,5-dimethoxytoluene [5] is formed in high yield by direct iodination of orcinol dimethyl ether, the 4-iodo-analogue has so far only been prepared from 4-nitroorcinol which is obtained in very low yield on nitration of orcinol. It is known, however, that resorcinol dimethyl ether reacts with phenyl lithium forming mainly the 2-lithium derivative [17]. Orcinol dimethyl ether when metalated in the same way and treated with iodine gave 4-iodo-3,5-dimethoxytoluene in an overall yield of 50 %.

Ullmann coupling of 4-iodo-3,5-dimethoxytoluene afforded 2,6,2′,6′-tetramethoxy-4,4′-dimethyldiphenyl [16]. On boiling with hydrobromic acid this compound was demethylated and dehydrated, giving 1,9-dihydroxy-3,7-dimethyldibenzofuran (IV), m.p. 190—192°, which was different from pannarol. Like its lower homologue, 1,9-dihydroxydibenzofuran, it showed abnormally high R_F-values (*cf.* Ref.[12]).

A mixed Ullmann coupling with 4-iodo-3,5-dimethoxytoluene and an excess of 2-iodo-3,5-dimethoxytoluene furnished a mixture of diphenyls. On prolonged boiling with hydrobromic acid the crude product gave a mixture of phenols, the main component of which proved paper-chromatographically identical with 3,7-dihydroxy-1,9-dimethyldibenzofuran (II). Paper chroma-

tography also revealed a minor component of the same high R_F-value as 1,9-dihydroxy-3,7-dimethyldibenzofuran (IV). On treatment of the chromatogram with Gibbs' reagent a third component appeared as a blue spot as would be expected for 3,9-dihydroxy-1,7-dimethyldibenzofuran (III). This spot behaved in all respects like that of pannarol.

The main part of the 3,7-dihydroxy-1,9-dimethyldibenzofuran was separated from the crude phenol mixture by crystallisation from benzene. The mother liquor was evaporated and the residue was chromatographed on a column of cellulose powder impregnated with dimethyl sulphoxide, using diethyl ether as the mobile phase. The fractions eluted were analysed by paper chromatography using the same solvent system [18]. 3,9-Dihydroxy-1,7-dimethyldibenzofuran, m.p. 184—185°, was shown to be identical with pannarol by mixed melting point determination as well as by comparison of the infra-red absorption curves. Methylation gave 3,9-dimethoxy-1,7-dimethyldibenzofuran, similarly identified as pannarol dimethyl ether.

From these results, pannaric acid may be formulated as (V) or (VI), the only structures which are consistent with biosynthesis from orsellinic acid. A strong indication in favour of structure (V) was given by the negative reaction of dimethyl pannarate with Gibb's reagent [15]. Moreover, the infra-red absorption curve of dimethyl pannarate in potassium bromide showed two separate carbonyl bands at 1 660 cm^{-1} and 1 690 cm^{-1}, respectively, indicating intramolecular as well as intermolecular hydrogen bonding. The absorption curve of dimethyl di-O-methyl pannarate, as expected, showed a single carbonyl band at 1 725 cm^{-1}.

A confirmation of structure (V) was obtained by permanganate oxidation of di-O-methylpannaric acid to a tetracarboxylic acid which on heating gave a yellow di-anhydride and on methylation gave a tetramethyl ester $(CH_3O)_2C_{12}H_2O(COOCH_3)_4$, m.p. 225—226°, undepressed on admixture with 3,9-dimethoxy-1,2,6,7-tetracarbomethoxydibenzofuran [9] previously obtained from porphyrilic acid (I). The identity was confirmed by comparison of the infra-red spectra.

Porphyrilic acid (I) from *Haematomma coccineum* and pannaric acid (V) from *Crocynea membranacea* contain the same carbon skeleton and are both major phenolic compounds of the respective lichens. It is noteworthy that no lichen acid containing the carbon skeleton (VI) has so far been found. This gives some support to the proposed mechanism for oxidative coupling of orsellinic acid homologues, involving intermediate radicals, stabilised by chelation, that are formed by participation of the most acidic phenolic hydroxyl group, that *para* to the carboxyl group [12].

Fertile specimens of *Crocynea membranacea* have never been found and the classification appears somewhat questionable. Other *Crocynea* species are well defined. From a chemical point of view a relationship with the genus *Haematomma* would appear probable.

EXPERIMENTAL

All melting points were determined on a Kofler micro hot stage. Whatman No. 1 paper was used for paper chromatography. Ultra-violet absorption spectra were measured with a Beckman spectrophotometer, model DU. The infra-red spectra were recorded with

a Perkin-Elmer double beam spectrophotometer, model 21 (sodium chloride prism; potassium bromide discs).

Isolation of pannaric acid. *Crocynea membranacea* (3.14 kg) was extracted with ether for 5 days in a Soxhlet apparatus. The ether extract (10 l) deposited a crystallisate (10 g, m. p. 172—181°) which was not further investigated *. The mother liquor was evaporated to dryness and the crystalline residue was extracted repeatedly with boiling petroleum ether (b. p. 100—120°). The material extracted was crystallised from ethanol giving roccellic acid (90 g), m. p. 130.5—132.0°, $[\alpha]_D^{20}$ +18.0 (EtOH, *c* 1.94). Lit. m. p. 131°, $[\alpha]_D$ +17.4 (EtOH) [19].

The insoluble fraction (45 g, m. p. 228—234°) on crystallisation from diacetone alcohol afforded crude pannaric acid as yellowish needles (25 g, m. p. 230—235°) of sufficient purity for most of the degradative work. A solution of the crude acid (1.2 g) in acetone (20 ml), water (0.5 ml) and ether (200 ml) was filtered through a 3 cm column of alkali-free alumina (5 g) and followed by 200 ml ether-acetone (10:1). The combined filtrates on evaporation afforded pannaric acid (700 mg), which crystallised as colourless needles (300 mg) from aqueous acetone. The purity of the acid was checked by chromatography on phosphate-impregnated paper [9,13] using a *n*-butanol-water solvent system. The acid showed the R_F-values 0.05 (0.1 M Na_3PO_4); 0.10 (0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4 1:1) and 0.65 (0.1 M NaH_2PO_4). The spots were visible in ultraviolet light (bluish-white fluorescence under alkaline conditions) and gave a blue colour with Gibbs' reagent and a dark red-brown colour with *bis*-diazotised benzidine [20] under alkaline conditions.

Pannaric acid was dried for analysis *in vacuo* for 1 h at 120°; loss of weight: 9.6 %; $C_{16}H_{12}O_7$, $2H_2O$ requires 10.2 %; m. p. 243—245°. The equivalent weight was determined by potentiometric titration in 50 % methanol. (Found: C 60.6; H 4.2; equiv. wt. 160. $C_{16}H_{12}O_7$ requires C 60.8; H 3.8; equiv. wt. required for a dicarboxylic acid, 158.)

Di-O-acetylpannaric acid. Pannaric acid (75 mg) was boiled for 5 min with acetic anhydride (1 ml) containing pyridine (0.05 ml). The solution was evaporated *in vacuo* and the residue crystallised from aqueous methanol to give the diacetate, colourless needles decomposing from 240°. (Found: C 59.6; H 4.0; CH_3CO 20.5. $C_{20}H_{16}O_9$ requires C 60.0; H 4.0; $(CH_3CO)_2$ 21.5.)

Dimethyl pannarate. A solution of pannaric acid (160 mg) in acetone containing 2 % of water (20 ml) was cooled to −10° and a slight excess of ethereal diazomethane added. After 2 min, the reaction was interrupted by the addition of acetic acid. The dimethyl ester crystallised from methanol as needles m. p. 254—255°, sparingly soluble in most solvents. (Found: C 62.7; H 4.6; OCH_3 18.0. $C_{18}H_{16}O_7$ requires C 62.8; H 4.65; $(OCH_3)_2$ 18.0.) The infra-red absorption curve showed carbonyl bands at 1 690 and 1 660 cm^{-1}.

The ester gave a light brown colour with *bis*-diazotised benzidine when tested on alkaline paper (Na_3PO_4) but no colour with Gibbs' reagent. A saturated solution of the ester in 96 % ethanol did not give any colour with ferric chloride. R_F-values (*n*-butanol-water): 0.78 (Na_3PO_4); 0.84 (Na_3PO_4 + Na_2HPO_4 1:1).

Dimethyl di-O-acetylpannarate. Dimethyl pannarate was warmed for a few minutes with acetic anhydride containing pyridine. The dimethyl di-O-acetylpannarate was crystallised from ethanol as needles, m. p. 162—164°. (Found: C 61.7; H 4.7; OCH_3 14.5; CH_3CO 21.1. $C_{22}H_{20}O_9$ requires C 61.7; H 4.7; $(OCH_3)_2$ 14.5; $(CH_3CO)_2$ 20.1.)

Dimethyl di-O-methylpannarate. Pannaric acid suspended in acetone containing methanol was treated for two days with an excess of ethereal diazomethane; the acid gradually dissolved. The reaction product crystallised from methanol to give dimethyl di-O-methylpannarate, needles, m. p. 166—168°. (Found: C 64.2; H 5.8; OCH_3 32.6; C—CH_3 (Kuhn-Roth) 7.55. $C_{20}H_{20}O_7$ requires C 64.5; H 5.4; $(OCH_3)_4$ 33.3; $(C-CH_3)_2$ 8.06.) The infra-red absorption curve showed carbonyl band at 1 725 cm^{-1}.

Di-O-methylpannaric acid. Dimethyl di-O-methylpannarate (2.25 g) was heated for 1 h on a boiling water bath with ethanol (25 ml) and 2 N aqueous sodium hydroxide (25 ml). Acidification with hydrochloric acid afforded a precipitate which was collected (1.9 g) and crystallised from ethanol to give di-O-methylpannaric acid, m. p. 240—246°

* It is not certain that these products are constituents of *Crocynea* since the material collected is always contaminated with small amounts of other lichens. It is, however, easy to prove by paper chromatography that the pure *Crocynea* contains pannaric acid.

(decomp.). R_F-values (*n*-butanol-water): 0.05 (Na_3PO_4); 0.10 (NaH_2PO_4, Na_2HPO_4 1:1); 0.75 (NaH_2PO_4). The spots were detected by their bluish-white fluorescence in ultraviolet light. (Found: C 62.3; H 4.8; OCH_3 16.8. $C_{18}H_{16}O_7$ requires C 62.8; H 4.65; $(OCH_3)_2$ 18.0.)

Pannarol (III). *a*) Pannaric acid (400 mg) was boiled for 12 h under reflux in an atmosphere of carbon dioxide with 88 % formic acid (10 ml). The dark solution was evaporated to dryness under reduced pressure and the residue dissolved in ether. The ether solution was extracted with aqueous sodium bicarbonate, dried and filtered through a small column of alkalifree alumina. Evaporation of the filtrate afforded pannarol (230 mg) which crystallised from aqueous ethanol as needles, m. p. 184—185°, undepressed on admixture with 3,9-dihydroxy-1,7-dimethyldibenzofuran, m. p. 184—185°, described below. The identity was confirmed by comparison of the infra-red absorption curves. (Found: C 74.0; H 5.6. $C_{14}H_{12}O_3$ requires C 73.7; H 5.3.) U.V. absorption (in 96 % ethanol): λ_{max} 233 mμ (log ε 4.74); 267 mμ (4.46); 287 mμ (4.40); 300 mμ (4.21); 312 mμ (4.39).

b) Pannaric acid (50 mg) placed in a small test tube was heated cautiously over a free flame under reduced pressure. It decomposed and sublimed. Loss of weight, 28.5 %. $C_{16}H_{12}O_7$ — 2 CO_2 requires a loss of weight of 27.8 %. The sublimate was identical with the pannarol obtained in the previous experiment.

Pannarol gave a red-brown colour with *bis*-diazotised benzidine, a blue colour with Gibbs' reagent, a red colour with bleaching powder but no colour with ferric chloride in ethanol. Paper chromatography (benzene-water): R_F 0.33. The spot was fairly elongated. A more convenient solvent system is described below (see 3,9-dihydroxy-1,7-dimethyldibenzofuran).

Di-O-methylpannarol. Pannarol (90 mg) in dry acetone (10 ml) was boiled under reflux with potassium carbonate (1.10 g) and dimethyl sulphate (0.38 g). After 6 h, excess dimethyl sulphate was destroyed by the addition of water and heating for 1 h. The solution was filtered and evaporated to dryness, the residue was dissolved in benzene and the solution was filtered through a small column of alumina. The filtrate was evaporated and the residue crystallised from aqueous methanol and finally distilled under reduced pressure to give di-O-methylpannarol, m. p. 94.5—95.5°, undepressed on admixture with the 3,9-dimethoxy-1,7-dimethyldibenzofuran, m. p. 94—95°, described below. The identity of the two compounds was confirmed by comparison of the I.R. absorption curves. (Found: C 74.7; H 6.0; OCH_3 24.2. $C_{16}H_{16}O_3$ requires C 75.0; H 6.3; $(OCH_3)_2$ 24.0.)

Di-O-acetylpannarol. Pannarol was acetylated at room temperature with acetic anhydride containing pyridine to give di-O-acetylpannarol which was crystallised from methanol, needles, m. p. 136.5—137.5°. (Found: C 68.6; H 5.1; CH_3CO 27.3. $C_{18}H_{16}O_5$ requires C 69.2; H 5.1; $(CH_3CO)_2$ 26.9). U.V. absorption (in 96 % ethanol) λ_{max} 226 mμ (log ε 4.38), 258 mμ (4.15), 285 mμ (4.28).

Permanganate oxidation of di-O-methylpannaric acid. Pannaric acid dimethyl ether (400 mg) in water (60 ml) containing potassium carbonate (700 mg) was heated on a boiling water bath and a solution of potassium permanganate (4 g) in water (100 ml) was added dropwise with stirring over 3 h. After heating for another 12 h, the excess permanganate was destroyed and the manganese sludge was filtered off and washed with water. The combined filtrates were concentrated to a volume of 40 ml and acidified with concentrated hydrochloric acid (2 ml). On standing in the refrigerator, the solution deposited a crystalline acid (A) which was collected, washed and dried at room temperature. Yield, 250 mg. It showed no definite melting point but readily formed a yellow anhydride. (Found: Equiv. wt. 94. $C_{18}H_{12}O_{11}$ requires equiv. wt. as a tetracarboxylic acid, 102.)

Dianhydride of the acid A. The acid (A) (80 mg) was dried *in vacuo* at 150° (loss of weight, 9.5 %. $C_{18}H_{12}O_{11}$ requires a loss of weight of 9.6 %) and the anhydride was purified by sublimation. M. p. 320—325°. (Found: C 58.5; H 2.1; OCH_3 16.1. $C_{18}H_8O_9$ requires C 58.7; H 2.2; $(OCH_3)_2$ 16.8.) The I.R. absorption curve showed carbonyl bands at 1 850 and 1 785 cm^{-1}.

Tetramethyl ester of the acid A. The acid (A) (80 mg) in methanol suspension was methylated with an excess of ethereal diazomethane. The ester crystallised from acetone as prisms, m. p. 225—226°, undepressed on admixture with 3,9-dimethoxy-1,2,6,7-tetracarbomethoxydibenzofuran [9], m. p. 225—226.5°. (Found: C 57.0; H 4.3; OCH_3 38.0.

$C_{22}H_{20}O_{11}$ requires C 57.4; H 4.3; $(OCH_3)_6$ 40.4.) The identity was confirmed by comparison of the I.R. absorption curves.

3,5-Dimethoxy-4-iodotoluene. Phenyllithium (20 mmole) in ether (10 ml) was added to a solution of orcinol dimethyl ether (10 mmole) in ether (10 ml) in an atmosphere of oxygen-free nitrogen. The orcinol-lithium compound started to crystallise after 24 h at room temperature. After another 5 days at 0° no further crystallisation was observed. The ethereal solution was decanted and the crystals were dissolved in dry tetrahydrofuran (10 ml). Iodine (8 mmole) was added and the solution was refluxed for 15 min. Excess iodine was destroyed with aqueous sodium thiosulphate and the solution was extracted with ether. Evaporation of the ether extract afforded a crystalline product which was crystallised from ethanol to give 3,5-dimethoxy-4-iodotoluene (1.4 g, 5 mmole), m. p. 96—97°, undepressed on admixture with an authentic specimen synthesised according to Posternak *et al.*[16]

1,9-Dihydroxy-3,7-dimethyldibenzofuran. 2,6,2′,6′-tetramethoxy-4,4′-dimethyldiphenyl was synthesised by Ullmann coupling of 3,5-dimethoxy-4-iodotoluene as described by Posternak *et al.*[16] The crude diphenyl (300 mg, m. p. 139—145°) was boiled with hydrobromic acid (10 ml; d, 1.54) for 6 h in an atmosphere of carbon dioxide. The reaction product was collected, washed with water, dried and dissolved in benzene. The solution was filtered through a short column of alumina, evaporated and the product was crystallised from dimethyl sulphoxide-water (1:4) giving 1,9-dihydroxy-3,7-dimethyldibenzofuran (100 mg), m. p. 190—192°. (Found: C 73.9; H 5.4. $C_{14}H_{12}O_3$ requires C 73.7 H 5.3.)

This phenol gave a blue colour with ferric chloride in ethanol and a blue colour with Gibbs' reagent.

3,9-Dihydroxy-1,7-dimethyldibenzofuran. Ullmann coupling of 3,5-dimethoxy-2-iodotoluene (7.5 g) and 3,5-dimethoxy-4-iodotoluene (0.8 g) with copper powder (25 g) followed by extraction of the reaction product with light petroleum afforded a crude mixture of diphenyls (3.2 g, 71 %) which was boiled for 16 h with hydrobromic acid (50 ml; *d*, 1.54) to effect demethylation and ring closure. The crude phenol mixture (2.2 g) was analysed by paper chromatography, using dimethyl sulphoxide as stationary phase [18] and either (A) diethyl ether or (B) toluene-petroleum ether b. p. 60—80° 3:1 as mobile phase. It was extracted with benzene overnight in a Soxhlet apparatus; the larger part of the main component (1.8 g), 3.7-dihydroxy-1,9-dimethyldibenzofuran, (R_F = (A) 0.68; (B) 0.18) crystallised in a fairly pure state (m. p. 239—242°; Shibata [7] gives m. p. 243°).

The mother liquor was evaporated to dryness and chromatographed on a column of cellulose powder (15 g) impregnated with 4 % dimethyl sulphoxide in ether, using 1 % dimethyl sulphoxide in ether as an eluant. The separation was followed by paper chromatography. The first fractions contained a small amount of 1,9-dihydroxy-3,7-dimethyldibenzofuran, described above (R_F = (A) 0.92; (B) 0.75.) The last fractions gave further amounts (150 mg) of 3,7-dihydroxy-1,9-dimethyldibenzofuran. The middle fractions gave 3,9-dihydroxy-1,7-dimethyldibenzofuran (250 mg) (R_F = (A) 0.79; (B) 0.21), characterised by a blue colour with Gibbs' reagent. It was crystallised from aqueous ethanol, needles, m. p. 184—185°. (Found: C 73.7; H 5.4. $C_{14}H_{12}O_3$ requires C 73.7; H 5.3.) U.V. absorption (96 % ethanol): λ_{max} 233 mμ (log ε 4.74); 267 mμ (4.46); 287 mμ (4.40); 300 mμ (4.21); 312 mμ (4.39).

3,9-Dimethoxy-1,7-dimethyldibenzofuran The phenol was methylated in boiling acetone with dimethyl sulphate and potassium carbonate giving the dimethyl ether, needles, m. p. 94.5—95.5°, from aqueous methanol. (Found: C 75.5; H 6.2. $C_{16}H_{16}O_3$ requires C 75.0; H 6.3.)

REFERENCES

1. Erdtman, H. *Biochem. Z.* **258** (1933) 172.
2. Asahina, Y. and Asano, J. *Ber.* **66** (1933) 689, 893, 1215.
3. Curd, F. H. and Robertson, A. *J. Chem. Soc.* **1937** 894; Schöpf, C. and Ross, F. *Naturwiss.* **47** (1938) 772; *Ann.* **546** (1941) 1; Barton, D. H. R., Deflorin, A. M. and Edwards, O. E. *J. Chem. Soc.* **1956** 530.
4. Erdtman, H. and Wachtmeister, C. A. *Chem. & Ind. (London)* **1957** 1042.
5. Wachtmeister, C. A. *Acta Chem. Scand.* **12** (1958) 147.

6. Shibata, S. *Acta Phytochim. (Japan)* **14** (1944) 177.
7. Shibata, S. *Acta Phytochim. (Japan)* **14** (1944) 9.
8. Erdtman, H. and Wachtmeister, C. A. *Nature* **172** (1953) 724; *Chem. & Ind. (London)* **1956** 960.
9. Wachtmeister, C. A. *Acta Chem. Scand.* **8** (1954) 1433; **10** (1956) 1404.
10. Barton, D. H. R. and Cohen, T. *Festschrift A. Stoll*, Birkhäuser, Basel 1957, p. 117.
11. Erdtman, H. and Wachtmeister, C. A. *Festschrift A. Stoll*, Birkhäuser, Basel 1957, p. 144.
12. Wachtmeister, C. A. *Svensk Kem. Tidskr.* **70** (1958) 117.
13. Wachtmeister, C. A. in Linskens: *Papierchromatographie in der Botanik* Springer, Berlin 1955, p. 99.
14. Hesse, O. *J. prakt. Chem.* **70** (1904) 1.
15. Gibbs, H. D. *J. Biol. Chem.* **72** (1927) 649.
16. Posternak, T., Ruelius, H. W. and Tcherniak, J. *Helv. Chem. Acta* **26** (1943) 2036.
17. Adams, R., Wolff, H., Cain, C. K. and Clark, J. H. *J. Am. Chem. Soc.* **62** (1940) 1770.
18. Wickberg, B. *Acta Chem. Scand.* **12** (1958) 615.
19. Kennedy, G., Breen, J., Keane, J. and Nolan, T. J. *Sci. Proc. Roy. Dublin Soc.* **21** (N.S.) (1937) 557.
20. Koch, J. E. and Krieg, W. *Chemiker-Ztg* **62** (1938) 140.

Received July 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1863–1868

Quantitative Paper Chromatography of Carotenoids

ARNE JENSEN and SYNNØVE LIAAEN JENSEN

Norwegian Institute of Seaweed Research, and Organic Chemistry Laboratories, Norges Tekniske Høgskole, Trondheim, Norway

A highly selective, rapid and simple method for the quantitative, circular chromatography of carotenoids on a commercially available paper with a kieselguhr filler is described. The R_F-values for a series of carotenoids are listed in Tables 1 and 3. The applications of the method are discussed.

The indisputable success of paper chromatography for the separation of complex mixtures of closely related compounds would lead one to expect that this method has been well developed also in carotenoid chemistry. One of the reasons for this not being the case may be the efficiency of column chromatography in the carotenoid field. The few published records of paper chromatography are concerned mainly with the separation of carotenoids from chlorophylls [1-3]. No simple and satisfactory system for separation of carotenoid mixtures on paper seems to have been developed.

Since kieselguhr columns have been used successfully in column chromatography of carotenoids [4], it was expected that paper with a kieselguhr filler might prove useful. The present work led to the development of a highly selective, rapid and simple method for the separation of carotenoid mixtures.

MATERIALS AND METHODS

Schleicher and Schüll filter paper No. 287 (Kieselguhrfilter, kieselguhr content about 20 %) was used throughout this work. The solvents were of analytical grade and used without further purification. The β-carotene, lutein and zeaxanthin were synthetic products obtained commercially. A sample of synthetic cryptoxanthin was kindly supplied by Hoffmann-La Roche & Co., a crystalline sample of chloroxanthin from a green mutant of *Rhodopseudomonas spheroides* by Dr. T. O. M. Nakayama and a crystalline specimen of rhodoxanthin from *Narthecium ossifragum* (L.) Huds, by Dr. A. Stabursvik. Crystalline samples of astaxanthin from lobster shells, lycopene from tomatoes, fucoxanthin from brown seaweeds and violaxanthin from *Viola tricolor* were provided by the Organic Chemistry Laboratories of this University. Crystalline bacterioruberine *a* was isolated from a *Halobacterium* sp. (Strain 1c, from the Department of Biochemistry, this University), whilst spirilloxanthin, P481, OH-P481 and rhodopin were isolated in the crystalline state from *Rhodospirillum rubrum* (Strain S1) and *Rhodopseudomonas palustris* (from dried cells kindly supplied by Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, Calif.);

Table 1. R_F-values for a series of carotenoids

Carotenoid	Member of the set	Structure	Conjugated double bonds	Isolated double bonds
β-Carotene	all-*trans*	bicyclic	11	0
Cryptoxanthin	all-*trans*	»	11	0
Zeaxanthin	all-*trans*	»	11	0
Lutein	all-*trans*	»	10	1
Violaxanthin		»	9	0
Astaxanthin	all-*trans*	»	11	0
Rhodoxanthin	all-*trans*	»	12	0
Fucoxanthin	all-*trans*			
γ-Carotene	all-*trans*	monocyclic	11	1
Lycopene	all-*trans*	aliphatic	11	2
P481 [6]	all-*trans*	»	12 [7]	1 [7]
	Neo A [8]	»	12	1
Spirilloxanthin [9]	all-*trans*	»	13	0
	Neo a [8]	»	13	0
	Neo b [8]	»	13	0
Chloroxanthin [10]	all-*trans*	»	9	2
Rhodopin (OH-Lycopene) [11]	all-*trans*	»	11	1
OH-P481 [6]	all-*trans*	»	12 [12]	0 [8]
	Neo A [8]	»	12	0
	Neo B [8]	»	12	0
Bacterioruberine α [14]	Neo U [8]	»	13	0
	all-*trans*	»	13	0
	Neo A [8]	»	13	0
	Neo B [8]	»	13	0

* acetone in pet. eth. b.p. 60—70°C.

γ-carotene was isolated from *Chlorobium thiosulfatophilum* (Strain L1, obtained from Dr. J. Lascelles, Department of Microbiology, University of Oxford).

The chromatographic separations were carried out on circular papers at room temperature in a Petri dish or a desiccator using the technique of Rutter [5]. The solvent system consisted of petroleum ether (b.p. 60—70°C) alone or mixed with various amounts of acetone. For the separation of chloroplast pigments of brown algae petroleum ether with 0.5—2 % *iso*propanol was found favourable. The carotenoid mixtures were dissolved either in acetone or in acetone-petroleum ether mixtures. An aliquot containing 10—100 μg of carotenoids was applied in portions to the centre of the paper by means of a capillary such that the diameter of the spot did not exceed 1 cm. After the application of each portion the solvent was evaporated at room temperature in a stream of pure nitrogen. When the whole aliquot had been put on, a small amount of acetone was applied to the paper on the wick just outside the edge of the spot. This caused the carotenoids to form a narrow ring shaped band surrounding the centre of the paper and was found to result in more regular zones. The separation took 15 min with a 12.5 cm and 20 min with an 18 cm diameter paper. For determination of R_F-values the papers were dried in a stream of warm air, the zones marked with a pencil and the position of the solvent front was determined in UV light (bluish fluorescence). Otherwise the chromatograms were not dried before elution of the pigments in order to avoid oxidation of the carotenoids. The coloured zones containing the carotenoids were cut out immediately after separation, tightly packed in small glass tubes with one end drawn out to a capillary and eluted with acetone, whereafter the extracts were made up to a known volume with acetone and the absorption spectrum determined in a Zeiss PMQ2 spectrophotometer. When separating compounds with low R_F-values the solvent was allowed to evaporate from the rim of the paper which extended outside the petri dish.

on Schleicher & Schüll No. 287 paper.

Functional groups	Character of OH-groups	R_F-value				
		0 %	2 %	5 %	10 %	20 % *
		0.95	0.98	0.98	0.98	
1 OH	secondary	0.29	0.62	0.81	0.91	
2 OH	secondary		0.09	0.30	0.59	0.87
2 OH	secondary			0.39	0.72	0.91
2 OH, 2–O–	secondary			0.18	0.44	0.83
2 OH, 2 C=O	secondary				0.57	0.85
2 C=O				0.10	0.27	0.72
>(2 OH, 2 C=O)					0.40	0.81
–		0.68				
–		0.53	0.86			
1 OCH_3[7]			0.35	0.60		
1 »			0.50	0.75		
2 OCH_3			0.18	0.40	0.76	
2 »			0.31	0.54		
2 »			0.48	0.73		
1 (?) OH	tertiary	0.13	0.46	0.73	0.90	
1 OH	tertiary			0.39	0.75	
1 OH[6], 1 OCH_3[13]	tertiary[8]				0.54	
1 » 1 »	»				0.78	
1 » 1 »	»				0.94	
2 (?) OH	tertiary[8]				0	0.36
»	»				0.02	0.44
»	»				0.06	0.51
»	»				0.10	0.57

APPLICATIONS

In the present work the method has been applied mainly to the separation of carotenoids from brown seaweeds and bacteria. The R_F-values obtained for a series of carotenoids are presented in Table 1.

1. Grouping and separation of carotenoids. Quantitative separation of bicyclic carotenes, mono-hydroxy-xanthophylls and dihydroxy-xanthophylls was accomplished as exemplified by the separation of the series β-carotene, cryptoxanthin and zeaxanthin. These groups could be further separated into their individual components in the cases investigated.

Within the group of aliphatic, bacterial carotenoids studied a similarly satisfactory separation of the different all-*trans* compounds was obtained.

2. Separation of stereoisomeric sets. Separation of *cis-trans* isomers belonging to the same set could easily be obtained as seen from Table 1.

3. Recoveries and quantitative determinations. Recoveries obtained by this method were determined by applying known amounts of pure carotenoids to the paper. After the chromatographic development the amount of carotenoid eluted was measured in the spectrophotometer. The results are presented in Table 2.

Table 2. Pigment recovery after chromatography.

Compound	Amount in μg	
	Applied	Recovered
β-Carotene	14.3	14.3
	7.2	7.1
Zeaxanthin	3.9	3.8
	7.8	7.8
	7.8	7.6

The recoveries obtained both with β-carotene and with zeaxanthin were between 97 and 100 %.

The carotene content of dried grass and of seaweed meal was determined by means of paper chromatography. Details of the procedure are to be published[15]. Using the paper chromatographic method, the values obtained for the carotene content were somewhat higher than those obtained with the conventional procedure. This may be a result of the higher over-all recoveries of the new method.

4. Separation of chloroplast pigments. Minor variations in the composition of the solvent system allowed a ready separation of the chloroplast pigments from various sources as shown in Table 3.

DISCUSSION

The efficiency of the Schleicher and Schüll filter paper No. 287 for the separation of carotenoids must be ascribed to its content of kieselguhr, since ordinary filter paper gave very poor separations. Used in circular chromatography the kieselguhr paper allowed a ready separation of carotenoid mixtures into carotenes, mono-hydroxy- and dihydroxy-xanthophylls. These groups could be separated further into their components provided they were more strongly adsorbed than β-carotene.

Table 3. Separation of chloroplast pigments.

Extract of		R_F-values	
		1.5 % *iso*propanol *	10 % acetone *
Ascophyllum nodosum	β-carotene	0.96	
	lutein	0.80	
	violaxanthin	0.36	
	fucoxanthin	0.33	
	chlorophyll a	0.55	
Dried grass	carotene		0.98
	lutein		0.73
	zeaxanthin		0.60
	chlorophyll a		0.41
	chlorophyll b		0.21

* in petroleum ether b.p. 60–70°C.

Probably the best demonstration of the efficiency of the method was the ready separation of *cis-trans* isomers of carotenoids belonging to the same stereoisomeric set. The method allowed quantitative studies of different *cis-trans* isomerization processes to be carried out much more easily, with greater accuracy and with much smaller amounts of pigments than are required for column chromatography. A mixture containing approximately 50 μg of carotenoid could be separated quantitatively into the main stereoisomers which were eluted and examined in the spectrophotometer. The rapidity of the method is particularly valuable for studies of carotenoids which isomerize readily or are otherwise very labile. Thus, it was possible to carry out a stereochemical investigation of bacterioruberine α[8] which was sterically too labile to allow exact *cis-trans* isomerization studies by means of column chromatography.

With slightly modified solvent systems the method gave satisfactory separation of the chloroplast pigments of both algae and grass extracts, and allowed quantitative determination of the major components.

When care was taken, the R_F-values of the compounds were reproducible within ± 0.01 units. The solvent mixtures should be used only once when determining R_F-values; the reason for this is probably that the paper removes relatively more of the acetone than of the petroleum ether. Evaporation of the solvent from the Petri dish during the chromatographic run must be avoided. Large amounts of fatty material in the extracts may influence the R_F-values of the pigments considerably. In such cases saponification or some other purification process should precede the chromatographic separation in order to obtain the characteristic R_F-values.

It may be seen from Table 1 by comparing the R_F-values of the bacterial carotenoids with those of carotenoids from other sources that the R_F-values were not influenced merely by the number of functional groups and the number of double bonds. The nature of the skeleton (aliphatic or cyclic) and the character of the hydroxyl groups (secondary or tertiary) obviously have a definite effect on the R_F-value. For example OH-P481 is more strongly adsorbed to the paper than zeaxanthin and lutein; the reverse is true for adsorption on alumina columns. Bacterioruberine α chromatographs very similarly to lutein on alumina, but is held more strongly than lutein on paper. Also for some of the other pigments investigated the order of adsorbtion on paper differed from that obtained with other adsorbents. The method thus offers an additional tool for identification of chloroplast pigments.

The recovery of pigments extracted from the paper was found to be quantitative, and the eluted pigments showed absorption spectra in visible light comparable with the best recorded in the literature. Quantitative determination of the main components of several complex carotenoid mixtures were successfully performed by means of the above method.

Paper chromatography is particularly useful for purity tests of small samples, for following chemical and biochemical transformations and for investigations in which only very small amounts of material are available. The fact that only small amounts of material are required, and that the method gives a high resolving power, makes it a very good test for identification of carotenoids when combined with co-chromatography of known substances.

Acknowledgements. We are indebted to Mr. Ian Dundas of the Department of Biochemistry for the cultivation of *Chlorobium thiosulfatophilum* and the *Halobacterium* sp., and to Professor N. A. Sørensen, Organic Chemistry Laboratories, for critically reading the manuscript. One of us (SLJ) gratefully acknowledges a maintenance grant from *Norges Tekniske Høgskole.*

REFERENCES

1. Bauer, L. *Naturwiss.* **39** (1952) 88.
2. Strain, H. H. *J. Phys. Chem.* **57** (1953) 638.
3. Angkapindu, A., Silberman, H., Tantivatana, P. and Kaplan, I. R. *Arch. Biochem. Biophys.* **75** (1958) 56.
4. Wilkes, J. B. *Ind. Eng. Chem. Anal. Ed.* **18** (1946) 702.
5. Rutter, L. *Nature* **161** (1948) 435.
6. Goodwin, T. W. and Osman, G. *Biochem. J.* **56** (1954) 222.
7. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 381.
8. Liaaen Jensen, S. *To be published.*
9. Polgár, A., van Niel, C. B. and Zechmeister, L. *Arch. Biochem. Biophys.* **5** (1946) 243.
10. Nakayama, T. O. M. *Arch. Biochem. Biophys.* **75** (1958) 352.
11. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 842.
12. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochem. et Biophys. Acta* **29** (1958) 477.
13. Liaaen Jensen, S. *Acta Chem. Scand.* **12** (1958) 1698.
14. Petter, H. F. M. *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Ardeel. Natuurk.* **34** (1931) 1417.
15. Jensen, A. *Acta Chem. Scand.* **13** (1959) 1259.

Received July 20, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1869–1876

Studies on Arabogalactans

III.* Degradation Products of Arabogalactan A from *Larix occidentalis* Nutt. and an Electrophoretic Examination of Arabogalactans from other *Larix* Species

HANS O. BOUVENG

Institutionen för Träkemi, Kungl. Tekniska Högskolan, Stockholm, Sweden

The arabogalactan A obtained from *Larix occidentalis*, on mild hydrolysis to remove the arabinose residues, gave two different galactan fractions which were easily separated by fractional precipitation of their cetyl-trimethylammonium borate complexes. On methylation and hydrolysis of the methylated derivatives, both galactans afforded 2-*O*-methyl-D-galactose, 4-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose though in significantly different proportions. Some arabogalactans from other *Larix* species were examined by electrophoresis and by fractionation and were found in all cases to be of the same complex nature as the *L. occidentalis* polysaccharide.

INVESTIGATION OF DEGRADED ARABOGALACTAN A

In part II of this series [1] an account was given of the fractionation of the arabogalactan fraction from Western larch (*Larix occidentalis* Nutt.) into two fractions (A and B) and of a methylation study of the component A with the higher degree of polymerisation and the higher electrophoretic mobility. Neither sedimentation analysis nor electrophoresis in borate buffer gave any indication of heterogeneity in the two polysaccharides. However, it was later observed that polysaccharide A after brief, mild hydrolysis gave two distinct components on electrophoresis, one with slightly higher electrophoretic mobility than arabogalactan A and one with about the same mobility as arabogalactan B.

In contrast to the behaviour of the material studied by Aspinall *et al.*[2], the arabinose could be removed by prolonged mild hydrolysis without unduly

* Part III and IV of this series were presented at the 136th Meeting of the American Chemical Society in Atlantic City, N. J., September 1959 (abstract 14E).

extensive break-down of the remaining polysaccharide. After removal of the low molecular weight oligosaccharides, a fractional precipitation with cetyltrimethylammonium hydroxide (CTA-OH) — boric acid gave two electrophoretically pure galactans, A'I, $[\alpha]_D^{20}$ +13°, M_G 0.84 and A'II, $[\alpha]_D^{20}$ +19°, M_G 0.66. The arabogalactans A and B had M_G values of 0.76 and 0.63 and rotations of +7° and +10°, respectively. An osmometric determination gave a $\overline{DP}_n$ value of 48 for A'II. An approximate calculation of the degree of polymerisation of A'I from the proportion of terminal groups appearing as tetramethylgalactose on hydrolysis of the methylated polysaccharide gave a value of 20. Arabogalactan B was degraded in the same manner to yield a single component (B') with the same electrophoretic mobility as the original polysaccharide and with a rotation of +21°.

The two galactans were methylated following the procedure described in part II[1] with the exception that A'I after the preliminary methylation with methyl sulphate and sodium hydroxide was methylated once with methyl iodide and barium oxide in dimethyl formamide solution as proposed by Kuhn *et al.*[3] for the methylation of low molecular weight carbohydrates. The barium oxide seemed to give slightly lower methoxyl content than silver oxide but may be useful since the oxidising silver oxide depolymerises more incompletely methylated polysaccharides [4].

The methylated polysaccharides were hydrolysed in a mixture of 30 % glacial acetic acid and 70 % 0.5 N hydrochloric acid and each mixture of methyl ethers obtained was resolved on a carbon-Celite column. Further separation, if required, was done on thick filter paper. The amount of each ether was estimated by hypoiodite oxidation [5]. The results are summarised in Table 1.

Table 1. Methyl ethers (in mole %) obtained from the methylated hydrolysed galactans A'I and A'II from degraded larch arabogalactan A.

Sugar	% (A'I)	% (A'II)
4-Methylgalactose	0.6	0.3
2-Methylgalactose	2.5	3.4
2,4-Dimethylgalactose	27.9	20.5
2,6-Dimethylgalactose	2.1	2.7
2,3,4-Trimethylgalactose	13.8	21.8
2,4,6-Trimethylgalactose	13.6	19.6
2,3,4,6-Tetramethylgalactose	39.6	31.7

These results show that A'I and A'II contain the same types of linkages but in different proportions. A'II is a more moderately branched polysaccharide, while A'I has the same heavily branched structure as the original polysaccharide. The 2-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose must be assigned at least some structural significance which implies the presence of a small fraction of 1,4-linkages partly combined with doubly branched units, but the 4-*O*-methyl-D-galactose is probably a result either of incomplete methylation or of demethylation during hydrolysis. The amount of 2,4,6-tri-

O-methyl-D-galactose is strikingly larger than that obtained from arabogalactans A and B. This indicates that the easily hydrolysed units of the original polysaccharide (L-arabinose, 3-*O*-β-L-arabopyranosyl-L-arabinose, D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose) are attached to the rest of the molecule mainly through 1,6-linkages as has been suggested by White [6] in the case of the arabinose.

These results indicate a possibility that the arabogalactan A could be heterogeneous. A separation of the material into the hypothetical components corresponding to the galactans A'I and A'II * by fractional precipitation with CTA-OH—boric acid did not appear feasible since no separation could be obtained on electrophoresis in borate buffer or by trial precipitations. On hydrolysis of arabogalactan A in 0.01 N acid at 100°C for 2 h only, it was found possible to isolate a small quantity of electrophoretically pure A II from the main fraction of the A I type (M_G *ca.* 0.85) by fractionation in the usual way. This fraction probaly contained still unchanged arabogalactan A beside A I. Chromatograms of hydrolysates indicated that these two fractions contained about the same proportions of arabinose. Electrophoretic examination of the material obtained from A I on hydrolysis for a further 2 h showed that more A II had been formed. The A I fraction after precipitation as the CTA-borate complex and isolation in the usual manner was subjected to hydrolysis under the same conditions for another 4 h. Further amounts of A II were again found.

It is not yet possible to decide whether A I and A II are fragments of a single molecular species or of two. It is however known that part of the galactopyranosidic linkages in the polysaccharide must be of a weaker type, possibly due to non-bonded steric interaction in the heavily branched structure (*cf.* the preceding paper in this series). If arabogalactan A is homogeneous a possible explanation of the appearance of A I and A II is that they correspond to two molecular fragments of different degrees of branching which are joined by linkages of this type. The observation, that the rates of formation of A I and A II and of galactose and 6-β-galactopyranosyl-galactose under these hydrolytic conditions are of the same order of magnitude, is in agreement with this view. A further indication of the homogeneity of arabogalactan A is the fact that on electrophoresis of the hydrolysed polysaccharides there is little trailing between the two spots corresponding to A I and A II. It therefore seems more probable that A I and A II are released as such during the hydrolysis from a homogeneous polysaccharide and not from separate macromolecules; in the latter case the M_G values should change gradually from the original value (0.76) to the final values (0.84 and 0.66 for A'I and A'II, respectively) with consequent trailing. The small difference between the M_G values for B and B' shows that the removal by hydrolysis of the simple oligosaccharide residues

* The designation A I will be used below to refer to any polysaccharide material obtained by mild hydrolysis of arabogalactan A that has an electrophoretic mobility of the same order as the original arabogalactan A (*ca.* 0.80). A II will be used to refer to any material obtained from arabogalactan A in the same way that has the same electrophoretic mobility as arabogalactan B (*ca.* 0.65). A'I and A'II will be used exclusively for the arabinosefree galactans (M_G values 0.84 and 0.66, resp.) obtained by prolonged mild hydrolysis of arabogalactan A.

containing arabinose and galactose [7] appearently has very little effect on the electrophoretic mobility of this type of polysaccharide.

Examination of the arabogalactans from a number of larches (*cf.* the preceding section of this paper) showed that the proportions of A I and A II as estimated from electrophoretograms were fairly constant but that the proportion of A to B varied considerably.

INVESTIGATION OF THE ARABOGALACTANS FROM OTHER *LARIX* SPECIES

In view of the complex nature of the arabogalactans from *Larix occidentalis* it seemed desirable to make a brief examination of the arabogalactans of other *Larix* species to see if they were of the same type. The following species were investigated (the yields of arabogalactan calculated on acetone-extracted, airdried heart wood are given in brackets; the results obtained from *L. occidentalis* are included for comparison):

Order *Multiseriales* Patschke [8]:
L. Potaninii Batal. (7.2 %)
L. occidentalis Nutt. (9.3 %)
L. Lyallii Parl. (7.7 %).
Order *Pauciseriales* Patschke:
L. leptolepis (Sieb. & Zucc.) Gord. (8.0 %)
L. laricina (Du Roi) K. Koch (3.9 %)
L. Sukaczevii Djil. (5.3 %)
L. decidua Mill. (3.4 %)
L. Gmelinii (Rupr.) Kuzeneva var. *japonica* (Reg.) Pilger (15.7 %).

EXPERIMENTAL

All melting points are corrected. Evaporations were done under reduced pressure.

Chromatography: Papers: Whatman 1 and Schleicher and Schüll 602 hP. Solvent: Butanol, ethanol, water, 10:3:5. Spray reagent: anisidine hydrochloride.

Electrophoresis. Papers: Whatman 1 and Schleicher and Schüll glass fibre paper. Buffer: 0.1 M borate buffer of pH 10. Spray reagents: anisidine hydrochloride and α-naphtol-sulphuric acid in butanol. In the electrophoresis it was not possible to determine the temperature of the papers accurately and the M_G values are therefore only relative. The variation in different measurements was about ± 0.03 units.

Hydrolysis of the arabogalactan. Arabogalactan A was hydrolysed in 0.01 N hydrochloric acid at 100°C for 18 h. After cooling the solution was neutralised with silver carbonate, filtered, concentrated to a small volume and poured into ethanol. The product was filtered off giving a white powder. This was freed from low molecular weight oligosaccharides by reprecipitation twice with ethanol. A chromatogram of a hydrolysate showed only faint traces of arabinose. On electrophoresis the hydrolysate gave two distinct spots, the faster of which was designated as A′I and the slower as A′II. Hydrolysis of arabogalactan B and isolation of the product in the same way gave the degraded polysaccharide (B′). On electrophoresis this gave only a single spot. The characteristics of these polysaccharides are shown in Table 2.

Table 2. Arabogalactans and corresponding galactans from *Larix occidentalis.*

Polysaccharide	$[\alpha]_D^{20}$	% arabinose (molar)	% galactose (molar)	M_G
A	+ 7°	19	81	0.76
B	+10°	21	79	0.63
A'I	+13°	trace	100	0.84
A'II	+19°	—	100	0.66
B'	+21°	trace	100	0.63

Separation of A'I and A'II. The results of a typical experiment are summarised in Table 3 which shows the fractionation of the galactan mixture (7.51 g = 46 mmole anhydrogalactose) dissolved in water (300 ml) and 0.6 M boric acid solution (50 ml).

Table 3. Fractionation of hydrolysed arabogalactan A (7.51 g).

CTH-OH added (mmoles)	NaOH added (mmoles)	Weight of fraction (g)	Components
2.0	—	2.50	A'I
2.0	1.5	2.08	A'I
4.0	3.0	1.83	A'II
Residue		0.42	A'II

A'I and A'II were obtained in the approximate ratio 2:1. A'I contained traces of arabinose.

Methylation of the galactans A'I and A'II. The galactans were methylated first with methyl sulphate and sodium hydroxide. The product from methylation of A'I was then subjected to a methylation with methyl iodide and barium oxide in dimethyl formamide as described by Kuhn *et al.*[3]. After a reaction time of 20 h the unreacted barium oxide was removed by filtration and the dimethyl formamide was evaporated. The product was freed from barium iodide by treatment with freshly precipitated silver acetate in alcoholic solution. This treatment raised the methoxyl content from 25.3 to 38.7 %. The partially methylated galactans were exhaustively methylated with methyl iodide and silver oxide in dimethyl formamide following the procedure described in part II [1]. No trace of unmethylated monomer was found on chromatograms of hydrolysates of the methylated derivatives.

Hydrolysis of the methylated galactans and fractionation of the hydrolysates. The two galactans were each hydrolysed at a concentration of about 1.5 % in a mixture of glacial acetic acid and 0.5 N hydrochloric acid (1:2) at 100°C for 18 h. The reaction mixture was treated with excess silver acetate, concentrated to a small volume, taken up in ethanol and filtered. The mixture of ethers obtained on evaporation of the ethanol was dissolved in water and adsorbed on a carbon-Celite column 3.5 × 46 cm.

A'I (0.47 g) was eluted with 4 l 6 → 25 % ethanol + 2 l 25 → 40 % ethanol + 1 l 50 % acetone, and A'II (0.65 g) with 5 l 6 → 35 % ethanol + 1 l 50 % acetone. Both were collected in 25 ml fractions. The amount of reducing sugar in each fraction was determined by hypoiodite oxidation [5] and the mixed fractions were further resolved on Schleicher and Schüll 602 hP filter papers for quantitative estimation as described in part II [1]. The results of the fractionations are summarised in Table 4.

*Characterisation of the methyl ethers. 2-O-Methyl-*D*-galactose, 4-O-methyl-*D*-galactose* and *2,6-di-O-methyl-*D*-galactose* were indistinguishable from authentic material by chromatography or electrophoresis.

Table 4. Fractionation of the methylated hydrolysed galactans A'I and A'II.

Fract. No.	mmoles(total)	Sugars	% of fraction	mmoles
Galactan A'I				
24—38	0.065	4-Methylgalactose	18	0.012
		2-Methylgalactose	82	0.053
52—100	0.558	2,4-Dimethylgalactose		
101—135	0.065	2,4-Dimethylgalactose	34	0.022
		2,6-Dimethylgalactose	66	0.043
136—174 *	0.287	2,3,4-Trimethylgalactose		
175—220	0.192	2,4,6-Trimethylgalactose		
Residue	0.915	2,4,6-Trimethylgalactose	10	0.092
		2,3,4,6-Tetramethylgalactose	90	0.823
Galactan A'II				
24— 30	0.098	4-Methylgalactose	9	0.009
		2-Methylgalactose	91	0.089
48— 76	0.518	2,4-Dimethylgalactose		
77— 84	0.091	2,4-Dimethylgalactose	23	0.021
		2,6-Dimethylgalactose	77	0.070
85—119	0.576	2,3,4-Trimethylgalactose		
120—164	0.391	2,4,6-Trimethylgalactose		
Residue	0.960	2,4,6-Trimethylgalactose	13	0.124
		2,3,4,6-Tetramethylgalactose	87	0.836

* Contained traces of arabinose methyl ethers.

2,4-Di-O-methyl-D-galactose was crystallised from acetone, m. p. 101°, sintering at 85°, aniline derivative m. p. 213—215° and mixed m. p. 212—215°.

2,3,4-Tri-O-methyl-D-galactose. Aniline derivative, m. p. and mixed m. p. 169—170°.

2,4,6-Tri-O-methyl-D-galactose was obtained in a crystalline state from galactan A'II, m. p. 96—98° after recrystallisation from ethanol-ethyl acetate. Aniline derivative, m. p. 176—178°, in both cases undepressed on admixture with authentic samples.

2,3,4,6-Tetra-O-methyl-D-galactose. Aniline derivative in both cases, m. p. 199—201° and mixed m. p. 198—200°.

Estimation of the degree of polymerisation of A'I and A'II. For *A'II* the estimation was made osmometrically using the same type of osmometer as Lindberg and Meier [9]. The measurements were made in aqueous solution with "Ultrafilter allerfeinst" membranes from Membrangesellschaft Göttingen, Germany. This gave a $\overline{DP_n}$ value of 48 but this is of course subject to inaccuracies due to the use of water as solvent. The methylation data did not permit any reliable estimate of the DP value.

A'I. This galactan leaked too much through the membrane to allow osmometric measurement. Calculation from the excess of endgroups found from the methylation data gave a DP value of 20. Although precautions were taken, small losses due to the volatility [10] of the tetramethylgalactose corresponding to these endgroups were probably unavoidable. Allowing for losses of the order of 5 % (*cf.* Part II of this series) would give a DP value of 17. These DP values of course depend on the hydrolysis time used in the preparation of the galactans. It appears however that the ratio of the DP values for A'I and A'II is about 1:3.

Isolation and electrophoresis of the arabogalactans from other larchwoods. The polysaccharides were isolated mainly as described in Part I [7]. Extraction 3—4 times with water at room temperature afforded about 95 % of the arabogalactan accessible to cold water extraction.

Electrophoresis on glass fibre sheets in borate buffer and fractionation experiments showed that the arabogalactans from the closely related *L. occidentalis* and *L. Lyallii* by containing a larger proportion of the arabogalactan B (25—30 %) differed distinctly from all the other species investigated, which contained only 5—10 % of arabogalactan B. Due either to lack of material or to the small amounts present, arabogalactan B was only isolated in a pure state from *L. Lyallii* and in about 90 % purity from *L. Gmelinii*.

Investigation of the A fractions. The A fractions were isolated as described in Part II [1] of this series. They were analysed for arabinose and galactose according to Saeman *et al.*[11] The results of these analyses and of the analyses of the B fractions isolated are summarised in Table 5.

Table 5. The composition of the arabogalactans from some *Larix* species.

Species	Fraction	Galactose (weight %)	Arabinose (weight %)	Moles galactose per mole arabinose
L Potaninii	A	89.7	10.3	7.3
L occidentalis	A	83.9	16.1	4.3
»	B	81.6	18.4	3.7
L. Lyallii	A	85.5	14.5	4.9
»	B	75.7	24.3	2.6
L. leptolepis	A	90.3	9.7	7.8
L. laricina	A	86.7	13.3	5.4
L. Sukaczevii	A	86.7	13.3	5.4
L. decidua	A	87.2	12.8	5.7
L. Gmelinii var. *japonica*	A	92.2	7.8	9.8
»	B	83.7	16.3	4.3

From these results it is evident that the arabinose content of larch arabogalactans from different sources is subject to large variations. The higher arabinose content of the B fractions may be partly due to a contaminating araban (*cf.* the preceeding paper of this series). The detection of traces of a slow-moving component on an electrophoretogram heavily spotted with the B fraction of *L. Lyallii* is in agreement with this view.

The M_G values of the A fractions lay within the range 0.81—0.86 except for the fraction from *L. occidentalis* which had $M_G = 0.76 \pm 0.03$. The low M_G value of this polysaccharide can be attributed to its comparatively high arabinose content, the main part of which is present as terminal arabofuranosidic residues incapable of forming borate complexes [12]. The B fractions had M_G values of 0.63 ± 0.03.

Hydrolytic degradation of the A fractions. Samples (1.5 g) of the A fractions were subjected to hydrolysis in 0.005 N hydrochloric acid at 100°C for 18 h, and the polysaccharide material in the reaction mixture was precipitated with ethanol. Estimations from electrophoretograms indicated that all samples gave both A I and A II in proportions varying between 2:1 and 3:1. These variations are probably not significant since a slight fractionation of the galactans may occur during isolation.

The author is indebted to Dr. B. Lindberg for his keen interest in this work and also to Dr. R. H. Farmer, Princes Risborough, Dr. T. E. Timell, Montreal, Dr. E. v. Rudloff, Saskatoon and Dr. E. Rennerfelt, Stockholm, for gifts of wood, to the late Dr. E. Söderberg for valuable botanical advice, to Miss Anita Myhrman for her skilful assistance and to *Fonden för Skoglig Forskning* for financial support.

REFERENCES

1 Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **12** (1958) 1977.
2. Aspinall, G. O., Hirst, E. L. and Ramstad, E. *J. Chem. Soc.* **1958** 593.
3. Kuhn, R., Baer, H. H. and Seeliger, A. M. *Ann.* **611** (1958) 236.
4. Croon, I. *Private communication.*
5. Hirst, E. L., Hough, L. and Jones, J. K. N. *J. Chem. Soc.* **1949** 57.
6. White, E. V. *J. Am. Chem. Soc.* **64** (1942) 1507.
7. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **10** (1956) 1515.
8. Ostenfeld, C. H. and Larsen, S. *Biol. Medd. (Copenhagen)* **9** (1930) 2.
9. Lindberg, B. and Meier, H. *Svensk Papperstidn.* **60** (1957) 785.
10. Gardiner, J. G. and Percival, E. *J. Chem. Soc.* **1958** 1414.
11. Saeman, J. F., Moore, W. E., Mitchell, R. L. and Millet, M. A. *TAPPI* **37** (1954) 336.
12. Foster, A. B. *J. Chem. Soc.* **1957** 1395.

Received June 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1877–1883

Studies on Arabogalactans

IV. A Methylation Study of Arabogalactan B from *Larix occidentalis* Nutt.

HANS O. BOUVENG

Institutionen för Träkemi, Kungl. Tekniska Högskolan, Stockholm, Sweden

Arabogalactan B, the lower molecular weight arabogalactan from Western larch *(Larix occidentalis)*, was methylated and hydrolysed, giving 2-*O*-methyl-D-galactose, 4-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, an unknown methyl-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,5-di-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-L-arabinose and 2,3,5-tri-*O*-methyl-L-arabinose.

The arabogalactans found in a number of species of *Larix* have been shown to consist of two main fractions (A and B) [1,2]. B, which has a lower degree of polymerisation, usually forms about 5—10 % of the total arabogalactan fraction but in *L. occidentalis* Nutt. and *L. Lyalli* Parl. it is about 30 % of the arabogalactan. The isolation of arabogalactan B on a preparative scale from the arabogalactan of *L. occidentalis* was described in part II of this series [1]. The polysaccharide was obtained as a white powder, $[\alpha]_D^{20}$ +10°, containing 21 % (molar) arabinose. It appeared from the results of paper electrophoresis and sedimentation analysis to be homogeneous.

Arabogalactan B was swollen in dimethyl formamide and then methylated with methyl iodide and silver oxide [3] to yield a methylated polysaccharide containing 35.6 % methoxyl. A second methylation raised the methoxyl content to 42.6 % but four more methylations in the same way raised the methoxyl content only to 43.0 %, the theoretical value being 44.6 %. The formation of a precipitate of silver halogenide on hydrolysis of the methylated polysaccharide indicated that it was contaminated with complex silver salts formed during the methylation [4]. Careful examination of a hydrolysate of the methylated polysaccharide showed that unmethylated monomer was absent, which is a good criterion of exhaustive methylation.

The methylated polysaccharide was hydrolysed and the mixture of methyl ethers obtained was resolved on a carbon-Celite column. The amount of reduc-

ing sugar in each fraction was determined by hypoiodite oxidation and the mixed fractions were further separated on filter papers both for preparative purposes and for quantitative estimation of the components present. The results are summarised in Table 1.

Table 1. Methylated sugars obtained from 2.0 g of methylated arabogalactan B.

Methyl ether	mmoles	molar %	M_G value
Unmethylated monomers	—	<0.1	
4-Methylgalactose	0.03	0.3	0.30
2-Methylgalactose	0.20	2.3	0.43
2-Methylarabinose	0.05	0.6	0.34
2,4-Dimethylgalactose	2.65	31.0	<0.05
2,6-Dimethylgalactose	0.33	3.9	0.38
2,3-Dimethylarabinose	0.04	0.5	<0.05
Unknown methylgalactose	0.06	0.7	0.30
2,3,4-Trimethylgalactose	1.01	11.8	
2,3,4-Trimethylarabinose	0.44	5.1	
2,5-Dimethylarabinose	0.69	8.1	
2,4,6-Trimethylgalactose	0.34	4.0	
2,3,4,6-Tetramethylgalactose	2.06	24.1	
2,3,5-Trimethylarabinose	0.65	7.6	

The appearance of 2-*O*-methyl-L-arabinose and 2,3-di-*O*-methyl-L-arabinose in approximately equimolecular quantities can be explained as due to the presence of about 1.7 % of an araban as an impurity in the original polysaccharide [5]. As arabofuranosidic residues should not form complexes with borate ions [6] such an araban would not be precipitated in the fractionation with cetyltrimethylammonium hydroxide—boric acid. It should therefore be found only in the unprecipitated fraction, which is in agreement with the total absence of the corresponding ethers in the hydrolysate of methylated arabogalactan A[1].

The isolation of 2-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose from hydrolysates of the methylated derivatives of arabogalactan A and of the galactans obtained on mild hydrolysis of arabogalactan A was reported in parts II [1] and III [2] of this series. The argument presented there on their structural significance must also be valid in the present case. The possibility that these ethers originate from a branched galactan built up of 1,4-linked units cannot be disregarded. However they constitute 4,6—6,6 mole % of the hydrolysates which would correspond to at least 10 % of such a galactan in the original polysaccharide. Such a quantity would not easily have been overlooked in the fractionation and electrophoresis and it therefore seems more probable that these ethers are derived from the arabogalactans and not from a contaminating galactan. The isolation of 2-*O*-methyl-D-galactose from methylated European larch arabogalactan [7] and of 2,6-di-*O*-methyl-D-galactose from the arabogalactans of White spruce [8] and Jack pine [9] suggests that a small proportion of 1,4-linkages is normally present in the arabogalactans from coniferous woods.

A comparison of the present results with those obtained on the methylated arabogalactan A shows that the chains of galactose residues forming the backbones of the two polysaccharides are very similar both in the types of linkage present and in their proportions. However, in making such a comparison the possibility must be born in mind that A may be heterogeneous. The amount of end groups as calculated from methylation data is 34.8 and 36.8 % for A and B, respectively. However, due to various deficiencies in the methods used these figures will be too low, especially in the case of A. Estimation of the amount of endgroups from the amount of branching points gives values of 42.3 and 39.0 % respectively. There is a higher proportion of 3-*O*-β-L-arabopyranosyl-L-arabofuranose units in fraction B than in A. The excess of 2,5-di-*O*-methyl-L-arabinose relative to 2,3,4-tri-*O*-methyl-L-arabinose shows that some arabinose must be present in 3-*O*-L-arabofuranosyl-L-arabofuranose residues. The existence of 3-*O*-L-arabofuranosyl-3-β-L-arabopyranosyl-L-arabofuranose is less probable as no 2,4-di-*O*-methyl-L-arabinose was found in the hydrolysate.

Tests, made by courtesy of Dr. M. Heidelberger, State University, New Jersey, USA, of the reactivity of the arabogalactans towards Type XIV antipneumococcal horse serum, showed differences between the two polysaccharides. Arabogalactan A not only reacted faster than B but precipitated considerably more antibody. It seems unlikely that the slight differences between A and B indicated by the methylation data are sufficient to account for the differences in their reactivity towards antipneumococcal serum and in their rate of migration on electrophoresis (M_G 0.76 for A and 0.63 for B). It is more probable that the two molecules are built up in a different manner with concomitant differences in the accessibility of those groups which are normally involved in the formation of borate complexes and of complexes with antipneumococcal serum (*cf.* Ref.[10]).

Some reference should be made to possible deficiencies in the methods used to obtain the methylation data given in Parts II, III and IV of this series. As was pointed out in Part II estimates of the number of endgroups present from the yield of tetramethylgalactose and trimethylarabinose give lower values than those required by estimates of the numbers of branching points from the yield of monomethyl and dimethyl galactose. This is partly due to the slight volatility of the tetramethylagactose and the trimethylarabinose. This discrepancy was greatly diminished by chloroform extraction of the distillates obtained on concentration of solutions of these highly methylated ethers and returning the recovered material to the ether mixture. The quantitative estimation of difficultly separated substances after fractionation on thick filter paper is of course subject to some inaccuracy. This was especially the case in the estimation of 2,6-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,5-di-*O*-methyl-L-arabinose and 2,3,4-tri-*O*-methyl-L-arabinose but since the relative error probably lies within the range 5—10 %, this inaccuracy should not seriously affect the results.

It appears from the analytical data (*cf.* Table 4, Part II [1] and Table 2 in the present paper) that some trailing occurred in the elution from the carbon column. It was possible to take this trailing into account only in the case of the most abundant components as the material trailing was only a few percent

of the total present in each case. In the investigation of degraded arabogalactan A [2] when a different batch of activated carbon was used in the column, practically no trailing occurred.

It was found that the mild hydrolysis of larch arabogalactan, in addition to removing the arabofuranose residues also splits off large amounts of galactose and 6-*O*-β-D-galactopyranosyl-D-galactose at the same time [12]. It was assumed that these units were originally attached to the rest of the molecule through furanosidic linkages. That galactofuranose units are present in larch arabogalactan has already been suggested by Jones [13]. Normal paper chromatographic methods proved inadequate for the separation of the expected 2,3,5-tri-*O*-methyl-D-galactose from the accompanying 2,3,4,6-tetra-*O*-methyl-D-galactose and of 2,3,5,6-tetra-*O*-methyl-D-galactose from 2,3,5-tri-*O*-methyl-L-arabinose. However the chromatographic technique developed by Wickberg [14] using paper impregnated with dimethyl sulphoxide as stationary phase and ether or benzene as mobile phase gave an excellent separation of these methyl ethers. This showed clearly that no methylated galactofuranoses were present in the hydrolysates from the methylated arabogalactans A and B.

The easy release of these galactose residues is unexpected. Estimation of the amounts of oligosaccharides formed on hydrolysis of unfractionated arabogalactan A in 0.01 N hydrochloric acid at 100°C[15] showed that after 8 h about 80 % of the arabinose is split off and that the oligosaccharide part of the hydrolysate contains 55 % arabinose and 3-β-arabopyranosyl-arabinose, 35—40 % galactose and 6-β-galactopyranosyl-galactose and 5—10 % of larger fragments containing galactose. Detectable amounts of 3-β-galactopyranosyl-galactose were found only after 10 h. This disaccharide is usually present in mixtures of oligosaccharides obtained on stronger hydrolysis of the arabogalactan [7,15]. These results indicate that a part of the galactopyranosidic linkages is hydrolysed with about the same rate as the arabofuranosidic linkages. Hydrolysis at those linkages leads to the loss of about 15 % of the galactose originally present.

A comparison of these results with those obtained from methylation studies on arabogalactans from *Larix decidua* [7] and from *Pinus* [9,16] and *Picea* [8] shows that the *Pinus* and *Picea* arabogalactans are of a different, less branched type with the arabinose present as single, terminal furanose residues. The arabogalactans from *Larix decidua* are appearently very similar in structure to those from *L. occidentalis* except for a slightly different arrangement of the arabinose residues.

EXPERIMENTAL

All melting points are corrected. All evaporations were done under reduced pressure.

Chromatography. Papers: Whatman No. 1 and 3 MM, Schleicher and Schüll 602 hP. Solvent: Butanol, ethanol, water, 10:3:5. Spray reagent: anisidine hydrochloride.

Methylation of arabogalactan B. Arabogalactan B ($[a]_D^{20}$ + 10° (*c*, 1.0 in water), 21 mole-% arabinose) was prepared as described in Part II. The polysaccharide (4.72 g) was swollen in dry dimethyl formamide (50 ml) overnight, silver oxide was then added and the mixture was stirred vigorously to give a suspension. Dry methyl iodide (25 ml) was distilled into the reaction flask in small portions over a period of 3 h and further silver oxide (14 g) was added during the same time in 2 g portions. The mixture was stirred for a further 15 h at room temperature and then worked up as described in Part II. The product

(5.30 g; OCH_3, 35.6 %) was then methylated five times in the normal way but the methoxyl content could not be raised above 43 % (theoretical value 44.6 %).

Fractionation of the hydrolysate of methylated arabogalactan B. Methylated arabogalactan B (2.0 g) was heated with formic acid (100 ml) at 100°C for 6 h. The solution was concentrated and the product was dissolved in aqueous 0.5 N hydrochloric acid (100 ml) and kept at 100°C for 14 h. This solution was then neutralised by filtering through an ion exchange column (IR 4-B), and concentrated to small volume. The aqueous distillate from this concentration was extracted three times with chloroform and the minute residue obtained on evaporation was dissolved in a few drops of water and combined with the concentrated hydrolysate. The same procedure was always used in the concentration of solutions containing free, highly methylated ethers.

The hydrolysate was adsorbed on a carbon-Celite column (3.5 × 43 cm) and separated by gradient elution with 5.5 1 6 — 24 % aqueous ethanol followed by 1 1 50 % acetone. 20 ml fractions were collected.

The results of the fractionation are summarised in Table 2. The amount of reducing sugar in each of the combined fractions was determined by hypoiodite oxidation [17]. The mixed fractions were further separated by chromatography of 10—15 mg samples on Schleicher and Schüll 602 hP paper for quantitative estimation [1] and by chromatography of the remainder for preparative purposes on Schleicher and Schüll 602 hP or Whatman 3 MM papers.

Table 2. Fractionation of methylated hydrolysed arabogalactan B.

Fract.	mmoles (tot.)	Sugars	%	mmoles
25— 27	<0.010	Arabinose Galactose		
28— 40	0.280	4-Methylgalactose 2-Methylgalactose 2-Methylarabinose	10 73 17	0.028 0.204 0.048
50— 81	2.52	2,4-Dimethylgalactose		
82— 91	0.120	2,4-Dimethylgalactose 2,3-Dimethylarabinose	67 33	0.08 0.040
92—106	0.370	2,4-Dimethylgalactose 2,6-Dimethylgalactose Unknown galactose ether	11 73 16	0.041 0.270 0.059
107—119	0.715	2,4-Dimethylgalactose 2,6-Dimethylgalactose 2,3,4-Trimethylgalactose 2,3,4-Trimethylarabinose	2 8 58 32	0.014 0.057 0.415 0.229
120—136	0.876	2,3,4-Trimethylgalactose 2,3,4-Trimethylarabinose 2,5-Dimethylarabinose	55 24 21	0.482 0.210 0.184
137—179	0.613	2,3,4-Trimethylgalactose 2,5-Dimethylarabinose	18 82	0.110 0.503
180—246	0.344	2,4,6-Trimethylgalactose		
Residue	2.71	2,3,4,6-Tetramethylgalactose 2,3,5-Trimethylarabinose	76 24	2.06 0.65

Characterisation of the methyl ethers. 4-O-Methyl-D-galactose. The ether was separated from the accompanying 2-*O*-methyl-D-galactose and 2-*O*-methyl-L-arabinose on Schleicher and Schüll 602 hP papers. The small quantity obtained (about 3 mg) was crystallised from ethanol, m. p. 195° and mixed m. p. 195—205°. It was chromatographically and electrophoretically indistinguishable from an authentic specimen of 4-*O*-methyl-D-galactose.

2-O-Methyl-D-galactose. The ether was recrystallised from ethanol, m. p. and mixed m. p. 150—152°, chromatographically and electrophoretically indistinguishable from authentic 2-*O*-methyl-D-galactose.

2-O-Methyl-L-arabinose. This ether gave only arabinose on demethylation and travelled on chromatograms at about 90 % of the rate of 2,4-di-*O*-methyl-D-galactose. On electrophoresis it migrated faster (M_G 0.34) than 4-*O*-methyl-D-galactose (M_G 0.30) but slower than 2-*O*-methyl-D-galactose (M_G 0.43). These characteristics make a 2-*O*-methyl-L-arabinose structure very probable.

2,4-Di-O-methyl-D-galactose. The ether was recrystallised from chloroform-moist ethanol, m. p. 97°, sintering at 85°; aniline derivative, m. p. 210—212° undepressed on admixture with an authentic specimen of 2,4-di-*O*-methyl-D-galactosyl-N-phenylamine.

2,3-Di-O-methyl-L-arabinose. The ether was obtained by separation of fractions 82—91 on thick filter paper. It gave arabinose only on demethylation and travelled slightly faster than 2,4-di-*O*-methyl-L-arabinose on chromatography. It was immobile on electrophoresis and therefore a 2,3-di-*O*-methyl-L-arabinose structure is the most probable.

2,6-Di-O-methyl-D-galactose. The ether was separated from the accompanying unknown methylgalactose and 2,4-di-*O*-methyl-D-galactose by fractionation of fractions 92—106 on thick filter paper. M. p. and mixed m. p. 116—118°. It was chromatographically and electrophoretically (M_G 0.38) indistinguishable from authentic 2,6-di-*O*-methyl-D-galactose.

Unknown methylgalactose. This ether, probably an artifact, gave galactose on demethylation and on chromatography moved about 20 % faster than 2,4-di-*O*-methyl-D-galactose. It had M_G 0.30 which shows that either the 1,2- or the 3,4-glycol grouping was unsubstituted [18]. Of the possible ethers with this structural feature 3,4-di-*O*-methyl-D-galactose would probably be more easily desorbed from the column. This indicates the alternatives 4,6-di- or 3,4,6-tri-*O*-methyl-D-galactose, the latter being in best agreement with the high R_F value.

2,3,4-Tri-O-methyl-D-galactose. This ether and the next two were obtained from fract. 107—179 by fractionation on thick filter paper. It was characterised as the aniline derivative, m. p. 170—172°, undepressed on admixture with an authentic specimen of 2,3,4-tri-*O*-methyl-D-galactosyl-*N*-phenylamine.

2,3,4-Tri-O-methyl-L-arabinose. The ether was converted to the phenylhydrazide of the corresponding acid which was recrystallised from ethyl acetate-*iso*propyl ether, m. p. 159—161°, undepressed on admixture with an authentic specimen of 2,3,4-tri-*O*-methyl-L-arabonic acid *N*-phenylhydrazide.

2,5-Di-O-methyl-L-arabinose. The ether was converted to the amide of the corresponding acid. After recrystallisation from ethyl acetate it had m. p. 133—134° in good agreement with values previously reported [19].

2,4,6-Tri-O-methyl-D-galactose. The ether was converted to the aniline derivative, m. p. 181—182°, undepressed on admixture with an authentic specimen of 2,4,6-tri-*O*-methyl-D-galactosyl-*N*-phenylamine.

2,3,4,6-Tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-L-arabinose. After removal of a suitable amount for quantitative estimation and chromatography, the fraction containing these ethers (550 mg) was boiled with aniline in ethanol. Crystalline 2,3,4,6-tetra-*O*-methyl-D-galactosyl-*N*-phenylamine (250 mg), m. p. and mixed m. p. 197—198° separated from the reaction mixture. The non-crystalline portion was heated with aqueous 0.5 N sulphuric acid (10 ml) for 3 h, neutralised and concentrated to dryness. After removal of a small quantity for chromatography, the remainder was fractionated on a small cellulose column which had previously been impregnated with the lower phase of the *n*-butanol-water system and then washed with the upper phase which was also used as eluant. The top fraction afforded pure 2,3,5-tri-*O*-methyl-L-arabinose which was converted to the amide of the corresponding acid, m. p. 138—139°, in good agreement with values previously reported [19].

Chromatography on paper impregnated with dimethyl sulphoxide (DMSO). For an attempted detection of furanosidic galactose methyl ethers, fractions 120—136, 137—179, 180—245 and the residue were chromatographed according to the technique described by Wickberg [14]. 20 % DMSO in toluene was used for impregnation of the papers. Ethyl ether-DMSO 24:1 was used as mobile phase for tetramethylgalactose and trimethylarabinose ethers and benzene-DMSO 20:1 for trimethylgalactose and dimethylarabinose ethers. The following R_F-values were obtained when ether was used as mobile phase: 2,3,5,6-tetra-*O*-methyl-D-galactose 0.72, 2,3,5-tri-*O*-methyl-L-arabinose 0.64, and 2,3,4,6-tetra-*O*-methyl-D-galactose (two spots with trailing) 0.52 and 0.47. When benzene was used as mobile phase the chromatograms were run for 2.5 h giving the following migration lengths: 2,3,4-tri-*O*-methyl-D-galactose 2.2 cm, 2,4,6-tri-*O*-methyl-D-galactose 3.5 cm, 2,3,5-tri-*O*-methyl-D-galactose 4.4 cm, 2.5-di-*O*-methyl-L-arabinose 3.0 cm, and 2,3,4-tri-*O*-methyl-L-arabinose 10 cm. No furanosidic methylgalactoses were found in the fractions investigated.

The author is indebted to Dr. B. Lindberg for his keen interest in this work, to Miss Anita Myhrman for skilful assistance, and to *Fonden för Skoglig forskning* for financial support.

REFERENCES

1. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **12** (1958) 1977.
2. Bouveng, H. O. *Acta Chem. Scand.* **13** (1959) 1869.
3. Kuhn, R., Trischmann, H. and Löw, I. *Angew. Chem.* **67** (1955) 32.
4. Kuhn, R. and Schretzmann, H. *Angew. Chem.* **67** (1955) 785.
5. Whistler, R. W. and Smart, C. L. *Polysaccharide Chemistry*, New York 1953, p. 200.
6. Foster, A. B. *J. Chem. Soc.* **1957** 1395.
7. Aspinall, G. O., Hirst, E. L. and Ramstad, E. *J. Chem. Soc.* **1958** 593.
8. Adams, G. A. *Can. J. Chem.* **36** (1958) 755.
9. Bishop, C. T. *Can. J. Chem.* **35** (1957) 1010.
10. Heidelberger, M. *J. Am. Chem. Soc.* **77** (1955) 4308.
11. Gardiner, J. G. and Percival, E. *J. Chem. Soc.* **1958** 1414.
12. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **10** (1956) 1515.
13. Jones, J. K. N. *J. Chem. Soc.* **1953** 1672.
14. Wickberg, B. *Acta Chem. Scand.* **12** (1958) 615.
15. Bouveng, H. O. *Unpublished results.*
16. Wadman, W. H., Anderson, A. B. and Hassid, W. Z. *J. Am. Chem. Soc.* **76** (1954) 4097.
17. Hirst, E. L., Hough, L. and Jones, J. K. N. *J. Chem. Soc.* **1949** 57.
18. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **10** (1956) 1283.
19. Maher, G. G. *Advances in Carbohydrate Chem.* **10** (1955) 263.

Received June 10, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1884–1889

Studies on a Galactan from Norwegian Spruce Compression Wood (*Picea abies* Karst.)

HANS O. BOUVENG and HANS MEIER

Träkemiska avdelningen, Svenska Träforskningsintitutet, Stockholm, Sweden

A galactan, containing about 13 % uronic acid residues, was isolated from Norwegian spruce compression wood. Partial hydrolysis gave D-galactose and a number of neutral oligosaccharides, which were shown by paper chromatography and paper electrophoresis to belong to the same homologous series. The first member of this series was crystalline and identified as 4-*O*-β-D-galactopyranosyl-D-galactose. The neutral part of the polysaccharide must therefore be composed essentially of linear chains of 1:4-β-linked D-galactose residues.

It has been known for some time that compression wood on hydrolysis yields a much higher proportion of galactose than normal wood [1]. In the present investigation a polysaccharide containing galactose residues was isolated from compression wood from Norwegian spruce (*Picea abies* Karst.) and characterised. Acetone-extracted, milled compression wood, on extraction with hot water, yielded only a small fraction (A). However, a large amount of polysaccharide was isolated from the solution obtained on delignification of the wood meal with chlorite. This fraction (B) contained about half the galactose residues in the wood. Further extraction of the holocellulose successively with hot water, 14 % potassium hydroxide and 24 % potassium hydroxide containing 3 % boric acid [2] yielded fractions C, D and E, respectively. The results of these extractions are summarised in Table 1.

Fractions B and C were subfractionated by precipitation with barium hydroxide [3], yielding precipitates B I and C I, and soluble fractions B II and C II. From analysis of hydrolysates of these fractions (Table 1) it was evident that B I was a fairly pure galactan containing 12.9 % uronic acids and contaminated with small amounts of glucomannan. This fraction, representing about 44 % of the galactose residues originally present in the wood, was used for the present investigation.

Arabogalactans have been isolated from several coniferous woods, including spruce wood [4], and the possibility was considered that the present galactan might be derived from an arabogalactan, from which arabinose residues had been hydrolysed during delignification. It was found, however, that a larch

Table 1. Carbohydrate analyses of polysaccharide fractions from spruce compression wood.

	Yield g	Galactose %	Glucose %	Mannose %	Arabinose %	Xylose %
Spruce compression wood, extracted with acetone (Klason lignin: 38.8 %)	391.0	19.6	55.6	11.5	2.6	10.7
A: H_2O-extract	1.3	74.3	4.5	16.1	4.1	0.9
B: Polysaccharides from the delignification solution						
B I: $Ba(OH)_2$ precipitate	21.4	97.3	trace	2.7	trace	trace
B II: Centrifugate	5.5	72.1	2.2	1.9	3.7	20.1
Holocellulose (Klason lignin: 1.1 %)	208.0	7.5	65.8	11.9	2.4	12.5
C: Polysaccharides from the H_2O extract of the holocellulose						
C I: $Ba(OH)_2$ precipitate	5.4	45.2	11.6	41.3	1.0	0.9
C II: centrifugate	2.5	33.1	8.4	2.8	5.8	49.9
D: Polysaccharides from the 14 % KOH extract	40.7	19.0	5.2	12.4	6.2	57.2
E: Polysaccharides from the 24 % KOH + 3 % H_3BO_3 extract						
E I: $Ba(OH)_2$ precipitate	6.6	6.7	23.3	68.3	0.8	0.9
Residue		1.5	91.3	6.1	—	1.0

arabogalactan, after similar treatment, could be recovered unchanged. Furthermore the arabogalactans have highly branched structures, while the present polysaccharide gave a fairly strong film, characteristic for a polysaccharide with an essentially linear structure [5a]. The infrared spectrum of the polysaccharide showed a strong band at 886 cm^{-1}, indicating a β-pyranosidic structure [6].

Hydrolysis gave D-galactose and a number of acidic components, four of which were isolated in a fairly pure state by chromatography on thick filter paper. One of these acids was very probably galacturonic acid. A preliminary examination by paper chromatography and electrophoresis of hydrolysates of the other three acids indicated that in addition to galactose, one of them contained galacturonic acid and the other two glucuronic acid.

The possibility that part of the galacturonic acid originated from pectic acid was examined but attempted fractionation with cetyl-trimethylammonium hydroxide (CTA-OH), which is known to precipitate acidic polysaccharides [7], was not successful. A spectrophotometric investigation of the polysaccharide revealed the presence of about 9 % lignin; by treatment with chlorine and extraction with ethanolic ethanolamine this value was reduced to 1 %. The delignified material on electrophoresis on glass paper gave a very elongated spot with M_G-value ranging from about 0.45 to 0.97. This indicated that the sample was highly polymolecular but no distinct components could be detected. Even after delignification the sample could not be separated by fractionation with CTA-OH into a neutral galactan and a pectic acid. This corroborates the results of the hydrolyses mentioned above, that showed that at least part

of the uronic acid is bound to the galactan. Whether these residues are present on the galactan chains as single residues or as chains of pectic acid type, as was recently suggested by Aspinall *et al.*[8] for a galactan and a pectic acid from sisal hemp, could not be decided.

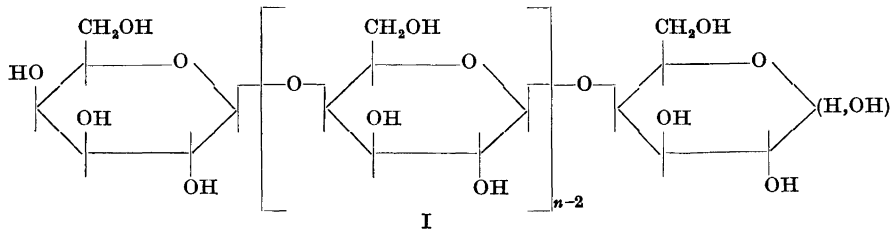
Since a nitrate ester of the galactan (N 11.8 %) was only partly soluble in acetone or butyl acetate, the osmotic pressure was determined on the sodium salt of the unsubstituted galactan in 0.1 N sodium chloride solution. The value obtained, $\overline{DP}_n$ 52, is however rather uncertain since the sample also contained lignin.

Partial hydrolysis of the galactan and removal of the acidic components left a number of neutral sugars, which were fractionated by carbon column chromatography. The first four components were completely separated but only a partial separation was obtained of four more components which followed

Table 2. R_F (solvent b) and M_G (borate buffer, pH 10) values of the neutral components.

Fraction No.	Component	R_F	M_G
34— 64	galactose	0.39	0.95
116—148	galactobiose	0.27	0.48
180—202	galactotriose	0.19	0.40
210—230	galactotetraose	0.14	0.35
232—246	galactopentaose	0.10	0.32
243—255	galactohexaose	0.07	0.31
253—270	galactoheptaose	0.05	0.30
262—281	galactooctaose	0.04	0.28

these. D-Galactose and a galactobiose were obtained crystalline. The galactobiose, $[\alpha]_D^{25}$ +67° showed the same paper chromatographic and electrophoretic data and the same X-ray diffraction pattern as the 4-*O*-β-D-galactopyranosyl-D-galactose isolated from white birch [9], a sample of which was obtained by courtesy of Dr. T. E. Timell. Further its melting point 210—212° was undepressed on admixture with Timell's sample. The R_F and M_G values of the sugars are given in Table 2. The R_M-values, plotted against n, give a straight line (Fig. 1), indicating that the sugars belong to a homologous series of the general formula I.



I

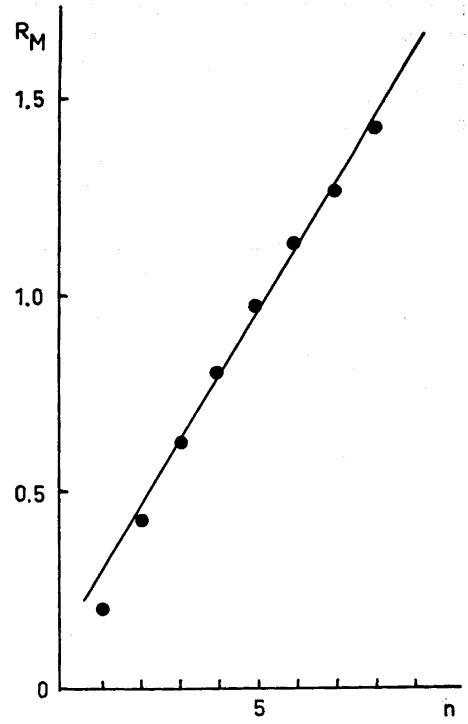


Fig. 1. Relation between R_M-values and chain length.

The electrophoretic data are also in agreement with this conclusion and each of the components on partial hydrolysis yielded all the lower oligosaccharides. Only traces of neutral oligosaccharides, other than those belonging to this series, were observed in the hydrolysate of the galactan, and the galactan must therefore be composed essentially of linear chains of β-1,4-linked D-galactose residues. Uronic acid residues are linked to some of these. The nature of these linkages and the question of whether some of the uronic acid residues are present in pectic acid chains must await further study.

The galactan from spruce compression wood is thus of similar type to that belonging to the pectic triad [5b]. A similar galactan has recently been obtained by Andrews *et al.*[10] from *Strychnos nux vomica* seeds and the isolation of 4-β-D-galactopyranosyl-D-galactose by Gillham and Timell [11] from a hydrolysate of white birch α-cellulose also indicates the presence of a polysaccharide of this type. The presence of pectic substances in the middle lamella of wood fibres has long been assumed, and it has recently been demonstrated [12,13], that the middle lamella of unripe aspen fibres and of pine tracheids contain a high percentage of polymers, built up from galactose, arabinose and galacturonic acids, which are the constituent sugars of the pectic triad. Although a large part of the galactans from the compression wood was extracted during the delignification, a considerable part remained undissolved even after treat-

ment with 14 % potassium hydroxide. This does not necessarily imply that the wood contains galactans that are structurally very different. The same difference has also been observed in spruce glucomannans [14], two extreme fractions of which showed some difference in DP but had eseentially the same chemical structure.

EXPERIMENTAL

Melting points are corrected. Optical rotations were determined in water: *c*, 1.5. The concentration of solutions was done under reduced pressure, at a bath temperature of about 40°.

Paper chromatograms were run on Whatman No. 1 and 3 MM papers, using the solvent systems (v/v):

a) Ethyl acetate-acetic acid-water, 3:1:3 (upper phase)

b) Ethyl acetate-pyridine-water, 2:1:2 (upper phase).

The method of Saeman *et al.*[15] was used for quantitative determinations. Paper electrophoreses were run on Schleicher and Schüll glass fibre papers and on Whatman No. 3 MM paper in 0.1 M borate buffer of pH 10 or 0.1 M acetate buffer of pH 4.

Isolation and fractionation of polysaccharides. Acetone-extracted wood meal (18—35 mesh) was extracted with hot water. Acidification and precipitation with ethanol, yielded fraction A. The wood meal was then delignified by the chlorite method at 60° and pH 4.7. The combined chlorite solutions were dialysed against running tap water for 11 days, acidified and precipitated with ethanol, yielding fraction B. The holocellulose was then extracted successively with hot water, 14 % potassium hydroxide and 24 % potassium hydroxide with 3 % boric acid. The latter two extractions were made under nitrogen, and the polysaccharides extracted were recovered by precipitation with ethanol (fractions C, D and E respectively).

Fractions B and C were dissolved in water and a saturated solution of barium hydroxide was added [3]. The material in the precipitates and in the supernatants was recovered, yielding fractions B I, C I and B II, C II respectively.

B I was delignified with chlorine and ethanolic ethanolamine, as described previously[16]. Treatment with cetyl-trimethylammonium hydroxide (CTA-OH) precipitated about 2/3 of the material. The precipitate and the material remaining in solution were isolated as described by Bouveng and Lindberg [17]. They had optical rotations of +80° and +55°, resp., the first fraction having a considerably higher content of uronic acid residues. Attempted fractionation of undelignified material with CTA-OH failed.

B I was readily soluble in water, $[\alpha]_D^{25}$ +57°. It contained about 9 % lignin [16] and 12.9 % of uronic acid residues [18], calculated as galacturonic acid. The $\overline{DP}_n$ was determined osmometrically as already described for an araboglucuronoxylan [16].

Hydrolysis of B I. A solution of B I (3 g) in 4 % sulphuric acid (800 ml) was kept at 120° for 1 h and then neutralised with barium hydroxide. The neutral solution was passed successively through columns of Dowex 50 (H^+) and Dowex 3 (free base) ion exchange resin. D-Galactose was recovered from the deionised solution. The acids were eluted from the anion exchange resin with 1 N ammonium hydroxide and the solution obtained was concentrated to a syrup. Electrophoresis and paper chromatography (solvent a) revealed the presence of galacturonic acid and of at least three acids with lower R_F values. The latter were separated by chromatography on thick filter paper. A chromatographic and electrophoretic examination of these acids after hydrolysis with 6 % sulphuric acid at 120° for 6 h indicated that one of them contained galactose and galacturonic acid while the other two were composed of galactose and, probably, glucuronic acid.

Partial hydrolysis of BI. A sample of BI (3 g) was heated with N sulphuric acid (200 ml) for 40 min on a steam bath, cooled and deionised. The solution was concentrated to 10 ml and then adsorbed on a carbon-Celite column (4 × 45 cm). Gradient elution with aqueous ethanol, 1 — 10 % (4 l) followed by 10—35 % (6 l) effected a separation of the components. The first four members, galactose to galactotetraose were completely separated. From the pentaose to the octaose they only partially separated, but relatively

pure top fractions were obtained of all components. The R_F- and M_G-values of the components are given in Table 2.

D-*Galactose* (346 mg), $[\alpha]_D^{25}$ +82°, m. p. 164—167°, undepressed on admixture of an authentic sample.

Galactobiose (278 mg), $[\alpha]_{25}^{D}$ +67°, m. p. 210—212°, alone or mixed with β-1,4-galactobiose, kindly supplied by Dr. T. E. Timell, Montreal. The X-ray diffraction pattern was identical with that reported by Gillham *et al.*[9] The M_G-value in borate buffer, 0.48, was, as expected, lower than the values reported for the corresponding β-1,3- and β-1,6-linked disaccharides [19] (0.69 and 0.84).

Galactotriose (213 mg), $[\alpha]_D^{25}$ +58°. Amorphous.

Galactotetraose (151 mg), $[\alpha]_D^{25}$ +53°. Amorphous.

Acknowledgment. The authors thank Docent B. Lindberg for his keen interest in this work.

REFERENCES

1. Stockman, L. and Hägglund, E. *Svensk Papperstidn.* **51** (1948) 269.
2. Jones, J. K. N., Wise, L. E. and Jappe, J. P. *Tappi* **39** (1956) 139.
3. Meier, H. *Acta Chem. Scand.* **12** (1958) 144.
4. Adams, G. A. *Can. J. Chem.* **36** (1958) 755.
5. a. Whistler, R. L. and Smart, C. L. *Polysaccharide Chemistry*, New York 1953, p. 32.
5. b. *Ibid.* p. 161 ff.
6. Barker, S. A., Bourne, E. J., Stephens, R. and Whiffen, D. H. *J. Chem. Soc.* **1954** 3468.
7. Scott, J. E. *Chem. & Ind. London* **1955** 168.
8. Aspinall, G. O. and Cañas-Rodriguez, A. *J. Chem. Soc.* **1958** 4020.
9. Gillham, J. K., Perlin, A. S. and Timell, T. E. *Can. J. Chem.* **36** (1958) 1741.
10. Andrews, P., Hough, L. and Jones, J. K. N. *J. Chem. Soc.* **1954** 806.
11. Gillham, J. K. and Timell, T. E. *Svensk Papperstidn.* **61** (1958) 540.
12. Sultze, R. F. *Tappi* **40** (1957) 985.
13. Meier, H. *Svensk Papperstidn.* **62** (1959) 687.
14. Croon, I. and Lindberg, B. *Acta Chem. Scand.* **12** (1958) 453.
15. Saeman, J. F., Moore, W. E., Mitchell, R. L. and Millet, M. A. *Tappi* **38** (1954) 336.
16. Meier, H. *Acta Chem. Scand.* **12** (1958) 1911.
17. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **12** (1958) 1977.
18. Johansson, A., Lindberg, B. and Theander, O. *Svensk Papperstidn.* **57** (1954) 41.
19. Bouveng, H. O. and Lindberg, B. *Acta Chem. Scand.* **10** (1956) 1283.

Received June 3, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1890–1896

Solvent Extraction of Metal Chelates

II. An Investigation on some 1-Phenyl-3-methyl-4-acyl-pyrazolones-5

BROR SKYTTE JENSEN

Danish Atomic Energy Commission, Risø, Denmark

The apparent mean complexity constants of various 1-phenyl-3-methyl-4-acyl-pyrazolones-5 with Be^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, UO_2^{2+}, La^{3+} and Th^{4+} in a chloroform-water system are compared and discussed.

A linear relationship between the logarithm of the distribution coefficient of an uncharged metal chelate and the logarithm of the distribution coefficient of the undissociated chelating agent is proposed, the slope of the line being characteristic of the metal ion in question.

The compounds investigated are compared with the more common extractive chelating agents with regard to capacity and separation possibilities in a chloroform-water system, and show promise as valuable reagents for analytical and radiochemical separations.

Among the extractive chelating agents the β-diketones, as a group, range among the most useful. Compounds such as acetylacetone and thenoyltrifluoroacetone are to be regarded as standard chemicals in laboratories performing analytical or radiochemical work.

Several investigations on the extractive properties of different chelating agents have been published previously [1,2].

In this paper the results of an investigation on a class of β-diketones, the 1-phenyl-3-methyl-4-acyl-pyrazolones-5, in a chloroform-water system will be reported.

The 1-phenyl-3-methyl-4-acyl-pyrazolones are readily accessible compounds [3]. Furthermore their physico-chemical constants such as acid dissociation constants, distribution coefficients *etc.* can be varied within a wide range by proper substitution in the molecules. Hence this class of compounds seems to serve well as a basis for a closer study of the influence of the physical and chemical properties of a chelating agent on its extractive and chelating behaviour.

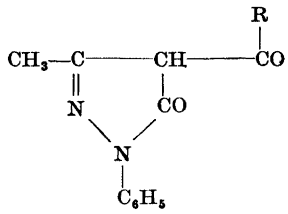


Fig. 1.

RESULTS AND DISCUSSION

The metal ion extractions have been investigated by means of a titration procedure [4], which has been described previously. By this method the chelating agent is characterized by a $pK_{a,E}$ value, which is the apparent pK_a value shown by an acid in the two-phase system under consideration. When only the undissociated acid HA is expected to distribute between the two phases with a distribution coefficient K_d, the following relation was shown to be valid [4,5].

$$pK_{a,E} = pK_a + \log\ (1 + K_d) \qquad (1)$$

where pK_a is the value of the acid dissociation constant in aqueous solution.

The chelating agent, metal ion, solvent systems are characterized by either the pH_{50} of 50 % extraction of the metal ion under consideration or by an apparent mean complexity constant K_{av}[4].

As K_{av} is a real constant of the system whereas pH_{50} depends on the actual concentrations involved, the former will be used to characterize the extractive behaviour of a specified metal ion in the system under consideration.

In the case where the uncharged species MA_N is the only one extracted with a distribution coefficient λ and a complexity product β_N, the following relation has been shown to be valid [4].

$$\log\ K_{av} = \tfrac{1}{N}\ \log\ \beta_N\ (1 + \lambda) \qquad (2)$$

where it is assumed that N is the maximum coordination number of the metal ion for the ligand.

By changing the pK_a value of a chelating agent by a proper substitution we generally also change the K_d value of a compound. Conversely it might be possible to change the K_d value of a compound without influencing the pK_a value to any larger degree.

In the acetyl-, propionyl-, butyryl-, valeryl- and capronyl substituted pyrazolones we have such a set of compounds where we expect the pK_a values to remain almost constant throughout the series.

In Fig. 2 is shown a plot of log K_{av} against $pK_{a,E}$ for these compounds and different metal ions. The points corresponding to a specified metal ion are seen to satisfy a linear relationship.

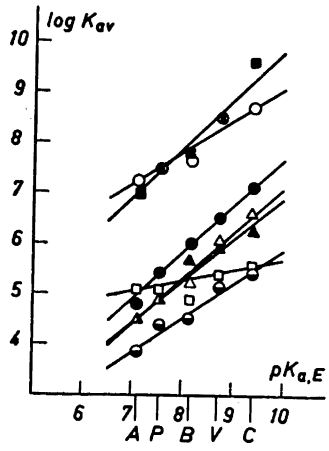


Fig. 2. The apparent mean complexity constants of the 4-acetyl (propionyl, butyryl, valeryl, capronyl) substituted pyrazolones (A, P, B, V, C) with different metal ions in a chloroform-water system plotted against the apparent $pK_{a,E}$ values of the chelating agents in this system.

■Th^{4+}, OUO_2^{2+}, ●Pb^{2+}, △Zn^{2+}, ▲La^{3+}, □Be^{2+}, ◒Mn^{2+}

As is well established [6] a relationship between $\frac{1}{N}\log\beta_N$ and pK_a for a series of related compounds can be expressed as:

$$\frac{1}{N}\log\beta_N = rpK_a + q \qquad (3)$$

This equation implies that for pK_a constant β_N will also be constant, and the plot in Fig. 2 should then be expected to represent a relationship between the distribution coefficients K_d and λ.

A linear relationship between the logarithm of the distribution coefficients as

$$\frac{1}{N}\log(1+\lambda) = s\log(1+K_d) + t \qquad (4)$$

when combined with (3) leads to (5)

$$\frac{1}{N}\log\beta_N(1+\lambda) = \log K_{av} = spK_{a,E} + (r-s)pK_a + (q+t) \qquad (5)$$

expressing the above observed linear relationship between $\log K_{av}$ and $pK_{a,E}$ when pK_a is assumed constant.

The behaviour of each metal ion in the above described systems may therefore be regarded as characterized by the combined action of eqns (3) and (4), and hence on the constants r, q, s, and t.

The magnitudes of s and t might be expected to depend much on the choice of organic solvent whereas r and q can be regarded as independent of this choice.

It is seen that the values of s in the order of magnitude of 0.85 to 1.00 are most common. For the uranyl and manganese ions values 0.60 to 0.65 are found and for the beryllium ion a value as low as 0.20.

That s and r are generally of different magnitude is exemplified in Fig. 3 which shows available data of $\log K_{av}$ for the beryllium ion and uranyl ion plotted against $pK_{a,E}$ for variously substituted pyrazolones. The change in

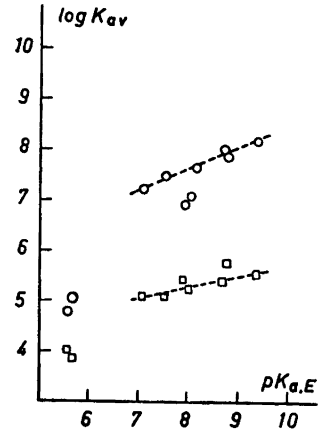


Fig. 3. The apparent mean complexity constants for variously substituted pyrazolones with the uranyl ion (O) and the beryllium ion (□) in a chloroform-water system plotted against the apparent $pK_{a,E}$ values of the chelating agents in this system. Data in Table 1.

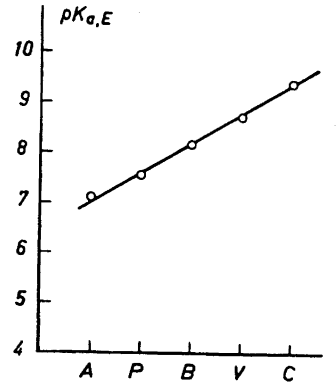


Fig. 4. The apparent $pK_{a,E}$ values of 4-acetyl (propionyl, butyryl, valeryl, capronyl) substituted pyrazolones (A, P, B, V, C) in chloroform plotted as a function of substitution.

$pK_{a,E}$ must be attributed to changes in either pK_a, K_d or in both. The plot contains the data used in the construction of Fig. 2, shown to lie on the dashed lines. In Fig. 3 no simple straight line relationship, apart from this, can be found.

To the author it seems to be too optimistic to expect the validity of eqn (5) to be strictly conserved in changing pK_a and K_d at will. No sharp definition of what is meant by "closely related compounds", can be set up. Many factors, such as steric hindrance, *etc.*, which may alter the complexity constants cannot be included in an equation like (5). Therefore an expression of general validity cannot be expected to be found.

It might be of interest to show the effect of substitution on the distribution coefficient K_d of a chelating agent.

In Fig. 4 is shown a plot of $pK_{a,E}$ in going from acetyl substitution on the pyrazolone through propionyl, butyryl, valeryl to capronyl substitution. A linear relationship is obvious. The slight alternating effect shown might be significant. As is to be expected, an increase of the aliphatic sidechain parallels an increase in lipophilic character of the chelating agent, and hence increases its distribution coefficient between an organic phase and water.

The data used in the construction of Figs. 2, 3 and 4 are collected in Table 1.

The empty places in the table indicate cases where the capacity of the systems has been exceeded by the concentrations used. This means that precipitations of chelates occurred during the titration, and hence no K_{av} values could be determined. The concentrations of chelating agents were 0.05 M, and those of the metal salts *ca.* 0.005 M, in the titration experiments [4]. With metals,

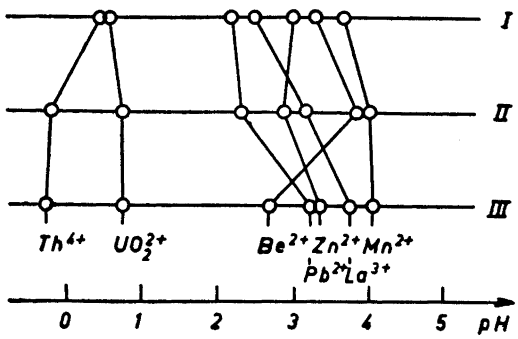


Fig. 5. The pH values of 50 % extraction of tracer amounts of different metals by 1 M solutions of 4-butyryl-1-phenyl-3-methyl-pyrazolone-5 (I) and 4-capronyl-1-phenyl-3-methyl-pyrazolone-5 (II) in chloroform are compared with those for thenoyltrifluoroacetone (III).

which form slightly soluble hydroxides, these were often precipitated when alkaline conditions were reached during the titration. These precipitations did not interfere with the determinations of the K_{av} values, as the pH changes of importance for this determination occurred in acid media.

For each system the pH_{50} of 50 % extraction of tracer amounts of the metal by 1 M solutions of the chelating agent in chloroform can be calculated by means of the following relation [1,4]

$$pH_{50} = pK_{a,E} - \frac{1}{N} \log \beta_N \ (1 + \lambda) \qquad (6)$$

This pH_{50} value is identical with the constant $\frac{1}{N} \log K$ used by Dyrssen to characterize the metal ion, chelate, solvent systems.

Inspection of Table 1 shows that, except for the beryllium ion, no large change in the pH_{50} is observed when varying the substituents in the 1-phenyl-3-methyl-pyrazolone-5 nucleus. Data for thenoyltrifluoroacetone (TTA) in chloroform, determined by the titration procedure, have been included in the table.

In Fig. 5 the pH_{50} values determined from eqn (6) for the butyryl- and capronyl-derivatives are compared with those of TTA.

CONCLUSIONS

It has been the aim of the present investigation to separate and clarify some of the various factors involved in metal ion extractions by chelating agents. The ultimate goal of such an investigation is to find chelating agents with properties which make them suitable for a large scale application, *e. g.* in the reprocessing of spent reactor fuels.

Chelating agents might be found which serve well in the laboratory where losses of chemicals and attainments of equilibria play a smaller role than in a large scale application, where a compound for those reasons might be useless.

Table 1.

Substituent R	$pK_{a,E}$	$\frac{1}{N} \log \beta_N (1 + \lambda)$						
		Th^{4+}	UO^{3+}	La^{3+}	Pb^{2+}	Zn^{2+}	Mn^{2+}	Be^{2+}
Acetyl	7.10 ± 0.1	7.00 ± 0.2	7.25 ± 0.2	4.50 ± 0.15	4.80 ± 0.15	4.50 ± 0.15	3.85 ± 0.15	5.1 ± 0.15
Propionyl	7.55 ± 0.1	7.50 ± 0.2	7.50 ± 0.2	4.90 ± 0.15	5.42 ± 0.15	4.90 ± 0.15	4.40 ± 0.15	5.1 ± 0.15
Butyryl	8.17 ± 0.1	7.75 ± 0.2	7.65 ± 0.2	5.70 ± 0.15	6.00 ± 0.15	5.20 ± 0.15	4.50 ± 0.15	4.9 ± 0.15
Valeryl	8.74 ± 0.1	8.5 ± 0.2	8.5 ± 0.2	5.9 ± 0.15	6.5 ± 0.15	6.1 ± 0.15	5.15 ± 0.15	5.4 ± 0.15
Capronyl	9.4 ± 0.1	9.65 ± 0.2	8.7 ± 0.2	6.25 ± 0.15	7.1 ± 0.15	6.6 ± 0.15	5.4 ± 0.15	5.55 ± 0.15
Ethoxy-carbonyl	7.95 ± 0.1	not meas.	6.95 ± 0.2	5.45 ± 0.15	5.65 ± 0.15	—	—	5.45 ± 0.15
Chloro-acetyl	5.7 ± 0.1	5.65 −0.2	5.05 ± 0.2	3.42 ± 0.15	4.1 ± 0.15	—	—	3.84 ± 0.15
Trifluoro-acetyl	5.6 ±0.15	not meas.	4.8 0.2	not meas.	not meas.	not meas.	not meas.	4.0 ±0.15
Benzoyl	8.1 ± 0.1	7.7 ± 0.2	7.1 ± 0.2	5.65 ± 0.15	6.25 ± 0.15	—	—	5.25 ± 0.15
p-Bromo-benzoyl	8.80 ± 0.1	8.50 ± 0.2	7.9 ± 0.2	6.5 ± 0.15	7.2 ± 0.15	—	—	5.25 ± 0.15
p-Nitro-benzoyl	7.6 ± 0.1	—	—	—	—	—	—	—
Thenoyltri-fluoroacetone	8.45 ± 0.1	8.75 ± 0.2	7.75 ± 0.2	4.7 ± 0.15	5.25 ± 0.15	5.1 ± 0.15	4.4 ± 0.15	5.6 ± 0.15

Many polyvalent metal ions show a large degree of hydrolysis even in rather acid solutions, so a chelating agent capable of extraction of these metals from strongly acid solutions would seem to be desirable, as then problems of adsorption and colloid formation could be avoided. Fortunately the polyvalent metal ions are also prone to complex formation so they will possibly combine with a chelating agent even if it is slightly dissociated, *i. e.* in acid solution.

By increasing the acid strength of a chelating agent a correspondingly larger concentration of the complexing anion can be obtained in acid solutions. But, as is well established, the increase in acid strength of an acid, within a series of closely related compounds, parallels a decrease in the metal ion affinity of the anion. The net result of a variation of pK_a in a series of chelating agents might therefore well be only a slight change in the pH_{50} of a metal ion when going from one compound to the other. This has in fact been shown to be the case for the compounds discussed in this paper.

With regard to the separation possibilities, inspection of Fig. 5 shows, that in some cases the acylated pyrazolones are inferior; in other cases superior to

TTA. On the average the capronyl substituted pyrazolone seems to offer even better separation possibilities than TTA for the metal ions investigated.

As the capacities of the systems investigated are rather large, and the separation possibilities adequate for many purposes, the compounds show promise as extractive chelating agents, for analytical and radiochemical work.

Acknowledgements. The author wishes to thank Mr. Ole Jørgensen for valuable technical assistance.

REFERENCES

1. Dyrssen, D. *Svensk Kem. Tidskr.* **68** (1956) 212.
2. Morrison, G. H. and Freiser, H. *Solvent Extraction in Analytical Chemistry.* John Wiley and Sons. New York 1957.
3. Skytte Jensen, B. *Acta Chem. Scand.* **13** (1959) 1668.
4. Skytte Jensen, B. *Acta Chem. Scand.* **13** (1959) 1347.
5. Dyrssen, D. *Svensk Kem. Tidskr.* **64** (1952) 213.
6. Martell, A. E. and Calvin, M. *Chemistry of the Metal Chelate Compounds,* Prentice Hall Inc. New York 1953.

Received June 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1897–1912

Short Communications

Synthesis of Disodium S-[2-(methoxycarbonyl)ethyl] Phosphorothioate and a Barium Salt of S-(carboxymethyl) Phosphorothioic Acid

STIG ÅKERFELDT

Eastern Pennsylvania Psychiatric Institute, Philadelphia, Pennsylvania, U.S.A.

Recently [1] it was shown that trisodium phosphorothioate can be used for the preparation of a S-substituted phosphorothioic acid. Thus 2-bromoethylamine was found to react quantitatively with trisodium phosphorothioate in the presence of N,N-dimethylformamide to yield a sodium S-(2-aminoethyl) phosphorothioate.

To investigate the usefulness of trisodium phosphorothioate as a starting material for the preparation of other S-substituted phosphorothioic acids the reaction between this compound and acrylic acid methyl ester and bromoacetic acid has now been investigated. In both cases a rapid reaction was observed without the presence of a catalyst. Bromoacetic acid was found to react with trisodium phosphorothioate in a manner analogous to that described for 2-bromoethylamine [1], whereas acrylic acid methyl ester adds the phosphorothioate to the double bond forming a S-[2-(methoxycarbonyl) ethyl] phosphorothioate:

$$CH_2 = CHCOOCH_3 + H_2O + Na_3SPO_3 \rightarrow Na_2PO_3SCH_2CH_2COOCH_3 + NaOH$$

The addition takes place in contradistinction to Markownikoff's rule. This behaviour is similar to that observed by Dahlbom [2] in the diethylamine catalyzed reaction between acrylic acid and hydrogen sulfide.

The prepared substances are easily hydrolyzed by acids to the corresponding thiols. Thus these compounds constitute intermediates in a route for the preparation of thiols.

Materials and methods. Trisodium phosphorothioate was prepared according to Yasuda and Lambert [3]. Acrylic acid methyl ester (stabilized with *p*-methoxyphenol) and bromoacetic acid were products of Eastman Kodak Company. Phosphate determinations were performed according to Gomori [4] after hydrolysis of the samples in 1 M hydrochloric acid at 100° for 30 min. When barium ions were present, the hydrolysis was carried out in the presence of sulfuric acid and the precipitated barium sulfate was removed before analysis.

Melting points are corrected.

Preparation of disodium S-[2-(methoxycarbonyl)ethyl] phosphorothioate. 10 g (56 mmole) of trisodium phosphorothioate were dissolved in 75 ml of water and 10 ml (110 mmole) of acrylic acid methyl ester were added. The mixture was vigorously stirred* for 5 min after which time all phosphorothioate had reacted (no black precipitate obtained with silver ions). 200 ml of ethanol were added and the precipitated oil was separated. 100 ml of methanol and 100 ml of ethanol were added to the oil in the order indicated under stirring. The precipitated substance was filtered off and dried in vacuum. Yield 10.2 g (68 %). (Found: C 17.6; H 3.7; P 11.4. Calc. for $Na_2PO_3SCH_2CH_2COOCH_3$, 1.5 H_2O (271.16): C 17.7; H 3.7; P 11.4.)

Preparation of barium S-(carboxymethyl) phosphorothioate. 9.0 g (50 mmole) of trisodium phosphorothioate were dissolved in 60 ml of

* The methoxycarbonyl group was found to be easily hydrolyzed when the reaction was carried out at temperatures above *ca.* 25°.

water and 7.7 g (55 mmole) of bromoacetic acid were added. The solution was stirred for 15 min at 0°. After that time all the phosphorothioate had reacted. A solution of 20 g (82 mmole) barium chloride dihydrate and 2.2 g (55 mmole) of sodium hydroxide in 75 ml of water was then added under stirring at 0°. The precipitate was filtered off and washed with 50 ml of ice cold water and with 100 ml of ethanol. 13.1 g (61 %) of substance were obtained after drying in vacuum. (Found: C 5.5; H 1.7; P 7.1; Ba 48.5. Calc. for $(BaPO_3SCH_2COO)_2Ba,6H_2O$ (858.34): C 5.6; H 1.9; P 7.2; Ba 48.0.) The prepared substance hydrolyzes spontaneously upon storage.

Acid hydrolysis of the prepared compounds. 7.61 mmole of $Na_2PO_3SCH_2CH_2COOCH_3$, 1.5 H_2O were hydrolyzed for 30 min in 50 ml of 1 M hydrochloric acid under nitrogen at 100°. Hydrogen sulfide or phosphorothioate were not detected after hydrolysis. The hydrolysate consumed 7.46 matom of iodine as titrated with a standardized iodine solution (98 % of theoretical amount). The oxidized hydrolysate was extracted with peroxide free ethyl ether giving 0.8 g of 3,3'-dithiodipropionic acid, m.p. 154—155° (from 4-methyl-2-pentanone), undepressed by admixture with an authentical sample.

3.37 mmole of $(BaPO_3SCH_2COO)_2Ba$, $6H_2O$ were hydrolyzed as described above. The hydrolysate consumed 6.41 matom of iodine (95 % of theoretical amount). Hydrogen sulfide or phosphorothioate could not be detected in the hydrolysate, which was then tested for glycolic acid by the two tests described by Feigl [5]. These were both negative. The thiol present in the hydrolysate was identified as mercaptoacetic acid by the paper chromatographic method recently described [6]. For chromatography the solvents Nos. 3 and 4 in Ref.[6] were used.

The author is indebted to Mrs. M. L. Kip for valuable technical assistance.

1. Åkerfeldt, S. *Acta Chem. Scand.* **13** (1959) 1479.
2. Dahlbom, R. *Acta Chem. Scand.* **5** (1951) 690.
3. Yasuda, S. K. and Lambert, J. L. *J. Am. Chem. Soc.* **76** (1954) 5356.
4. Gomori, G. *J. Lab. Clin. Med.* **27** (1942) 955.
5. Feigl, F. *Spot Tests in Organic Analysis*, Elsevier Publishing Company, Amsterdam 1956, p. 346.
6. Åkerfeldt, S. *Acta Chem. Scand.* **13** (1959) 627.

Received September 29, 1959.

The Isolation of S-Methylcysteinesulphoxide and *S-n*-Propylcysteinesulphoxide from Onion *(Allium cepa)* and the Antibiotic Activity of Crushed Onion

ARTTURI I. VIRTANEN and E. J. MATIKKALA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Fresh homogenized onions *(Allium cepa)* have a strong antimicrobial effect. 50 to 100 mg of crushed onions in 1 ml of nutrient solution inhibits the growth of the *Staphylococcus aureus* strain used in our experiments completely, and 15 mg still has a retarding effect on the growth. When the enzymes in whole onions *(Allium cepa)* are inactivated using different methods (heating to about 100°C in a sealed glass tube, or keeping in boiling ethanol for a shorter time, or homogenizing with ethanol after freezing with CO_2 ice) the antimicrobial effect of the extracts is again very low. A total inhibition is not then yet achieved with extracts corresponding to 1 g of onion in 1 ml of nutrient solution. Most of the antimicrobial activity is thus formed in onion through enzymatic reactions.

In a lecture the senior author [1] earlier mentioned that according to investigations in this laboratory onion contains both S-methylcysteinesulphoxide (MCSO) and S-*n*-propylcysteinesulphoxide (PCSO) from which the corresponding thiolsulphinates are formed enzymatically. These have a strong antibiotic effect against many microbes. This effect is generally somewhat weaker than that of allylthiolsulphinate formed from S-allylcysteinesulphoxide present in garlic *(Allium sativum)*, but nevertheless of the same order of magnitude [2].

PCSO was isolated from onion in the following way: 3 kg of chilled onions grown in Finland were extracted with cold methanol (added methanol + the water contained in the onion = 80 % methanol). The free amino acids were separated on an Amberlite IR-120 column and fractionated with water on a 2.2 × 95 cm column filled with Dowex 1 resin (the resin in acetate form was washed with water).

When 100 ml of solution had been let through, fractions of 8 ml each were taken.

water and 7.7 g (55 mmole) of bromoacetic acid were added. The solution was stirred for 15 min at 0°. After that time all the phosphorothioate had reacted. A solution of 20 g (82 mmole) barium chloride dihydrate and 2.2 g (55 mmole) of sodium hydroxide in 75 ml of water was then added under stirring at 0°. The precipitate was filtered off and washed with 50 ml of ice cold water and with 100 ml of ethanol. 13.1 g (61 %) of substance were obtained after drying in vacuum. (Found: C 5.5; H 1.7; P 7.1; Ba 48.5. Calc. for $(BaPO_3SCH_2COO)_2Ba,6H_2O$ (858.34): C 5.6; H 1.9; P 7.2; Ba 48.0.) The prepared substance hydrolyzes spontaneously upon storage.

Acid hydrolysis of the prepared compounds. 7.61 mmole of $Na_2PO_3SCH_2CH_2COOCH_3$, 1.5 H_2O were hydrolyzed for 30 min in 50 ml of 1 M hydrochloric acid under nitrogen at 100°. Hydrogen sulfide or phosphorothioate were not detected after hydrolysis. The hydrolysate consumed 7.46 matom of iodine as titrated with a standardized iodine solution (98 % of theoretical amount). The oxidized hydrolysate was extracted with peroxide free ethyl ether giving 0.8 g of 3,3'-dithiodipropionic acid, m.p. 154—155° (from 4-methyl-2-pentanone), undepressed by admixture with an authentical sample.

3.37 mmole of $(BaPO_3SCH_2COO)_2Ba$, $6H_2O$ were hydrolyzed as described above. The hydrolysate consumed 6.41 matom of iodine (95 % of theoretical amount). Hydrogen sulfide or phosphorothioate could not be detected in the hydrolysate, which was then tested for glycolic acid by the two tests described by Feigl [5]. These were both negative. The thiol present in the hydrolysate was identified as mercaptoacetic acid by the paper chromatographic method recently described [6]. For chromatography the solvents Nos. 3 and 4 in Ref.[6] were used.

The author is indebted to Mrs. M. L. Kip for valuable technical assistance.

1. Åkerfeldt, S. *Acta Chem. Scand.* **13** (1959) 1479.
2. Dahlbom, R. *Acta Chem. Scand.* **5** (1951) 690.
3. Yasuda, S. K. and Lambert, J. L. *J. Am. Chem. Soc.* **76** (1954) 5356.
4. Gomori, G. *J. Lab. Clin. Med.* **27** (1942) 955.
5. Feigl, F. *Spot Tests in Organic Analysis*, Elsevier Publishing Company, Amsterdam 1956, p. 346.
6. Åkerfeldt, S. *Acta Chem. Scand.* **13** (1959) 627.

Received September 29, 1959.

The Isolation of S-Methylcysteinesulphoxide and *S-n*-Propylcysteinesulphoxide from Onion *(Allium cepa)* and the Antibiotic Activity of Crushed Onion

ARTTURI I. VIRTANEN and E. J. MATIKKALA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Fresh homogenized onions *(Allium cepa)* have a strong antimicrobial effect. 50 to 100 mg of crushed onions in 1 ml of nutrient solution inhibits the growth of the *Staphylococcus aureus* strain used in our experiments completely, and 15 mg still has a retarding effect on the growth. When the enzymes in whole onions *(Allium cepa)* are inactivated using different methods (heating to about 100°C in a sealed glass tube, or keeping in boiling ethanol for a shorter time, or homogenizing with ethanol after freezing with CO_2 ice) the antimicrobial effect of the extracts is again very low. A total inhibition is not then yet achieved with extracts corresponding to 1 g of onion in 1 ml of nutrient solution. Most of the antimicrobial activity is thus formed in onion through enzymatic reactions.

In a lecture the senior author [1] earlier mentioned that according to investigations in this laboratory onion contains both S-methylcysteinesulphoxide (MCSO) and S-*n*-propylcysteinesulphoxide (PCSO) from which the corresponding thiolsulphinates are formed enzymatically. These have a strong antibiotic effect against many microbes. This effect is generally somewhat weaker than that of allylthiolsulphinate formed from S-allylcysteinesulphoxide present in garlic *(Allium sativum)*, but nevertheless of the same order of magnitude [2].

PCSO was isolated from onion in the following way: 3 kg of chilled onions grown in Finland were extracted with cold methanol (added methanol + the water contained in the onion = 80 % methanol). The free amino acids were separated on an Amberlite IR-120 column and fractionated with water on a 2.2 × 95 cm column filled with Dowex 1 resin (the resin in acetate form was washed with water).

When 100 ml of solution had been let through, fractions of 8 ml each were taken.

Fractions 15—42 were combined, evaporated, and the extract obtained was fractionated on a column (3 × 105 cm) containing Dowex 50 resin in the H-form (1 N HCl). Fractions of 20 ml each were taken. PCSO emerged in fractions 172—218 together with some other amino acids. These fractions were combined, the amino acids separated in an Amberlite IR-120 column and refractionated in a 3.3 × 80 cm column containing Dowex 50 resin in equilibrium with 1.5 N HCl. 470 ml of the eluant emerging first from the column were discarded, then fractions of 20 ml each were taken. PCSO emerged in fractions 205—230. These fractions were combined and the amino acids separated in an Amberlite IR-120 column. Besides PCSO the solution still contained valine. The solution, freed from ammonia, was therefore fractionated on a cellulose powder column (3.2 × 34 cm) using water-saturated butanol as solvent. PCSO emerged in pure form in fractions 67—85 (12 ml/30 min). It was crystallized from a water-acetone solution. The crystals (30 mg) were small needles. When this substance and synthetic PCSO were run on a two-dimensional chromatogram (butanol-acetic acid-water and phenol-water-NH_3) the spots covered each other completely. (Found: C 40.63; H 7.71. Calc. for $C_6H_{13}O_3NS$: C 40.20; H 7.31.)

The thioether formed from PCSO on reduction was also isolated in crystalline form. On paper chromatographic comparison with synthetic S-*n*-propylcysteine both compounds showed to be identical. (Found: N 8.60. Calc. for $C_6H_{13}O_2S$: N 8.58.)

In order to ascertain that the PCSO isolated from onion is an *n*-propyl and not an *iso*-propyl derivative, S-*iso*-propylcysteine was prepared from L-cysteine and *iso*-propylbromide according to Stoll and Seebeck [3]. It was further oxidized with H_2O_2 to the sulphoxide. When comparing the travelling of PCSO with that of the obtained S-*iso*-propylcysteinesulphoxide on a one-dimensional paper chromatogram (solvent butanol-acetic acid-water), the *iso*-propyl compound was found to move so much slower than PCSO that when the substances had reached the middle of a Whatman sheet the spots separated from each other. Synthetic S-*n*-propylcysteinesulphoxide moved with the same speed as the PCSO isolated from onion. On the basis of this the structure of PCSO can be established as S-*n*-propylcysteinesulphoxide.

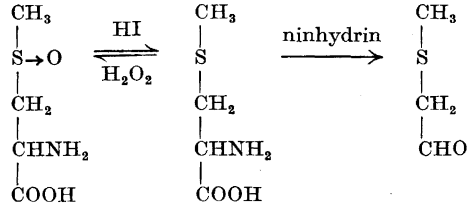
$$CH_3 \cdot CH_2 \cdot CH_2 \cdot SO \cdot CH_2 \cdot CHNH_2 \cdot COOH \underset{H_2O_2}{\overset{HI}{\rightleftharpoons}} CH_3 \cdot CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CHNH_2 \cdot COOH$$

S-*n*-propylcysteinesulphoxide (PCSO) or dihydroalliin

S-*n*-propylcysteine (PCS)

The isolation of MCSO. A methanol extract was prepared from 3 kg of onions in the same way as when PCSO was isolated. Amino acids were also separated on an Amberlite IR-120 column as before. The first fractionation was performed on a Dowex 1 resin (column 2.5 × 83 cm) in 0.5 N acetic acid form. When 150 ml of the eluant had emerged, fractions of 9 ml/20 min were taken. Fractions 3—30 were evaporated and then fractionated with a solvent of butanol-acetic acid-water on a cellulose powder column (5.7 × 80 cm). Because of the thick syrupy consistence the evaporated solution had to be placed on the column in 100 ml, and hence the separation of amino acids was not good. Fractions which contained MCSO and *cyclo*-alliin [4] were evaporated and fractionated on Dowex 50 resin in 0.5 N HCl form (column 3.1 × 112 cm). Fractions of 10 ml/25 min were first taken and then 350 fractions of 15 ml/20 min, *i. e.* 570 fractions in all. The solvent was still 0.5 N HCl. MCSO emerged in pure form in fractions 419—445. It was crystallized with ethanol from water solution. Yield 149 mg. It was identified by paper chromatography with S-methylcysteinesulphoxide which had earlier been isolated in this laboratory from *Capsella bursa pastoris* and chemically characterized. (Found: N 9.23. Calc. for $C_4H_9NSO_3$: N 9.27.) *Cyclo*alliin emerged in fractions 529—560. 4.6 g were obtained as crystals.

The structure of MCSO isolated from onion was confirmed by first reducing it to S-methyl-



S-Methylcysteine-sulphoxide S-Methyl-cysteine Methylthio-acetaldehyde

R_F-Values (descending chromatograms, Whatman No. 1 paper).

	Butanol-acetic acid-water	Phenol-water (NH_3-atm.)
S-Methylcysteinesulphoxide, synth.	0.10	0.73
» from onion	0.10	0.73
S-Methylcysteine	0.30	0.74
S-*n*-Propylcysteinesulphoxide, synth.	0.26	0.81
» from onion	0.26	0.81
S-*n*-Propylcysteine	0.56	0.86
S-*iso*Propylcysteinesulphoxide, synth.	0.23	0.81
S-*iso*Propylcysteine	0.54	0.88
Alanine	0.20	

cysteine and oxidizing this with ninhydrin to S-methylthioacetaldehyde according to Virtanen *et al.*[5]

30 mg of MCSO were added to 1 ml of 57 % HI. The solution was allowed to stand for 3 h after which a small amount of water was added. After extraction with ether the water solution was passed through a column containing Dowex 1 in acetate form (1.3 × 13 cm, resin 200—400 mesh). S-Methylcysteine was eluted with 100 ml of water. The solution was evaporated to dryness *in vacuo*, and the residue washed with 20 ml into a distillation flask which contained 3 g of KH_2PO_4, 4.5 g of NaCl, and 75 mg of ninhydrin. 10 ml of water was added, and the mixture was distilled with steam for 40 min. The distillate, 380 ml, was taken up in 20 ml dimedone solution. The crystals formed during 24 h were separated by filtration. They were recrystallized two or three times from an alcohol-water solution. The crystals melted at 126—127°C. The m. p. of the methylthioacetaldehyde, obtained in a similar way from synthetic S-methylcysteinesulphoxide, was the same, and so was the mixed m. p. The IR-spectra also confirmed the identity of the compounds.

Semi-quantitative estimation of MCSO and PCSO. 100 g of chilled, peeled onions were extracted in 85 % methanol. After 24 h the extract was filtered, and the procedure was repeated twice. The residue was pressed as dry as possible. The combined methanol solutions were passed through an Amberlite IR-120 column, and amino acids were eluted from the resin by 1 N ammonia. Two-dimensional chromatograms were run (butanol-acetic acid-water and phenol-water-NH_3) with different amounts of the evaporated extract. Parallel chromatograms were run with different concentrations of MCSO and PCSO. By comparing the colour of the spots it was possible to calculate that the onions used had contained about 50 μg of PCSO per 1 g of fresh material. The error of determination hardly exceeds ± 10 %. Using the above solvents MCSO travels in much the same way as does glutamine. Another solvent system had therefore to be used in the determination of MCSO. Substituting butanol-acetic acid-water with the solvent butanol-benzylalcohol (1:1)-water, the time of running being 9 days, MCSO could be separated from glutamine. 200 μg of MCSO were then found in 1 g of fresh onion. In another batch of onion 60 μg of PCSO and 240 μg of MCSO were found.

The financial support of the *Rockefeller Foundation* is gratefully acknowledged. Thanks are due to Mrs. Anneli Lehtinen for skilful technical assistance.

1. Virtanen, A. I. *Schweiz. Z. allgem. Pathol. u. Bakteriol.* **21** (1958) 970.
2. Small, L. V. D., Bailey, J. H. and Cavallito, C. J. *J. Am. Chem. Soc.* **69** (1947) 1710.
3. Stoll, A. and Seebeck, E. *Helv. chim. Acta* **32** (1949) 866.
4. Virtanen, A. I. and Matikkala, E. J. *Suomen Kemistilehti B* **29** (1956) 134; Matikkala, E. J. and Virtanen, A. I. *Ibid.* **30** (1957) 219; Virtanen, A. I. and Matikkala, E. J. *Ibid.* **31** (1958) 191; *Acta Chem. Scand.* **13** (1959) 623.
5. Virtanen, A. I., Laine, T. and Toivonen, T. *Z. physiol. Chem. Hoppe-Seyler* **266** (1940) 193; Virtanen, A. I. and Rautanen, N. *Biochem. J.* **41** (1947) 101.

Received October 12, 1959.

Microbiological Activity of Some Mixed Disulfides of Thiamine for *Lactobacillus fermenti*

Z. G. BÁNHIDI

Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Thiamine, its ortho-, and pyrophosphate form (as well as other phosphorylated forms) are equally active for growth promotion of *Lactobacillus fermenti* strain 36 (ATCC 9338) in the absence of cystine and cysteine. [1,2] Under these conditions, however, the homomeric disulfides of thiamine and its phosphorylated derivatives will not be utilized by the bacterial cells. Addition of cysteine, ascorbic acid and other reducing agents or reduction of the disulfides by heating is necessary for these substances to satisfy the absolute requirement of thiamine for *L. fermenti*. *

Our results obtained by growth analysis in living cells, were in full agreement with reports based on enzymatic model systems, in which the homomeric disulfide of thiamine pyrophosphate (cocarboxylase) was found to require activation by reduction for function as coenzyme during anoxidative or oxidative pyruvate-decarboxylation [3-5]. Both the data obtained with our growth test and those of the enzymatic model system must be considered as *per se* counter-evidences against a sulfhydryl-disulfide function of thiamine in the cells, according to

$$2\ T_{ol} \rightleftharpoons T\text{-}SS\text{-}T \quad \ldots\ldots\ldots\ a$$

or more correctly

$$2\ TPP_{ol} \rightleftharpoons TPP\text{-}SS \quad \ldots\ldots\ldots\ b$$

Nevertheless, none of these results exclude the probability that thiamine or its phosphates function as carriers of alien sulfhydryl compounds by forming heteromeric mixed disulfides in connection with some physiological process. Extended work on the biological effect of the mixed disulfides was justified because of the discovery of formation of several allithiamine derivatives in the garlic [6] and the report on the superiority of thiamine propyl disulfide toward thiamine in the treatment of some thiamine deficiency symptoms [7]. For the test, our growth system was used instead of enzymatic models, partly because the mixed disulfides of thiamine are more readily available than those of thiamine pyrophosphate, and because the utilization of a substituted nutrient by a homogeneous population of growing cells offers more general information than an isolated enzymatic model system, which has unfortunately not yet reached the desirable purity.

Experimental. The term »utilization» of some substances related to thiamine was intermittently used by us to denote the growth promoting ability of a compound in a defined medium. In this sense the compound in question was considered as a nutrient and not as an established metabolite. We tacitly assume, however, that the inability of the *L. fermenti* cells to grow on a certain thiamine derivative might depend on several different reasons, *e. g.*, on the cell wall barrier, or on the lack of enzymes

Table 1.

	Thiamine equivalent 2 μg			4 μg		
Thiamine *	40	42	43	36	35	35
T—SS—T	83	82	81	81	82	84
T—SS—T incubated with cell mash of *L. fermenti*	90	92	94	85	84	97
as above plus cysteine added	40	39	38	35	33	35
T—SS—T plus cysteine	38	38	50	38	37	39
T—SS—T incubated with intact cells of *L. fermenti*	90	90	91	87	86	91
as above plus cysteine added	66	93	34	47	59	42
The pH of incubation	*4.5*	*6.0*	*8.0*	*4.5*	*6.0*	*8.0*

* Numerical values are means of two turbidimetric readings at each concentration and are expressed as per cent of light transmitted at 5 950 Å.

* *Abbreviations* not explained in the legend of Fig. 1.: The thiol form of thiamine (or its derivatives) T_{ol}; the thiazole form of the same, T_{tz}; the homomeric disulfide of thiamine T—SS—T, and that of thiamine pyrophosphate TPP—SS, while cocarboxylase is denoted as TPP.

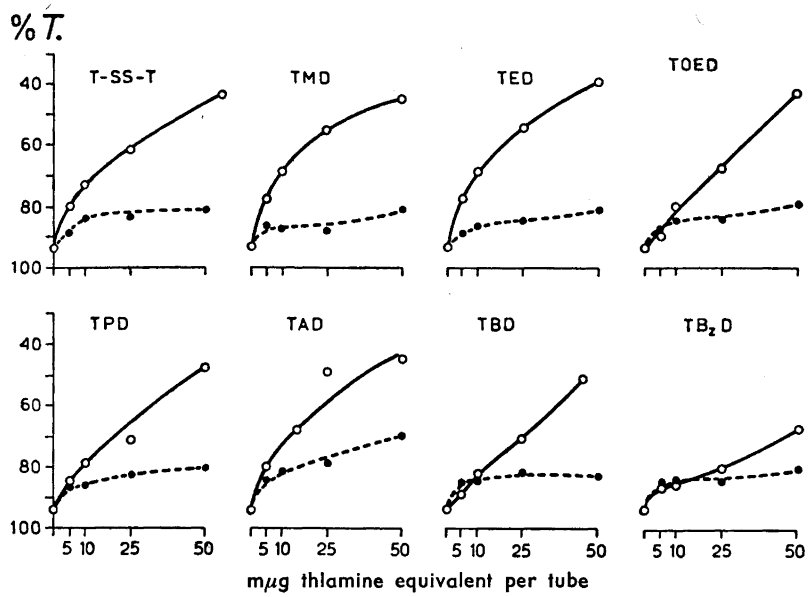


Fig. 1. Growth test of different disulfide derivatives of thiamine with *Lactobacillus fermenti* strain 36 on fluid medium. Abbreviations not occurring in the text are in accordance with the terminology proposed by Matsukawa [13], *viz.*, TMD, thiamine methyl disulfide; TED, thiamine ethyl disulfide; TOED, thiamine hydroxyethyl disulfide; TPD, thiamine propyl disulfide. TAD, thiamine allyl disulfide; TBD, thiamine butyl disulfide; TB_ZD, thiamine benzyl disulfide. Dotted lines represent test in absence of cysteine and full lines were used when 500 μg cysteine was added to each ml of the medium. All thiamin derivatives were added aseptically to the autoclaved medium in order to protect the disulfides from reduction. Growth was measured turbidimetrically in dublicates, and expressed as per cent transmittance (% *T.*).

able to convert the disulfides to a form available for thiaminokinases.

With these possibilities in mind we tried to split the homomeric disulfide of thiamine with the aid of cell free extracts of *L. fermenti* as well as with large amounts of living bacterial cells.

One day old culture of *L. fermenti*, grown on a complete medium (micro inoculum broth, Difco) was washed twice in saline, frozen in acetone-dry ice mixture and subjected to desintegration, according to Hughes [6]. Disruption of the cells was registered microscopically. Ten mg of the homogenized cell mash was Seitz-filtered and incubated with one mg of T—SS—T at 37°C for 30 min in 1/15 M phosphate buffer at different pH's, as seen in Table 1.

Intact washed cells were also subjected to incubation with T—SS—T for comparison. In each case 10 mg wet cell sediment was used as starting material and combined with 1 mg T—SS—T in 5 ml buffer solution. Similar batches were supplemented with 400 μg cysteine, for controls. The batches, containing intact cells, were Seitz-filtered after incubation in order to ensure aseptic test. Activity tests with *L. fermenti* were performed, according to former experiments on fluid media lacking cysteine [9,2] by using suitable dilutions of the above preparations. The results show that neither the cell mash nor the intact cells were able to liberate thiamine activity from T-SS-T under conditions where cysteine easily performed the activation of T—SS—T to growth factor for *L. fermenti*.

An enzymatic test which shows inability of cells or cell free preparations to perform an expected reaction must be considered cautiously since physical conditions might have been unfavorable during the experiment. In the present case, however, we are dealing with a substrate (T—SS—T) which is labile enough

at the conditions used (at least at slightly basic pH) to be easily converted to free thiamine as was shown by controls containing cysteine.

As an extension of the enzymatic work, *L. fermenti* was cultivated on the synthetic basal medium supplemented with thiamine sufficient for more than half-maximum growth. Cells were then harvested and washed twice by centrifuging and Seitz-filtration in order to make available the thiamine factors of the bacteria for analysis. Batches of the cell mash were chromatographed on paper in several solvent systems and afterwards developed bioautographically according to the procedure detailed in the subsequent paper [10]. The bioautographs of the bacterial cell mash revealed the presence of four distinct spots tentatively identified as thiamine pyrophosphate, thiamine orthophosphate, free thiamine-thiazole and a minor fraction (less than about one tenth of any of the former) behaving in all respect as T−SS−T.

An explanation for the contradiction between the cell mash experiment showing inability to split T−SS−T and the bioautographic analysis showing the presence of minute but distinct zones of T−SS−T in the cell mash, would be the theory that T−SS−T represents the end product of the thiamine/cocarboxylase metabolism.

In the hope of obtaining more information about the possible role of the disulfides of thiamine in the cells, experiments were performed with some mixed disulfides of thiamine.

The disulfides of thiamine subjected to microbial growth test were T−SS−T (prepared according to Zima *et al* [11] and seven different allithiamine homologues prepared by and obtained from Matsukawa [12,13]. Description of the compounds, explanation of abbreviations and the results obtained are given in Fig. 1. The conditions of the tube test for growth of *L. fermenti* on a chemically defined basal medium as well as details of cultivation of stock culture and the technique of turbidimetric readings were the same as in our earlier work [9,2]. Each substance was tested in both cysteine free medium and with added cysteine. Under these conditions thiamine was active in both cases giving quantitative results rather similar to that of T−SS−T with cysteine. (For this reasons thiamine values were left out from Fig. 1.) On the other hand only six of the thiamine alkyl disulfides supported the growth of *L. fermenti* to an extent comparable with T−SS−T on molar basis. All these derivatives required, however, activation by cysteine in a similar way as T−SS−T, demonstrating that the mixed disulfides of thiamine tested in this work, are not directly utilizable by the cells of *L. fermenti*. Thiamine benzyl disulfide showed very little increase in activity after addition of cysteine. This suggests the presence of a structural hindrance in the molecule for splitting the disulfide linkage with cysteine or for replacement of the benzylmercaptan with the same.

Conclusion. Intact or disrupted cells of *Lactobacillus fermenti* are probably not able to liberate thiamine from thiamine disulfide

Several different alkyldisulfides of thiamine behave similary to the homomeric disulfide of thiamine in a growth test with *L. fermenti* and serve as nutrient for the cells only after activation by cysteine or other reducing agents in contrast to thiamine, which supports growth without activation. Thiamine benzyl disulfide resists cysteine activation.

Acknowledgements. The author is indebted to Professor T. Matsukawa and the Takeda Pharmaceutical Industries, Ltd., Juso-Nishinocho Higasiyodogawa-ku, Osaka (Japan) for the generous gift of the allithiamine derivatives used in this work. The valuable assistance of Mrs Eva Börje-Lööv, chem. eng., is gratefully acknowledged. This work was sponsered by *The Swedish Natural Science Research Council* and by the *Faculty of Mathematics and Natural Sciences* of the *University of Stockholm.*

1. Bánhidi, Z. G. *Biochem. Z.* **332** (1959) 77.
2. Bánhidi, Z. G. *J. Bacteriol.* **79** (1960). *In press.*
3. Karrer, P. and Viscontini, M. *Helv. Chim. Acta* **29** (1946) 711 and 198.
4. Engelhardt, V. A. and Kanopkaite, S. I. *Biochimija* **22** (1957) 21.
5. von Holt, C., Wiethoff, E. O., Kröner, B., Leppla, W. and von Holt, L. *Biochem. Z.* **329** (1957) 119.
6. Matsukawa, T., Yurugi, Sh. and Matsuoka, T. *Science* **118** (1953) 325.
7. Inouye, K. *J. Vitaminol.* **4** (1958) 68.
8. Hughes, D. E. *Brit. J. Exptl. Pathol.* **32** (1951) 97.
9. Bánhidi, Z. G. *Acta Chem. Scand.* **12** (1958) 517.
10. Bánhidi, Z. G. *Acta Chem. Scand.* **13** (1959) 1904.
11. Zima, O., Göttmann, G., Hoffmann, A., Hepding, L. and Hotovy, R. E. *Merck's Jber. Pharm.* **67** (1953) 1.
12. Matsukawa, T., Kawasaki, H., Iwatsu, T. and Yurugi, Sh. *J. Vitaminol.* **1** (1954) 13.
13. Matsukawa, T. *J. Vitaminol.* **4** (1958) 65.

Received October 26, 1959.

Thiamine Pantetheine Disulfide as a Microbial Growth Factor

Z. G. BÁNHIDI

Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

In connection with our study on the nutritional requirements of *Lactobacillus fermenti*, strain 36 (ATCC 9338), a growth enhancement with thiamine was observed, when yeast extract or pantethine was added to the basal medium [1]. The stimuli could, however, be compensated by addition of cysteine or other reducing agents to the medium. *

More detailed investigation showed later that both the homomeric disulfide of thiamine and several mixed disulfides of the allithiamine type are inactive as growth factors for *L. fermenti*, except when reduced or treated with cysteine [2-4]. It remained to establish whether a postulated thiamine pantetheine disulfide could account for the observed enhancement with pantethine. If so, this would require rather different physiological aspects for the thiamine pantetheine derivative when compared with the allithiamines.

Experimental. The mixed disulfide of the thiol form of thiamine and pantetheine (the mercaptoethanolamine of pantothenic acid) was first mentioned by Sahashi *et al.*[5] who observed a new spot on the chromatograms of a mixture of the two vitamins. It was presumed that the two compounds form a mixed disulfide:

$$\text{T}-\text{SS}-\text{T} + 2\,\text{LBF}-\text{SH} \rightleftharpoons 2\,\text{T}-\text{SS}-\text{LBF} \quad \ldots a$$

We prepared T−SS−LBF through incubation of equimolar amounts of the parent compounds (on sulfhydryl basis) in a 1/15 M phosphate buffer solution of pH 7.5−8.5 for 20−30 min at 37°C.

T−SS−T was prepared according to Zima *et al.*[6] After repeated recrystallization from aqueous acetone the product was tested bioautographically for purity as below.

Commercial pantethine (Nutritional Biochemicals Inc.) was reduced to LBF−SH by forcing the solution of 100 mg LBF−SS in 5 ml 0.5 N H_2SO_4 through a 0.8 × 12 cm column of freshly amalgamated 20 mesh zinc granules (Jones reductor) with the aid of nitrogen gas, according to the reduction of coenzyme-A by Buyske *et al.*[7]

In analogy with the work of Matsukawa and Yurugi [8] on the thiamine cysteine mixed disulfides we prepared T−SS−LBF also through

$$2\,\text{T}_{\text{ol}} + \text{LBF}-\text{SS} \rightleftharpoons 2\,\text{T}-\text{SS}-\text{LBF} \quad \ldots\ldots b$$

Chromatographic analysis and growth test gave similar results with both preparations according to equations *a* and *b*, in agreement with the presumed analogy.

The reaction mixtures were subjected to paper chromatographic separation in several solvent systems (*viz.*, *n*-butanol : water : acetic acid 125:125:30; *n*-butanol: ethanol : acetic acid:water 4:1:1:10; *iso*-butanol: pyridine : water 3:2:5; and *tert.*-butanol : water : methylethylketone 2:2:1). Ascending chromatograms were carefully dried in hot air current, and developed bioautographically by laying on the surface of large agar plates in which the basal medium was completed with 2 % agar and seeded with a heavy suspension of *L. fermenti*. The technique was otherwise similar to our agar cup plate method described elsewhere [9].

Separation of the T−SS−T reaction mixture resulted in more than two spots as shown in the corresponding columns of Fig. 1. This was also the case with the other solvent systems used. Among the spots the factor next to the fastest moving component was identified as T−SS−T by its R_F, and by its disappearance on the bioautograms which were developed with a basal medium lacking cysteine. Under these conditions all components other than T−SS−T were active including the fastest moving factor. Thus the fastest moving factor behaved differently as the starting materials used for the synthesis, or as the other predictable compounds of known structure (*e. g.*, T−SS−T) and must be considered to be the actual T−SS−LBF complex.

Meanwhile, the slowest moving spots of columns II and III in Fig. 1, are evidently representing the thiol form of thiamine since this appears only at slightly alkaline pH according to the equilibrium:

$$\text{T}_{\text{tz}} \rightleftharpoons (\text{T}_{\text{pseudobase}}) \rightleftharpoons \text{T}_{\text{ol}} \quad \ldots\ldots\ldots c$$

Considering eqn. *c* as well as eqns. *a* and *b*, the partition in column III reflects the simultaneous presence of all factors of the three equilibria.

* *Abbreviations:* T, T_{tz}, T_{ol} and T−SS−T denote thiamine, the thiazole (amino) form of thiamine, the thiol form of the same, and the homomeric disulfide form. For pantethine and pantetheine, respectively, the letters LBF−SS and LBF−SH were applied in view of the early name of these factors (Lactobacillus Bulgaricus Factor) while the mixed disulfide of thiamine with pantetheine is represented with T−SS−LBF.

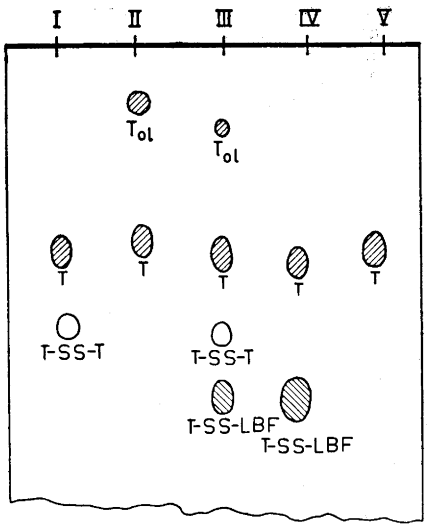


Fig. 1. Selective bioautography of thiamine pantethein disulfide (T—SS—LBF). Ascending chromatography on Whatman No. 1 paper in *n*-butanol : ethanol : acetic acid : water 4:1:1:10 solvent system (upper layer). Bioautographic development was made with *L. fermenti* 36 by using media with or without cysteine. Unshaded zones do not support growth in absence of cysteine.

Attention should be paid in this connection to the fact that in the agar plate bioautography, T—SS—LBF is active for *L. fermenti* both in presence and in absence of cysteine. This is also the case with the thiol form of thiamine, the activity of which could not be decided with the tube test [3].

The fastest moving spot of T—SS—LBF marked in column III of Fig. 1, was subjected to elution with ethanol water mixture and rechromatographed. Column IV of Fig. 1 shows that thiamine will be easily liberated from the eluate despite the mild conditions of the elution and that of the chromatographic procedure. Also slight acidity or addition of traces of cysteine will shunt reaction *b* to the left.

Batches of the eluate of the T—SS—LBF spot were subjected to quantitative test with the agar cup plate method and the tube method using *L. fermenti*. Analysis in absence and in presence of cysteine in the medium showed that T—SS—LBF i fully active as a growth factor (tube test in Fig. 2) in contrast to our experience with the homomeric and heteromeric disulfides of thiamine [4]. It is, however, not decided at present whether the thiamine like activity of thiamine pantetheine disulfide can be considered as a physiological peculiarity or should be attributed to the chemical lability of this compound.

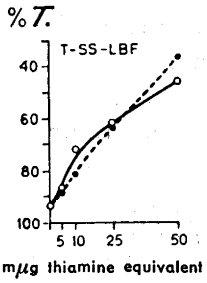


Fig. 2. Quantitative test of thiamine pantetheine disulfide with *L. fermenti* 36 on fluid media with 500 μg cysteine per ml and without cysteine (the latter marked with dotted line). Growth was measured turbidimetrically in dublicates at 5 950 Å and is expressed in per cent transmittance (% *T.*).

Conclusion. Thiamine pantetheine disulfide functions as a growth factor for *Lactobacillus fermenti* similarly to the thiazole and thiol forms of thiamine under experimental conditions which are unfavourable for the utilization of thiamine disulfide or allithiamine analogues.

Acknowledgements of the author are given to Mrs. Eva Börje-Lööv, chem. eng., for her valuable assistance and to the *Swedish Natural Science Research Council* and *Faculty of Mathematics and Natural Sciences of the University of Stockholm* for financial support.

1. Bánhidi, Z. G. *Acta Chem. Scand.* **12** (1958) 517.
2. Bánhidi, Z. G. *Biochem. Z.* **332** (1959) 77.
3. Bánhidi, Z. G. *J. Bacteriol.* **79** (1960). *In press.*
4. Bánhidi, Z. G. *Acta Chem. Scand.* **13** (1959) 1901.
5. Sahashi, Y., Funahashi, S., Yamashita, K. and Akatsuka, T. *J. Biochem.* (*Tokyo*) **41** (1954) 463.
6. Zima, O., Göttmann, G., Hoffmann, A., Hepding, L., and Hotovy, R. E. *Merck's Jber. Pharm.* **67** (1953) 1.
7. Buyske, D. A., Handschumacher, R. E., Schilling, E. D. and Strong, F. M. *J. Am. Chem. Soc.* **76** (1954) 3575.
8. Matsukawa, T. and Yurugi, Sh. *Science* **118** (1953) 109.
9. Bánhidi, Z. G. *Analyst* **84** (1959).

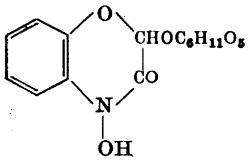
Received October 26, 1959.

The Precursors of 6-Methoxybenzoxazolinone in Maize and Wheat Plants, their Isolation and Some of their Properties

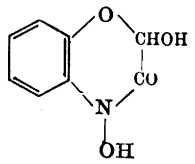
ÖRN WAHLROOS and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

In preliminary papers from this laboratory it was reported that 6-methoxybenzoxazolinone (MBOA) and benzoxazolinone (BOA), which were earlier isolated from wheat and maize plants (MBOA) and from rye plants (BOA), were formed from precursors in these plants [1]. A glucoside $C_{14}H_{17}O_9N$ was isolated in crystalline form from rye seedlings after inactivation of the enzymes present in intact plants. From this glucoside an aglucone $C_8H_7O_4N$ was enzymatically formed when fresh plants were crushed [2]. For the glucoside and the aglucone the following structures have been presented[3] by Virtanen and Hietala[3].



Glucoside
$C_{14}H_{17}O_9N$



Aglucone
precursor of BOA
2,4-dihydroxy-1,4-benzoxazin-3-one

The glucoside

The isolation of the glucoside from maize seedlings. 2.8 kg fresh weight of maize seedlings (grown in a greenhouse, about 10 days old, plants about 10 cm high, green parts) were put into 7 l of boiling water, keeping the temperature of the water above 80°C. When all seedlings were added (about 15 min), the mixture was cooled with tap water. It was filtered, the seedlings were homogenized in water of room temperature and filtered. The combined filtrates were evaporated *in vacuo* to about 0.5 l. The concentrate was extracted six times with *n*-butanol. The butanol extract was evaporated *in vacuo*, water was added to 0.5 l, and the suspension was extracted five times with ether. 56 g of carbon were added to the water phase (the carbon had previously been washed with 2 N hydrochloric acid and water, deactivated with cyanide according to Schramm and Primosigh [4] and washed with water). It was mixed for 30 min and filtered (washed cellulose powder as filter aid). The carbon was washed on the filter with 0.5 l of distilled water, and was eluted with 1.5 l of a mixture containing benzene, methanol, and water 3:7:1 v/v. The eluate was evaporated to dryness *in vacuo*, the residue dissolved in a small amount of water, and chromatographed on a polyamide column (5.5 × 34 cm) using water as solvent. The polyamide powder was mixed into water and allowed to stand for 2 h, occasionally stirring, before it was poured into the column. It was washed in the column with 700 ml of methanol and 1.5 l of water before chromatographing. At the solvent front a small amount of ninhydrin-positive material appeared, and immediately after that the precursor. Other UV-absorbing material remained in the column. The red coloured fractions containing the precursor were pooled, evaporated to dryness *in vacuo* and rechromatographed on a cellulose powder column (7.5 × 27 cm) with *n*-butanol saturated with water. The cellulose powder was washed with 2 N hydrochloric acid, distilled water, hydroxy-quinoline-containing methanol, to remove heavy metal ions, and methanol, and was air-dried before packed into the column. First a red front emerged from the column. It contained part of the precursor. After it a colourless front emerged, containing the precursor. This crystallized from the colourless fraction when evaporated *in vacuo*. It was recrystallized from absolute ethanol. Yield: 1.23 g of red material, containing a large amount of the precursor, 1.22 g of crystalline crude preparation (pink), and 0.143 g of colourless narrow prisms, m. p. 168—170°C (decomp.).

During the isolation of the glucoside its presence in different fractions was followed by treating the samples with fresh, crushed wheat seedlings, after which MBOA was determined. The homogenate was prepared by grinding 20 g of 7 to 10-day-old wheat seedlings in a homogenizer with 50 ml of water, and was sieved to obtain an even suspension. Each sample to be tested for the glucoside was mixed with 10 ml of this suspension. 10 ml of the suspension was used as a blank. The samples were allowed to stand for 30 min to 1 h at room temperature. They were kept in boiling water for 15 min and filtered. The filtrates were extracted with equal amounts of ether. Equal parts of the ether extracts were chromatographed one-dimensionally on paper with the solvent *n*-propanol-ammonia (25 %), 7:3. Pieces of the

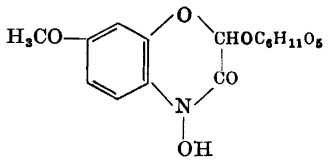
same size were cut out between R_F-values 0.6 and 0.8. They were eluted with equivalent volumes of absolute ethanol. The UV-spectra between 230 and 310 mμ were measured against the ethanol eluate of the chromatographic spot of the blank. The spectrum thus obtained shows the MBOA formed from the precursor in the preparation added.

A comparison of the glucoside in maize and wheat plants. Wheat seedlings (grown in a greenhouse, about 10 days) were put into boiling water. They were cooled, ground in water of room temperature, and filtered. The filtrate was extracted with ether and the waterphase remaining six times with *n*-butanol. A butanol extract was prepared in the same way from maize seedlings. Two-dimensional chromatograms were made from the butanol extracts obtained from maize and wheat, as well as from their mixture, with the solvents water-saturated *n*-butanol and 2 % acetic acid. In this solvent system the glucoside of maize and wheat moved as one spot. By chromatography it was also established that this precursor is not present in wheat seeds.

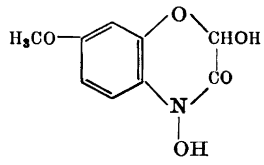
Properties of the glucoside. When dissolved in 25 % ammonia, the glucoside turns dark brown in a day. With ferric chloride it gives a blue colour. The UV-spectrum in water: Max. 270 mμ (K = 29.6). A knee at 282–283 mμ (K = 27.6). Min. 237 mμ.

Identification of the sugar part: The glucoside was kept for 2 h in a boiling water bath in 2 N hydrochloric acid. It was cooled, and extracted with ether. In the water phase the chloride-ions were exchanged to acetate-ions by pouring through a Dowex 1 column in acetate form. The effluent was evaporated to dryness *in vacuo*, dissolved in a small amount of water, and chromatographed parallel with glucose on Whatman No. 4 paper (aniline hydrogen phthalate spray). The sugar formed on hydrolysis was characterized as glucose by using different solvent systems: a) water-saturated *n*-butanol (ascending), b) *n*-butanol-glacial acetic acid-water, 63 : 10 : 27 v/v (descending), and c) water-saturated phenol (descending).

The nitrogen content of the glucoside (Kjeldahl determination, using permanganate). (Found: N 4.13. Calc. for $C_{15}H_{19}O_{10}N$: 3.85.) The methoxy content of the glucoside: 8.1 %, calc. for $C_{15}H_{19}O_{10}N$: 8.3. The molecular weight calculated from the obtained methoxy value was 382, calc. for $C_{15}H_{19}O_{10}N$: 373.



Glucoside
$C_{15}H_{19}O_{10}N$



Aglucone
precursor of MBOA
2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one

The growth-inhibiting effect of the glucoside on *Fusarium nivale* was negligible. 6 mg per 1 ml of oat-glycerol nutrient solution, pH 6.5–6.8, caused about 50 % inhibition.

The aglucone

The isolation of the aglucone. 400 g fresh weight of maize seedlings (grown as mentioned above) were treated in a juice press. After 30 min the press juice was centrifuged for 40 min at 10 000 *g* and a temperature between 0 and 10°C. The clear supernatant was decanted and extracted twice with ether. The ether solution was dried for about 2 h with sodium sulphate, filtered, and evaporated at room temperature to a small volume. The precipitate thus obtained was recrystallized several times from methanol and then from an ether-*cyclo*hexane mixture. 75 mg of pink needle-like crystals, m. p. 156–157°C (decomp., gas evolution), were obtained.

Properties of the aglucone. Conversion into MBOA: A solution of the aglucone was made up in water to a concentration of 10 μg per ml. The UV-spectrum was measured: Maxima at 267 mμ (K = 34.7) and 292 mμ (K = 32.5), minima at 237 mμ (K = 5) and 280 mμ (K = 29.4). This solution was heated under reflux for 30 min in a boiling water bath and its UV-spectrum was measured once more.

Table 1. The growth-inhibiting effect of the aglucone on *Fusarium nivale*. Oat-glycerol nutrient solution, pH 6.5–6.8. Numbers refer to the diameter of the colony in mm.

Concentration mg/ml	Growth +						
	1	2	3	4	5	6	days
0 (contr.)	12	14	14	16	22	29	
0.5	10	10	14	15	16	22	
1.0	?	+	+	+	+	+	
1.5	+?	+?	+?	0	0	0	
2.0	0	0	0	0	0	0	
2.5	0	0	0	0	0	0	
3.0	0	0	0	0	0	0	

Table 2. R_F-values of the glucoside and the aglucone. Whatman No. 4 paper.

Solvent	R_F	
	The glucoside	The aglucone
H_2O-sat.*n*-BuOH	0.4–0.6 (tailing)	0.72–0.78
n-BuOH:AcOH:H_2O, 8:2:2	0.33	0.81
n-BuOH:MeOH:Bz:H_2O, 3:1:1:1	0.53	0.84
2 % AcOH in water	0.90	0.74
n-PrOH:NH_3(25 %), 7:3	0.30	decomposes

The spectrum then obtained was identical with the spectrum of MBOA, $E_{286} = 0.266$. Because the molar extinction of MBOA is 5 690 this absorbtion corresponds to 7.7 μg of MBOA per ml. From this concentration and that of the aglucone the following molecular weight for the aglucone is obtained: $\frac{10}{7.7} \cdot 165 = 214$, calc. for $C_9H_9O_5N = 211$.

When dissolved in 25 % ammonia, the aglucone turns yellow-brown within a few minutes. After 30 min the solution is green, and after about 2 h it is dark brown. This solution was chromatographed, and one of the products was chromatographically and spectrophotometrically identified with MBOA. With ferric chloride the aglucone gave a blue-violet colour.

The nitrogen content of the aglucone was 6.91 %, calc. for $C_9H_9O_5N$: 6.63. The methoxy content of the aglucone was 15.01 %; the molecular weight calculated from the methoxy content was 207, calc. for $C_9H_9O_5N$: 211.

The growth-inhibiting effect of the aglucone is nearly as high as that of MBOA and BOA (Table 1). The effect is mainly fungistatic. R_F-values can be seen in Table 2.

The support given by the *Rockefeller Foundation* is gratefully acknowledged.

1. Virtanen, A. I. and Wahlroos, Ö. *Suomen Kemistilehti B* **31** (1958) 402.
2. Virtanen, A. I. and Hietala, P. K. *Ibid.* **32** (1959) 38, 138.
3. Virtanen, A. I. and Hietala, P. K. *Ibid.* **32** (1959) 252.
4. Schramm, G. and Primosigh, J. *Ber.* **76** (1943) 373.

Received October 20, 1959.

Mixed Solvent Effect with Dibutyl Phosphate

Modification of a Method[1] for the Preparation of Carrier-free Yttrium-90

DAVID DYRSSEN and SOLVEIG EKBERG

Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Dialkyl phosphoric acids have been used[1,2] for the separation of ^{90}Y from its parent ^{90}Sr. Dibutyl phosphate (= HA) is preferred by us since the equilibrium constant, $K = [YA_3(HA)_3]_{org}\,[Y^{3+}]^{-1}[H^+]^3[H_2A_2]_{org}^{-3}$, is larger for the dibutyl phosphate (DBP) – chloroform system[1] (log $K = 3.22 \pm 0.13$) than for the dioctyl phosphate-toluene system[3] (log $K \sim 1.3$), and since the equilibria for DBP have been more fully investigated[4,5]. Dioctyl phosphate (DOP) has a lower solubility in water[3] than DBP due to the fact that it contains larger hydrocarbon groups. Consequently, DOP will have a larger distribution ratio than DBP. However, with chloroform the distribution ratio of DBP is 74 for a 0.1 M solution, and the loss of DBP into 0.1 M HNO_3 is not a serious problem.

This suggested modification of the method previously given by Dyrssen[1] allows ^{90}Y to be back-extracted into HNO_3 with better recovery at lower acidity and less contamination of DBP. This is accomplished by the addition of methyl *iso*butyl carbinol (hexol) to the chloroform phase containing 0.1 M DBP and the ^{90}Y extracted from $^{90}Sr + {}^{90}Y$ in 0.1 M HNO_3.

This mixed solvent effect may be explained by the formation of a 1 : 1 complex between DBP (HA) and hexol (B) which reduces the concentration of HA and H_2A_2, and thus the extraction of Y^{3+} according to equations given below. There is no reason to believe that a trialkyl phosphate is formed, *i.e.* $(BuO)_2((CH_3)_2CHCH_2CH(CH_3)O)PO$, in the presence of an aqueous phase. These conclusions were drawn from a study[5] of the extraction of Eu^{3+} and Am^{3+} by DBP in different solvents carried out at this institute, as well as a study[5] of DBP itself in the same set of solvents.

The effect of addition of hexol on the distribution of ^{90}Y (org/aq) is shown in Table 1. It is possible from these data to calculate $[H_2A_2]_{org}$ from

$$[H_2A_2]_{org}^3 = qK^{-1}[H^+]^3 \,;\, \log K = 3.03$$

and C_B from the known volume of hexol added:

$$C_B = v(v + 15)^{-1}\,7.95$$

Using the following relations

$$C_B = [HAB]_{org} + [B]_{org}$$

$$0.1 \cdot 15(v + 15)^{-1} = 2\,[H_2A_2]_{org} + [HAB]_{org}$$

$$10^{4.61} = [H_2A_2]_{org}[HA]_{org}^{-2} \quad \text{(Ref.}^5\text{)}$$

we could calculate the value of

$$K_B = [HAB]_{org}[HA]_{org}^{-1}[B]_{org}^{-1}$$

as log $K_B = 2.21$ for hexol and DBP in chloroform. This value is larger than for TBP in chloroform[1], probably because TBP and $CHCl_3$ form a complex.

With these constants and equations it is possible to calculate the distribution of ^{90}Y and DBP for the following two pairs of organic–aqueous solvents (*cf.* Fig 1):

0.1 M DBP in $CHCl_3$/0.1 M HNO_3:

$$\log q_Y = +2.13$$
$$\log q_A = +1.87$$

hexol + 0.1 M DBP in $CHCl_3$ (1 + 5)/1 M HNO_3:

$$\log q_Y = -3.43$$
$$\log q_A = +2.58$$

Recommended procedure: 5 ml of a 0.1 M HNO_3 solution containing ^{90}Sr is shaken

Table 1. Distribution of ^{90}Y at 25°C between 15 ml 0.1 M DBP in chloroform (alcohol-free) and 15 ml 0.1 M HNO_3, for various amounts of hexol added. The two phases were shaken for one minute by hand and then separated by centrifugation.

ml hexol	0	0.05	0.1	0.2	0.5	1	2	5
log q	(+2.13)[a]	+2.055	+2.012	+1.897	+1.523	+1.040	+0.154	−1.655

[a] extrapolated value, measured value in Ref.[5]: 2.17.

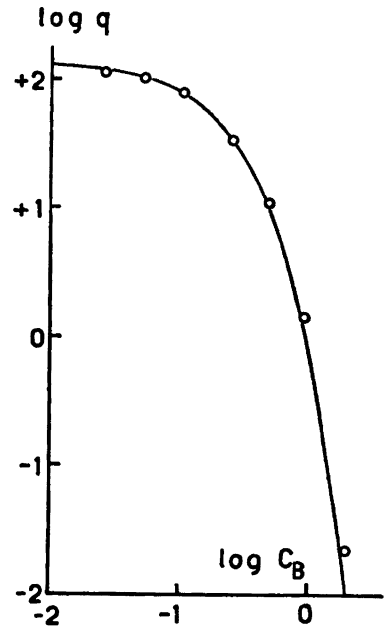


Fig. 1. Distribution of ^{90}Y between a solution of 0.1 M DBP in chloroform and 0.1 M HNO_3 as a function of the conc. of hexol in the chloroform phase. The curve is calculated with equations in the text assuming that the lowering of q is due to formation of a complex between DBP and hexol.

with 5 ml of 0.1 M DBP in alcohol-free chloroform. The amount of ^{90}Sr extracted is less than 10^{-2} %. This amount may be reduced by a factor of 10^4 by washing the organic layer with 5 ml of 0.1 M HNO_3; 1 % of ^{90}Y is lost for each wash. The ^{90}Y is quantitatively back-extracted into 5 ml of 1 M HNO_3 after the addition of 1 ml of methyl *iso*butyl carbinol (hexol). The aqueous phase then contains about 0.0003 M DBP. The DBP conc. can, however, be lowered by a factor of 400 by washing the 1 M HNO_3 phase with 1 ml of hexol + 5 ml of chloroform. In this way the conc. of DBP can be reduced to a very low level and the final solution will only contain volatile substances (HNO_3), water, chloroform and hexol) in addition to the ^{90}Y.

The authors wish to thank professor Lars Gunnar Sillén for his comments on this work, which has been supported by *Statens råd för atomforskning. (Swedish Council for Atomic Research)*. The English has been revised by Mr. Fred Holmes.

1. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1277.
2. Peppard, D. F., Mason, G. W. and Moline, S. W. *J. Inorg. & Nuclear Chem.* **5** (1957) 141.
3. Peppard, D. F. et al. *J. Inorg. & Nuclear Chem.* **4** (1957) 334; **7** (1958) 276.
4. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1771.
5. Dyrssen, D. and Liem Djiet Hay. *Acta Chem. Scand.* **14** (1960). *In the press.*

Received October 29, 1959.

The Composition of Rat Milk Fat

AAGE JART, J. P. FUNCH and H. DAM

Danish Fat Research Institute, Copenhagen, Denmark

Rats are extensively used in experiments on nutrition, *e.g.* for the study of the role of fats. It is therefore of interest to know the composition of the fat in the milk consumed by the suckling young rat.

Investigations on this problem have been carried out by Hallanger and Schultze [1], who found it possible to increase the content of polyenoic acids (mainly dienoic) by increasing the content of such fatty acids in the diet.

The aim of the present study was to determine not only the polyenoic fatty acids but also the chain length of the fatty acids in milk fat of rats receiving the stock diet commonly used in our laboratory.

To study this problem the fat fraction of rat milk was isolated and converted into methyl esters which were analyzed by means of gas-liquid chromatography [2]. The column consisted of 0.9 m Celite 545 with silicone elastomer E 301. The carrier gas used was nitrogen at a rate of 1 l per hour, and the temperatures applied ranged from 160°C to 225°C. Assuming the areas lying under the recorded curves to be proportional to the molar percentages of the individual components, the following composition (Table 1) was found (in per cent w/w of the fatty acid mixture).

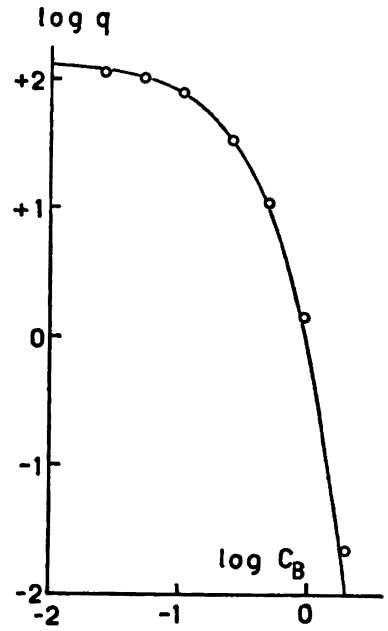


Fig. 1. Distribution of ^{90}Y between a solution of 0.1 M DBP in chloroform and 0.1 M HNO_3 as a function of the conc. of hexol in the chloroform phase. The curve is calculated with equations in the text assuming that the lowering of q is due to formation of a complex between DBP and hexol.

with 5 ml of 0.1 M DBP in alcohol-free chloroform. The amount of ^{90}Sr extracted is less than 10^{-2} %. This amount may be reduced by a factor of 10^4 by washing the organic layer with 5 ml of 0.1 M HNO_3; 1 % of ^{90}Y is lost for each wash. The ^{90}Y is quantitatively back-extracted into 5 ml of 1 M HNO_3 after the addition of 1 ml of methyl *iso*butyl carbinol (hexol). The aqueous phase then contains about 0.0003 M DBP. The DBP conc. can, however, be lowered by a factor of 400 by washing the 1 M HNO_3 phase with 1 ml of hexol + 5 ml of chloroform. In this way the conc. of DBP can be reduced to a very low level and the final solution will only contain volatile substances (HNO_3), water, chloroform and hexol) in addition to the ^{90}Y.

The authors wish to thank professor Lars Gunnar Sillén for his comments on this work, which has been supported by *Statens råd för atomforskning. (Swedish Council for Atomic Research)*. The English has been revised by Mr. Fred Holmes.

1. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1277.
2. Peppard, D. F., Mason, G. W. and Moline, S. W. *J. Inorg. & Nuclear Chem.* **5** (1957) 141.
3. Peppard, D. F. et al. *J. Inorg. & Nuclear Chem.* **4** (1957) 334; **7** (1958) 276.
4. Dyrssen, D. *Acta Chem. Scand.* **11** (1957) 1771.
5. Dyrssen, D. and Liem Djiet Hay. *Acta Chem. Scand.* **14** (1960). *In the press.*

Received October 29, 1959.

The Composition of Rat Milk Fat

AAGE JART, J. P. FUNCH and H. DAM

Danish Fat Research Institute, Copenhagen, Denmark

Rats are extensively used in experiments on nutrition, *e.g.* for the study of the role of fats. It is therefore of interest to know the composition of the fat in the milk consumed by the suckling young rat.

Investigations on this problem have been carried out by Hallanger and Schultze [1], who found it possible to increase the content of polyenoic acids (mainly dienoic) by increasing the content of such fatty acids in the diet.

The aim of the present study was to determine not only the polyenoic fatty acids but also the chain length of the fatty acids in milk fat of rats receiving the stock diet commonly used in our laboratory.

To study this problem the fat fraction of rat milk was isolated and converted into methyl esters which were analyzed by means of gas-liquid chromatography [2]. The column consisted of 0.9 m Celite 545 with silicone elastomer E 301. The carrier gas used was nitrogen at a rate of 1 l per hour, and the temperatures applied ranged from 160°C to 225°C. Assuming the areas lying under the recorded curves to be proportional to the molar percentages of the individual components, the following composition (Table 1) was found (in per cent w/w of the fatty acid mixture).

Table 1.

Acid	%
C_7	<0.05
C_8	2
C_9	0.1
C_{10}	6
C_{11}	0.15
C_{12}	4
C_{13}	0.1
C_{14}	6
C_{15}	0.5
C_{16}	22
C_{17}	0.1
C_{18}	59

It will be seen that the analyzed fat from rat milk contains about 1 % of fatty acids with an uneven number of carbon atoms in the chain, and that it is a characteristic feature that it contains appreciable quantities (about 0.5 %) of a fatty acid with a 15-carbon atom chain. In agreement with other investigations [3], butyric acid and caproic acid were not found in the rat milk fat.

To determine the content of unsaturated fatty acids the fats isolated from another milk sample (with approximately the same distribution of the fatty acids as regards chain length) was examined by alkali isomerization analysis [4]. The following results were obtained (unsaturated linkages being assumed to occur chiefly in the C_{18}-fraction):

linoleic acid	11	%
linolenic acid	4	»
conjugated dienoic acids	0.9	»

It appeared, moreover, from the iodine value (65.7 for the methyl esters) that the triglycerides of rat milk contain about 40 % oleic acid.

Experimental. The milk was collected 15—18 days after parturition from 15 rats which had been fed on the diets listed in Table 2. The milk donors were separated from the litter in the afternoon preceding milk collection the following morning. The rats were anesthetized with 0.1—0.2 ml of a 6 % Nembutal sodium solution (Abbott), given subcutaneously. The milk was expressed by the thumb and forefinger, and the droplets were collected in a pipette. About 1 ml was collected from each rat.

To a 10—12 ml sample of the collected milk was added 1 ml 10 % ammonium hydroxide and 10 ml ethanol (96 %), and the mixture was shaken vigorously. Now 25 ml ethyl ether were added and the mixture again shaken; the treatment was repeated with 25 ml petroleum ether, whereupon the mixture was allowed to stand over night for complete separation. The ether phase was separated and evaporated to dryness — finally in vacuum. The residue was dissolved in 20 ml methanol, and 20 mg sodium methoxide were added in the form of 2 ml of a methanolic solution. The reaction mixture was refluxed for a couple of hours and then set aside over night. After addition of 20 ml ethyl ether, just sufficient distilled water was added dropwise to cause the solution to separate into two phases (*i. e.* about 20 ml distilled water). The ether phase with the methyl esters was separated, and the aqueous phase was extracted once more with 25 ml ethyl ether. The two ether phases were combined, and an equal

Table 2. Percentage composition of the diets.

Diet No. 1 *		Diet No. 2 *	
Oat	42	Oatmeal	77.7
Wheat	42	Wheat	8.0
Pulverized sea shells	8	Hempseed	8.0
Gritted charcoal	2	Linseed	4.0
Cod-liver oil	6	Calcium carbonate	1.3
		Sodium chloride	1.0

* Diet No. 1 was given on Tuesday, Thursday, Saturday and Sunday. Diet No. 2 was given on Monday, Wednesday and Friday. — Water was given during the day and whole milk during the night *ad lib.*

volume of petroleum ether (30—35 ml) was added to cause water to separate out. The ether solution was finally dried by means of anhydrous sodium sulphate, and the ether was removed by evaporation. The last traces of volatile foreign matter were removed from the methyl esters by treatment for 1 hour at 60°C in vacuum produced by a water pump.

1. Hallanger, L. E. and Schultze, M. O. *Proc. Soc. Exptl. Biol. Med.* **96** (1957) 473.
2. Jart, Aa. *Acta Chem. Scand.* **13** (1959) 1723.
3. Lucky, T. D., Mende, T. J. and Pleasants, J. *J. Nutrition* **54** (1954) 345.
4. American Oil Chemists' Society. *Official and Tentative Methods of Analysis*, 2nd Ed. Rev. to 1958. Chicago 1946—58. Cd 7—58.

Received October 31, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1913–1918

The Osmotic Pressure of Macroions

JOHN GRAAE

Kemisk Laboratorium B, Polyteknisk Læreanstalt, Copenhagen, Denmark

and

KJELD MARCKER

Universitetets Fysisk-Kemiske Institut, Copenhagen, Denmark

A theoretical study on the colloidal osmotic pressure and the Donnan-effect is presented. 1) An expression for osmotic pressure under ideal conditions is derived. 2) Deviations from the expression as regards colloidal osmotic pressure are explained by an "excluded volume" theory. 3) Deviations from the expression as regards the Donnan-effect are explained by a lowering of the effective charge of the macroion at salt concentrations greater then 0.1 M and in the case of hyaluronic acid by the formation of a network of hyaluronate molecules at salt concentrations lower then 0.1 M.

In this paper will be presented some theoretical considerations about the osmotic pressure of macroions, both in the case where the Donnan-effect is eliminated by high salt concentrations as where this is not so. The paper will fall in three parts: 1) The derivation of the osmotic pressure under ideal conditions; 2) The deviations from ideality when the Donnan-effect may be neglected; 3) The deviations from ideality as regards the Donnan-effect and the possibility of explaining this by accepting a lowering of the effective charge of the macroion when the salt concentration is increased. In all three cases the calculations will be illustrated with experimental data published earlier from this laboratory.

1. Let us denote the macroion R^- with the charge $-\nu$, and further:

Substance		H_2O	K^+	Cl^-	R^-
Symbol		M_o	M_+	M_-	M_2
Mole fractions	outer liquid	$1-2y$	y	y	0
	inner liquid	$1 - x_+ - x_- - x_2$	x_+	x_-	x_2

The condition of electroneutrality yields:

$$x_+ = x_- + \nu\ x_2 \tag{0}$$

Assuming ideal conditions we have:

$$\mu_i = \mathrm{J}_i + RT\ln z_i$$

where z_i is the mole fraction of M_i and J_i is independent of all the different mole fractions.

Denoting chemical potentials of the two liquids μ' and μ, respectively, and denoting the pressures by p^+ and p, respectively, we get:

$$\mu'_o = \mathrm{J}^+_o + RT\ln\ (1 - 2y)$$

$$\mu_o = \mathrm{J}_o + RT\ln\ (1 - x_+ - x_- - x_2)$$

and from

$$u'_o = u_o:$$

$$1 - x_+ - x_- - x_2 = (1 - 2y)e^{-\alpha_o}$$

where

$$\alpha_o = (\mathrm{J}_o - \mathrm{J}^+_o)/RT = (p - p^+)V_o/RT = PV_o/RT$$

P is osmotic pressure and V_o the molar volume of pure water at the temperature in question. As P in the experiments is only a minute fraction of an atmosphere, it never exceeds 20 cm water, and is usually, much lower, we can substitute $1 - \alpha_o$ for $e^{-\alpha_o}$. Consequently we get:

$$x_+ + x_- + x_2 - 2y\ (1 - \alpha_o) = \alpha_o \tag{1}$$

Next we express that the potential μ_1 of KCl must be the same on both sides of the membrane. With $\mathrm{J}_+ + \mathrm{J}_- = \mathrm{J}_1$ we get:

$$\mu'_1 = \mathrm{J}^+_1 + 2RT\ln y$$

$$\mu_1 = \mathrm{J}_1 + RT\ln(x_+x_-)$$

proceeding in the same way as above, and substituting $e^{-\alpha_1}$ with $(1 - \alpha_1)$, where $\alpha_1 = P(V_+ + V_-)/RT = PV_1/RT$ we get:

$$x_+x_- = (1 - \alpha_1)y^2 \tag{2}$$

Expressing (0) by the two equations:

$$x_+ = Z + \tfrac{1}{2}\ \nu\ x_2$$
$$x_- = Z - \tfrac{1}{2}\ \nu\ x_2$$

we get from (1)

$$Z = \tfrac{1}{2}\ (\alpha_0 - x_2) + y\ (1 - \alpha_0) \tag{1a}$$

and from (2)

$$Z^2 = y^2\ (1 - \alpha_1) + \tfrac{1}{4}\ \nu^2\ x_2^2 \tag{2a}$$

Squaring (1a) and accepting that α_0 is so much smaller than 1 that α_0^2 can be neglected compared to 1, we arrive at the equation:

$$0 = \tfrac{1}{4}(x^2)(\nu^2-1) + x_2[\tfrac{1}{2}\alpha_0 + y(1-\alpha_0)] - y\alpha_0(1-2y+\alpha_0) - y^2\alpha_1 - \tfrac{1}{4}\alpha_0^2 \qquad (3)$$

In the case where $y = 0$ this equation becomes:

$$x_2^2\ (\nu^2-1) + 2x_2\alpha_0 - \alpha_0^2 = 0$$

which gives:

$$P = RTx_2\ (\nu + 1)/V_0$$

If we now consider the case where $\alpha_0 \ll 1$ and $\alpha_0 \ll y$ eqn. (3) is reduced to:

$$\tfrac{1}{4}\ x_2^2(\nu^2-1) + x_2y - \alpha_0(y-2y^2) - y^2\alpha_1 = 0 \qquad (3a)$$

and introducing again that $\alpha_0 = PV_0/RT$ and $\alpha_1 = PV_1/RT$ we find for the osmotic pressure:

$$P = \frac{\tfrac{1}{4}x_2^2(\nu^2-1) + x_2y}{\frac{V_0}{RT}(y - 2y^2) + \frac{V_1}{RT}\ y^2}$$

or by rearrangement

$$P = \frac{RTx_2}{V_0(1-2y) + V_1y}\left[1 + \frac{x_2(\nu^2-1)}{4y}\right] \qquad (4)$$

Eqn. (4) differs from a commonly accepted formula [1] by having the term (ν^2-1) instead of ν^2. In the case of hyaluronic acid where the mean charge exceeds several hundred this is of course insignificant, but in the case of proteins near the isoelectric point the difference may be significant.

2. It is a well known fact that even when the salt concentration is so high that the figure in the brackets in (4) is 1, deviations from the theoretical expression are found in many cases. Jensen and Marcker [2] have demonstrated that dextran obeys the theoretical laws with good accuracy, but this is definitely not true when measurements are performed with hyaluronic acid, where Jensen and Marcker [2] find the law

$$c/p = A\ (1-Bc)$$

to be the one which fits the experimental results best. (c is concentration of hyaluronate in g/l, P is osmotic pressure in cm water and A and B are constants, of which A is proportional to the molecular weight of the sample in question and B is the same for different samples.)

The problems concerned with the dependence of osmotic pressure on concentration have been studied theoretically by Güntelberg and Linderstrøm-Lang [3] and Scatchard [4] specially in the case where the high molecular substance is a protein. Here we shall present a simple explanation of the experimental results obtained earlier in this laboratory.

The assumption which is the basis of the following calculations is that the volume of the solvent should be corrected by subtraction of the "effective" volume of the dissolved molecules. In the case of dextran this volume could be expected to be relatively small at the concentrations used by Jensen and

Marcker, but in the case of hyaluronate where we have molecules with high negative charges the correction may be important either because of the repulsive forces of the charges or because of the ionic clouds which make it impossible for the molecules to come so close to each other, as when uncharged.

The following symbols will be used: c_g = weight concentration, P = osmotic pressure, N = number of molecules, v = total volume of solution, M = molecular weight of preparation used.

Let us have N molecules in a total volume of v. The "effective" volume of the molecules is then BNM, where B is a constant that includes the "effective" volume of the charged molecular unit. We then assume that the osmotic pressure is proportional to the "effective" concentration, *i. e.*:

$$P = A' \frac{N}{v - BNM}$$

substituting $N = c_g v/M$ and rearranging we get:

$$c_g/P = AM\ (1 - Bc_g) \qquad (5)$$

which is identical with the expression found empirically by Jensen and Marcker [2].

It must be supposed that B varies with the salt concentration, pH and other factors influencing the charge of the molecule. In the case of ampholytes as, *e. g.*, proteins, it must further be assumed that B has its smallest value at the isoelectric point in good agreement with Christiansen, Jensen and Marcker [5] who measured the osmotic pressure of bovine plasma albumin at different pH values and found a minimal osmotic pressure at pH 4.64.

Let us suppose that in this case the constant B could be written $B = D(1 + E\ \Delta\text{pH})$, where ΔpH is the difference between the actual pH and the isoelectric pH, and D and E are constants. Introducing this in eqn (5) we find at constant concentration

$$K'P - L\ P\ |\Delta\text{pH}| - 1 = 0$$

where K' and L are new constants. This means that p plotted *versus* pH should be a hyperble, in good agreement with Fig. 2 in Christiansen, Jensen and Marcker [5]. This could be re-written as:

$$1/P = K - L\ |\Delta\text{pH}|$$

and $1/P$ plotted *versus* pH should yield a straight line. This is shown in Fig. 1, using the points from the right half of Fig. 2 in Christiansen, Jensen and Marcker. (Note in this figure the point at about pH 3.6 which makes it reasonable to believe that the curve should have shown a higher degree of symmetry.)

3. We have now accounted for the osmotic pressure in the case where the Donnan-effect has been eliminated by a suitably high concentration of salt. However measurements performed by Robinson and Jensen [6] show that the salt concentration necessary to eliminate the Donnan effect is much smaller than calculated from (4). A fraction of hyaluronate with a molecular weight of 500 000 will have ν about 1 000. Inserting this in (4) gives that a concentra-

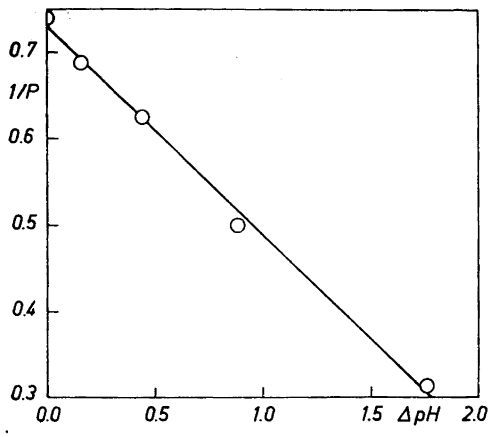


Fig. 1. A graph of $1/P$ cm water^{-1} plotted against ΔpH for the experiment recorded in Fig. 2 in Christiansen, Jensen and Marcker [5].

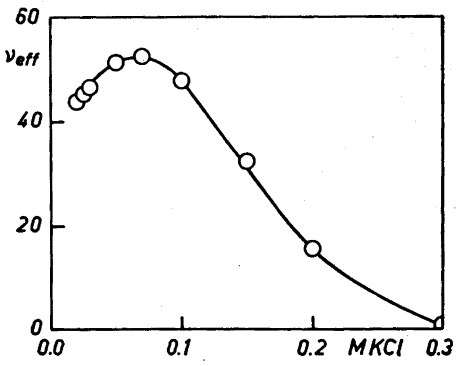


Fig. 2. A graph of ν_{eff} calculated from (7) using figures from Robinson and Jensen [6] plotted against concentration of KCl. Molecular weight of sample is about 120 000, $\nu_{theor.} \simeq 240$.

tion of hyaluronate at 10 g/l demands about 100 M KCl to reduce the Donnan effect to 5 % of the measured osmotic pressure. In this case, however, Robinson and Jensen find that there is no change in osmotic pressure when salt concentration varies from 2 M to 1 M and only when salt concentration is lower than 1 M is an increase in osmotic pressure observed. It seems reasonable to explain this by assuming an effective charge of the hyaluronate lower than the theoretical charge, due to the presence of potassium ions. To see whether this assumption is justified we shall use the figures recorded by Robinson and Jensen [6] to calculate the effective charges necessary to make eqn (4) fit with the experimental results.

We change (4) to

$$P = P_{min}\left[1 + \frac{x_2(\nu^2-1)}{4y}\right] \tag{6}$$

where P_{min} is the osmotic pressure measured at salt concentrations so high that the addition of more salt would cause no change. By calculating the concentration of hyaluronate, x_2 we use the apparent molecular weight at the concentration used by Robinson and Jensen (10 g/l) and do not try to extrapolate to infinite dilution, as this procedure should automatically correct for volume effect and possibly other deviations from the van't Hoff theory. For the same reason we simply use P_{min} as the factor, and do not try to calculate this factor. Solving (6) in respect of ν gives us

$$\nu_{eff} = \sqrt{\frac{4y}{x_2}\left(\frac{P}{P_{min}} - 1\right) + 1} \tag{7}$$

If we now calculate ν_{eff} we do not find as expected that ν_{eff} increases with decreasing salt concentration, with the theoretical value as the upper limit, but we find as demonstrated in Fig. 2 that this is only the case when the salt concentration is higher than about 0.1 M, and that below this concentration ν_{eff} decreases with decreasing salt concentration.

It should be noticed that a change in the behaviour of hyaluronic acid at salt concentrations about 0.1—0.2 M is observed in other properties of hyaluronic acid [6,7]. This may be explained by the assumption that hyaluronic acid at low salt concentrations forms some sort of a network and only at higher salt concentrations is it dissolved as single molecules. This too can explain the results found here, as this would cause a decrease in x_2, which would make the values of ν_{eff} calculated from (6) higher than those recorded in Fig. 2.

Acknowledgements. We are very much indebted to Professor, Dr. J. A. Christiansen for his interest in this work and for his many valuable suggestions.

The authors' thanks are due to Dr. C. E. Jensen who has kindly allowed us to use his experimental results in our calculations and has given us valuable information about the behaviour of hyaluronic acid, as well as about the immense literature on the subject.

Financial support from *NOVO Terapeutisk Laboratorium* and *Købmand i Odense Johann og Hanne Weimann, født Seedorf's foundation* is acknowledged by Kjeld Marcker.

REFERENCES

1. Overbeek, J. Th. G. *The Donnan Equilibrium*, in Kruyt, H. R. (ed.) *Colloid Science I.* Elsevier Publishing Co. Amsterdam 1952, p. 190.
2. Jensen, C. E. and Marcker, K. *Acta Chem. Scand.* **12** (1958) 855.
3. Güntelberg, A. V. and Linderstrøm-Lang, K. *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **27** (1949) 1.
4. Scatchard, G. *J. Am. Chem. Soc.* **68** (1946) 2315.
5. Christiansen, J. A., Jensen, C. E. and Marcker, K. *Acta Chem. Scand.* **11** (1957) 402.
6. Robinson, M. and Jensen, C. E. *To be published.*
7. Jensen, C. E. *Studier over hyaluronsyre.* (Diss.) University, Copenhagen 1957.

Received August 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1919–1926

Chromatographic Separation of Chondrosin and Hyalobiuronic Acid on an Acidic Resin

ANUND HALLÉN

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

Aminopolysaccharides, consisting of chondroitin sulphuric acid, hyaluronic acid, keratosulphate, β-heparin and ordinary heparin, as well as tissue specimens, were submitted to mild acid hydrolysis, and the hydrolysate chromatographed on a cation exchange resin. The ninhydrin-positive components obtained were identified as chondrosin, hyalobiuronic acid, glucosamine and galactosamine. A component obtained from β-heparin showed the same chromatographic behaviour as chondrosin. The front material possibly contained hexosamine ester sulphate derivatives.

It seems to be relatively well documented that aldobiuronic acids containing β-D-glucuronide bonds are fairly resistant to acid hydrolysis. For instance, Heidelberger and Goebel [1] boiled Type III pneumococcus polysaccharide for 5 h in 3 % sulphuric acid in order to obtain cellobiuronic acid. This has also proved to apply to hexosamine-containing mucopolysaccharides. The hexosamine-hexuronic acid containing disaccharide chondrosin obtained by Schmiedeberg [2] after boiling chondroitin sulphuric acid *(C.S.A.)* or chondroitin with 2—4 % nitric acid for 1—1.5 h was shown in Levene's laboratory [3] to consist of glucuronic acid and galactosamine in glycosidic junction, and in Masamune's [4] and Meyer's [5] laboratories to be 3-*O*-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-galactopyranose. A similar disaccharide, mucosin [6] or hyalobiuronic acid [7,8], was obtained by Isikawa [6] in Masamune's laboratory by boiling hyaluronic acid (*H.A.*) with 55 % oxalic acid for 5 h. Independently it was obtained in Meyer's laboratory [7,8] by boiling *H.A.* with 0.5 M sulphuric acid for 3 h. It has been shown to be 3-*O*-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucopyranose [9]. As indicated by the investigations of Jeanloz [10] the resistance of the glucuronide bond is also encountered in acidic methanol solution of methylated mucopolysaccharides.

Both the D-galactoside bond in keratosulphate and the L-iduronide bond in β-heparin are comparatively acid-labile [11]. Ordinary heparin is unique in having very acid-stable glucosaminide bonds [12].

During unsuccessful attempts to isolate an oligosaccharide fraction from mild acid hydrolysates of keratosulphate according to Cifonelli and Dorfman [11] I observed that chondrosin and hyalobiuronic acid could be separated from one another by chromatography on Zeo-Karb 225. Whereas the behaviour of unsubstituted hexosamine on acidic resins has been studied by many authors [13-19] there are only sporadic reports on partial hydrolysis products [5,11,16]. This paper deals with an ion exchange method for quantitative determination and for isolation of the mild acid hydrolysis products from mucopolysaccharides. For the same purpose Fischer and Dörfel [20] have described a paper-chromatographic technique.

EXPERIMENTAL

Preparation of the polysaccharides. *C.S.A.* and *H.A.* were prepared as Mg salts from bovine cartilage and vitreous body, respectively, using the method of Scott [21], including digestion with papain and fractionation with cetyl pyridinium chloride. Chondroitin was obtained from *C.S.A.* by hydrolytic removal of ester sulphate groups in acidic methanol, followed by mild alkaline hydrolysis of the methyl ester groups, according to Kantor and Schubert [22]. Keratosulphate was obtained from human nucleus pulposus with the method of Gardell [23], including digestion with pancreatic and intestinal enzymes, phenol extraction and fractionation with alcohol. Crude heparin was prepared essentially according to Charles and Scott [24], and was then precipitated as the insoluble Ba salt. Heparin monosulphuric acid was obtained from the heparin mother liquor, according to Jorpes and Gardell [12]. β-Heparin was prepared from lung heparin mother liquor, according to Marbet and Winterstein [25], and was then further purified by hyaluronidase digestion according to Rodén and Dorfman [26].

Hydrolysis of the polysaccharides. 125 mg of the *H.A.* preparation (ash 16 %, moisture 15 %) was dissolved in 250 ml of hot 0.6 M hydrochloric acid, and put in a strongly boiling water bath. Every 10 min, a 5-ml sample was taken, cooled, and neutralized by the addition of 10 drops of pyridine. Suitable amounts were taken for determination of the reducing power [27] (2.5 ml), Elson-Morgan colour [28,16] (0.5 ml) and ninhydrin colour [29] (0.3 ml). Crystalline glucosamine-HCl was used as standard. The results of these analyses are summarized in Fig. 1, where the values are expressed as μmole glucosamine equivalents per 2 ml of solution (containing 1 mg of *H.A.*).

For the chromatographic experiments, 0.5—10 mg of the polysaccharide preparations were hydrolyzed with reflux in 10 ml of 0.6 M hydrochloric acid. The hydrolysate was evaporated *in vacuo* to a small volume, and dried in a desiccator over concentrated sulphuric acid and sodium hydroxide pellets.

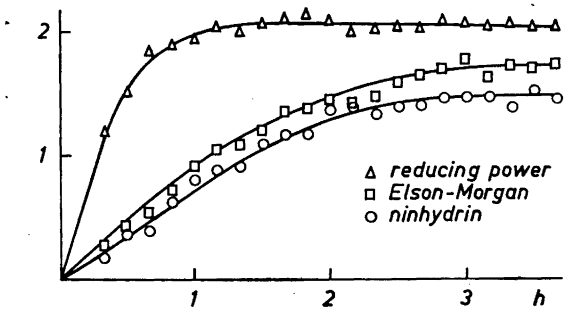


Fig. 1. Course of hydrolysis of *H.A.* in 0.6 M hydrochloric acid at 100°C. The values are expressed as μmole glucosamine equivalents per mg of *H.A.* preparation.

In experiments with some tissues, such as the annulus fibrosus of the intervertebral disc, it proved necessary first to gelatinize the collagen by boiling in water for 30 min and, after grinding, to make a dry powder with alcohol and ether. Humins were removed by filtration.

For hexosamine determinations, hydrolysis was performed in sealed tubes with 0.5—1 ml of 6M hydrochloric acid for 8 h [16].

The dried hydrolysate was put on the column in the usual way, dissolved in a total volume of 0.2—0.4 ml of the buffer.

Chromatography of the hydrolysate. The sodium form of Zeo-Karb 225 X 8, 200—400 mesh, was used in 0.6 × 40 cm columns with a flow rate of 1 ml/h. The effluent was collected in 0.5- or 1.0-ml fractions.

To study the influence of pH on the chromatography of the components several experiments were performed, where different amounts of sodium carbonate had been added to the eluting hydrochloric, sulphuric or phosphoric acids. It was found that in the pH range 0.5—7.0 the hexosamines (analyzed with the Elson-Morgan method [28,16]) chromatographed fairly independently of changes in pH, the ionic strength being the only factor of importance. This was in sharp contrast to the amino acids (analyzed with ninhydrin [29]), which were extremely sensitive to changes in pH. Thus, above pH 2.4 the hexosamines appeared among the amino acids, whereas at lower pH they appeared in front of the amino acids. At pH 2.3, the effluent volume of the hexosamines was only slightly less than that of hydroxyproline (recognized by its yellow tint in the ninhydrin test), the first amino acid to appear.

0.10—0.15 M $NaHSO_4/Na_2SO_4$ buffer, pH 1.9 (*i.e.*, the pK_s of HSO_4^-) was found suitable for the present purpose. The effluent volume of the hydroxyproline was then about twice that of the hexosamines. The ammonium ion came after the hydroxyproline as did other components in protein hydrolysates.

The buffer was easily prepared, for instance by adding sodium hydroxide pellets (60 g) to 0.10 M sulphuric acid (10 l) until 10 ml of the buffer needed 5 ml 0.10 M sodium hydroxide for neutralization. Toluene, 0.5 ml per liter of buffer, was added to prevent growth of microorganisms. At room temperature it could be stored in stoppered vessels for at least one month. Before use, dissolved gases were removed by a water suction pump.

Analysis of the fractions. The ninhydrin method of Moore and Stein [29] proved to be more sensitive and to give better standard curves than both the Elson-Morgan [28,16], the Dische-Borenfreund [30] or the Tracey [31] methods. 0.5 ml of the ninhydrin reagent [29] was added to each fraction. Although it was verified that leucine could have been used as a standard [18,29], crystalline glucosamine-HCl was chosen instead. This was because the colour development of this reagent, dissolved in the buffer described above, was constant during one month's storage in the refrigerator.

RESULTS

Conditions of hydrolysis. From Fig. 1 it was inferred that when using boiling 0.6 M hydrochloric acid 2.5 h should be a highly appropriate time for obtaining partial hydrolysis products. This was because the ninhydrin colour development then reached a plateau, indicating maximal liberation of amino-groups under the mildest possible conditions. It also proved to be useful in view of the fact that maximal yields of the disaccharides were then obtained in the chromatograms.

Identification of the peaks. Fig. 2 shows a typical chromatogram of the *C.S.A.* and *H.A.* hydrolysates. Several peaks, labelled 1, 2a, 2b, 3a and 3b, were present. In this chromatogram, in which 2 mg of each of the polysaccharides had been hydrolyzed for 4 h in 0.6 M hydrochloric acid, the components were obtained in the following amounts: component 1, 0.19 μmole; 2a, 1.14 μmole; 2b, 1.12 μmole; 3a, 0.34 μmole and 3b, 0.22 μmole glucosamine (or leucine) equivalents. All the components contained hexosamine, as indicated

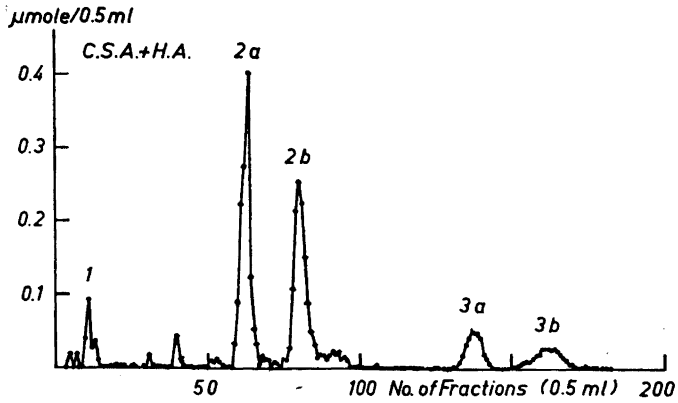


Fig. 2. 2 mg each of the *C.S.A.* and *H.A.* preparations hydrolyzed for 4 h in 0.6 M hydrochloric acid, chromatographed on Zeo-Karb 225 with 0.10 M $NaHSO_4/Na_2SO_4$ buffer, pH 1.9, and analyzed with ninhydrin. Component 1 identified as hexosamine ester sulphate derivatives (?), 2a as chondrosin, 2b as hyalobiuronic acid, 3a as glucosamine and 3b as galactosamine.

by a positive Elson-Morgan [28,16] and Dische-Borenfreund [30] reaction. Components 1, 2a and 2b gave a positive Dische carbazol reaction [32], indicating the presence of uronic acid. When chondroitin was substituted for *C.S.A.*, component 1 was lacking. When the proportions of *C.S.A.* and *H.A.* were varied, component 2a proved to vary in quantity parallel with the amount of *C.S.A.* (see Table 1). Glucosamine-HCl added to the hydrolysate increased the amount of component 3a, and galactosamine-HCl that of 3b. Such simple experiments made identification possible with a high degree of probability, of component 2a as chondrosin, 2b as hyalobiuronic acid, 3a as glucosamine and 3b as galactosamine.

In order to study the components in more detail, chromatograms were run on a larger scale. The fractions belonging to the same peak were pooled, and suitable amounts were taken from the solutions thus obtained for determination of the ninhydrin colour [29], reducing power [27], Elson-Morgan colour [28], Dische carbazole colour [32] and ester sulphate. Leucine, glucose, glucosamine and glucurone, respectively, were used as standard.

Table 1. Amount of components 2a and 2b obtained from different *C.S.A.* and *H.A.* mixtures hydrolyzed for 2.5 h in 0.6 M hydrochloric acid.

C.S.A.	*H.A.*	2a	2b
mg	mg	µmole	µmole
—	2.0	—	1.30
0.2	2.0	0.17	1.39
1.2	2.0	1.05	1.56
2.0	2.0	1.70	1.54
2.0	—	1.60	—

Table 2. Analysis of the components. The values are referred on a molar basis to the ninhydrin colour.

Component	Reducing power	Elson-Morgan *	Dische carbazole	Ester sulphate
1 **	4.7	3.5	3.8	2.1
2a	0.9	1.1	1.1	—
2b	1.0	1.1	1.1	—
3a	1.0	1.0	—	—
3b	0.9	1.0	—	—

* After hydrolysis.
** From a chromatogram with *C. S. A.* only.

In accordance with Rapport *et al.*[7], it was found that the Elson-Morgan values of components 2a and 2b were about half the theoretical ones. Hydrolysis in 3 M hydrochloric acid for 6 h was therefore performed for these determinations. The acid was removed in the desiccator. For the ester sulphate determination, an excess of barium chloride was added, and the resulting barium sulphate precipitate was centrifuged down, washed and discarded. After addition of the washing water to the clear solution, one volume of concentrated hydrochloric acid was added, and the solution boiled for 4 h. On the next day the precipitate was collected on a platinum-iridium Gooch filter, ignited and weighed.

The results of these analyses are presented in Table 2. For convenience the values have been referred, on a molar basis, to the ninhydrin colour.

Within the errors of the methods, components 2a and 2b (see Table 2) seemed to contain amino groups, reducing groups, hexosamine and uronic acid in equimolar amounts. This indicated that these components were aldobiuronic acids. After N-acetylation with acetic anhydride [33], the Morgan-Elson reaction [34,33] was positive, implying [35,36] that components 2a and 2b did not contain 4-*O*-substituted hexosamines. The 3-*O*-substitution was indicated by a modified Elson-Morgan reaction [17], showing a 510 mμ maximum typical of this substitution [11].

For preparative purpose it was found convenient to use the resin in hydrogen cycle and elute the components with dilute volatile acids [5,16]. In

Table 3. Components obtained from different polysaccharides hydrolyzed for 2.5 h in 0.6 M hydrochloric acid.

Polysaccharide	% recovery of component				
	1	2a	2b	3a	3b
C.S.A.	7	65	—	—	6
H.A.	—	—	35	5	—
Keratosulphate	7	—	—	65	—
β-Heparin	12	20	—	—	40
Ordinary heparin	5	—	—	—	—
Heparin monoacid	—	—	—	—	—

one experiment complete separation was obtained on a 4 × 50 cm column of the components 2a and 2b from the hydrolysate of about 0.2 g each of *C.S.A.* and *H.A.* by eluting with 0.05 M hydrochloric acid. The effluent volume of component 2a was 1.2—1.7 l, that of 2b 1.8—2.5 l. The fractions belonging to the same peak were pooled and evaporated in vacuo to a small volume. On freeze-drying there was some tendency to syrup formation, but when redissolved in water and freeze-dried again the components were obtained as white powders; 2a 63 mg, 2b 44 mg. The component 2a obtained in this way proved also to be component 2a in the sulphate buffer chromatogram. The hexosamine of component 2a was identified as galactosamine, that of 2b as glucosamine with the chromatographic method of Gardell [16] after hydrolysis with 6 M HCl for 8 h. The identities were secured by the effluent volumes and by the fact that glucosamine added to the hydrolysate of component 2a yielded two peaks, whereas only one peak was obtained when it was added to the hydrolysate of component 2b.

As indicated by a high reducing power and by containing ester sulphate (see Table 2) component 1 could be composed of low molecular hexosamine ester sulphate derivatives (with a free amino group capable of reacting with ninhydrin). This was also supported by the fact that it was obtained from sulphated polysaccharides, but not from *H.A.* or chondroitin. It was prepared in an impure state by hydrolyzing *C.S.A.* in 0.6 M hydrochloric acid for 2.5 h in the presence of excess barium chloride. The hydrolysate was evaporated *in vacuo* to a small volume and put on Zeo-Karb 225 in hydrogen cycle. Component 1 was eluted with distilled water. After boiling with strong hydrochloric acid and filtration a heavy barium sulphate opalescence was obtained on the addition of barium chloride. The component also contains N-acetyl groups since the Morgan-Elson reaction [34] was positive.

Behaviour of the different polysaccharides. The recovery of the components varied somewhat in different experiments. In Table 3, the quantity recovered (in per cent) is referred to the hexosamine content after hydrolysis for 8 h in 6 M hydrochloric acid. It is evident that *C.S.A.* yielded essentially only chondrosin (2a) in the chromatograms, *H.A.* only hyalobiuronic acid (2b), keratosulphate glucosamine (3a) and β-heparin galactosamine (3b) together with a small amount of component 2a.

On hydrolysis with strong hydrochloric acid, the keratosulphate and heparin monosulphuric acid preparations yielded a small amount of galactosamine, indicating contamination by *C.S.A.* For this reason traces of component 2a obtained in the analysis of these polysaccharides are not included in the table. Nor was the β-heparin pure, since it contained glucosamine. In this case, however, component 2a probably originated essentially from the β-heparin, since Cifonelli *et al.*[37] isolated such a disaccharide in small amounts after hydrolysis of β-heparin in M hydrochloric acid for 1 h.

Behaviour of certain tissues. Up to 50 mg of tissue could be analyzed with this technique. A chromatogram of the hydrolysate of 10 mg of freeze-dried nucleus pulposus is shown in Fig. 3. The following amounts of the different components were obtained from a specimen of nucleus pulposus of a 19-year-old subject: component 1, 0.76 μmole; 2a, 2.90; 2b, 0.0; 3a, 1.60; 3b, 0.32 μmole glucosamine equivalents. The corresponding values for the specimen

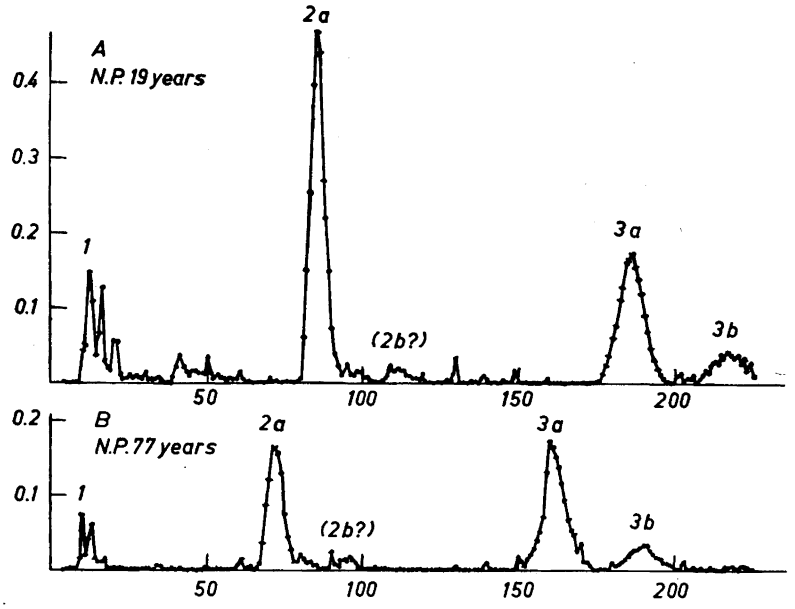


Fig. 3. Chromatogram of 10 mg of nucleus pulposus hydrolyzed for 2.5 h, otherwise as in Fig. 2. A from a man aged 19 years, B 77 years.

from a 77-year-old subject were: component 1, 0.24 μmole; 2a, 1.05; 2b, 0.0; 3a, 1.48; 3b, 0.27 μmole. These results are in agreement with the presence of *C.S.A.* and keratosulphate, and with the increase in the ratio of keratosulphate/*C.S.A.* with rising age postulated in an earlier paper [38]. Thus, the ratio of 3a/2a increased from 0.55 in the 19-year-old subject to 1.41 in the 77-year-old.

From 44 mg of annulus fibrosus, the following amounts were obtained: component 1, 0.50 μmole; 2a, 1.32; 2b, 0.0; 3a, 0.84; 3b, 0.36 μmole, indicating the presence chiefly of *C.S.A.* and keratosulphate.

From 55 mg of human adult rib cartilage, the corresponding amounts were: 1, 0.16 μmole; 2a, 1.65; 2b, 0.0; 3a 2.64; 3b, 0.63 μmole, indicating that *C.S.A.* and keratosulphate were present, as is also indicated by the investigation of Kaplan and Meyer [39].

From 38 mg of umbilical cord, the yields were; component 1, 0.10 μmole; 2a, 0.22; 2b, 0.75; 3a, 0.50; 3b, 0.40 μmole, indicating the presence of *H.A.* in particular, but of other polysaccharides as well.

Acknowledgements. I am greatly indebted to Docent Lennart Rodén for valuable discussions, and to Mrs. Ruth Flagell for skilful technical assistance.

The investigation has been aided by grants from *Karolinska Institutets reservationsanslag.*

REFERENCES

1. Heidelberger, M. and Goebel, W. F. *J. Biol. Chem.* **74** (1927) 613.
2. Schmiedeberg, O. *Arch. exptl. Pathol. Pharmakol. Naunyn-Schmiedeberg's* **28** (1891) 355; **87** (1920) 47.
3. Levene, P. A. *J. Biol. Chem.* **140** (1941) 267.
4. Masamune, H., Yosizawa, Z. and Maki, M. *Tôhoku J. Exptl. Med.* **55** (1951) 47.
5. Davidson, E. A. and Meyer, K. *J. Am. Chem. Soc.* **76** (1954) 5686.
6. Isikawa, T. *Tôhoku J. Exptl. Med.* **53** (1951) 217; **54** (1951) 81, 197; **55** (1951) 23.
7. Rapport, M. M., Weissmann, B., Linker, A. and Meyer, K. *Nature* **168** (1951) 996.
8. Weissmann, B., Rapport, M. M., Linker, A. and Meyer, K. *J. Biol. Chem.* **205** (1953) 205.
9. Weissmann, B. and Meyer, K. *J. Am. Chem. Soc.* **76** (1954) 1753.
10. Jeanloz, R. W. *Helv. Chim. Acta* **35** (1952) 262.
11. Cifonelli, J. A. and Dorfman, A. *J. Biol. Chem.* **231** (1958) 11.
12. Jorpes, J. E. and Gardell, S. *J. Biol. Chem.* **176** (1948) 267.
13. Freudenberg, K., Walch, H. and Molter, H. *Naturwiss.* **30** (1942) 87.
14. Partridge, S. M. *Biochem. J.* **45** (1949) 459.
15. Moore, S. and Stein, W. H. *J. Biol. Chem.* **192** (1951) 663; **211** (1954) 893.
16. Gardell, S. *Acta Chem. Scand.* **7** (1953) 207.
17. Boas, N. F. *J. Biol. Chem.* **204** (1953) 553.
18. Eastoe, J. E. *Nature* **173** (1954) 540.
19. Crumpton, M. J. *Biochem. J.* **72** (1959) 479.
20. Fischer, F. G. and Dörfel, H. *Z. physiol. Chem. Hoppe-Seylers* **301** (1955) 224.
21. Scott, J. E. *The assay of acidic polysaccharides from tissues.* Thesis, Manchester University, 1956.
22. Kantor, T. G. and Schubert, M. *J. Am. Chem. Soc.* **79** (1957) 152.
23. Gardell, S. *Acta Chem. Scand.* **8** (1954) 362; **11** (1957) 668.
24. Charles, A. F. and Scott, D. A. *J. Biol. Chem.* **102** (1933) 425.
25. Marbet, R. and Winterstein, A. *Helv. Chim. Acta* **34** (1951) 2311.
26. Rodén, L. and Dorfman, A. *J. Biol. Chem.* **233** (1958) 1030.
27. Somogyi, M. *J. Biol. Chem.* **160** (1945) 61.
28. Elson, L. A. and Morgan, W. T. J. *Biochem. J.* **27** (1933) 1824.
29. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
30. Dische, Z. and Borenfreund, E. *J. Biol. Chem.* **184** (1950) 517.
31. Tracey, M. V. *Biochem. J.* **52** (1952) 265.
32. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
33. Roseman, S. and Daffner, I. *Anal. Chem.* **28** (1956) 1743.
34. Morgan, W. T. J. and Elson, L. A. *Biochem. J.* **28** (1934) 988.
35. Kuhn, R., Gauhe, A. and Baer, H. H. *Chem. Ber.* **87** (1954) 1138.
36. Jeanloz, R. W. and Trémège, M. *Federation Proc.* **15** (1956) 282.
37. Cifonelli, J. A., Ludowieg, J. and Dorfman, A. *J. Biol. Chem.* **233** (1958) 541.
38. Hallén, A. *Acta Chem. Scand.* **12** (1958) 1869.
39. Kaplan, D. and Meyer, K. *Nature* **183** (1959) 1267.

Received July 28, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1927–1935

Studies on Rennin

II. On the Crystallisation, Stability and Proteolytic Activity of Rennin

BENT FOLTMANN

Chr. Hansen's Laboratory, Ltd., Copenhagen, Denmark

Rennin has been crystallised from commercial preparations as well as from laboratory extracts from calf stomachs after clarification with $Al_2(SO_4)_3$ and Na_2HPO_4 followed by 2 precipitations with NaCl.

Solutions of crystalline rennin are shown to have optimum stability at pH 5.5 to 6.0. The stability is also good at pH 2, whilst at pH around 3.5 and above 6.5 the enzyme is unstable.

Proteolytic experiments have shown that casein is digested by rennin over a wide range of pH (2–6.5), whereas bovine serum albumin is digested only in a rather narrow interval around pH 3.4.

1. INTRODUCTION

Crystallisation of the milk-clotting enzyme, rennin, has been described by several authors [1–9], but only few systematic studies have been published on the chemistry and properties of rennin. Alais [5] has stated that crystalline rennin is homogeneous on free electrophoresis in phosphate buffer 0.1 M pH 6.8, whilst Ernstrom [9] found that crystalline rennin on electrophoresis in phosphate buffer, pH 6.8, ionic strength 0.2, consisted of a main component and a component of slower mobility representing about 3.8 % of the total protein. According to Schwander *et al.*[10], crystalline rennin prepared by Berridge [1] was electrohomogeneous on paper electrophoresis. The preparation was found to be not quite homogeneous in the ultracentrifuge and by diffusion measurements. Schwander *et al.*[10] also confirmed Hankinson's [2] observation that the isoelectric point of rennin is about pH 4.6.

No investigations concerning the stability of rennin * solutions in relation to pH have been published, but it has been stated earlier that rennet is rapidly inactivated by alkali, and that its maximum stability lies between pHs 5.3 and 6.3 (Holwerda [11], Porcher [12], Ege and Lundsteen [13]).

* Here and in the following rennin means the enzyme purified by crystallisation, and rennet means a preparation containing rennin and other proteins.

Having isolated the crystalline rennin, Berridge [1] proved that it was a proteolytic enzyme with pH optimum at 3.7 against hemoglobin as substrate. Using the same substrate, de Baun *et al.*[3] have confirmed Berridge's statement. The proteolytic degradation of casein by rennin from pH 2 to pH 7 has been studied by Nitschmann and Bohren [14]. However, these investigations have mostly concerned the primary proteolytic action which, according to Alais *et al.*[15], is thought to be the essential change for the coagulation of casein with calcium as observed in the ordinary clotting of milk by rennet.

In the course of the present work, 19 preparations of crystalline rennin have been made. The raw materials for the crystallisations have been Chr. Hansen's commercial rennet preparations, as well as extracts made in the laboratory from calf stomachs directly. On electrophoresis by the moving-boundary method, the preparations appeared electrohomogeneous at pH 5.19, 5.78 and 6.35, ionic strength 0.2. The stability experiments have shown that solutions of rennin have optimum stability in the same interval of pH as stated earlier for rennet. The experiments on proteolytic degradation of casein and serum albumin with rennin have shown maximum proteolytic activity at pH 3.4 to 4.0.

2. METHODS AND RESULTS

2.1. Determination of the milk-clotting activity

The activity is expressed in rennin units (RU) according to Berridge [1]. This unit is defined as the rennin activity which clots 10 ml of reconstituted skim milk in 100 sec at 30°C. The substrate consists of 12 g spray-dried skim milk powder reconstituted in 100 ml 0.01 M $CaCl_2$.

Though the differences in clotting ability of various skim milk powders are diminished to some extent by reconstitution in 0.01 M $CaCl_2$, the powders may still show differences from batch to batch. Instead of using correction factors to overcome this difficulty, as suggested by Berridge [16], it was considered preferable to carry out the routine milk-clotting tests as relative tests. The activity of unknown samples was compared with that of a sample of liquid rennet as reference standard. The stability of this reference standard was checked at intervals by means of a basic standard consisting of freeze-dried rennin crystals stored at −15°C.

The crystals of the basic standard were prepared as described below. After crystallisation, most of the salt from the mother liquor was removed by rapid washing with ice cold water. After centrifugation, the crystals were suspended in water and freeze-dried as a suspension. Using a sample of good-quality spray-dried skim milk powder, the activity of the basic standard was determined using Berridge's [16] method, 900 RU/mg N being obtained.

The routine assays were performed at 35°C, and took place in a water thermostat similar to that described by Sommer and Matsen [17]. 50 ml of fresh skim milk were pipetted into wide-necked, 125-ml glass bottles. The bottles were placed on rollers in the thermostat. They had a 20° inclination, and the opening was just above the surface of the water. During the experiments, the bottles were slowly rotated (18 r.p.m.). 1 ml of rennin dilution was added to each bottle and thoroughly mixed with the contents. The clottingpoint,

indicated by the appearance of the first grains in the film flowing down the sides of the bottles, was noted. It was arranged that the rennin concentration chosen would give a clotting-time of 6—8 min. This could generally be achieved by using rennin concentrations of about 4 RU/ml.

The dilutions were made so that the difference between the clotting-time of the unknown sample and that of the reference standard was less than 20 sec. The rennin activity of the unknown sample was then calculated on the assumption of inverse proportionality between the clotting-time and rennin concentration (Storch and Segelcke[18]).

Care was taken that the unknown sample and the reference standard used in the milk-clotting test had the same buffer composition, as even small amounts of buffers with pH different to that of the skim milk could influence the clotting-time.

2.2. Preparation of crystalline rennin

28 dried calf stomachs (450 g) were finely cut and extracted at room temperature under continuous stirring in 7 litres 1 N NaCl. After 4 h, the suspended tissue was separated by passing through a coarse strainer. 5.5 litres of raw extract was obtained (pH 5.8, pre-formed activity 9.8 RU/ml).

Clarification and activation were carried out in one process: 150 ml of 1/3 M $Al_2(SO_4)_3$ was added to the raw extract; pH after addition, 3.65. Immediately after, 150 ml of 1 M Na_2HPO_4 was added and the resulting pH was 4.6. In order to diminish the dilution caused by clarification, concentrated solutions of alum and phosphate, temperature 40°C, were used. The preparation was left to stand 24 h at room temperature, after which the suspension was neutralised to pH 5.6 with 70 ml 1 M Na_2HPO_4, and the precipitate formed was discarded after centrifugation. The last traces of turbidity disappeared after filtration. The volume of the clarified extract was 5 400 ml. Yield and activity of this extract and of the extract in the following steps of the preparation are illustrated in Table 1.

The clear yellow extract was precipitated by saturation with NaCl. After 24 h, the precipitate was collected by means of centrifugation. It was re-dissolved in 450 ml 1/20 M phosphate buffer, pH 6.3. The volume of the resulting solution was 500 ml, pH 5.5. The second precipitation took place in a refrigerator (2°C) as a slow salting-out according to McMeekin[19]. A dialysing bag

Table 1. Crystallisation of rennin.

	ml	RU/ml	mgN/ml	RU/mgN	RU thousands	% recovery
Clarified extract	5 400	164	2.40	68.4	886	100
Dissolved 1st precipitation	500	1 240	2.88	431	620	70
» 2nd »	140	3 830	7.06	542	536	60
» 1st crystallisation	90	3 880	4.30	902	349	39
» 2nd »	75	3 640	3.84	948	273	31
Suspension 3rd »	50	4 300	4.59	937	215	24

(Visking cellulose) with solid NaCl was slowly rotated whilst submerged 2 cm in the solution. 130 g of NaCl was dissolved in the course of 5 days. The bag was removed, and the globular precipitate was collected by centrifugation. It was dissolved in 115 ml 1/20 M phosphate buffer pH 6.3, and after one day in the refrigerator, a little insoluble residue was removed by means of centrifugation.

The clear solution (140 ml, pH 5.4) was left in the refrigerator. After 3 days, the first traces of crystals appeared, and the following week a rapid crystallisation took place. After 18 days, the suspension was centrifuged. The activity of the supernatant was 930 RU/ml. The crystals were washed in distilled water (2°C). The activity of the washings (100 ml) was 236 RU/ml. The crystals were dissolved in distilled water, and left to stand overnight in the refrigerator. A small, insoluble residue was discarded.

Re-crystallisation took place after addition of 5.4 g NaCl, and after 3 weeks in the refrigerator, the activity of the clear supernatant had fallen to 180 RU/ml. The crystals were once more collected, dissolved, and recrystallised as above. After the third crystallisation, the crystals were washed three times in 2 N, 3 N and 4 N NaCl respectivly. The crystals were stored in 4 N NaCl at 2°C.

Regarding the shape of rennin crystals, various statements have been published by different authors. Berridge [1], Alais [5] and Ernstrom [9] have published photos of rectangular crystals which appear to be identical with most of the preparations made in this laboratory. Hankinson [2] and Oeda [6] describe their preparations as needles. De Baun *et al.*[3] state that their crystals resemble Hankinson's, whilst Hostettler [7] has published a photo of some crystal fragments.

Nearly all our preparations of rennin have crystallised in rectangular plates or blocks, but two smaller samples crystallised in needles or long thin rods. The preparations were stored in a freezer at −15°C, and after 4 months, the needle-formed crystals had been transformed into rectangular plates. In spite of several attempts, it has not been possible to reproduce the needles, whether by re-crystallisation or in other preparations.

The homogeneity of the preparations was controlled by means of moving-boundary electrophoresis in a Tiselius apparatus equipped with the Svensson

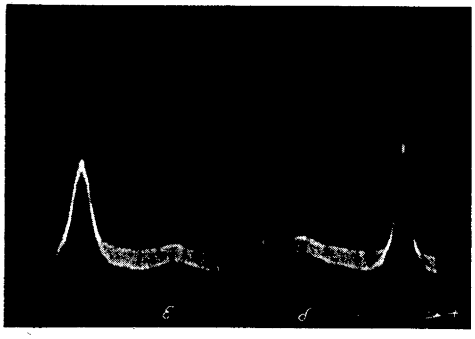


Fig. 1. Electrophoretic pattern of twice re-crystallised rennin. Concentration 0.29 % in phosphate buffer pH 5.78, ionic strength 0.1 + 0.1 M NaCl. The pattern is shown after 31 440 sec of migration, field strength 4.1 V/cm. Temperature in the bath 1.3°C. Svensson's optical system with parallel slit was used.

Table 2. Stability of rennin in solutions.

	pH	Ionic strength	Initial activity	Activity after: 2	4	7	11	days
2°C			RU/ml	RU/ml				
Citrate buffer	3.5	0.05	196	160	150	137	130	
Phosphate buffer	7.0	0.05	196	184	181	169	158	
25°C				6	24	48	96	hours
Citrate buffer	3.5	0.05	196	164	133	112	92	
Phosphate buffer	7.0	0.05	196	176	147	113	95	

optical system. The runs were made at 1.3°C in phosphate buffers, ionic strength 0.1, to which was added NaCl to a total ionic strength of 0.2, pH 5.19, 5.78 and 6.35. The runs were made with a field strength of 3.7 to 4.4 V/cm. The mobility of the descending boundary was determined and found to be -2.05, -3.44 and -3.53 10^{-5} cm^2 volt^{-1} sec^{-1}. Under these conditions, the rennin appeared to be electrohomogeneous, Fig. 1. A solubility test also showed that the enzyme was free of significant contamination with other proteins (Foltmann [20]).

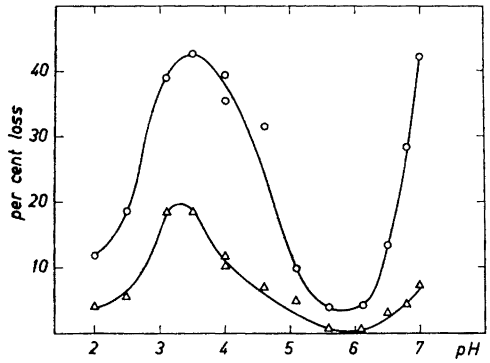


Fig. 2. The influence of pH on the stability of rennin expressed as per cent loss of initial activity after 48 h at 25°C (○) and 48 h at 2°C (△). Ionic strength of all solutions 0.05, pH 2—4 citrate buffers, pH 4—5.1 acetate buffers, pH 5.1—7 phosphate buffers. The upper points at pH 4 represent loss in citrate buffers, and the lower loss in acetate buffers.

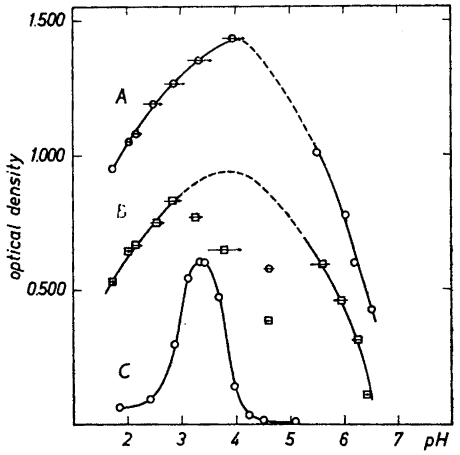


Fig. 3. Proteolytic activity of rennin. Substrate: A and B, 0.89 % casein; C, 0.86 % bovine serum albumin. Enzyme activity per ml reaction mixture: A, 5.33 RU; B, 1.33 RU; C, 14 RU. All experiments, 1 h reaction at 32°C. Ordinate, optical density at 275 mμ of supernatant after precipitation with TCA.

2.3. The influence of pH on the stability of rennin solutions

A 10-ml suspension of rennin crystals was centrifuged. The crystals were washed with cold, distilled water, and after suspension in water, were dialysed in a Visking bag against 3 × 2 litres distilled water at 2°C (6 + 20 + 24 h). By conductivity measurement, it was established that the final concentration of NaCl was less than 10^{-4} N. The activity of the solution was, after dilution to 200 ml, 392 RU/ml, pH 6.0. Equal volumes (5 ml) of buffers, ionic strength 0.1, and rennin solutions were mixed, and to each tube were added three drops of toluene. The tubes were stoppered and divided into two series, one being placed in a water thermostat at 25°C, and the other being in a refrigerator at 2°C. The result of the experiment, expressed as the per cent loss of the initial activity after two days, is illustrated in Fig. 2. Table 2 shows the course of the inactivation at pH 3.5 and 7.0.

The stability experiments were performed in citrate buffers, pH from 2.0 to 4.0. Acetate buffers were used from 4.0 to 5.1, and phosphate buffers for pH over 5.1. At pH 4.0, acetate and citrate buffers were tested in parallel. In all experiments, citrate buffers gave slightly higher losses in activity than acetate buffers at pH 4.0. As the inactivation of rennin at pH below 5 may be considered to be an autolysis, it is of interest to note that similar results have been obtained with digestion of serum albumin in citrate and acetate buffers. The former gave a more rapid degradation than the latter, indicating that citrate has an activating effect upon the proteolytic activity of rennin. At pH 5.1, experiments were made in parallel with acetate and phosphate buffers, but no difference in stability was noted.

2.4. The proteolytic activity of rennin

Digestion of casein by rennin. The casein was precipitated from fresh skim milk by adding 0.1 N HCl to pH 4.6, redissolved in water by slowly adding 0.1 N NaOH to pH 6.7, and was then re-precipitated twice. After washing with distilled water, the casein was dried with alcohol and ether. For the experiments, the casein was dissolved by slowly adding 0.1 N NaOH to pH 6.7. Two ml of a 2 % casein solution was mixed with 2 ml of diluted HCl (the concentration of HCl required to give a suitable pH was determined in preliminary experiments). Digestion took place in a water thermostat at 32°C. The casein mixtures were stirred for 60 min by mechanical inversions of the tubes (three inversions per min). 0.5 ml of rennin was added to each tube, and after a further 60 min, during which time the inversions were continued, 3 ml of the reaction mixture was precipitated with 3 ml 1 M trichloroacetic acid (TCA). The precipitate was removed by centrifugation, and the optical density of the supernatant was determined at 275 mμ. A blank test, in which rennin was added after addition of TCA, was run at each pH. The pH was measured at the beginning and at the end of the reaction. The results are illustrated in Fig. 3 A and B. In most of the tubes the pH was displaced during the reaction as illustrated by the length of the arrows.

Due to the insolubility of casein around the isoelectric point pH 4.6, the proteolytic activity gives irregular results in this range of pH. In Fig. 3, the branches of the curves outside the range of pH in which casein is precipitated, are connected with dotted lines in order to obtain regular curves with only one maximum.

Digestion of serum albumin by rennin. The substrate in these experiments was "bovine serum albumin — Armour". The substrate was supplied as a 30 % solution and was diluted down to 6 % before use. 1 ml substrate solution was mixed with 5 ml citrate buffer, ionic strength 0.1. The reaction took place at 32°C. The substrate mixtures were placed in the thermostat 15 min before addition of 1 ml rennin dilution to each tube. The reaction was stopped after 60 min by adding 5 ml 1 M TCA to each tube, and after centrifugation, the optical density of the supernatant was determined at 275 mμ. A blank test was run at each pH. No displacement of pH was noticed during the reaction. The results are shown in Fig. 3 C.

3. DISCUSSION

As the clotting ability of re-constituted skim milk depends to a great extent upon the milk powder used, it has been very difficult to compare the enzymic activity of preparations of rennin crystals made at different laboratories, even if the activity has been expressed in rennin units according to Berridge [1].

Alais [5] made an attempt to compare his crystals to those of Berridge but came to rather divergent results, probably owing to the above-mentioned difficulties. Fortunately, the activity of several preparations of crystals has been compared against Chr. Hansen's commercial rennet, and the author has therefore been able to convert the activities to the same RU used in this work.

The author is grateful to C. Alais for personal communication of results on crystals made after salt fractionation and after preparative electrophoresis on agar gel(published [21]). The author is likewise indebted to M. T. Sode-Mogensen and J. Thomasov, and to M. T. Sode-Mogensen and N. Tofte Jespersen for permission to publish their analyses of a sample of Berridge's crystals (prepared 1945), analysed in 1951 and 1955. H. Dan of Chr. Hansen's Laboratory, Inc., Milwaukee, has independently prepared a sample of rennin crystals, which has been analysed at this laboratory. Ernstrom's result is taken from his publication [9]. The various results appear in Table 3.

Table 3. Comparison between the rennin activity per mg N of different preparations of crystalline rennin.

Preparation	Analysed by	RU/mg N
Average this work	B. Foltmann	944
H. Dan	B. Foltmann	1 066
C. A. Ernstrom [9]	C. A. Ernstrom	922
N. J. Berridge [1] (1945)	M. T. Sode-Mogensen and J. Thomasov (1951)	889
N. J. Berridge [1] (1945)	M. T. Sode-Mogensen and N. Tofte Jespersen (1955)	731
C. Alais [5] (prepared by salt fractionation)	C. Alais	662
C. Alais [21] (prepared by electrophoresis)	C. Alais	858

Though there are appreciable differences between the various preparations, the activity of all is of the same order of magnitude. It should be added that deviations in the preparations made at this laboratory have been from 880 RU/mgN to 1 000 RU/mgN. Alais, analysing a sample of our crystals, found a deviation of only 4 % from that found here.

The stability of solutions of crystalline rennin is considerably less than that of commercial liquid rennet. This is often met with in the purification of enzymes (Anson [22]). The results of the present work confirm the well-known fact that rennin is very sensitive to alkaline or even neutral reaction. Around pH 3.5, the enzyme is unstable. As this range coincides with that of the maximum of proteolytic activity, it indicates that the loss may be due to autolysis. When comparing the inactivation of rennin at 2° and 25°C it is noticed that by raising the temperature, the inactivation at pH 7 is increased considerably more than at pH 3.5.

Casein is the natural substrate for rennin, but as it is only very slightly soluble around its isoelectric point (pH 4.6) it is not well suited to characterise the proteolytic activity of rennin in relation to pH. The precipitated casein is not particularly accessible to the proteolytic activity of rennin, and this will cause irregular observations around pH 4.6. On deeper degradation, these irregularities will be reduced to some extent. However, if the observations plotted in Fig. 3 are connected in such a way that regular curves with only one maximum are obtained, the "maximum" of proteolytic activity is found at pH 4. As it was observed that citrate precipitates casein over a greater range of pH than does HCl, the experiments have been performed without addition of buffer, although this had the disadvantage of the pH being displaced during the reaction. On comparing the degradation of casein and serum albumin, it was noticed that, 1: serum albumin is only degraded in a rather narrow interval around pH 3.4, and 2: that the degradation of serum albumin proceeds much more slowly than that of casein. However, this cannot be regarded as a special case for casein and rennin, as casein in general is more easily digested by proteolytic enzymes than are globular proteins.

Though the general proteolytic activity of rennin is only feeble at pH 6.6—6.7, which is the normal pH of fresh skim milk, Alais *et al.*[15] have shown that the clotting of casein starts with a rapid release of non protein nitrogen (NPN) soluble in 12 % TCA. A study on the enzymatic and the coagulation stages of the clotting of milk by rennin (Foltmann [23]) has supported the point of view that the increase of NPN soluble in 12 % TCA corresponds to the enzymatic phase proper. The lack of linearity between the rennin concentration and the reciprocal of the clotting time may be explained by the time lag of the coagulation stage, when the clotting point observed occurs some time after the increase of NPN comes to an end.

Acknowledgements. The electrophoretic experiments were carried out at the Protein Laboratory of the University of Copenhagen by Dr. N. Harboe. The author is grateful to Dr. N. Harboe for his kind help and for many inspiring discussions. The author wishes to thank the management of Chr. Hansen's Laboratory Ltd. for permission to publish the results. Thanks are also due to the staff of the laboratory for their kind interest in the work, especially to Mrs. K. M. Djurtoft for her valuable co-operation and to Mrs. I. M.

Kristensen for skilled technical help. The author is indebted to Prof. C. Faurholt, Dr. R. Djurtoft and Dr. N. Harboe for critical review of the manuscripts. Mr. R. Gillman has kindly assisted in the translation to English.

REFERENCES

1. Berridge, N. J. *Biochem. J.* **39** (1945) 179.
2. Hankinson, C. L. *J. Dairy Sci.* **26** (1943) 53.
3. De Baun, R. M., Connors, W. M. and Sullivan, R. A. *Arch. Biochem. Biophys.* **43** (1953) 324.
4. Berridge, N. J. and Woodward, C. *J. Dairy Res.* **20** (1953) 255.
5. Alais, C. *Lait* **36** (1956) 26.
6. Oeda, M. *XIV Intern. Dairy Congr.* Rome 1956 **2** (2) 367.
7. Hostettler, H. *Milchwissenschaft* **10** (1955) 40.
8. Foltmann, B. *Acta Chem. Scan.* **12** (1958) 343.
9. Ernstrom, C. A. *J. Dairy Sci.* **41** (1959) 1663.
10. Schwander, H., Zahler, P. and Nitschmann, H. *Helv. Chim. Acta* **35** (1952) 553.
11. Holwerda, B. J. *Biochem. Z.* **134** (1923) 381.
12. Porcher, C. *Lait* **10** (1930) 47.
13. Ege, R. and Lundsteen, E. *Biochem. Z.* **268** (1934) 164.
14. Nitschmann, H. and Bohren, H. U. *Helv. Chim. Acta* **38** (1955) 1953.
15. Alais, C., Mocquot, G., Nitschmann, H. and Zahler, P. *Helv. Chim. Acta* **36** (1953) 1955.
16. Berridge, N. J. *Analyst* **77** (1952) 57.
17. Sommer, H. H. and Matsen, H. *J. Dairy Sci.* **18** (1935) 741.
18. Storch, V. and Segelcke, Th. *Milch-Ztg.* **3** (1874) 997.
19. McMeekin, T. L. *J. Am. Chem. Soc.* **61** (1939) 2884.
20. Foltmann, B. *Acta Chem. Scand.* **13** (1959) 1936.
21. Alais, C., Grabar, P. and Cristol, G. *Intern. Symp. Enzyme Chem.* Tokyo 1957, Pergamon Press, London 1958, p. 523.
22. Anson, M. L. *Les Protéines*, 9éme Conseil de Chimie, Stoops, Bruxelles 1953 p. 213.
23. Foltmann, B. *XV Intern. Dairy Congr.* London 1959 **2**, p. 655.

Received July 15, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1936–1942

Studies on Rennin

III. On the Solubility of Rennin

BENT FOLTMANN

Chr. Hansen's Laboratory, Ltd., Copenhagen, Denmark

Rennin crystals show a slow solubility rate. They are very soluble in distilled water (more than 8 %) and very slightly soluble in concentrated salt solutions (about 0.02 % in 1 N NaCl, pH 5.5). Amorphous precipitates prepared of crystallised rennin fulfil the requirements of the phase rule for a single-component system at the pH of optimum stability. Amorphous rennin shows a solubility minimum in Na-acetate buffers at ionic strength 0.1, pH 5.45. In NaCl solutions, ionic strength 2.5 to 4.85, the log.sol. decreases linearly with ionic strength. The solubility around the isoelectric point (pH 4.6) is small, 0.01 % at ionic strength 0.005, increasing with rising ionic strength.

1. INTRODUCTION

Previous studies on rennin have only contained few remarks on its solubility. Hankinson and Palmer [1] have stated that purified, non-crystallised rennin behaves like a globulin, soluble in a dilute NaCl solution, precipitating at pH 4.5—4.7 (the isoelectric point), insoluble in saturated salt solutions. Berridge [2] has stated that rennin crystals have a slow solubility rate and a very low solubility in salt solutions. Berridge [2] also made a solubility test for homogeneity using an $MgCl_2$/Na-acetate buffer as the liquid phase.

As rennin preparations are most often made using precipitations with NaCl, and as divalent metal ions will interfere in the milk-clotting test, the following experiments are made with NaCl-solutions buffered with Na-acetate.

The crystalline rennin used in these experiments was prepared and recrystallised twice as described earlier (Foltmann [3]). During the experiments, it was very difficult to get exact, reproducible results when working with rennin crystals, as these have a very slow rate of solubility in salt solutions, and as the final phase of the crystallisation also proceeds slowly. The experiments in the sections 3.2, 3.3 and 3.4 were therefore performed using amorphous precipitates of rennin prepared in the following way: Rennin crystals were dissolved by dialysis against distilled water, and by rapid mixing of the rennin solution with the solvent in question, the rennin was precipitated in an amorphous state.

2. EXPERIMENTAL

The rennin activity was assayed and expressed in rennin units (RU) as previously described [3]. The results may be converted to mg rennin after dividing by 140, as the rennin used had an activity of 140 RU/mg dry weight. The optical density of the solutions was determined on a Beckman spectrophotometer DU at 277.5 mμ.

The solubility experiments were made in 2—3 ml centrifuge tubes closed by a rubber stopper. In order to avoid air-bubbles in the tubes during the experiments, the tubes were closed, using a thin syringe needle — through which the air could escape — beside the rubber stopper. However, it was very difficult to expel all the air, and during most of the experiments, air was present in the tubes. Control experiments have shown that the loss of enzymatic activity due to surface inactivation on the air-bubbles in the tubes was negligible.

The tubes were attached to a rectangular grid by means of a rubber band. For experiments with amorphous precipitates, equilibrium between the solid and the liquid phases was ensured by mechanical inversion of the tubes (3 inversions per min) during 18—20 h. For experiments with the crystalline form, the inversions were continued for up to 10 days. The experiments at 2°C were performed in a refrigerator in which the temperature was controlled to ± 1°.

The solid phase was separated from the liquid phase by means of centrifugation in the refrigerator. A possible rise of temperature during the centrifugation could not be observed. For experiments at 25°C, equilibration took place in a water thermostat (± 0.1°C). Centrifugation took place at room temperature, 23°C ± 2°.

As the precipitates of amorphous rennin were formed by mixing rennin solutions with a solvent, the equilibrium was in most cases arrived at from the supersaturated side. However, by salting-out in concentrated salt solutions, where rennin in the amorphous state is more soluble at 2° than at room temperature, the equilibrium was arrived at from both unsaturated and supersaturated solutions.

The unsaturated conditions were established by mixing the rennin and salt solutions at room temperature, and letting them stand at this temperature for 3 h before the inversion process was started in the refrigerator. The supersaturation was obtained by cooling the rennin and salt solution to 2°C before mixing and inversion.

3. RESULTS

3.1. Solubility of rennin crystals

Exact determinations of solubility of rennin crystals in solutions of NaCl are very difficult to carry out. The solubility rate is very slow. When working with a slight surplus of solid phase, even 8 days of inversion of the tubes in the refrigerator will not be sufficient to establish equilibrium. However, it is observed that rennin crystals are very soluble in distilled water. The most concentrated solution we have analysed has had an activity of 11 000 RU/ml, corresponding to an 8 % solution, pH of the solution 5.5. This solution was made by dialysis of the crystals against distilled water. During the process of dialysis, semi-dissolved crystals formed a heavy bottom phase, somewhat like honey; the viscous liquid was not analysed, but it was obviously much more concentrated than the final 8 % solution. However it shall be added, that by electrodialysis of the solution pH was displaced to 4.6 and the rennin was precipitated in an amorphous state.

In salt solutions, the solubility of the crystals decreases very rapidly when the salt concentration is raised. In 0.2 N NaCl, the solubility is around 200 RU/ml, in 1 N NaCl 30 RU/ml and in 4 N NaCl 5 RU/ml, corresponding to 1.43, 0.21 and 0.04 mg rennin/ml. The pH of the liquid phase has been 5.5 ± 0.1

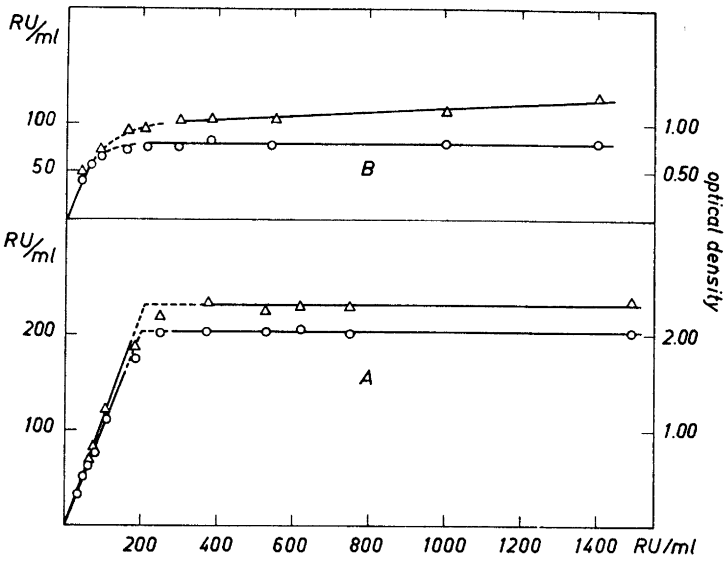


Fig. 1. Solubility test for the homogeneity of rennin. Solid phase: Amorphous precipitates of rennin. Liquid phase: A, NaCl-acetate, pH 5.5, ionic strength 4. B, Na-acetate buffer, pH 4.6, ionic strength 0.05. Abscissa: Total rennin activity of the suspensions. Ordinate: (△) optical density of the liquid phase at 277.5 mμ. (○) rennin activity of the liquid phase. All determinations made at 2°C.

during all these determinations. Contrary to precipitates of amorphous rennin, the rennin crystals seem to be more soluble at 25°C than at 2°C.

In the experiments on the solubility rate of rennin crystals, it was noted that the solubility rate did not only depend on the total amount of the solid phase. Also of importance was whether or not the crystals had been used in earlier solubility experiments. If the crystals had been "extracted" once, the rate of solubility would be much slower in a second experiment, almost as if a superficial layer of rennin molecules was more loosely bound in the crystals than the deeper placed molecules. A constant ratio between the rennin activity and the optical density of the liquid phase suggested that no fractionation took place owing to repeated "extraction" of the crystals.

3.2. Solubility test for homogeneity of rennin (Fig. 1)

Solubility tests for the homogeneity of rennin were made at the pH of optimum stability (pH 5.5) and at a pH near the isoelectric point (pH 4.6). All experiments in this section were made at 2°C. These experiments were performed using amorphous precipitates of rennin as the solid phase. The precipitates were formed by rapid mixing of dialysed rennin solutions with the solvent.

In experiments made at pH 5.5, 0.4 ml rennin solution was mixed with 1.6 ml of a stock salt solution consisting of 117 g NaCl + 41 g Na-acetate + 45

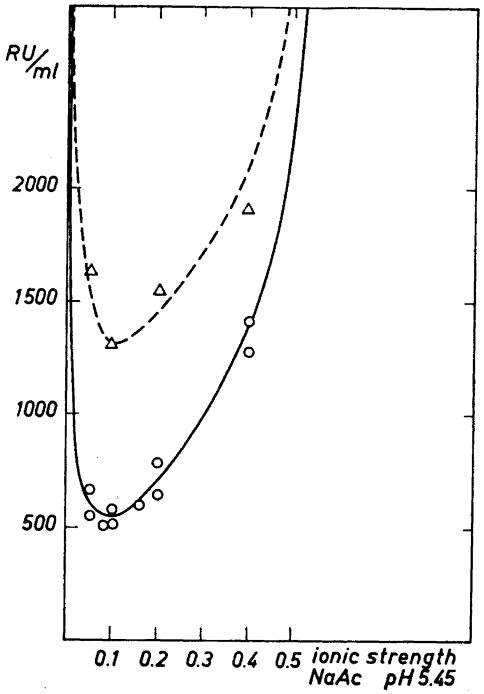


Fig. 2. Solubility of amorphous precipitates of rennin at low salt concentrations. Liquid phase: Na-acetate buffers pH 5.45 ± 0.05. Dotted line, solubility at 25°C. Full line, solubility at 2°C.

ml 1 N acetic acid made up with water to 500 ml. The pH of the solution was 5.5 and the resulting ionic strength of the suspensions was 4.0. The suspensions were equilibrated by 18 h of inversion of the tubes. The activity and the optical density of the supernatants after centrifugation are illustrated in Fig. 1 A. At pH's near the isoelectric point rennin is not quite stable (Foltmann [3]), but as further experiments (section 3.4) were made in this range of pH, the homogeneity was also tested at pH 4.6. In these experiments, 1 ml of rennin solution was mixed with 1 ml Na-acetate buffer pH 4.6, ionic strength 0.1. The activity and optical density of the supernatants after centrifugation are shown in Fig. 1 B.

3.3. Salting-out of rennin (Figs. 2 and 3)

As previously stated (Foltmann [4]), rennin in the amorphous state has a solubility minimum at ionic strength 0.1, pH 5.4. In order to localise this solubility minimum in dilute salt solutions, experiments were made with suspensions of amorphous rennin in solutions of ionic strength from 0.05 to 0.4. To control pH, Na-acetate buffers were used in the liquid phase. When preparing the buffers, the relative concentration of acetic acid was adjusted so as to maintain

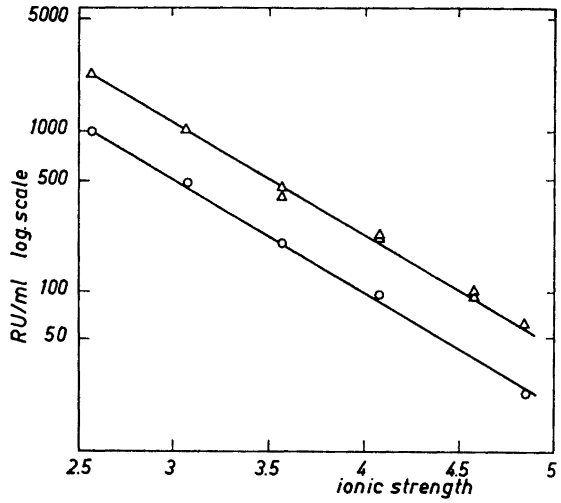


Fig. 3. Salting-out of amorphous rennin by NaCl. pH of the liquid phase 5.3 ± 0.05. Upper line (△) solubility at 2°C. Lower line (○) solubility at 25°C.

the pH at 5.45 ± 0.05. The experiments were made at 2°C and at 25°C. Results are shown in Fig. 2.

In the experiments on salting-out of rennin in concentrated salt solutions, NaCl was dissolved in Na-acetate buffer, pH 5.6, ionic strength 0.1, giving ionic strength of the mixtures from 2.5 to 4.85. The resulting pH, 5.3 ± 0.05. Results are shown in Fig. 3, and it is seen that the enzyme in equilibrium with amorphous precipitates is more soluble in concentrated salt solutions at 2°C than at 25°C.

In Fig. 3, the results are illustrated by log RU/ml, as RU/ml was the quantity observed directly. The logarithm of the solubility decreases linearly with the ionic strength according to Cohn's equation [5], $\log s = B - Ks \cdot \frac{\Gamma}{2}$. After the concentration of soluble rennin was converted from RU/ml to g/litre, the constants of Cohn's equation were calculated by the method of the least squares. The following values were obtained:

$$2°,\ B = 3.00,\ Ks = -0.703$$
$$25°,\ B = 2.67,\ Ks = -0.708$$

As experience has shown that crystallisation often starts at glass surfaces, these experiments were performed in polyethylene tubes, assuming the polyethylene surface to be more inactive than the glass surface. In spite of this, it has not been possible to determine the solubility of amorphous rennin at ionic strengths from 0.4 to 2.5. Within this range, the solutions were so supersaturated with respect to crystals, that crystallisation started immediately. In experiments at ionic strengths 2.5 and 3, a microscopic examination of the precipitate disclosed a few crystals, but the granular, amorphous precipitate

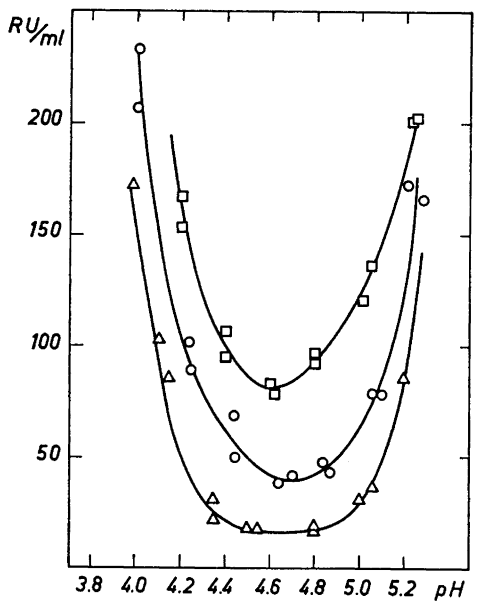


Fig. 4. The influence of pH and ionic strength on the solubility of amorphous precipitates of rennin near the isoelectric point. Liquid phase: Na-acetate buffers, △: ionic strength 0.005; ○: 0.025; □: 0.05.

dominated. The great surplus of the most soluble form will have fixed the concentration of the liquid phase. Experience has also shown that at rennin concentrations below 500 RU/ml, crystallisation starts very slowly, although the solutions are supersaturated with respect to crystals.

3.4. Solubility of rennin at the isoelectric point (Fig. 4)

The experiments were made using acetate buffers; ionic strengths of the mixtures: 0.005, 0.025 and 0.05. In each determination, 1 ml of dialysed rennin solution has been mixed with 1 ml of buffer. The dialysed rennin solution had a pH of 5.5. In experiments at low ionic strength especially, the buffer capacity of the rennin was sufficient to displace the pH of the buffer. The pH of every mixture was therefore checked after equilibrium was obtained, and the final pH was used when plotting the results in Fig. 4. All experiments referred to in Fig. 4 were made at 2°C.

Results from experiments made at 25°C are rather uncertain, due to the instability of the enzyme in this range of pH. However, it seems that under these conditions, the solubility is higher at 25°C than at 2°C. At pH 4.6, ionic strengths 0.025 and 0.05, the solubility at 25°C was determined as 54 and 114 RU/ml.

4. DISCUSSION

The solubility test for homogeneity of the rennin, made with 4 N NaCl-acetate as the liquid phase (Fig. 1 A), nearly fulfilled the requirements of the phase rule for a single-component system, although the point of inflection is not absolutely sharp. When using Na-acetate buffer, ionic strength 0.05, pH 4.6, as the liquid phase (Fig. 1 B), the results do not follow the phase rule for a single-component system. The »point of inflection» is round, and the optical density especially will rise with rising concentration of the solid phase. Here, it must be remembered that the enzyme is not stable at pH 4.6, and the deviations may be ascribed to the beginning of degradation. This experiment cannot be regarded as a test for homogeneity, but as further experiments were made at pH's around the isoelectric point, it was considered expedient to report these results.

At pH near the isoelectric point rennin is rather insoluble (Fig. 4), but the instability below pH 5 greatly complicates a theoretical interpretation of the solubility curves. The second solubility minimum at ionic strength 0.1 (Fig. 2), further complicates a theoretical examination. Because of this second solubility minimum the solubility at ionic strength 0.1 will be diminished, relative to lower ionic strength, on raising pH from 4.6 to 5.4. This means that the rising branches of the solubility curves, above pH 4.6, will intersect between pH 5.2 and pH 5.4. The point of intersection has not yet been fixed.

At pH 5.3—pH 5.5, the solubility of amorphous rennin is greater at 25°C than at 2°C, this when the ionic strength is below 0.4, whilst the opposite is the case at ionic strengths above 2.5. However, it has not been possible to determine the point of intersection of the curves owing to crystallisation of the rennin.

In Figs. 2, 3 and 4, the results of the experiments are only illustrated by means of rennin activity curves, this for purposes of clarity. However, the ratio between the milk-clotting activity and the optical density at 277.5 mμ has been checked in all experiments. As could be expected (Fig. 1B), this ratio was found not to be constant in the experiments around the isoelectric point (Fig. 4). In the other experiments made at 2°C, the ratio was constant ± 5 %. However, the ratio decreased up to 30 % in the least active supernatants from experiments at 25°C. As a corresponding increase in the same ratio was not found after dissolution of the remaining solid phase, the decrease of the enzymatic activity of the liquid phase was probably due to beginning inactivation and not to fractionation.

REFERENCES

1. Hankinson, C. L. and Palmer, L. S. *J. Dairy Sci.* **25** (1942) 277.
2. Berridge, N. J. *Biochem. J.* **39** (1945) 179.
3. Foltmann, B. *Acta Chem. Scand.* **13** (1959) 1927.
4. Foltmann, B. *Acta Chem. Scand.* **12** (1958) 343.
5. Cohn, E. J. *Physiol. Rev.* **5** (1925) 349.

Received July 15, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1943–1954

Conjugate Additions of Grignard Reagents to alpha,beta-Unsaturated Esters

V. Additions of *n*-Butylmagnesium Bromide to *sec*-Butyl Esters of Maleic, Fumaric, Citraconic, and Mesaconic Acids

EIGIL BJERL NIELSEN, JON MUNCH-PETERSEN, PEER MØLLER JØRGENSEN and SUSANNE REFN

Organisk-kemisk Laboratorium, Polyteknisk Læreanstalt, Copenhagen, Denmark

n-Butylmagnesium bromide reacts with *sec*-butyl maleate, fumarate, citraconate and mesaconate by addition to the carbon-carbon double bond (1,4-addition) as well as by addition to one of the carbonyl groups (1,2-addition). 1,4-Additions lead to substituted succinic esters, while 1,2-additions followed by 1,4-additions result in saturated lactones. With maleic and fumaric esters the yields of simple 1,4-addition product (*n*-butylsuccinic ester) are rather low, but the yield from the maleic ester is greatly improved by cuprous chloride catalysis. The yields from citraconic and mesaconic esters are quite satisfactory with respect to the amounts of 1,4 addition product formed, but the products are mixtures of the two isomeric methyl-*n*-butylsuccinic esters. From mesaconic ester the major product is the α,α'-disubstituted ester, whereas the product from citraconic ester is mainly the α,α-disubstitute. In the presence of cuprous chloride citraconic and mesaconic esters are to a large extent reduced (to pyrotartaric ester). The effect of inverse addition of the Grignard reagent has been studied with certain esters. The mechanisms of the reactions are considered.

This paper is concerned with the behaviour of the *sec*-butyl esters of maleic, fumaric, citraconic and mesaconic acids in reactions with Grignard reagents under certain conditions which have previously been found favourable for conjugate addition in the reactions of esters of mono-carboxylic α,β-unsaturated acids such as *sec*-butyl crotonate[1]:

$$CH_3CH{=}CH{-}COO\text{-}sec\text{-}C_4H_9 \xrightarrow{n\text{-}C_4H_9MgBr} CH_3\underset{\displaystyle n\text{-}C_4H_9}{\underset{|}{C}}H{-}CH_2COO\text{-}sec\text{-}C_4H_9$$

Reactions between maleic and fumaric esters and metalloörganic compounds have occasionally been described in the literature [2-6] and maleic anhydride has also been included in these studies [4-6]. In most cases methyl or ethyl esters have been employed, and the reactions have generally taken place as 1,2-additions (to the carbonyl group), although 1,4-additions (to the carbon-carbon double bond) have often been reported as well. The products obtained have thus been either unsaturated or saturated diketones or tertiary alcohols (*cf.* Ref.[7]). Usually, no yields are given.

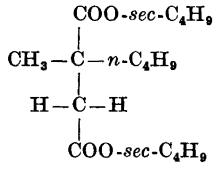
Marvel *et al.*[6], however, have obtained about 30 % yield of diethyl *iso*propylsuccinate from equivalent amounts of either diethyl maleate or fumarate and *iso*propylmagnesium bromide, when the Grignard reagent was added to the ester (no special mention was made of addition time or temperature). Furthermore, the same authors report that the 1,4-addition of *iso*propylcadmium reagent to diethyl maleate also gives a 30 % yield of diethyl *iso*propylsuccinate; in this case the ester was added to the reagent at 0° during 3 1/2 h. This latter result is remarkable, since usually cadmium reagents are believed to react by nucleophilic substitutions rather than as additions, and are consequently quite unreactive towards double bonds [8].

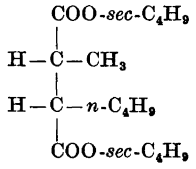
Also a zinc reagent has apparently been used in reactions of this type, since Michael [9] report the formation in 20—25 % yield of diethyl ethylsuccinate and diethyl α-methyl-α'-ethylsuccinate by the prolonged action of ethyl iodide and zinc upon diethyl fumarate and citraconate, respectively. No details are mentioned.

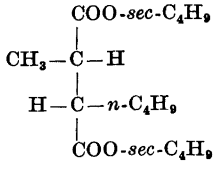
Much better results have been reported [10] with tetraethyl ethylenetetracarboxylate to which methyl-, ethyl- and phenylmagnesium bromides have been added (in yields of 79, 56 and 71 %, respectively) without taking any special precautions (cooling or prolonged addition time).

In the present investigation, elucidation of the following three questions has been attempted: 1. Does conjugate addition to the double bond take place and in what yield? 2. Could any difference in this respect be found between geometrically isomeric esters (such as maleic and fumaric esters)? 3. Which would be the orientation (if any) of addition to unsymmetrically substituted esters (such as citraconic and mesaconic esters)? The possible catalytic effect of cuprous chloride on the course of the reactions has also been investigated.

From maleic and fumaric esters only one simple 1,4-addition product is possible: *d,l-n*-butylsuccinic ester; but from citraconic and mesaconic esters, in fact, three different racemic methyl-*n*-butylsuccinic esters are conceivable: *d,l-α*-methyl-*α*-*n*-butylsuccinic, *d,l-erythro-* and *d,l-threo-α*-methyl-*α'*-*n*-butylsuccinic esters:





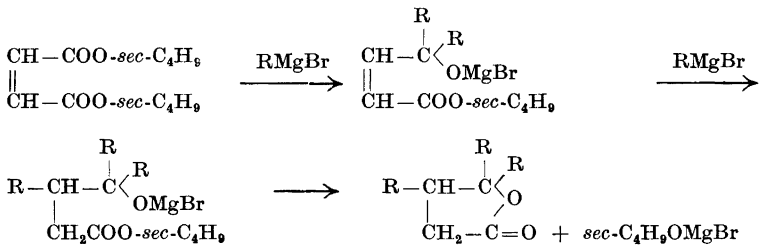


Since the addition process can hardly be visualized as being stereospecific (*cf.* below), the possibility that only one of the two different stereo-isomers (or a mixture of unequal amounts thereof) is formed has not been considered *.

The results of the addition reactions with *n*-butylmagnesium bromide are given in Table 1.

Non-catalyzed additions. When the general procedure [1] is followed, the maleic and fumaric esters give rather unsatisfactory yields (30—40 %) of the conjugate addition product (*n*-butylsuccinic ester), considerable amounts (17 %) of 1,2-addition product (lactone, see below) being formed. From the citraconic and mesaconic esters, on the other hand, quite satisfactory yields (66 and 55 %, resp.) of addition products are obtained, with only little lactone being produced. In one run with mesaconic ester a small quantity of reduction product (se below) was isolated.

The high-boiling by-products (II, IV and VII) are believed to be lactones, which are formed by initial 1,2-addition to one ester group, followed by 1,4-addition and ring-closure, according to the following scheme (*e.g.* with maleic ester):



The lactone structure of II, IV and VII has been confirmed by analyses and infra-red spectroscopy. The formation of small amounts of lactones has also been observed by earlier workers [2,5].

The reduction process is discussed below.

Cuprous chloride catalyzed reactions. Since cuprous chloride has been previously found to catalyze 1,4-addition in competition with 1,2-addition [7,12] and reduction [13], this catalytic procedure was also tried in the present work, but the results were not entirely consistent with the earlier findings.

In the case of the maleic ester the yield of the conjugate addition product was indeed considerably increased (to 67 %); very little lactone was now produced. The reaction with the fumaric ester was little affected by the presence of cuprous chloride; the amount of 1,4-addition product was somewhat increased at the expense of the lactone.

In the case of the citraconic and mesaconic esters, however, the yield of the 1,4-addition product was strongly decreased (to 30 and 20 %, resp.) by the presence of cuprous chloride, and the reduction of the double bond (leading to

* It is quite likely, however, that one of the stereo-isomeric α,α'-disubstituted acids has been isolated in a comparatively pure form (by the recrystallizations during the identification work, see experimental part), since the melting point of our α,α'-compound is rather sharp and considerably higher (136°) than that reported in the literature [11]: 85—105°. No attempt has been made to determine the configuration.

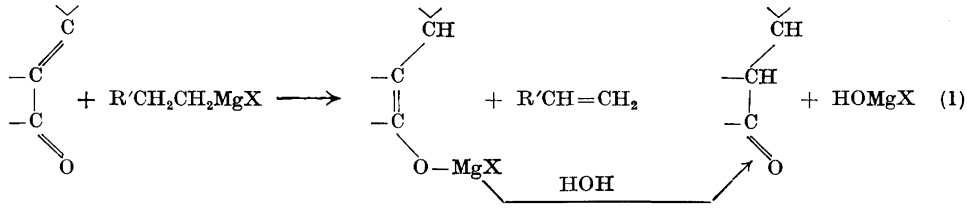
Table 1. Additions of *n*-butylmagnesium bromide to *sec*-butyl esters of maleic (mal), fumaric (fum), citraconic (cit) and mesaconic (mes) acids.

Ester	Catalyst	Products		b. p., °C/mm	n_D^{20}	yield %	remarks
mal	none	I[a]	(1,4-add.)	106—107/0.6	1.4353	39	
		II[b]	(lactone)	143/1	1.4600	17	
mal	CuCl	I	(1,4-add.)			57	ice-salt cooling; ester added during 1.5 h.
		II	(lactone)			6	
mal	CuCl	I	(1,4-add.)			67	
		II	(lactone)			very little	
fum	none	I[c]	(1,4-add.)	113/1	1.4341	19	
		II[d]	(lactone)	131/0.7	1.4600	10	
fum	CuCl	I	(1,4-add.)			32	ester added during 3 h.
		II	(lactone)			17	
fum	CuCl	I	(1,4-add.)			38	
		II	(lactone)			13	
cit	none	III[e,f]	(1,4-add.)	125/2	1.4390	62—70	
		IV[g,h]	(lactone)	110—120/1	1.4665	4	
cit	CuCl	V[i,f]	(reduct.)	87— 92/0.9	1.4283	31—22	
		III	(1,4-add.)	108—110/0.6	1.4372	38—46	
		V[j,f]	(reduct.)	81/0.6	1.4275	9—0	
mes	none	VI[k]	(1,4-add.)	109/1	1.4355	42—55	
		VII[h]	(lactone)	129/0.7	1.4597	4—0	
		V	(reduct.)	87/0.9	1.4261	40	
mes	CuCl	VI	(1,4-add.)	108/0.7	1.4354	21	

I: Di-*sec*-butyl *n*-butylsuccinate[a]. II: β,γ,γ-Tri-*n*-butyl-γ-butyrolactone[b]. III: Di-*sec*-butyl methyl-*n*-butylsuccinate, 90 % α,α-, 10 % α,α'-isomer[e,f]. IV: Believed to be $\beta(\alpha)$-methyl-β,γ,γ-tri-*n*-butyl-γ-butyrolactone[g,h]. V: Di-sec-butyl methylsuccinate (pyrotartrate)[i,f]. VI: Di-*sec*-butyl methyl-*n*-butylsuccinate, 30 % α,α-, 70 % α,α'-isomer[k,f]. VII. Believed to be $\alpha(\beta)$-methyl-β,γ,γ-tri-*n*-butyl-γ-butyrolactone[h].

[a] Calc. for $C_{16}H_{30}O_4$ (286.40): C 67.09; H 10.56. Found: C 67.85; H 10.84. [b] Calc. for $C_{16}H_{30}O_2$ (254.41): C 75.53; H 11.89. Found: C 75.05; H 11.69. [c] Calc. for $C_{16}H_{30}O_4$ (286.40): C 67.09; H 10.56. Found: C 67.55; H 10.40; infra-red spectroscopy showed identity between I_{mal} and I_{fum}. [d] Calc. for $C_{16}H_{30}O_2$ (254.41): C 75.53; H 11.89. Found: C 75.35; H 11.43; infrared spectroscopy showed identity between II_{mal} and II_{fum}. [e] Calc. for $C_{17}H_{32}O_4$ (300.43): C 67.96; H 10.74. Found: C 69.00; H 11.02; the product contaminated with IV. By saponification of III and distillation methyl-*n*-butylsuccinic anhydride was obtained. Calc. for $C_9H_{14}O_3$ (170.20): C 63.51; H 8.29. Found: C 63.95; H 8.12. [f] Infra-red spectra showed great similarity between I, III, V, and VI. [g] This product was obtained in only one run and only as a by-product from the saponification of III. [h] Infra-red spectrum showed great similarity to II; the product was not further investigated. [i] Infra-red spectra showed identity between V_{cit} and V_{mes}. [j] Calc. for $C_{13}H_{24}O_4$ (244.32): C 63.90; H 9.90. Found: C 64.65; H 9.98. [k] Calc. for $C_{17}H_{32}O_4$ (300.43): C 67.96; H 10.74. Found: C 68.10; H 10.88.

methylsuccinic (pyrotartaric) ester) now became correspondingly dominating. This reaction could be written:



(1)

The mechanism of the reduction process is considered below (eqn. 5). The observation that cuprous chloride promotes reduction in competition with 1,4-addition of the Grignard reagent was surprising in view of the above-mentioned findings by Brändström and Forsblad [13], who report a pronounced effect of this catalyst to favour addition over reduction in the reaction of Grignard reagents with alkylidenecyanoacetic esters. This apparent inconsistency is discussed below.

Table 2. Ratios of isomeric methyl-*n*-butylsuccinic esters formed from citraconic and mesaconic esters, determined by infra-red spectroscopy (IR) and by gas-liquid chromatography* (GLC).

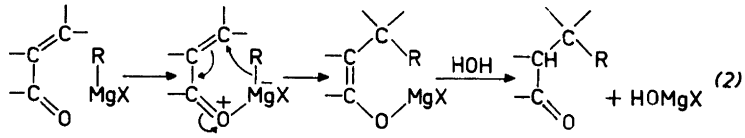
		Uncatalyzed		CuCl-catalyzed		CuCl-catalyzed inverse addition	
		IR	GLC	IR	GLC	IR	GLC
Citraconic	*α,α*	>80 %	90 %	>80 %	90 %	>80 %	90—95 %
	α,α'	<20 »	10 »	<20 »	10 »	<20 »	10— 5 »
Mesaconic	*α,α*	25 %	30 %	25 %	30 %		
	α,α'	75 »	70 »	75 »	70 »		

* Carried out and interpreted by A. Jart, Danish Fat Research Institut, Copenhagen.

*The formation of isomeric methyl-*n*-butylsuccinic esters from citraconic and mesaconic esters.* Regarding the question of the methyl-*n*-butylsuccinic esters obtained by the addition processes with citraconic (*cis*) and mesaconic (*trans*) esters, it was found that both isomers (*α*-methyl-*α*-*n*-butylsuccinic and *α*-methyl-*α'*-*n*-butylsuccinic esters) were formed, but in very different quantities in the two cases. More than 80 % of the *α,α*-isomer and less than 20 % of the *α,α'*-isomer was found in the product from the citraconic ester, while the product from the mesaconic ester contained the isomers in an approximately opposite ratio (Table 2). The ratios were determined by comparing the infra-red spectra of the crude mixtures of isomeric acids, obtained by saponification of the addition products, with the spectra of known mixtures of authentic acids. Although this procedure does not lead to exact values for the ratios, the difference in reactivity of the two geometrically isomeric esters with respect to orientation is quite clear. Somewhat more accurate values were obtained by gas chromatography, see experimental part.

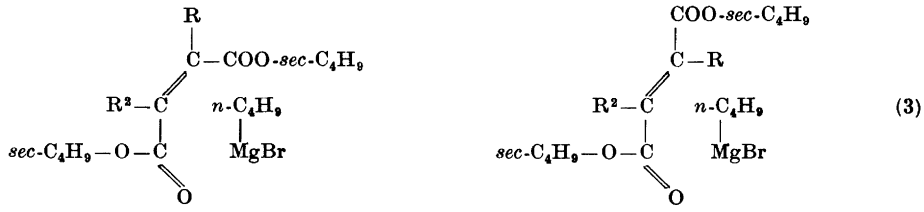
DISCUSSION

For the conjugate addition of Grignard reagents to α,β-unsaturated systems the following cyclic mechanism has been proposed [14]:



(2)

In the case of the esters under consideration, the first step in the above scheme should be represented as follows:



(3)

a. R = R^2 = H: Maleic
b. R = H, R^2 = CH_3: Citraconic
c. R = CH_3, R^2 = H: »
d. R = R^2 = H: Fumaric
e. R = H, R^2 = CH_3: Mesaconic
f. R = CH_3, R^2 = H: »

From this mechanism no stereospecificity could be expected and, in cases where α-methyl-α'-*n*-butylsuccinic esters (3*b* and *e*, above) are formed, the reaction products should be mixtures of nearly equal parts of *erythro*- and *threo*-isomers.

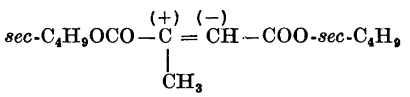
In the reactions with maleic and fumaric esters (3*a* and *d*), two factors should be important for the yield; the reactivity of the double bond and the steric conditions for the cyclic mechanism. Obviously, the configuration of the fumaric ester is more favourable in the latter respect than that of the maleic ester. However, the double bond of the maleic ester is probably much more reactive than that of the fumaric ester, since maleic esters are under considerable steric strain because of the two ester groups in the *cis*-position, which prevent co-planarity between the two carbonyl groups and the ethylenic bond. This strain would be particularly high in the case of the *sec*-butyl esters, and this factor, apparently, is the more important one.

In the reactions with citraconic and mesaconic esters (3*b* or *c* and *e* or *f*) the same two factors, mentioned above for maleic and fumaric esters, should be operative, again resulting in a higher yield of addition product from the *cis*-isomer (citraconic ester) than from the *trans*-isomer (mesaconic ester).

One further point should be mentioned, which may be significant. Whereas the yield of addition product from fumaric ester was considerably less than from maleic ester, the total yield of addition *and* reduction products from mesaconic ester was only little less than the corresponding yield from citraconic ester. This was true of both the uncatalyzed and the catalyzed reactions.

These findings are in agreement with the view that the strain caused by the groups in the *cis*-position is an enhancing factor in the addition reaction since mesaconic ester may be expected to be under considerably more strain than fumaric ester, although less than citraconic and maleic esters.

Considering the above mechanism, an understanding may also be reached, as to why the two — structurally different — isomers predominate in the ester-mixtures produced by the two additions to citraconic and mesaconic esters. Here, again, two factors may be important: the polarization of the double bond and the steric conditions for the cyclic mechanism. In the case of citraconic ester (*cis*) the two ester groups are equal in stereo-chemical respect. The polarization of the double bond:



then, is apparently a more important directing factor than the overcrowding at the methyl-substituted carbon atom (situation 3*c*, above). Thus the α,α-disubstituted succinic ester is the main product. In the case of mesaconic ester (*trans*), the steric conditions for situation 3*e* (above) is much more favourable than situation 3*f*. The polarization factor then becomes of minor importance with the α,α'-compound now being produced in the larger quantity.

Effects of cuprous chloride catalysis. While the orientation of the addition to citraconic and mesaconic esters is rather unaffected by the presence of cuprous chloride during the addition process (*cf.* Table 2), the reduction of these esters (to pyrotartaric ester), which in the uncatalyzed reaction is only of minor importance in comparison to the 1,4-addition, becomes a dominating side-reaction when cuprous chloride is present.

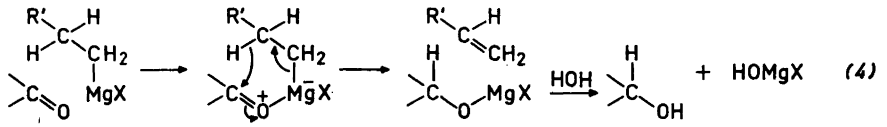
A general statement, that this catalyst appears to enhance the electrophilic reactivity of the carbon-carbon double bond, does not provide any explanation for this experimental fact. Tentatively, one might suggest that an understanding may be sought in the concept of a regular complex formation between cuprous chloride and the carbon-carbon double bond. A great deal of evidence for such complexes can be found in the literature [15], and they also have acquired some technical interest [16]. Andrews and his co-workers [17] have studied the partition of cuprous chloride between water and an organic solvent containing alkenes, unsaturated alcohols (such as allylalcohol) and a number of α,β-unsaturated acids (including those, the *sec*-butyl esters of which have been studied in the present series). Cuprous chloride appears usually to form 1:1 complexes with the unsaturated compounds; the bonding is presumed to occur between the cuprous ion and a carbon atom, an electron pair being furnished by the carbon-carbon double bond. It appears reasonable that, by this coordination, the reactivity of the double bond towards nucleophilic reagents should be augmented. There seems to be no general agreement as to the complexing properties of *cis*, *trans* isomers *, but the negligible effect of cuprous

* Basolo and Pearson (Ref.[15], p. 352 -353) state that *cis*-olefins form more stable complexes than do *trans*-olefins.

chloride on the reaction with fumaric ester indicate that the *trans* compound in this case is the poorer complexing agent, corresponding to the generally lower reactivity mentioned above.

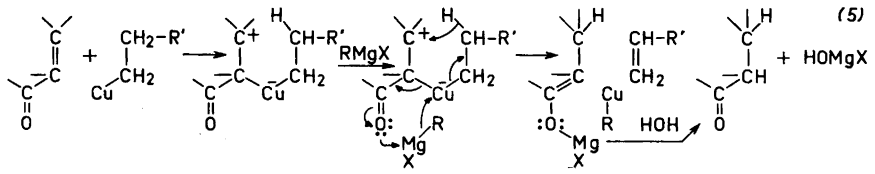
By the coordination also the overcrowding at the double-bonded carbon atoms should be increased. This might favour sterically less demanding reactions, in this case the reduction.

While the cyclic mechanism mentioned above for the 1,4-addition should still be valid in the case of cuprous chloride catalysis, this is not possible for the corresponding mechanism, which has been suggested [18] for the reduction of carbonyl compounds by means of Grignard reagents:



Reduction of the *carbon-carbon* double bond of an α,β-unsaturated carbonyl compound could not take place according to such a mechanism involving a *six*-membered ring and the coordination of the Grignard magnesium with *oxygen*. Provisionally, the following cyclic concerted mechanism is put forward (R = $R'CH_2CH_2$, *cf.* the over-all reaction (eqn. 1)):

$$RMgX + CuCl \rightleftharpoons RCu + MgXCl$$



This mechanism involves an exchange reaction between cuprous chloride and the Grignard reagent leading to the formation of a copper-alkyl compound [19], which is then supposed to coordinate with carbon instead of with oxygen. These synchronous shifts also account for the fact that the effect of cuprous chloride is a catalytic one, with the copper-alkyl being continuously regenerated. Nothing could be stated about the orientation of the attack of the cuprous chloride at the double bond, since the polarization effect and the steric effect have an opposite influence, and in this case the nature of the reaction product does not, of course, give any information.

Effects of using inverse addition procedure. As mentioned in the foregoing, the reduction-promoting action of cuprous chloride appeared to be in contrast to the effect found by Brändström and Forsblad [13]. However, these authors emphasize the importance of inverse addition (that is the addition of the Grignard reagent *to* the ester containing the cuprous chloride). With the three

Table 3. Yields of 1,4-addition and reduction products from reactions of *n*-butylmagnesium bromide with α,β-unsaturated esters under different conditions.

Ester	Product	Normal addition no cat.	Normal addition CuCl	Inverse addition no cat.	Inverse addition CuCl
Ethyl *iso*propylidene-cyanoacetate	1,4-add.[a]	51 %	50 %	45 %	77 %
	reduct.[b]	17 »	18 »	14 »	5 »
Ethyl *iso*propylidene-malonate	1,4-add.[c]	60 %	42 %	61 %	89 %
	reduct.[d]	22 »	40 »	20 »	3 »
sec-Butyl citraconate[e]	1,4-add.	66 %	38—46 %		20 %
	reduct.	0 »	31—22 »		0 »

[a] Ethyl 2-cyano-3,3-dimethylheptanoate (ethyl 1,1-dimethylpentyl-cyano-acetate), b.p. 128°/13 mm, n_D^{20} 1,4412. Calc. for $C_{12}H_{21}O_2N$ (211.30): C 68.21; H 10.02. Found: C 68.05; H 9.80. [b] Ethyl *iso*propylcyanoacetate, b.p. 92—93°/13 mm, n_D^{20} 1.4238. This product did not give the correct analyses; it is probably contaminated with ethyl cyanoacetate, which may arise by a reversal of the condensation of this ester with acetone (the formation of ethyl *iso*propylidene-cyanoacetate). [c] 1,1-Dicarbethoxy-2,2-dimethylhexane (ethyl 1,1-dimethylpentyl-malonate), b.p. 132—133°/13 mm, n_D^{25} 1.4360. Calc. for $C_{14}H_{26}O_4$ (258.35): C 65.08; H 10.14. Found: C 65.50; H 9.98. [d] Ethyl *iso*propylmalonate, b.p. 96—98°/13 mm, n_D^{25} 1.4223. Calc. for $C_{10}H_{18}O_4$ (202.24): C 59.38; H 8.97. Found: C 59.60; H 8.77. [e] See Table 1.

esters of Table 3 we have, therefore, investigated the influence of changing conditions in this respect.

Our results with *iso*propylidenecyanoacetic ester are, in fact, in complete accordance with those of Brändström and Forsblad: cuprous chloride has no effect upon the addition/reduction ratio in the normal addition, but when inverse addition is used, the reduction is largely suppressed and the addition favoured.

With citraconic ester, on the other hand, the normal, uncatalyzed addition procedure appear to be the method which gives the best yields of 1,4-addition. The presence of cuprous chloride strongly favours the reduction when normal addition procedure is used (*cf.* also Table 1), and the catalyzed, inverse addition method, although preventing reduction, gives very low yield of 1,4-addition product, a non-destillable residue being the main reaction product.

Finally, with *iso*propylidenemalonic ester cuprous chloride promotes reduction in the normal addition procedure, but prevents reduction in the inverse procedure, whereas the yields in the uncatalyzed reactions are independent of the addition method.

The results with *iso*propylidenecyanoacetic and citraconic esters with respect to the competition between 1,4-addition and reduction are obscured by the rather low yields of the products in question. The results with *iso*-propylidenemalonic ester, on the other hand, may perhaps be taken as typical, since here, apparently, other reactions take place only to a small extent.

These results are not inconsistent with the above suggested mechanisms. The increased reduction in the normal addition procedure is already considered (eqn.5). In the inverse addition, on the other hand, the cuprous chloride is mixed with the ester, and the Grignard reagent is then slowly added. The catalyst could thus be expected to coordinate with the double bond of the ester, and, by the subsequent slow addition of the Grignard reagent, the ordinary cyclic mechanism (eqn. 2) could be operative, only with an activated double bond.

EXPERIMENTAL

Microanalyses are by Mr. Preben Hansen, Microanalytical Division, The Chemical Laboratory, The University of Copenhagen. Fractional distillations were through a simple 45 cm × 8 mm Podbielniak-type column with a tantalum wire spiral, a heated jacket and a partial reflux head. All products were checked by infra-red spectroscopy using a Beckman IR 2 single-beam spectrophotometer with sodium chloride optics. The spectra of the solid compounds (*i. e.* the methylbutylsuccinic acids) were recorded using the potassium bromide technique, whereas those of the liquids were measured in solutions in carbon tetrachloride.

Preparation of starting materials. sec-*Butyl* esters were prepared from commercial acids according to the general esterification procedure previously described [20]: *maleinate*, b.p. 85°/0.8 mm, n_D^{20} 1.4432, yield 85 %; *fumarate*, b. p. 80°/0.5 mm, n_D^{20} 1.4430, yield 32 % (65 % fumaric acid recovered); *citraconate*, b. p. 86–87°/0.5 mm, n_D^{20} 1.4457, yield 72 %; *mesaconate*, b. p. 89.5°/0.6 mm, n_D^{20} 1.4491, yield 93 %. The identity of the esters was checked by elementary analyses. Infra-red spectra of *sec*-butyl maleate and fumarate agreed with those published in the literature [21]. The spectra of citraconic and mesaconic esters differed from one another, which was taken as evidence that the stereochemical structures were retained during the esterification processes.

The spectra of these four esters, as well as of those products mentioned in the following, which are saturated *sec*-butyl esters * have all been found to show a strong absorption, split in three peaks, around 1 111 cm^{-1} (9.00 μ). This group of bands is, therefore, considered to be a characteristic absorption for *sec*-butyl esters of carboxylic acids.

Ethyl isopropylidenecyanoacetate, b. p. 113°/17 mm, n_D^{25} 1.4653, was prepared according to Wideqvist [22], *diethyl isopropylidenemalonate*, b. p. 108–110°/13 mm, n_D^{20} 1.4484, according to Cope and Hancock [23].

Additions of n-*butylmagnesium bromide to* sec-*butyl esters of maleic, fumaric, citraconic and mesaconic acids.* The uncatalyzed reactions were carried out as described previously [1]; during 1.5–2 h, 0.1 mole of ester, dissolved in 100 ml of ether, was added to a Grignard solution, prepared from 6.3 g (0.26 g-atom) of magnesium turnings, 42.5 g (0.31 mole) of *n*-butyl bromide and 120 ml of ether, and cooled in ice-water during the addition.

In the cuprous chloride catalyzed reactions [20,7,12] the same amounts of the reagents were used, but 0.3 g of cuprous chloride was added in one portion after the Grignard solution had been cooled in ice-water for 15 min. The ester was dissolved in 200 ml of ether and added over a 2–3 h period. In one case with maleic ester cooling in ice-salt was applied during the addition of cuprous chloride and the ester, but the yield was no better than that obtained when only ice-water was used.

In the inverse addition reactions the Grignard solution was transferred to a siphoning vessel. From this it was added, by means of nitrogen pressure, dropwise to the ester cooled in ice-water during 1.5–2 h under vigourous stirring. When the inverse addition reaction was cuprous chloride catalyzed, the catalyst was added to the ester before adding the Grignard solution.

The inverse reactions with *iso*propylidenecyanoacetic ester were somewhat disturbed by the almost instantaneous formation of a viscous, sticky precipitate when the addition of the Grignard reagent was started. The addition of another 100 ml of ether and the

* And also of all other *sec*-butyl esters recorded during the work of this series.

interruption of the cooling seemed to improve dissolution of the precipitate and the effeciency of the stirring.

Identification of products. The products were isolated by fractional distillations and investigated by microanalyses and infra-red spectroscopy (*cf.* Table 1).

The spectra of the addition products, esters I, III and VI, are very similar and can hardly be distinguished significantly. The spectrum of the reduction product V shows a strong resemblance to those of the three addition products. These four spectra all show a carbonyl band at 1 725 cm^{-1} and the group of absorption bands at 1 111 cm^{-1}, which is considered to be characteristic for *sec*-butyl esters (se above), but no absorption was found for C=C or OH. These data, as well as the low refractive indices constitute strong evidence that the products are saturated *sec*-butyl esters.

The spectra of the high-boiling products II, IV and VII have a carbonyl peak at 1 772 cm^{-1} (5.64 μ), which is within the area reported [24] to be characteristic for saturated γ-lactones (1 780—1 760 cm^{-1}); there was no absorption found for saturated ester (1 750—1 735 cm^{-1}), saturated ketone (1 725—1 705 cm^{-1}), hydroxyl group or carbon-carbon double bond. Also an attempt at alkaline hydrolysis indicated a lactone structure: a very unsharp first end-point in the back-titration gave an equivalent weight of about 225 for II (calc. 254.4), but after some time more acid was required until eventually an amount of acid was used, which was equivalent to the amount of base employed.

The addition products from citraconic and mesaconic esters, III and VI, were saponified according to the saponification procedure previously described [20], except that the saponification time was prolonged to 24 h. The crude acids were obtained as oils, which in the case of VI slowly crystallized completely, whereas the acid-mixture from III only after long standing (a week or more) partially solidified. Crude crystalline products (m. p. range 85—110°) were obtained by recrystallization from ethanol-water. For identification purposes authentic samples of α-methyl-α-*n*-butylsuccinic acid, m. p. 86—88°, and α-methyl-α'-*n*-butylsuccinic acid, m. p. 121—132°, were prepared (see below). The infra-red spectra of these isomers could be distinguished from each other, especially by a difference in absorption around 1 190—1 330 cm^{-1}. Comparison of the spectra of the acids from III and VI with those of the authentic samples, showed the product from citraconic ester ($acid_{III}$) to be the α,α-isomer, whereas the product from mesaconic ester ($acid_{VI}$) was the α,α'-isomer.

By subsequent numerous recrystallizations from very diluted ethanol, the following melting points could indeed be obtained. $Acid_{III}$: m. p. 91—94° (neutr. equiv. 93.6); authentic α,α-isomer: m. p. 91—93° (neutr. equiv. 95.5); $acid_{VI}$: m. p. 132—137° (neutr. equiv. 95.5); authentic α,α'-isomer: m. p. 136—138° (neutr. equiv. 95.2). Mixed melting points of the two pairs of compounds showed no depressions; calc. neutr. equiv. 94.11.

In later experiments, however, also the α,α'-isomer was isolated from runs with citraconic ester, and both addition products (III and VI) might thus presumably be mixtures. The above-mentioned difference in the infra-red absorption groups around 1 190—1 330 cm^{-1} was utilized for estimating the approximate ratio of the isomers formed, by comparing the spectra of the crude acid-mixtures with spectra of suitable mixtures of authentic acids (Table 2).

The proportions of isomers in the mixtures were further checked by gas-liquid chromatography (Table 2), kindly performed and interpreted by Mr. Aage Jart, M.Sc., of the Danish Fat Research Institute, Copenhagen. The acids were transformed into their methyl esters by means of diazomethane. It was found that the retention time was rather strongly dependent on the size of the sample. Therefore, that mixture-component which was present in the smaller concentration was appearing first on the chromatogram in all cases. The ratios were estimated accurate to the nearest 5 %.

Preparations of authentic methyl-n-butylsuccinic acids. α-Methyl-α-*n*-butylsuccinic acid was prepared as described in literature [25]. The addition of hydrogen cyanide to ethyl α-cyano-β-*n*-butyl-β-methylacrylate (below) was followed by hydrolysis and decarboxylation of the dicyano-compound. The latter (ethyl α,β-dicyano-β-methylheptanoate) showed the b.p. 96—100°/1.5 mm (n_D^{23} 1.4399), whereas Miller and Long [25] report the b.p. 141—145°/2.3 mm. Ethyl α-cyano-β-*n*-butyl-β-methylacrylate, b.p. 142—144°/14 mm, n_D^{25} 1.4672, was obtained according to Cope *et al.*[26] by the condensation of methyl *n*-butyl ketone with ethyl cyanoacetate. The α-methyl-α-*n*-butylsuccinic acid had the m. p. 91—93° (rep.[25]: 93°) and the neutr. equiv. 95.5.

α-Methyl-α'-*n*-butylsuccinic acid was synthesized by the alkylation of ethyl *n*-butylmalonate [27] with ethyl α-bromopropionate, followed by hydrolysis and decarboxylation. The procedures used were those described in Organic Syntheses [28]. The α-methyl-α'-*n*-butylsuccinic acid had the m. p. 136—138° (rep.[11]: 85—105°) and neutr. equiv. 95.2.

The authors want to express their gratitude towards *Otto Mønsteds Fond* and *Det Teknisk-Videnskabelige Forskningsraad* for financial support.

REFERENCES

1. Munch-Petersen, J. *Acta Chem. Scand.* **12** (1958) 967.
2. Purdie, T. and Arup, P. S. *J. Chem. Soc.* **97** (1910) 1537.
3. Tarbell, D. S. *J. Am. Chem. Soc.* **60** (1938) 215.
4. Weizmann, C. and Bergmann, F. *J. Am. Chem. Soc.* **60** (1938) 2647.
5. Scholtis, K. *Ann.* **557** (1947) 82.
6. Marvel, C. S., Myers, R. L. and Saunders, J. H. *J. Am. Chem. Soc.* **70** (1948) 1694.
7. Munch-Petersen, J. *Acta Chem. Scand.* **12** (1958) 2007.
8. Cason, J. *J. Org. Chem.* **13** (1948) 231; Cason, J. and Smith, R. D. *J. Org. Chem.* **18** (1953) 1202.
9. Michael, A. *Ber.* **29** (1896) 1791.
10. Hsing, C. and Li, L. *J. Am. Chem. Soc.* **71** (1949) 774.
11. Marvel, C. S. and Fuller, J. A. *J. Am. Chem. Soc.* **74** (1952) 1508.
12. Munch-Petersen, J. *Acta Chem. Scand.* **12** (1958) 2046.
13. Brändström, A. and Forsblad, I. *Arkiv Kemi* **6** (1954) 561; *cf.* Hook, W. H. and Robinson, R. *J. Chem. Soc.* **1944** 152.
14. Lutz, R. E. and Reveley, W. G. *J. Am. Chem. Soc.* **63** (1941) 3180; *cf.* Johnson, J. R. in Gilman, *Organic Chemistry*, Vol. **II**, John Wiley & Sons, 2nd. ed. 1943, p. 1881—1882; Royals, E. E. *Advanced Organic Chemistry*, Prentice-Hall, 1954, p. 699 ff.; Alexander, E. R. *Principles of Ionic Organic Reactions*, John Wiley & Sons, 1950, p. 193—194.
15. For a review, see Douglas, B. E. in Bailar, *The Chemistry of the Coordination Compounds*, Reinhold Publishing Corp. 1956, p. 494; Basolo, F. and Pearson, R. G. *Mechanisms of Inorganic Reactions*, John Wiley & Sons 1958, p. 350 ff. For references see also Slade, P. E. and Jonassen, H. B. *J. Am. Chem. Soc.* **79** (1957) 1277.
16. Atkinson, E. R., Rubinstein, D. and Winiarczyk, E. R. *Ind. Eng. Chem.* **50** (1958) 1553 and further references given therein.
17. Keefer, R. M., Andrews, L. J. and Kepner, R. E. *J. Am. Chem. Soc.* **71** (1949) 2381, and further references given therein.
18. Mosher, H. S. and La Combe, E. *J. Am. Chem. Soc.* **72** (1950) 3994; *cf.* Swain, C. G. and Boyles, H. B. *J. Am. Chem. Soc.* **73** (1951) 870.
19. *cf.* Gilman, H. and Straly, J.M. *Rec. trav. chim.* **55** (1936) 821.
20. Munch-Petersen, J. *J. Org. Chem.* **22** (1957) 170.
21. Walton, W. L. and Hughes, R. B. *Anal. Chem.* **28** (1956) 1388.
22. Wideqvist, S. *Acta Chem. Scand.* **3** (1949) 303.
23. Cope, A. C. and Hancock, E. M. *J. Am. Chem. Soc.* **60** (1938) 2645.
24. Bellamy, L. J. *The Infra-Red Spectra of Complex Molecules*, Methuen, 2nd. ed. 1958, p. 179.
25. Miller, C. A. and Long, L. M. *J. Am. Chem. Soc.* **75** (1953) 375.
26. Cope, A. C., Hofmann, C. M., Wyckoff, C. and Hardenbergh, E. *J. Am. Chem. Soc.* **63** (1941) 3452.
27. Adams, R. and Kamm, R. M. *Organic Syntheses* Coll. Vol. **I** (1941) 250.
28. Adkins, H., Isbell, N. and Wojcik, B. *Organic Syntheses* Coll. Vol. **II** (1943) 262; Vliet, E. B., Marvel, C. S. and Hsueh, C. M. *Organic Syntheses* Coll. Vol. **II** (1943) 416.

Received July 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1955–1959

Conjugate Additions of Grignard Reagents to alpha,beta-Unsaturated Esters

VI. The Course of Reactions with Allylic Grignard Reagents

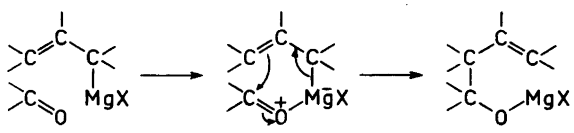
JON MUNCH-PETERSEN, PEER MØLLER JØRGENSEN and SUSANNE REFN

Organisk-kemisk Laboratorium, Polyteknisk Læreanstalt, Copenhagen, Denmark

In contrast to ordinary Grignard reagents, allylic Grignard reagents with *sec*-butyl esters of crotonic, tiglic and cinnamic acids give exclusively and in high yields tertiary alcohols as a result of additions to the carbonyl group (1,2-addition). Benzylmagnesium chloride, on the other hand, adds to the carbon-carbon double bonds in crotonic and cinnamic esters (1,4-addition), whereas tiglic ester with this Grignard reagent gives, in low yields, mixtures of both 1,2- and 1,4-additions products. Cuprous chloride has no effect in the reactions here described.

Grignard reagents from allylic halides differ in several respects from ordinary alkyl- and arylmagnesium halides. Not only must special measures usually be applied by their preparation (*cf.* below, experimental part) in order to prevent nucleophilic attack by the reagent on unreacted halide leading to hydrocarbons of the type biallyl, bibenzyl *etc.*, but also the Grignard reagent itself in its reactions may show a reactivity different from that of other Grignard reagents *. Thus, with allylic Grignard reagents very often the formation of "inverted" products (involving an allylic "rearrangement") is encountered and furthermore, many reactions, which take one course with ordinary Grignard reagents, give rise to other reaction products or to different yields with allylic Grignard reagents. Particularly the reactions of the so-called butenyl Grignard reagent (prepared from crotyl bromide) were thoroughly studied by Young, Roberts and co-workers [2]. These authors give extensive evidence that allylic Grignard reagents react by a cyclic mechanism, involving a synchronous electron shift, resulting in inversion of the allylic group:

* Reactions of allylic Grignard reagents, especially the work of Young, Roberts *et al.* (*e. g.* Ref.[2]), are discussed in several advanced treatises of organic chemistry [1].

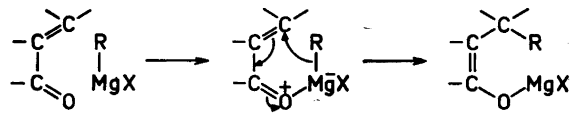


(1)

Consistent with this mechanism are the facts that addition of allylic Grignard reagents to the carbonyl group of sterically hindered ketones is facilitated, compared to the addition of other Grignard reagents, and that the allylic grouping is inverted during the addition process.

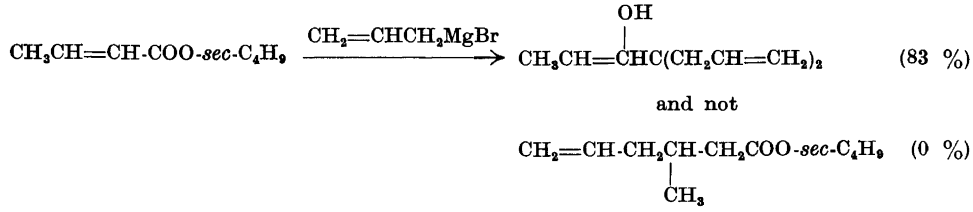
The present paper describes the reaction between *sec*-butyl esters of certain α,β-unsaturated acids and some allylic Grignard reagents under conditions which have previously been found favourable for conjugate addition (to the carbon-carbon double bond) of ordinary Grignard reagents [3]. As model substances have been used crotonic, tiglic and cinnamic esters, and as allylic halides: allyl, methallyl and crotyl (butenyl) bromides and benzyl chloride. The results are given in Table 1. They are seen to be in agreement with the findings of Young and Roberts [2], that 1,2-addition to the carbonyl group is greatly favoured. In contrast to the corresponding reactions with *n*-butylmagnesium bromide and other Grignard reagents, the reactions with allyl-, methallyl- and crotylmagnesium bromides all give tertiary alcohols in high yields. Benzylmagnesium chloride, on the other hand, gives 1,4-addition (ester) with crotonic and cinnamic esters, whereas it gives low yields and mixtures of ester and impure tertiary alcohol with tiglic ester *.

In the conjugate addition of Grignard reagents to α,β-unsaturated carbonyl compounds a different cyclic mechanism is generally believed to operate [4]:



(2)

but in the case of allylic Grignard reagents the above-mentioned cyclic electron shift (1) competes successfully with (2), unsaturated ketones and tertiary alcohols being formed in stead of "saturated" esters, *e. g.*



(83 %)

and not

(0 %)

* The tertiary alcohols formed by these reactions are presumably rather unstable, eliminating water easily. Many of them, after standing for a few weeks, turned into viscous polymers, insoluble in most solvents.

Table 1. Products and yields from reactions of *sec*-butyl esters of α,β-unsaturated acids with allylic Grignard reagents (RMgX).

Ester \ R	Allyl	Methallyl	Crotyl	Benzyl
Crotonic	tert. alcohol [a] 83 %	tert. alcohol [b] 85 %	tert. alcohol [c] 87 %	ester [d] (53 %)
Tiglic	tert. alcohol [e] 70 %	tert. alcohol [f] 94 %	tert. alcohol [g] 91 %	ester [h], 10–20 % tert. alcohol [i], (25–35 %)
Cinnamic	tert. alcohol [j] 65 %	tert. alcohol [k] 48 %	tert. alcohol [l] 67 %	ester [m] 63 %

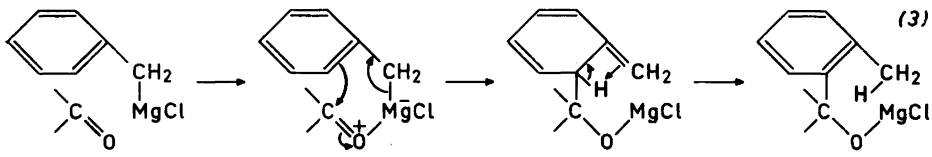
[a] Propenyldiallylcarbinol, b. p. 93.5°/35 mm, n_D^{20} 1.4707; calc. for $C_{10}H_{16}O$ (152.23): C 78.89; H 10.60. Found: C 78.90; H 10.64. [b] Propenyldimethallylcarbinol, b. p. 89°/12 mm, n_D^{20} 1.4695. Calc. for $C_{12}H_{20}O$ (180.28): C 79.94; H 11.18. Found: C 79.85; H 10.90. [c] Propenyldibutenylcarbinol, b. p. 87°/13 mm, n_D^{25} 1.4674. Calc. for $C_{12}H_{20}O$ (180.28): C 79.94; H 11.18. Found: C 79.40; H 11.33. [d] *sec*-Butyl β-methyl-γ-phenylbutyrate, b. p. 95–96°/0.5 mm. The product is not pure; by saponification was obtained a 70 % yield of β-methyl-γ-phenylbutyric acid, b. p. 124–125°/0.8 mm, n_D^{20} 1.5132. Calc. for $C_{11}H_{14}O_2$ (178.22): C 74.13; H 7.92. Found: C 74.20; H 7.92. [e] 1-Methylpropenyl-diallylcarbinol, b. p. 81–82°/11 mm, n_D^{20} 1.4765. Calc. for $C_{11}H_{18}O$ (166.25): C 79.46; H 10.92. Found: C 78.95; H 10.84. [f] 1-Methylpropenyl-dimethallylcarbinol, b. p. 105°/15 mm, n_D^{20} 1.4770. Calc. for $C_{13}H_{22}O$ (194.31): C 80.35; H 11.41. Found: C 80.25; H 11.39. [g] 1-Methylpropenyl-dibutenylcarbinol, b. p. 96°/12 mm, n_D^{20} 1.4810. Calc. for $C_{13}H_{22}O$ (194.31): C 80.35; H 11.41. Found: C 79.70; H 11.44. [h] *sec*-Butyl α,β-dimethyl-γ-phenylbutyrate, b. p. 102–103°/0.8 mm, n_D^{20} 1.4866. Calc. for $C_{16}H_{24}O_2$ (248.35): C 77.37; H 9.74. Found: C 77.72; H 9.83. [i] The product, b. p. 141–142°/0.8 mm, n_D^{25} 1.5621, was not obtained pure. According to the infra-red spectrum, it is essentially alcohol: 1-methylpropenyl-dibenzylcarbinol. [j] Styryldiallylcarbinol b. p. 117–118°/0.9 mm, n_D^{20} 1.5770. Calc. for $C_{15}H_{18}O$ (214.29): C 84.07; H 8.47. Found: C 83.90; H 8.96. [k] Styryldimethallylcarbinol, b. p. 123°/1 mm, n_D^{20} 1.5597. Calc. for $C_{17}H_{22}O$ (242.35): C 84.25; H 9.15. Found: C 83.60; H 8.25. [l] Styryldibutenylcarbinol, b. p. 119°/0.7 mm, n_D^{20} 1.5479. Calc. for $C_{17}H_{22}O$ (242.35): C 84.25; H 9.15. Found: C 84.50; H 9.16. [m] *sec*-Butyl β,γ-diphenylbutyrate, b. p. 157°/1 mm, n_D^{20} 1.5333. Calc. for $C_{20}H_{24}O_2$ (296.39): C 81.04; H 8.16. Found: C 81.00; H 8.05.

These results constitute further evidence, that the enhanced reactivity of allylic Grignard reagents towards carbonyl groups is caused by a special mechanism operating and is not merely due to a generally greater reactivity of these Grignard reagents as compared to others. Thus, they lend support to the cyclic mechanism of Young and Roberts *.

The fact that benzylmagnesium chloride does give the "normal" conjugate addition product (*i. e.* not reacting as an allylic reagent) is not incompatible

* In fact, Young and Roberts have themselves tried the reaction between butenylmagnesium bromide and *tert*-butyl cinnamate [5]. Very little 1,4-addition product was found, but the main reaction product was not identified.

with these considerations. As pointed out above, the Young-Roberts mechanism involves the inversion of the allylic system, which is well known to occur also in the case of the benzyl grouping, *e.g.* the formation of *o*-methylbenzylalcohol from benzylmagnesium chloride and formaldehyde [7]. However, this inversion must imply a transitory loss of the aromaticity of the ring and, consequently, require a rather high energy of activation:



In this case, therefore, the conjugate addition process (2) is the one mostly favoured. Indeed, the results with the benzyl reagent may thus be considered to be consistent with the Young-Roberts mechanism.

Cuprous chloride, which is known to catalyze 1,4-addition of Grignard reagents in competion with 1,2-addition [8], has been found to have no effect in the cases of allylic Grignard reagents here described.

During this work no attempt was made to determine the structure of the butenyl group in the tertiary alcohols formed. According to Young and Roberts [2], they are assumed to have the α-methylallyl structure.

EXPERIMENTAL

Microanalyses are by Mr. Preben Hansen, Microanalytical Division, The Chemical Laboratory, The University of Copenhagen. Fractional distillations were through a simple 45 cm × 8 mm Podbielniak-type column with a tantalum wire spiral, a heated jacket and a partial reflux head.

sec-*Butyl esters* were prepared as previously described [9]. Allyl bromide was obtained according to Organic Syntheses [10]. Methallyl (β-methylallyl) bromide, b. p. 95—96°, n_D^{20} 1.4718, was synthesized from the alcohol (Fluka, pract.) according to Jones *et al.*[11] Crotyl bromide, b. p. 102—105°, n_D^{20} 1.4775, was the Fluka, purum, product, which was purified by washing with sodium bicarbonate solution, drying and destillation. Benzyl chloride was the commercial product (Fluka, purum), which was redistilled, b. p. 64°/14 mm, n_D^{20} 1.5398. The magnesium turnings used were those from May & Baker; this product is much more finely cut than ordinary Grignard magnesium, and it has been found particularly suitable for the preparation of allylic Grignard reagents (below).

The Grignard reagents were prepared according to the general procedure of Gilman and McGlumphy [12], which consists in using a great excess of magnesium, effective stirring and slow addition of the halide.

The reactions between the Grignard reagents and the α,β-unsaturated esters and the isolation of the products were carried out as previously described [3]. The products are given in Table 1.

The identities of the reaction products were further checked by infra-red spectroscopy. The spectra were measured with a Beckman IR2 spectrophotometer with sodium chloride prisms, using 5 % solutions in carbon tetrachloride. The alcohols were identified by their hydroxyl band in the 3 μ region and by the absence of a carbonyl absorption. The spectra of the esters (from benzylmagnesium chloride) showed — besides a carbonyl band — the

group of absorption bands (around 1 111 cm^{-1}), which has previously been found to be characteristic for *sec*-butyl esters [13].

The authors want to express their gratitude towards *Otto Mønsteds Fond* and *Det Teknisk-Videnskabelige Forskningsraad* for financial support.

REFERENCES

1. See, *e. g.*, Alexander, E. R. *Principles of Ionic Organic Reactions*, John Wiley & Sons, Inc., New York 1950, p. 287; Royals, E. E. *Advanced Organic Chemistry*, Prentice-Hall, Englewood Cliffs, N. J. 1954, p. 696.
2. Young, W. G. and Roberts, J. D. *J. Am. Chem. Soc.* **68** (1946) 1472 and further references given there; Wilson, K. W., Roberts, J. D. and Young, W. G. *J. Am. Chem. Soc.* **72** (1950) 218.
3. Munch-Petersen, J. *Acta Chem. Scand.* **12** (1958) 967.
4. See, *e. g.*, Lutz, R. E. and Reveley, W. G. *J. Am. Chem. Soc.* **63** (1941) 3180; Johnson, J. R. in Gilman, *Organic Chemistry*, 2nd. ed., Vol. II, John Wiley & Sons, New York 1943, p. 1881—1882; Alexander, E. R. *Principles of Ionic Organic Reactions*, John Wiley & Sons, New York 1950, p. 193—194; Royals, E. E. *Advanced Organic Chemistry*, Prentice-Hall, Englewood Cliffs, N. J. 1954, p. 699.
5. Young, W. G. and Roberts, J. D. *J. Am. Chem. Soc.* **68** (1946) 649.
6. Johnson, J. R. *J. Am. Chem. Soc.* **55** (1933) 3029.
7. Tiffeneau, M. and Delange, R. *Compt. rend.* **137** (1903) 573 [*Chem. Zentr.* **1903** II, 1117]; Reichstein, T., Cohen, A., Ruth, M. and Meldahl, H. F. *Helv. Chim. Acta* **19** (1936) 412; *cf.* Young, W. G. and Siegel, S. *J. Am. Chem. Soc.* **66** (1944) 354.
8. Munch-Petersen, J. *Acta Chem. Scand.* **12** (1958) 2007, 2046.
9. Munch-Petersen, J. *J. Org. Chem.* **22** (1957) 170.
10. Kamm, O. and Marvel, C. S. *Organic Syntheses* Coll. Vol. **I** (1941) 27.
11. Jones, W. J., Davies, W. C., Bowden, S. T., Edwards, C., Davis, V. E. and Thomas, L. H. *J. Chem. Soc.* **1947** 1448.
12. Gilman, H. and McGlumphy, J. H. *Bull. soc. chim. France* [4] **43** (1928) 1322. [Shirley, D. A. *Preparation of Organic Intermediates*, John Wiley & Sons, New York 1951, p. 5.]
13. Bjerl Nielsen, E., Munch-Petersen, J. Møller Jørgensen, P. and Refn, S. *Acta Chem. Scand.* **13** (1959) 1943.

Received July 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1960–1970

On Vitamins in Sewage Sludge

X. Production of Vitamin B_{12} by Sulphate-Reducing Bacteria

HALINA Y. NEUJAHR

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

Vitamin B_{12} and some other growth factors were determined in sewage sludge fermented by sulphate-reducing bacteria according to Butlin [1] and in an extract of dry *Desulphovibrio desulphuricans* cells *.

The vitamin B_{12} activity, calculated as cyanocobalamin in *E. coli* cup plate assay, of sludge fermented by sulphate reducers was found to be approximately 0.2—0.3 μg/ml or 10—15 μg/g dry solids which is of the same order of magnitude as the corresponding activity of sludge fermented by methane bacteria. Bioautographic studies revealed the presence of cyanocobalamin and also some factor or factors with an R_c-value (R_F-value of the factor relative to that of cyanocobalamin) = 0.6—0.7 the nature of which was not elucidated.

The fermented sludge contained only small amounts of folic and folinic acid activity.

The dry cell extracts were found to contain 9 μg vitamin B_{12} activity/g dry cell material when assayed by the *E. coli* cup method but 15 μg/g when assayed by the *E. coli* turbidimetric method. The reasons for this discrepancy are discussed and some attempts to elucidate them are described.

The *E. coli* activity of the dry cell extract represents a mixture of at least four factors. Cyanocobalamin is present in small amounts. The predominating factor(s) may be identical with factor A and some unidentified factor(s) which behave slightly basically upon paper electrophoresis at pH 2.5. Factors: B(Ford), [19] aetiocobalamin phosphoribose (Bernhauer) [11], 2-methyl-mercapto-adenin-cobalamin (Bernhauer) [18] and factors Z2 + Z3 (Neujahr) [8] are absent or present only in very small amounts.

Three or four desoxyriboside factors were also found to be present in the cell extracts.

The cell extract was found to contain three factors which had a growth inhibiting activity for *L. leichmannii 313*. Attempts to purify these factors are described and their possible nature is discussed.

Butlin and co-workers [1] have prepared enrichment cultures of sulphate-reducing bacteria which can be successfully used for the production of sulphide from sulphate-enriched sewage sludges. When such sulphide fermentation

* The dry cell material was kindly provided by Dr. John Postgate of National Chemical Laboratory, Teddington, England.

takes place in sewage sludge, the otherwise spontaneously occurring methane fermentation is inhibited and the production of methane gas ceases. Butlin and co-workers have shown that the inhibition of methane fermentation by sulphide fermentation in combined, methane- and sulphide-producing fermentations depends a.o. on the preferential utilization of nutrients or (and) reducing material by sulphate-reducing bacteria [1]. In their subsequent work Butlin and co-workers [21] have found also certain other reasons for the inhibition of the methane fermentation.

Furthermore, the above cited authors have shown that pure cultures of laboratory strains of *Desulphovibrio desulphuricans* inoculated into sterile raw sludge produced negligible sulphide in contrast to crude enrichment cultures which were very active in this respect [1].

It has been reported from this laboratory [2,4] as well as from several others [3,5] that considerable amounts of vitamin B_{12} activity are formed during the methane fermentation of sewage sludge. It has also been reported that many of the organisms active during the methane fermentation of sewage sludge produce vitamin B_{12} activity when cultivated in synthetic media [6]. It was therefore considered to be of interest to investigate if vitamin B_{12} was formed also during the sulphide fermentation of sewage sludge and if it was formed by pure strains of *Desulphovibrio desulphuricans* grown in synthetic media.

EXPERIMENTAL

Using an active sulphide-producing crude culture of sulphate-reducing bacteria kindly provided by Dr. Butlin, the author performed two semicontinuous fermentations of raw sulphated sewage sludge according to the technique described by Butlin and co-workers [1]. Raw sludge was homogenized in a Turmix blendor and sieved through a sieve with a 0.80 mm mesh. The material obtained in this way contained 2 % dry solids. It was sulphated (50 g $CaSO_4$/l) and 1 l batches were inoculated with the active culture. Samples of 50 ml were removed daily and replaced by 50 ml raw sulphated sludge (2.5 g $CaSO_4$/50 ml). Nitrogen gas was blown through the fermentation flask. The H_2S formed was trapped in a solution of $CdCl_2 \cdot 5H_2O$.

The vitamin B_{12} activity of the removed samples was investigated using *E. coli 113—3* in plate assay and by bioautography as described elsewhere [7]. The growth activity for *S. faecalis ATCC 8043* and *L. citrovorum ATCC 8081* was also determined in some samples and calculated as folic acid and folinic acid activity, respectively. The results of the two series can be seen in Tables 1 and 2.

The determination of the ability of a pure strain of *Desulphovibrio desulphuricans* to produce vitamin B_{12} activity was performed on dry cell material *. The cells were autoclaved for 10 min at 121°C in water containing 0.01 % KCN at pH 6.5. The proportion of the dry cell material and water was 1:30. The autoclaved mixture was centrifuged and the supernatant used as cell extract for the different assays. For assaying desoxyriboside factors, the extract was concentrated tenfold by evaporation under reduced pressure at 30—40°C.

The values found for vitamin B_{12} activities, calculated as cyanocobalamin, are given in Table 3.

* *Cf.* footnote on p. 1960.

RESULTS AND DISCUSSION

Vitamin B_{12} activity of sulphate-enriched sludge fermented by the crude culture of sulphate-reducing bacteria

It is seen in Tables 1 and 2 that approximately 0.2—0.3 mg/l of vitamin B_{12} activity was produced in a sludge containing 2 % dry solids. This corresponds to 10—15 mg/kg dry solids which is of the same order of magnitude as in sludge which has undergone methane fermentation (10—15 mg/kg).

Paper chromatography followed by bioautography on *E. coli* plates gave two spots, one of which was identified as cyanocobalamin. The second spot, R_c = 0.6—0.7, could not be conclusively identified since, as reported before [8], it may correspond to several vitamin B_{12} factors having R_c-values (= R_F-values relative to that of cyanocobalamin) in the range 0.6—0.7. It can further be seen in Table 2 that the sludge fermented by sulphate-reducing bacteria exhibited only a small activity for *S. faecalis* and *L. citrovorum*. These activities were considerably higher in fresh sludge. A similar relation has been observed earlier in the methane fermentation of sewage sludge [2,4].

Table 1. Semi-continuos fermentation of raw sulphated sludge (ca 2 % dry solids content) by sulphate reducing bacteria.
R_c-value = R_F of a factor relative that of cyanocobalamin.

Days of fermentation	pH	*E. coli* activity	
		μg/ml *	Factors detected by paper chromatography **
0	8.1	0.03	
1	6.3	0.16	
2	6.3	0.17	Two chromatographic
3	6.3	0.19	spots were always present.
6	6.1	0.22	
7	6.2	0.21	1) R_c = 1.0
9	6.4	0.22	corresponding to cyanocobalamin
11	6.5	0.20	2) R_c = 0.6—0.7
13	6.6	0.20	the nature of which was
15	7.1	0.17	not elucidated
16	7.5	0.15	Factor B (R_c = 1.4) and
17	8.2	0.19	factors Z (R_c 0—0.4)
18	8.2	0.21	could not be detected at all.
20	7.9	0.16	
(21)	(8.1)	(0.11)	
24	7.8	0.20	
25	8.1	0.17	
27	8.0	0.17	
30	7.8	0.17	
31	7.8	0.17	

* calculated as cyanocobalamin in plate assay

** solvent system: butyl alcohol: water: acetic acid = 75 : 24 : 1 containing 0.01 % KCN, 48 h development, descending technique.

Table 2. Semi-continuous fermentation of raw sulphated sludge (ca. 2% dry solids content) by sulphate-reducing bacteria.
R_c-value = R_F of a factor relative to that of cyanocobalamin.

Days of fermentation	pH	*E. coli* activity		*S.f.* ***	*L.c.* ****
		µg/ml *	Factors detected by paper chromatography **	Activity µg/ml	
0	7.5	0.03		0.013	0.021
1	6.6	0.11			
2	6.8	0.13			
3	7.4	0.14	Two chromatographic spots		
4	7.6	0.21	were always present:	<0.003	<0.003
7	7.6	0.21	1) R_c = 1.0		
8	7.8	0.23	corresponding to		
9	7.8	0.21	cyanocobalamin		
10	7.8	0.21		0.004	0.005
11	7.8	0.21	2) R_c = 0.6–0.7		
12	7.8	0.16	the nature of which		
13	7.6	0.21	was not elucidated		
14	7.7				
15	8.0	0.20			
16	8.1	0.18	Factor B (R_c = 1.4) and factors		
17	8.0	0.27	Z (R_c 0–0.4) could not be		
19	8.0	0.22	detected at all.		
20	8.0	0.22		0.007	0.004
21	8.3	0.20			
22	8.0	0.20			
23	8.0	0.21			
24	8.1	(0.64)			
26	8.3	0.30			
27	8.3	0.30			
28	8.4	0.27			
29	8.4	0.20			
30	8.5	0.20			
31	8.4	0.20			
33	8.4	0.21			
34	8.5	0.24			
44	8.5	0.30			
59	8.3				

* calculated as cyanocobalamin in plate assay
** solvent system: butyl alcohol: water: acetic acid = 75 : 24 : 1 containing 0.01 % KCN, 48 h development, descending technique.
*** *S. faecalis* activity calculated as folic acid in plate assay.
**** *L. citrovonum* acitivity calculated as folinic acid in plate assay.

Vitamin B_{12} activity of dry cells of a pure strain of *Desulphovibrio desulphuricans.*

It can be seen in Table 3 that extracts of *Desulphovibrio desulphuricans* cells exhibit a greater vitamin B_{12} activity when assayed with *L. leichmannii* than when assayed with *E. coli*. The difference is expecially pronounced in the cup

plate assay. It was found that the higher activity values obtained with *L. leichmannii* could be accounted for by the presence of certain desoxyribosides which are also active for this organism. Paper chromatography in two solvent systems [7] followed by bioautography on agar plates seeded with *L. leichmannii*, and *L. acidophilus* R-26, ATCC 11506 gave three spots with R_F-values close to those of the desoxyribosides of adenine, uracil and 5-methylcytosine and/or guanine. These substances were present in the cell extracts in amounts of about 5—10 μg/ml as estimated by comparison with spots obtained on the bioautographs with standard solutions, *i.e.* they may constitute about 150—300 μg in 1 g dry cell material. Other desoxyriboside factors, if present, may occur in much smaller amounts than the three (or four) mentioned above since they could not be detected even in the tenfold concentrated cell extract.

Table 3. Vitamin B_{12} activity of *Desulphovibrio desulphuricans* cell extracts.

Test organism	Assay method	Vit. B_{12} activity calculated as cyanocobalamin μg/g dry cell material *
E. coli 113-3	cup plate	9
» » *113-3*	turbidimetry	15
L. leichmannii 313	cup plate	30
» »	turbidimetry	17

* average of three different determinations.

It can further be seen in Table 3 that the extracts of *Desulphovibrio desulphuricans* cells exhibit a greater vitamin B_{12} activity when assayed with *E. coli* by the turbidimetric method than by the cup plate technique. This phenomenon is quite unusual compared with previous experience. The vitamin B_{12} activity of natural materials investigated hitherto was always found to be greater when assayed by the cup plate technique than by the turbidimetric one. This is generally attributed to the presence in natural materials of different vitamin B_{12} factors together with cyanocobalamin.

In his work on the vitamin B_{12} requirement in certain soil bacteria Ford suggests that certain vitamin B_{12}-like factors, the "inactive analogues may slow down the rate at which vitamin B_{12} is absorbed and utilized by the cells" [9]. The presence of the "inactive analogues" in relatively high concentrations together with vitamin B_{12} gives, according to Ford [9], rise to larger and correspondingly less dense zones of growth in the plate assays than those obtained with vitamin B_{12} alone. A similar relation may apply also to *E. coli 113—3* and the reasoning of Ford [9] could thus give a plausible explanation of the often much larger and more diffuse, as compared with pure solutions of cyanocobalamin, growth zones obtained in *E.coli* plates with solutions containing a mixture of several vitamin B_{12} factors. However, a number of vitamin B_{12}-like factors, even those which lack the nucleotide part, *e.g.* factor B, are active for *E.coli*. Many of those "active analogues" give also larger and less dense zones

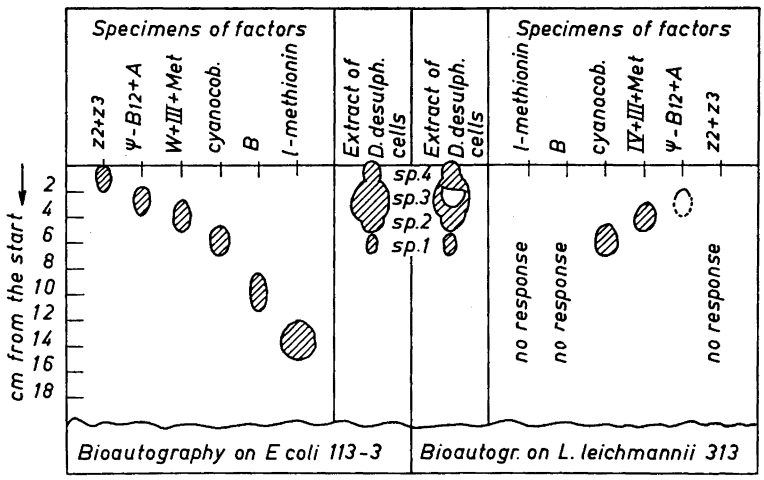


Fig. 1. Chromatographic separation of the vitamin B_{12} activity present in cell extracts of *Desulphovibrio desulphuricans*. Solvent system I [7], development: 18 h at 24°C.

of growth than cyanocobalamin when assayed with *E.coli* by the plate method, even when a solution of a single vitamin B_{12} factor is applied (*cf.* Ref. 8). Coates and Ford [20] suggest that cyanocobalamin may be utilized more rapidly by the *E.coli* cells than the other vitamin B_{12} factors active for this organism. The other factors may thus have time to diffuse further away from the cup until they can be utilized for growth than is the case with cyanocobalamin. This may perhaps explain why the solutions of such factors give rise to larger but less dense growth zones than those obtained with cyanocobalamin.

Reasoning in a similar way may lead to the supposition that the greater *E.coli* activity of *Desulphovibrio desulphuricans* cells exhibited in tube assay than in cup plate assay may depend on the presence in the cell extracts of a vitamin B_{12} factor which is still more rapidly utilized by the test organism cells than cyanocobalamin. Furthermore, this factor may be a dominant one in the cell extract.

Paper chromatography in two-solvent systems [7] combined with bioautography on *E.coli* and *L. leichmannii* plates gave four spots equally active for *E.coli* and *L. leichmannii*. The chromatographic separation after 18 h of development in solvent system I [7] is represented in Fig. 1. A consideration of Fig. 1 leads to following conclusions.

1) factor B is not present in the cell extract.

2) the spot corresponding to cyanocobalamin (spot 1), if identical with this factor, indicates that cyanocobalamin is present only in a small amount.

3) spot 2 and the largest spot of vitamin B_{12} activity (spot 3) cannot be identified after chromatographic separation during 18 h since they have R_c-values which may correspond to several vitamin B_{12} factors.

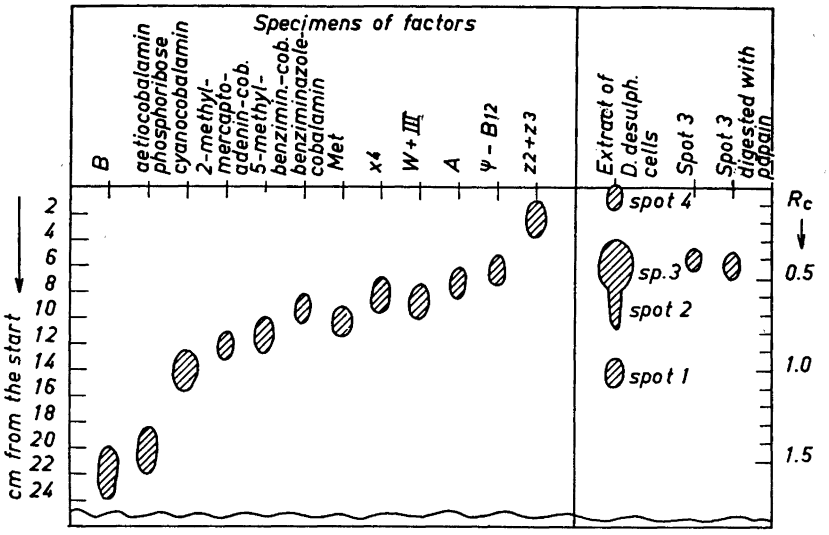


Fig. 2. Chromatographic separation of the *E. coli* activity exhibited by cell extracts of *Desulphovibrio desulphuricans*. Solvent system I [7], development: 48 h at 24°C.

4) spot 4 which corresponds on the *E.coli* bioautogram to factors Z (Z2 + Z3) cannot be identical with these factors since it is also active for *L. leichmannii* which is not the case with specimens of factors Z2 and Z3.

5) the extract of *Desulphovibrio* cells contains a growth inhibitor for *L. leichmannii* with an R_F-value close to that of spot 3.

By electrophoresis in 2 M Hac containing 0.01 % KCN according to Holdsworth [10], the *E. coli* activity of the cell extract could be separated into four spots, one slightly basic or almost neutral, one basic and two acidic. The largest part of the *E. coli* activity was represented by the basic and neutral factors while the acidic factors were present only in lesser amounts.

In order to identify spots 2 and 3, further chromatographic and electrophoretic studies were performed using for comparison specimens of several vitamin B_{12} factors kindly provided by Prof. Bernhauer. Fig. 2 shows the chromatographic separation of the *E. coli* activity after 48 h at 24°C in solvent system I [7].

From Fig. 2, the following conclusions can be drawn:

1) a prolonged period of development gives once again only four spots but provides, on the other hand, a better possibility for characterizing the spots obtained.

2) factor B (Ford) [19], aetiocobalamin phosphoribose (Bernhauer) [11] and factors Z2 + Z3 (Neujahr) [8] are not present in the cell extract.

3) spot 4 thus corresponds to some factor (or factors) which could not be identified with any of the available specimens. Its immobile character upon development with solvent system I [7] ressembles the behaviour of factor C_2 [12]. The possible identity of spot 4 with factor C_2 was not excluded by the results

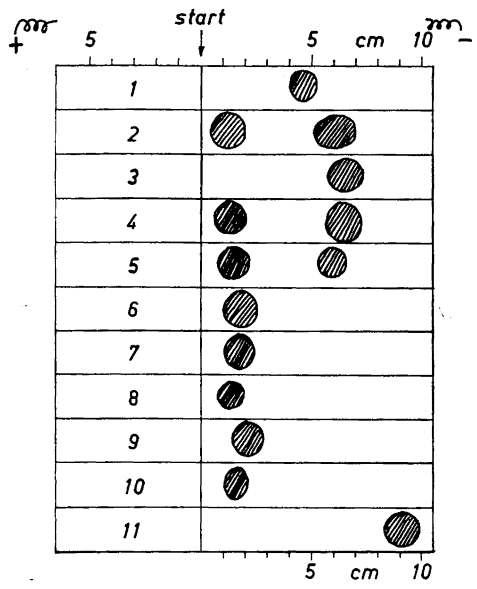


Fig. 3. Electrophoretic separation of the *E. coli* activity exhibited by cell extracts of *Desulphovibrio desulphuricans*. 2 M HAc containing 0.01 % KCN, pH 2.5, 10 V/cm, 18 h at +4°C; 1. ψ $-B_{12}$; 2. cell extract*; 3. factor A; 4. "spot 3" eluted from chromatograms; 5. "spot 3" after digestion with papain; 6. factor III; 7. cyanocobalamin; 8. benziminazole cobalamin; 9. factor W; 10. factor Met; 11. factor B.

of the electrophoretic studies since, as mentioned above minor quantities of two acidic factors could be observed, at pH 2.5, in the cell extract.

4) spot 1 most probably represents cyanocobalamin — this is in agreement with the presence of an electrophoretically almost neutral factor at pH 2.5.

5) Spot 2 is more difficult to identify. It can be seen in Fig. 2 that it may correspond to at least 6 factors, *viz.* benziminazole cobalamin (Bernhauer) [3], factor Met (Neujahr) [13], factor X [4] (Neujahr) [8], factor W (Neujahr) [8], factor 111 (Bernhauer) [3] and possibly others. The elongated character of this spot indicates also that it is a mixture of several factors. Further identification of this spot by means of paper electrophoresis was not possible since the six factors mentioned above behave similarily upon electrophoresis.

6) The main spot of *E. coli* activity (spot 3) seems at first sight to correspond to ψ-B_{12} as judged by its R_c-value. However, this is excluded by the results of electrophoretic studies shown in Fig. 3 where once again factor A seems to be present in the cell extract. Thus the nature of spot 3 could not be conclusively determined.

Due to a suspicion that spot 3 may represent some combined form of vitamin B_{12}, a number of spots 3 was cut out from the chromatograms, eluted with water and concentrated under reduced pressure. A part of the concentrated solution was digested with papain and another part autoclaved with KCN (1 mg/ml) for 15 min. The vitamin B_{12} activity of the resulting solutions was determined in plate assay and tube assay using *E. coli* and *L. leichmannii* and

* With the amount of cell extract applied in this experiment the two acidic factors mentioned in text (*cf.* p. 1966) did not appear. It was, however, couvenient to use this amount in order to identify the two basic factors.

also by chromatography and electrophoresis. The *E. coli* activity of the eluted spots 3 did not show the same relation between cup plate and tube assay values as the whole extracts. On the contrary, the activities found in tube assay were now lower than those found in the cup plate assay. Spots 1, 2 and 4 were also cut out from the chromatograms, eluted with water and assayed for *E. coli* activity alone and together with each other and with spot 3, but no evidence for synenergetic phenomena could be obtained. The problem could not be more thoroughly investigated because of the small amount of material available. The vitamin B_{12} activity values of the eluted spots 3 found with *L. leichmannii* in cup plate assay were once again higher than those found with *E. coli*. Upon electrophoresis in 2 M HAc at pH 2,5, the chromatographically homogenous spot 3 separated into two spots, one almost neutral and one basic with a mobility corresponding to factor A. Autoclaving with KCN (1 mg/ml) destroyed the greater part of the *E. coli* activity. Digestion with papain did not alter the position of spot 3 on the chromatograms noteworthly (Fig. 2) nor the position of the resulting two spots on electrophorograms (Fig 3), although the *E. coli* activity found in tube assay was now once again much higher than the activity found in cup plate assay. It is possible that the discrepancy of the values was at least to some extent due to methionine derived from the papain preparation.

Since the supposition that spot 3 represents some combined form of vitamin B_{12} could not be confirmed, it may be assumed that it is a mixture of factor A and some factor(s) which behave slightly basically upon electrophoresis at pH 2,5.

Inhibition factor.

It was mentioned above and, in addition, can be seen in Fig. 1 that the extract of *Desulphovibrio desulphuricans* cells contains a substance inhibiting the growth of *Lactobacillus leichmannii*. In solvent systems I and II[7], the substance had R_c-values corresponding to those of spot 3 of *E. coli* activity in these solvent systems. On electrophoresis in 2 M HAc at pH 2.5, it was neutral or slightly basic. Attempts were made to purify the substance. 6 g of dry cell material were autoclaved in 180 ml water containing 100 μg KCN/ml. After centrifugation the supernatant was evaporated to dryness under reduced pressure at 40°C. The dry residue was then extracted with 70 % EtOH several times and the combined extracts as well as the residue were assayed for the inhibiting activity using *L. leichmannii* plates for vitamin B_{12} assay but supplemented with cyanocobalamin (1 μg/ml). Almost all the growth inhibiting activity was found in the extracts. The combined extracts were then evaporated to dryness under the conditions described above. The dry residue was dissolved in a small amount of water and chromatographed on Wh 1 paper sheets of 18 × 38 cm for 48 h in solvent system I[7]. After drying, chromatogram strips of 1 cm width were cut at the right and the left side of each sheet and the position of the inhibition factor determined by putting the strips on *L. leichmannii* plates for vitamin B_{12} which contained cyanocobalamin. On this occasion, it was found that not one but three distinctly different inhibition zones were given by the extract. The three zones: Inh 1, Inh 2, Inh 3 had approximately following R_c-values:

Inh 1	1.10
Inh 2	0.65
Inh 3	0.37

These R_c-values are thus closely related to the R_c-values of many vitamin B_{12} factors [8]. Inh 1 seems to be present in much greater amounts than the other two inhibition factors. The three inhibition zones were cut out from chromatograms and eluted with water. The eluates were concentrated by evaporation under reduced pressure at 40°C. Attempts were made to determine absorption spectra of the three solutions thus obtained, but they were not successful.

The solutions were not sufficiently purified and concentrated. The experiments could not be continued due to the small amount of material available. However, even if the solutions had been sufficiently purified and concentrated the determination of their absorption spectra would have been quite uncertain since they contained considerable amounts of vitamin B_{12} activity. The separation of this activity from the inhibitor factors by the conventional chromatographic and electrophoretic techniques may be a rather difficult task as can be understood from the fact that the R_c-values of the inhibitor factors are very closely related to the R_c-values of many vitamin B_{12} factors (*cf.* Fig. 2) and that their neutral or slightly basic electrophoretic character at pH 2.5 ressembles that of cyanocobalamin and several other vitamin B_{12} factors.

The occurrence of bacterial inhibition factors in bacterial cell extracts has been reported by several authors. Bolinder [14] in this laboratory has found that several purine nucleotides, notably adenine ribotides and desoxyribotides, possess a growth inhibiting activity for *L. leichmannii* 313 in agar plates for the assay of vitamin B_{12}, desoxyribotides and desoxyribosides. However, the identity of the inhibitor factors found in *Desulphovibrio desulphuricans* cells with any of the compounds found by Bolinder [14] to have an inhibitory activity is unlikely since they behave quite differently upon chromatography.

Juillard [15] has isolated from a *Bacillus megaterium* mutant a growth inhibition factor for *E. coli 113—3* and identified it chromatographically as the monocarboxylic acid of factor B. This inhibition factor behaved as a competitive antimetabolite for *E. coli 113—3*.

Smith *et al.*[16] and Cuthbertson *et al.* [17] describe the antimetabolic activities for a B_{12}-requiring *E. coli* mutant of several compounds structurally closely related to vitamin B_{12}. In their experiments they used rigorously purified substances obtained by the chemical degradation of cyanocobalamin. As mentioned above, a further purification of the inhibition factors extracted from *Desulphovibrio desulphuricans* cells was not possible. The fact that the preparations did not exhibit any growth inhibiting activity on *E. coli 113—3* plates (*cf.* Fig. 1) may depend upon their high content of vitamin B_{12} which may overcome their possible growth inhibiting activity for *E. coli* whereas in *L. leichmannii 313* plates the growth inhibiting activity could not be overcome. Thus it cannot be decided whether the inhibition factors found in *Desulphovibrio* cell extracts are of the kind described by Juillard [15], Smith *et al.* [16] and Cuthbertson *et al.* [17], although their chromatographic and electrophoretic behaviour points to the suggestion that they may be structurally closely related to vitamin B_{12}.

Acknowledgements. The author is greatly indebted to dr. K. R. Butlin of the National Chemical Laboratory, Teddington, England, for his generosity in providing the active crude culture of sulphate-reducing bacteria and to dr. J. Postgate for the dried cells of *Desulphovibrio desulphuricans*. The author is also greatly indebted to Professor K. Bernhauer for the generous gift of specimens of different vitamin B_{12} factors. The author wishes to thank Professor H. Lundin, Head of the Division, for his encouraging interest in this work and to dr. D.A. Callieri for kind assistance during the first part of this investigation.

REFERENCES

1. Butlin, K. R., Selwyn, S. C. and Wakerley, D. S. *J. Appl. Bacteriol.* **19** (1956) 3.
2. Sjöström, A. G. M., Neujahr, H. Y. and Lundin, H. *Acta Chem. Scand.* **7** (1953) 1036.
3. Bernhauer, K. and Friedrich, W. *Angew. Chem.* **66** (1954) 776.
4. Neujahr, H. Y *Acta Chem. Scand.* **9** (1955) 622.
5. Janicki, J., Pawetkiewicz, J. and Nowakowska, K. *Acta Biochim. Polon.* **3** (1956) 161.
6. Neujahr, H. Y. and Callieri, D. A. *Acta Chem. Scand.* **13** (1959) 1453.
7. Neujahr, H. Y. *Acta Chem. Scand.* **11** (1957) 1191.
8. Neujahr, H. Y. *Acta Chem. Scand.* **10** (1956) 917.
9. Ford, J. E. *Can. J. Microbiol,* **3** (1957) 319.
10. Holdsworth, E. S. *Nature* **171** (1953) 148.
11. Dellweg, H. and Bernhauer, K. *Arch. Biochem. Biophys.* **69** (1957) 74.
12. Kon, S. K. *Other factors related to vitamin B_{12}, Biochemical Society Symposia* No. **13** (1955) 23.
13. Neujahr, H. Y. and Callieri, D. A. *Acta Chem. Scand.* **12** (1958) 1153.
14. Bolinder, A. E. *Acta Chem. Scand.* **11** (1957) 1610.
15. Juillard, M. *Zentr. Bakteriol. Parasiten. II. Abteilung 2, 1957, 701.*
16. Smith, E. L., Parker, L. F. J. and Gant, D. E. *Biochem. J.* **62** (1956) 14 P.
17. Cuthbertson, W. F. J., Gregory, J., O'Sullivan, P. and Pegler, H. F. *Biochem. J.* **62** (1956) 15 P.
18. Friedrich, W. and Bernhauer, K. *Chem. Ber.* **90** (1957) 1066.
19. Ford, J. E., Kon, S. K. and Porter, J. W. G. *Biochem. J.* 50 (1951) ix.
20. Coates, M. E. and Ford, J. E. *Methods of Measurement of Vitamin B_{12}, Biochemical Society Symposia* No. **13,** Cambridge (1955) 39.
21. Butlin, K. R. *Private communication.*

Received August 8, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1971–1996

Equilibrium Studies of Polyanions

VI. Polyvanadates in Alkaline Na(Cl) Medium *

NILS INGRI and FELIPE BRITO

Department of Inorganic Chemistry, The Royal Institute of Technology, Stockholm 70, Sweden

Equilibria in alkaline vanadate(V) solutions have been studied at 25°C with 0.5 Na(Cl) as ionic medium. Emf measurements were made using a glass or a hydrogen electrode, which together gave concordant results. For an average charge, z, per vanadium, between -0.6 and -1.0 ("instability range"), equilibrium was attained very slowly. Reproducible equilibria were obtained from $z = -1$ to $z = -2$; in this range z was determined as a function of h for various total concentrations, B, of vanadium between 0.080 and 0.00062_5 M.

An analysis of the data indicated that the principal species in this range are B^{2-} $[VO_2(OH)_3^{2-}, HVO_4^{2-}]$, HB_2^{3-} $[(VO_2)_2(OH)_5^{3-}, HV_2O_7^{3-}]$ and $H_3B_3^{3-}$ $[(VO_2)_3(OH)_6^{3-}, V_3O_9^{3-}]$. There was also evidence for a mononuclear complex HB^- $[VO_2(OH)_2^-, H_2VO_4^-, VO_3^-]$. The following formation constants were obtained:

$$2B^{2-} + H_2O \rightleftharpoons HB_2^{3-} + OH^- \quad \log \beta_{12} = -3.18 \pm 0.05$$
$$3B^{2-} + 3H_2O \rightleftharpoons H_3B_3^{3-} + 3OH^- \quad \log \beta_{33} = -10.42 \pm 0.04$$
$$\text{and } B^{2-} + H_2O \rightleftharpoons HB^- + OH^- \quad \log \beta_1 = -6.0 \pm 0.1$$

In this paper also tentative structures are given for the polynuclear complexes formed. The structures are based on five-coordination around vanadium and may give justification for the formulas $VO_2(OH)_3^{2-}$, $(VO_2)_2(OH)_5^{3-}$ and $(VO_2)_3(OH)_6^{3-}$.

The polyanions of vanadium have received a large amount of attention, but information concerning them has been most chaotic because no two workers have agreed and many different classifications, species, and types of reactions have been proposed. Among the methods used for studying these complicated equilibria the emf method seems to be the one that gives data of the highest accuracy. In a careful emf study using glass electrode, Francis and Hazel

* The experimental part of this work was started by Nils Ingri of this laboratory, and then carried out jointly by Nils Ingri and Dr. Felipe Brito, of the University of La Laguna (Canary Islands, Spain), who is working here as a guest research associate. It was clear immediately from

Rossotti [1] investigated the polyvanadates formed in acid solutions. From these data they concluded that VO_2^+ is the only species in strong acid solution and that with increasing pH it reacts with H_2O forming a series of decavanadates. They could explain the data with the following reactions and constants (1 M $NaClO_4$, 25°C)

	log K
$10\ VO_2^+ + 8H_2O \rightleftharpoons H_2V_{10}O_{28}^{4-} + 14H^+$	-6.75 ± 0.15
$H_2V_{10}O_{28}^{4-} \rightleftharpoons HV_{10}O_{28}^{5-} + H^+$	-3.6 ± 0.3
$HV_{10}O_{28}^{5-} \rightleftharpoons V_{10}O_{28}^{6-} + H^+$	-5.8 ± 0.1

The existence of such decavanadate groups has also been indicated by X-ray investigations of crystals (Lindqvist [2], Evans, Mrose and Marvin [3]). Newman and Quinlan [4] found that their spectrophotometric data on equilibrium solutions can be explained by assuming the formation of the decavanadate ions proposed by Rossotti and Rossotti [1].

It is generally believed that these highly condensed anions are broken down with increasing pH, and that the end product in this process is the mononuclear orthovanadate ion, VO_4^{3-}. Our intention when we started this work was to use the same method as Rossotti and Rossotti but to go from the opposite direction and study the reactions taking place when H^+ is added to VO_4^{3-}; in this way we had hoped to find out the formulas and the equilibrium constants for the intermediate complexes formed when VO_4^{3-} is transformed to a decavanadate.

However, experimental difficulties reduced the range available, and the average charge per vanadium, z, could only be varied between -2 and -1. At higher z-values an "instability range" exists giving slow equilibria, and at lower z-values it is difficult to measure z accurately enough. The range studied corresponds to the transition from "pyrovanadate" ($z = -2$) to "metavanadate" ($z = -1$) following the common misleading nomenclature; the position on the pH-scale is shown in Fig. 1.

---

the data that the predominant species in the range studied are those denoted here as B^{2-}, $H_3B_3^{3-}$, and HB_2^{3-}. Some deviations showed, however, that other species must be present also. After the experimental work was completed and they had agreed upon a reliable set of data, Felipe Brito and Nils Ingri decided to make the following calculations separately and then to compare their results. When they parted, they had been inclined to try to ascribe the disturbances to $H_2B_2^{2-}$, "$V_2O_6^{2-}$".

When each of them had completed his calculations both had come to the same conclusion, namely that the deviations were due to HB^-, and their values for the equilibrium constants agreed within the limits of error given; this is shown in Tables 3 and 4. Although both had used methods taken out of the general arsenal of methods used in this laboratory, their approach had been quite different, and the fact that their results agree seems to give added confidence in the data and methods. It was agreed that the work should be published in Spanish [1a] (giving the approach of Felipe Brito), and in English (giving the approach of Nils Ingri). After finishing the calculations, they have discussed each others' work quite thoroughly, and so it is only fair that they should be joint authors to the two papers. Since there would be no point in giving values for the log(equilibrium constants) differing by 0.01 or so (thus within the limits of error given), they have agreed on a single set of constants, which is given after Tables 3 and 4. May I use this opportunity of thanking them for their very pleasant cooperation.

Lars Gunnar Sillén.

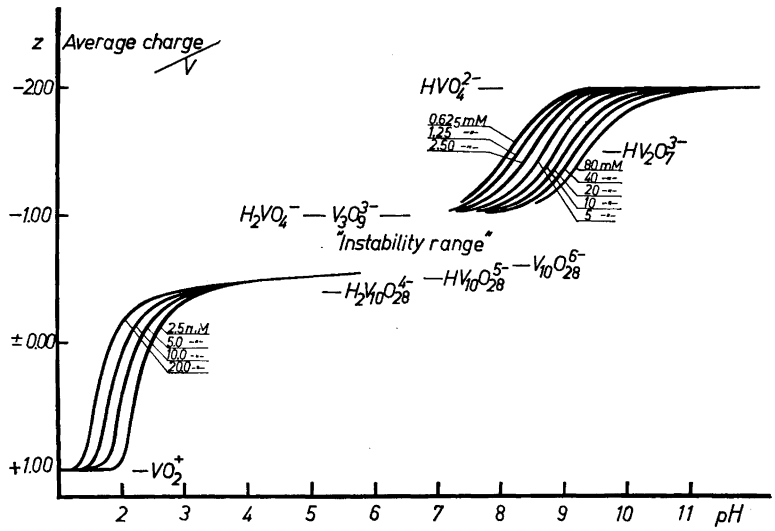


Fig. 1. Survey of data for vanadate (V) solutions: average charge, z, versus pH for various total concentrations, B. Curves on acid side from Rossotti and Rossotti [1] (1 M (Na) ClO_4). Curves on alkaline side from the present work. Between them is an "instability range", where equilibria are slowly attained. Note that the "phosphate" nomenclature has been used.

Like earlier work (Parts I[1], II[5], III[6] and IV[7] in this series) the present study was carried out as a number of potentiometric titrations at 25°C. In each titration the hydroxide ion concentration $[OH^-]$ was varied, and the total vanadium concentration, B, was kept constant. $[OH^-]$ was measured with a glass electrode with an accuracy of $\pm$ 0.3 mV. (Some experiments were also carried out using a hydrogen electrode). The variation of activity factors has largely been eliminated by keeping $[Na^+]$ = constant = 0.5 M. The experimental emf gave $[OH^-]$ (see eqn. 8), and the analytical composition of the solution gave B and H. H is the analytical excess concentration of hydrogen ions, H^+, assuming all vanadium to be in the form with $z = -2$, B^{2-} (= $VO_2(OH)_3^{2-}$, HVO_4^{2-}). From these quantities one could then calculate Z, the average number of protons H^+ bound to B^{2-}, the dominating species at $z = -2$, from the relation

$$BZ = H + [OH^-] \tag{1}$$

From Z we obtained z, the average (negative) charge per vanadium;

$$z = Z - 2 \tag{2}$$

and Z_+, the average number of OH^- bound to every VO_2^+ group (the quantity used by Rossotti and Rossotti [1]) from

$$Z_+ = 3 - Z = 1 - z \tag{3}$$

For the mathematical treatment the experimental data have been plotted (Fig. 2) with Z and z against $-\log [OH^-]$, with a separate curve for each B-value.

NOTATIONS

The way of writing the formulas. The relationship between phosphates and vanadates was deduced from the isomorphism of the minerals vanadinite, $Pb_5(VO_4)_3Cl$, and apatite, $Ca_5(PO_4)_3F$, by the work of Rammelsberg [8]; in addition, both Rammelsberg [8] and Roscoe [9] prepared a number of crystalline vanadates with analytical formulas similar to the corresponding phosphates. From these experiments Roscoe concluded that the solid vanadates could be classified in analogy to the phosphates, and he gave the following names and formulas to three groups of vanadates.

VO_4^{3-}	$V_2O_7^{4-}$(or HVO_4^{2-})	VO_3^-(or $H_2VO_4^-$)
"orthovanadate"	"pyrovanadate"	"metavanadate"

These formulas, which are based upon the idea of VO_4^- tetrahedra, were soon used [10] also to describe vanadium species in aqueous solutions, and this nomenclature is still used frequently; when the "metavanadate" ions have proved to be polynuclear, formulas like $V_3O_9^{3-}$ and $V_4O_{12}^{4-}$ have been proposed, in analogy with the polymetaphosphate ions.

In this paper the "VO_4"-nomenclature will be used sometimes, but more often the species found in this work will be written as complexes of VO_2^+ and OH^-. Thus $H_2VO_4^-$ will be written as $VO_2(OH)_2^-$, HVO_4^{2-} as $VO_2(OH)_3^{2-}$, $HV_2O_7^{3-}$ as $(VO_2)_2(OH)_5^{3-}$, and $V_3O_9^{3-}$ as $(VO_2)_3(OH)_6^{3-}$. These formulas may have some structural justification.

In our discussion about vanadates crystallized from solution and investigated with X-ray methods, analytical formulas like $(NH_4)_6V_{10}O_{28}(H_2O)_4$, $Ca_3V_{10}O_{28}(H_2O)_{16}$, KVO_3H_2O and KV_3O_8 will be used.

In the mathematical discussion, however, it will prove advantageous, for brevity and simplicity, to treat the complexes as composed of H^+ and B^{2-}, where B^{2-} is the mononuclear complex of charge 2—, thus HVO_4^{2-}, or $VO_2(OH)_3^{2-}$. Anticipating our results, the major complexes found by us in alkaline solutions may be written as

$$B^{2-} = VO_2(OH)_3^{2-} = HVO_4^{2-}$$
$$HB_2^{3-} = (VO_2)_2(OH)_5^{3-} = HV_2O_7^{3-}$$
$$H_3B_3^{3-} = (VO_2)_3(OH)_6^{3-} = V_3O_9^{3-}$$

Other complexes are

$$HB^- = VO_2(OH)_2^- = H_2VO_4^- = VO_3^-$$
$$H_{-1}B^{3-} = VO_2(OH)_4^{3-} = VO_4^{3-}$$

Dubious species are $H_2B_2^{2-}$, *etc.*

In essential parts the symbols are the same as in AB II[11] and Part II[5] in this series but will be repeated in order to simplify the reading.

a = $[OH^-]^{-1} = h\,K_w^{-1}$; corresponds to reagent A = $-OH^-$ (= H^+)
B^{2-} = species with one V and charge 2−, "HVO_4^{2-}" or $VO_2(OH)_3^{2-}$; $b = [B^{2-}]$
B total vanadium concentration
$\boldsymbol{B}$ a normalized quantity corresponding to B
E the measured emf in mV
E_{oa} = $E + 59.15 \log [OH^-] + E_j$. (8)
E_j = const. $[OH^-]$, liquid junction emf.
H total analytical concentration of H^+ over B^{2-} and H_2O; H may be negative
h = $[H^+]$
l = $\beta_{11}\beta_{12}^2\beta_{33}^{-1}$; (27)
n number of links (H_tB) per complex $B(H_tB)_n$
N unique value for n
P unique value for the number of H in H_PB_Q
$\bar{p}$ average number of H per complex formed from B^{2-} by addition of H^+
Q unique value for the number of B in H_PB_Q
$\bar{q}$ average number of B per complex formed from B^{2-} by addition of H^+
R^{-1} the average degree of condensation of B^{2-}
t number of H per B group in the link (H_tB)
u normalized quantity corresponding to a (24)
s number of B^{2-} in predominating complex B_s^{2s-}
v normalized quantity corresponding to ab (24)
w normalized quantity corresponding to a^2b. (15 c)
x $\log B - 1.5 \log [OH^-]$ (17)
y $1.5^{-1}Z$ (17)
z the average negative charge per vanadium (2)
Z the average number of H^+ bound to the dominating species of $z = -2$. (1)
Z_+ the average number of OH^- bound to every VO_2^+ group (the Z used by Rossotti and Rossotti [1]) (3)

The formation of a complex $H_pB_q^{(2q-p)-}$ can be written with H^+, or OH^- as reactant. We shall denote the corresponding equilibrium constants by $*\beta_{pq}$ and β_{pq}, to conform with the IUPAC tables of stability constants [12]:

$$pH^+ + qB^{2-} \rightleftharpoons H_pB_q^{(2q-p)-}\,;\ [H_pB_q^{(2q-p)-}] = *\beta_{pq}h^pb^q \qquad (4)$$

$$pH_2O + qB^{2-} \rightleftharpoons H_pB_q^{(2q-p)-} + pOH^-,\ [H_pB_q^{(2q-p)-}] = \beta_{pq}a^pb^q \qquad (5)$$

Since $[OH^-]$ is determined directly whereas h contains the uncertainty of K_w, it seems preferable to use instead of h the quantity

$$a = [OH^-]^{-1} = hK_w^{-1} \qquad (6)$$

which is proportional to h. It is seen from (4), (5) and (6) that

$$*\beta_{pq} = \beta_{pq}K_w^{-p} \qquad (7)$$

Chemical symbols are in Roman, concentrations in italic type. Concentrations and equilibrium constants will be expressed in M (moles per liter).

EARLIER WORK

The orthovanadate ion, VO_4^{3-}, was studied by Düllberg [10] using conductometric titrations. He found that, when solid orthovanadate is dissolved in water, an equivalent amount of OH^- is released. From that he concluded that the orthovanadate ion is such a strong base that it cannot exist in aqueous solution but reacts thus: $VO_4^{3-} + H_2O \rightleftharpoons HVO_4^{2-} + OH^-$.

In subsequent potentiometric studies, Britton, Robinson [13] and Ducret [14] came to the same conclusion.

However, in another paper Britton and Robinson [15] say that 49 % of the vanadate ions are hydrolysed in a solution where the total vanadium concentration = 83.3 mM.

In solubility measurements carried out by Britton and Robinson [16], an orthovanadate solution was titrated with a silver solution, and the silver concentration in the solution was measured with a silver electrode. The data obtained were in agreement with the solubility product: $[Ag^+]^3[HVO_4^{2-}][OH^-] = 10^{-24}$.

In a recent spectrophotometric investigation, Newman [17] and coworkers could explain their data on vanadium(V) in strongly alkaline solution by assuming a mixture of VO_4^{3-} and HVO_4^{2-}. They give $K = 0.097 \pm 0.005$ M for $VO_4^{3-} + H_2O \rightleftharpoons HVO_4^{2-} + OH^-$ at an ionic strength of 3.0 M.

The existence of a "*pyrovanadate*" *ion*, $V_2O_7^{4-}$, has been claimed by many authors. In an early cryoscopic investigation, Düllberg [10] found evidence for a dimeric vanadate species in solutions containing $2OH^-/V$. Jander and Jahr [18] reached the same conclusion from diffusion data.

Electrometric titrations [16] of sodium vanadate solution with Ag^+ (using a silver electrode) gave evidence only for the species HVO_4^{2-}, and the data could be explained assuming the solubility product $[Ag^+]^2[HVO_4^{2-}] = 10^{-13.7}$.

The solubility of $Ag_4V_2O_7$ in various solutions of sodium vanadate, $z = -2$, was measured by Souchay [19] and he found that the solubility was in accordance with the solubility product $[Ag^+]^2[HVO_4^{2-}] = \text{const}$. However, in more concentrated solutions this constant could not explain the data which were in better agreement with $[Ag^+]^4[V_2O_7^{4-}] = \text{const}$.

From spectrophotometric work [17] Newman claimed that the following dimerisation took place: $2HVO_4^{2-} \rightleftharpoons V_2O_7^{4-} + H_2O$ with $K = 48 \pm 5$.

From conductometric studies, Trujillo and Beltrán [20] concluded that the dimer in solutions with z between -1 and -2 has the charge -3. Emf measurements by Lefebvre [21] indicated the same complex together with its dimer.

The studies of pyrovanadate solutions by Russel and Salmon [22] with an ion exchanger also indicate that solutions with $z = -2$ contain a dimer with the charge -3.

Schwarzenbach and Parissakis [23] dissolved small amounts of $Na_4V_2O_7$ in a $Na_2SO_4(H_2O)_{10}$ melt and studied the change of the transition point. The data obtained were used for calculating the average number of vanadium atoms per complex. In concentrated solutions the ion $V_2O_7^{4-}$ was claimed to be formed whereas diluting gave equilibria $H_2O + V_2O_7^{4-} \rightleftharpoons 2HVO_4^{2-}$.

An early cryoscopic investigation *on the condensation of* "*metavanadate*" carried out by Düllberg [10] indicated a trimer. Jander and Jahr [18] concluded

from their diffusion data that the metavanadate ion should be a tetramer. Souchay [24] has criticized Jander and Jahr's conclusion and maintains that the solution contains trimers.

On the other hand Britton and Robinson [16] titrated vanadate solutions with Ag^+ using a silver electrode. The data could be explaned assuming $[Ag^+][VO_3^-] = 10^{-6.3}$ and not by $[Ag^+]^3[V_3O_9^{3-}] = \text{const}$. Souchay [25] says, however, that Britton and Robinson's result may be incorrect since, using the same method, he could explain the data assuming a trimer at high concentrations, and VO_3^- at low concentrations.

By studying the solubility of ammonium metavanadate Trujillo and Tejera [26] concluded that the metavandate is tetrameric. Also, Lefevbre [21] came to the same conclusion in his treatment of vanadate data with the method of "potentiometric surface".

Hazel, McNabb, and Santini [27] postulated, from emf measurements, that a complex $H_2V_2O_7^{2-}$ dimerizes to a tetramer.

From their cryoscopic studies in melting Glauber salt, Schwarzenbach and Parissakis [23] came to the conclusion that a tetramer, $V_4O_{12}^{4-}$, was formed in a range where the present work indicates a trimer, ($z = -1$).

Russel and Salmon's ion exchange work [22] indicated a trimer with the charge 3−.

From determination of vapor pressures over solutions with $z = -1$, Robinson and Sinclair [28] could calculate the average degree of condensation in the solution, which they found to be 3.

EXPERIMENTAL DETAILS

Reagents and analysis

Sodium hydroxide free from carbonate was prepared and analysed as described in Part II [5].

Sodium chloride, Merck p.a. was used after drying at 200−300°C without further purification.

Hydrochloric acid, Kebo p.a. was used. Standardisation against $KHCO_3$ and standard NaOH agreed within 0.1 %.

Vanadium(V)oxide, LKB p.a. was used. The vanadium(V)oxide was analysed by reducing an acid suspension of V_2O_5 with SO_2, boiling off the excess SO_2, and titrating with standard $KMnO_4$. Analysis of the product always gave > 99.93 % V_2O_5. In order to check if the oxide contained some impurities, which could affect the result of our titrations, V_2O_5 was prepared also from sodium vanadate according to the method proposed by Rossotti and Rossotti [1], and used for preparing the solutions in some titrations. The commercial product and that one prepared from sodium vanadate gave identical titration curves.

Apparatus

As in earlier work [5–7] in alkaline solution, Jena Geräte glass has been used for titration vessels and burets.

The electrode vessel and the salt bridge were of the same type as described Part II[5]. The glass electrode was a Radiometer (Copenhagen) type G 202 BT and the emf values were read to ±0.1 mV with a Radiometer PHM3 valve

potentiometer. The hydrogen electrode and the silver reference electrode cell were prepared as described in Part IV[7].

The cells including the titration vessel were kept in an oil thermostat at 25°C ± 0.1°C and the whole equipment kept in a thermostated room at 25°C. The solutions were introduced into the titration cell from burets calibrated by weighing water. The equilibrium solution was stirred by means of commercial nitrogen freed from O_2 which had passed through 10 % H_2SO_4, 10 % NaOH and 0.5 M NaCl.

The emf measurements

In our titrations a constant ionic medium has been maintained in order to keep the activity factors as constant as possible and thus use concentrations instead of activities in the law of mass action. To conform with earlier investigations it would be desirable to use 3 M $Na(ClO_4)$. However, in this work 0.5 M Na(Cl) has been used. The concentration 0.5 M has been chosen because of the low solubility of vanadate in stronger Na^+ media.

In this work the total vanadium concentration has been varied between $0.62_5 \times 10^{-3}$ and 80.0×10^{-3} M and as the concentration of the vanadate ions and $[OH^-]$ never exceed 20 % of the total anions, we assumed, following Biedermann and Sillén [29], the activity factors for all species to be approximately constant.

The determination of the equilibrium value for $[OH^-]$ was made with a glass or hydrogen electrode in combination with the half-cell

SE = 0.5 M NaCl | 0.5 M NaCl saturated with AgCl | AgCl, Ag

The cell used was thus:

— glass electrode | vanadate solution | SE +

or — Pt, H_2 (1 atm) | vanadate solution | SE +

Assuming the activity factors to be constant the emf of these cells can be written

$$E = E_{oa} + 59.15 \log [OH^-] + E_j = E_{oa} - 59.15 \log a + E_j \qquad (8)$$

where E_{oa} is a constant and E_j the liquid junction potential. The constant E_{oa} was determined from a solution of known $[OH^-]$ and with $[Na^+] = 0.5$ M as described in Part II[5]. Before and after every titration, the glass electrode was checked in a solution with known $[OH^-]$. It was found that this check was necessary as E_{oa} sometimes could change during a titration by several millivolts, in which case the whole titration was rejected.

For the liquid junction potential we found $E_j \approx 41\ [OH^-]$mV[7]; since $[OH^-]$ was usually < 0.001 M, E_j could be neglected in the range studied.

TITRATION PROCEDURE AND ATTAINMENT OF EQUILIBRIA

The present investigation started with a number of "bottle experiments". To a number of polythene bottles containing about 50 ml strongly alkaline vanadate solution with $z \approx -3.0$ (uncolored solutions), various amounts of acid vanadate solution with $z \approx +1.0$ (yellow solution) were added. After

the addition, all solutions were at first yellow. In some of the bottles the color faded immediately, in others it faded in the course of several days, but in the bottles containing most acid the color did not change. It was found out that colorless solutions could always be obtained as long as the average charge per vanadium, z, in the mixture was $\leqq -1.0$; the time for discoloration increased when z approached -1.0. By mixing two solutions, both with $z < -1$, uncolored solutions were always obtained.

The experiments described above were then carried out as titrations where the acid solution was added with a buret and the alkaline solution kept in a titration vessel; the change of $[OH^-]$ was followed with a *hydrogen* electrode. As long as the measured solution with $z \leqq -1$ was yellow the emf crept appreciably, whereas an uncolored solution showed a nearly constant potential. On mixing two uncolored solutions with $z \leqq -1.0$, stable potentials were obtained rapidly, and only a creep of the order of 0.06 mV per hour caused by very slow reduction of vanadium(V) was observed.

From these preliminary observations we concluded that when the two solutions are mixed in such a way that the average charge per vanadium, z, is allowed even momentarily to increase above -1, equilibrium is slowly obtained, but as long as all solutions have $z \leqq -1$ equilibrium is momentarily obtained.

Rossotti and Rossotti [1] found in their study of acid vanadate solutions that rapid equilibria could always be obtained when $z > -0.6$. Thus we concluded that equilibrium is obtained slowly for solutions with z ranging between -0.6 and -1.0, and in the following this range will be denoted as the "instability range" (see Fig. 1).

Even if the vanadate(V) solutions were slowly reduced by hydrogen, reproducible emfs were obtained if one consistently used the E value obtained after 20—30 min (the time usually required for H_2 electrodes to reach equilibrium) and did not allow the titration to take more than one day. However, for several reasons we decided to use the *glass* electrode for our final data. The data for our first titrations, using the hydrogen electrode, together with the glass electrode data are given in Table 1 and a comparison shows that the two electrodes give the same results within ± 0.3 mV. This agreement seems to show that no serious systematic error was involved in using glass electrodes in our alkaline vanadate solutions, which was most welcome since we do not pretend to understand how the glass electrode works.

Remembering the fact that rapid equilibria can always be obtained when $z \leqq -1$ for all solutions, the following two titration procedures have been worked out, and both have been used.

1) $z(\log[OH^-])_B$-titrations. In these titrations the total concentration of vanadium, B, has been kept constant and log $[OH^-]$ and thus z has been varied. The total hydroxide ion concentration was varied usually by decreasing z; then the solution in the titration vessel had $z \approx -1.03$ and was titrated with a more alkaline solution with $z < -2$. In a check titration with increasing z, the solution with $z \approx -1.03$ was in the buret and the alkaline solution in the titration vessel. The points so obtained, (see Fig. 2, 0.010 M points □) were on the same titration curve as the others (■), and there was no difference in the time for attainment of equilibria.

2) $B(\log[OH^-])_z$-titrations. In these titrations two ways for changing the total concentration of vanadium have been used. In one of them the solution in the titration vessel was 0.5 M Na(Cl), and a solution with known z and B was added; in the other way the solution containing the vanadium (known z and B) was kept in the titration vessel, and 0.5 M Na(Cl) was added by buret. From eqn. (1) and (2) it can be seen that during these titrations z was nearly constant, (In the range studied $[OH^-]$ can be neglected in comparison with H).

All these methods for getting experimental data have given consistent results, and equilibrium was always obtained rapidly.

Preparation of the vanadate solutions

Solution for the titration vessel. A weighed quantity of vanadium(V) oxide was added to a volumetric flask (of Jena-Geräte glass) and a calculated amount of standard sodium hydroxide was added. The addition of NaOH was chosen so as to make $z \approx -1.03$. In the preparation of the solutions with the lowest vanadium concentrations, the oxide dissolved rapidly giving an apparently colorless solution, but for higher concentrations it was necessary to dilute with de-aerated distilled water and raise the temperature to about 50—80°C for a short time. After dissolution of the vanadium(V) oxide, calculated amounts of solid NaCl were added. After waiting until the temperature had fallen to 25°C, the flask was carefully filled to the mark with de-aerated water. It was checked that the volume of the flask had not changed after heating and cooling. The manipulations described above were always carried out in a CO_2-free N_2 atmosphere.

The buret solution was prepared as described above except that the solutions were made more alkaline.

TREATMENT OF EXPERIMENTAL DATA

The primary experimental data: the emf values and the analytical composition of the solution were recalculated using eqns. (1), (2) and (8), to give z $(\log[OH^-])_B$. The data are given in this form in Fig. 2 and Table 1.

If mononuclear complexes only were formed, z would be a function of $[OH^-]$ only. From Fig. 2 it is seen that z is a function of both B and $[OH^-]$, and so, apparently, one polynuclear complex at least must be formed.

The $z(\log[OH^-])_B$ curves range between two z limits, $z = -1$ and $z = -2$. The complex or the complexes predominating at $z = -2$ and $z = -1$ may be polymers, and for our analysis it is convenient to start with one of these limiting complexes. As a concentrated vanadate solution became more acid on dilution (see Fig. 2) we concluded that $(B^{2-})_s$, the complex at $z = -2$, is of lower nuclearity than the complex at $z = -1$. To conform with previous methods we decided to study a condensation reaction and thus analyse the reactions taking place when H^+ is added to $(B^{2-})_s$. Before starting this analysis it is, however, necessary to find the value for s.

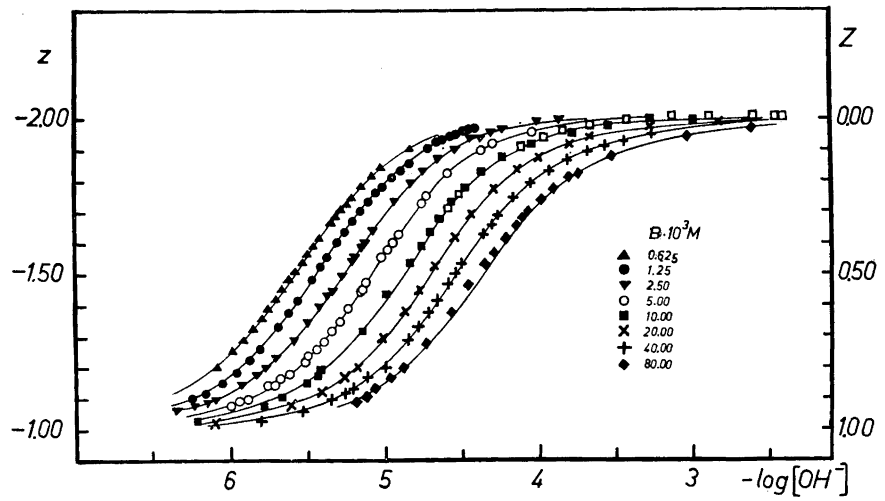


Fig. 2. Experimental data plotted as z ($\log[OH^-]$)$_B$. The average number, $Z = (2-z)$, of H^+ bound to B^{2-} is also given. The curves are calculated assuming the formation of HB_2^{3-} ($\log \beta_{12} = -3.19$) $H_3B_3^{3-}$ ($\log \beta_{33} = -10.42$) and HB^- ($\log \beta_1 = -6.04$). The open symbols for $B = 0.010$ M represent a back titration.

Table 1. Vanadates at 25°C in 0.5 M Na(Cl).

Data $z(\log[OH^-])_B$ obtained with glass electrode.

B = 0.000625 M. $-z$, $-\log[OH^-]$: 1.202, 6.086; 1.250, 5.990; 1.286,5.920; 1.323, 5.860; 1.354, 5.811; 1.386, 5.762; 1.416, 5.713; 1.450, 5.669; 1.480, 5.625; 1.509, 5.581; 1.538, 5.544; 1.565, 5.501; 1.590, 5.466; 1.614, 5.425; 1.669, 5.348; 1.685, 5.319; 1.704, 5.285; 1.723, 5.249; 1.744, 5.214; 1.781, 5.151; 1.813, 5.087; 1.842, 5.030.

B = 0.00125 M. $-z$, $-\log[OH^-]$: 1.018, 6.697; 1.062, 6.363; 1.112, 6.159; 1.145, 6.056; 1.180, 5.973; 1.219, 5.885; 1.259, 5.812; 1.282, 5.770; 1.328, 5.694; 1.374, 5.628; 1.418, 5.567; 1.486, 5.474; 1.526, 5.418; 1.566, 5.364; 1.602, 5.314; 1.632, 5.263; 1.668, 5.212; 1.701, 5.160; 1.733, 5.109; 1.760, 5.058; 1.782, 5.006; 1.811, 4.952; 1.832, 4.896; 1.851, 4.840; 1.925, 4.663; 1.934, 4.627; 1.945, 4.578; 1.953, 4.538; 1.962, 4.490; 1.969, 4.453; 1.974, 4.416.

B = 0.00250 M. $-z$, $-\log[OH^-]$: 1.080, 6.233; 1.112, 6.078; 1.148, 5.932: 1.176, 5.839; 1.232, 5.701; 1.288, 5.593; 1.340, 5.500; 1.392, 5.423; 1.444, 5.347; 1.492, 5.276; 1.544, 5.212; 1.644, 5.079; 1.740, 4.940; 1.836, 4.751; 1.904, 4.549; 1.944, 4.389; 1.960, 4.308; 1.968. 4.238; 1.996, 4.012; 2.008, 3.866.

B = 0.005 M. $-z$, $-\log[OH^-]$: 1.093, 5.911; 1.148, 5.701; 1.179, 5.620; 1.258, 5.443; 1.442, 5.157; 1.540, 5.030; 1.603, 4.951; 1.754, 4.736; 1.922, 4.300; 1.978, 3.614; 2.016, 3.207, 2.892, 2.015; 2.016, 2.800; —1.708, 6.004; 1.152, 5.695; 1.219, 5.521; 1.282, 5.397; 1.344, 5.296; 1.449, 5.144; 1.546, 5.019; 1.630, 4.914; 1.727, 4.768; 1.826, 4.593; 1.899, 4.375; 1.960, 4.042; 1.993, 3.661; 2.005; 3.369; 2.008, 3.178; 2.009, 2.929; 2.009, 2.975; 2.009, 2.929; 2.009, 2.838; 2.011, 2.767.

B = 0.010 M. $-z$, $-\log[OH^-]$: 1.078, 5.794; 1.101, 5.674; 1.147, 5.513; 1.172, 5.444; 1.316, 5.148; 1.484, 4.907; 1.537, 4.841; 1.593, 4.768; 1.634, 4.716; 1.682, 4.652; 1.735, 4.573; 1.785, 4.483; 1.832, 4.375; 1.878, 4.236; 1.920, 4.042; 1.957, 3.788; 1.977, 3.543; 1.990,

3.278; 1.996, 2.994; 2.001, 2.703; 2.002, 2.456; --- 2.005, 2.005; 2.000, 2.039; 2.004, 2.081; 2.003, 2.125; 2.006, 3.129; 1.996, 3.424; 1.980, 3.667; 1.960, 3.846; 1.943, 3.968; 1.914, 4.118; 1.824, 4.397; 1.761, 4.521; 1.713, 4.597; (titration in reversed direction).

B = *0.020 M.* $-z$, $-\log [OH^-]$: 1.018, 6.098; 1.071, 5.610; 1.117, 5.407; 1.165, 5.258; 1.211, 5.156; 1.291, 5.010; 1.378, 4.872; 1.448, 4.776; 1.525, 4.675; 1.615, 4.550; 1.690, 4.436; 1.773, 4.296; 1.834, 4.144; 1.874, 4.000; 1.914, 3.794; 1.943, 3.567.

B = *0.040 M.* $-z$, $-\log [OH^-]$: 1.028, 5.804; 1.059, 5.542; 1.098 ,5.352; 1.119, 5.258; 1.128, 5.210; 1.164, 5.117; 1.208, 5.011; 1.248, 4.894; 1.291, 4.850; 1.332, 4.786; 1.375, 4.720; 1.415, 4.663; 1.467, 4.593; 1.502, 4.546; 1.529, 4.507; 1.593, 4.421; 1.629, 4.369; 1.661, 4.303 1.692, 4.269; 1.747, 4.164; 1.795, 4.052; 1.834, 3.932; 1.866, 3.811; 1.895, 3.672; 1.912, 3.564; 1.928, 3.447; 1.938, 3.538; 1.948, 3.261; --- 1.094, 5.368; 1.155, 5.146; 1.205, 5.016; 1.247 4.933; 1.316, 4.813; 1.363, 4.739; 1.429, 4.649; 1.487, 4.566; 1.543, 4.495; 1.615, 4.396; 1.658, 4.330; 1.719, 4.227; 1.782, 4.095; 1.890, 3.718; 1.940, 3.395; 1.980, 2.818; 1.986, 2.594.

B = *0.080 M.* $-z$, $-\log [OH^-]$: 1.094, 5.181; 1.109, 5.124; 1.130, 5.059; 1.170, 4.970; 1.207, 4.869; 1.279, 4.731; 1.465, 4.446; 1.530, 4.352; 1.576, 4.282; 1.619, 4.218; 1.657, 4.154; 1.681, 4.107; 1.702, 4.069; 1.740, 3.987; 1.773, 3.905; 1.825, 3.743; 1.878, 3.522; 1.946, 3.038; 1.975, 2.607; 1.989, 2.240.

Data $B(\log[OH^-])_z$ obtained with glass electrode

$z \approx 1.09$. $B.10^3$, $-\log [OH^-]$, $-z$; 0.62_5, 6.543, 1.091; 1.29, 6.343, 1.092; 2.50, 6.154, 1.091; 5.04, 5.951, 1.091; 10.00, 5.757, 1.091; 19.97, 5.560, 1.092; 40.00, 5.368, 1.093.

$z \approx 1.29$. $B.10^3$, $-\log [OH^-]$, $-z$; 0.62_5, 5.919, 1.282; 1.250, 5.779, 1.288; 2.50, 5.591, 1.290; 5.00, 5.403, 1.292; 10.00, 5.212, 1.294; 20.00, 5.023, 1.296; 40.00, 4.837, 1.296.

$z \approx 1.43$. $B.10^3$, $-\log [OH^-]$, $-z$; 0.62_5, 5.741, 1.422; 1.25, 5.566, 1.429; 2.51, 5.380, 1.434; 5.00, 5.189, 1.437; 10.00, 4.996, 1.440; 20.00, 4.801, 1.442; 40.00, 4.614, 1.444;

$z \approx 1.59$. $B.10^3$, $-\log [OH^-]$, $-z$; 0.62_5, 5.444, 1.585; 1.27, 5.312, 1.587; 2.50, 5.150, 1.588; 5.02, 4.972, 1.589; 10.03, 4.778, 1.589; 21.34, 4.568, 1.590.

$z \approx 1.90$. $B.10^3$, $-\log [OH^-]$ $-z$; 0.62_5, 4.825, 1.903; 1.25, 4.725, 1.903; 2.50, 4.588, 1.903.

Data $z(\log[OH^-])_B$ obtained with hydrogen electrode

B = *0.040 M.* $-z$, $-\log [OH^-]$: 1.59, 4.43; 1.61, 4.41; 1.71, 4.25; 1.84, 3.92; 1.94, 3.38; 1.99, 2.60; 2.00, 2.44; 2.00, 2.33; ---- 1.09, 5.37; 1.16, 5.15; 1.21, 5.02; 1.25, 4.93; 1.32, 4.81; 1.36, 4.74; 1.43, 4.65; 1.49, 1.57; 1.54, 4.50; 1.62, 4.40; 1.66, 4.33; 1.72, 4.23; 1.78, 4.10; 1.89, 3.72; 1.94, 3.40; 1.98, 2.82; 1.99, 2.59.

B = *0.010 M.* $-z$, $-\log [OH^-]$: 1.07, 5.76; 1.21, 5.33; 1.47, 4.94; 1.56, 4.82; --- 1.70, 4.62. 1.80, 4.42; 1.86, 4.23; 1.97, 3.27.

Data $B(\log[OH^-])_z$ obtained with hydrogen electrode

$z \approx 1.59$. $B.10^3$, $-\log [OH^-]$, $-z$: 40.00, 4.42, 1.59; 35.00, 4.45, 1.59; 30.00, 4.49, 1.59; 2.50, 4.54, 1.59; 20.00, 4.60, 1.59; 10.00, 4.78, 1.59; 5.10, 4.88, 1.59; 2.50, 4.92, 1.59.

The nuclearity at $z = -2$

For $B = 0.0025-0.0200$ M and for z from -1.0 to -1.5 the experimental $z(\log[OH^-])_B$-curves are parallel within the limits of experimental errors, and according to AB I[29] the complex or the complexes formed may be written in the "core and links" form $(B^{2-})_s[(B^{2-})_sH_t]_n^{[2s+n(2s-t)]-}$ where t and n are quantities

which can be determined independently of the value of s. In our "core + links" analysis, which will be described in a following section, we found $t = 1.5$ and $n = N = 2$. With these values for t and n the upper limiting value for z may be expressed as a function of s

$$z_{\text{lim}} = \frac{t\ N - 2(N+1)s}{(N+1)s} = \frac{1-2s}{s}$$

and for $s = 1$, $z_{\text{lim}} = -1.0$; $s = 2$, $z_{\text{lim}} = -1.5$; $s = 3$, $z_{\text{lim}} = -1.67$. Experimentally, $z_{\text{lim}} = -1.00$ (see Fig. 2) and consequently our data are in agreement only with $s = 1$, and the species at $z = -2$ must be monomeric with the charge -2, B^{2-}, whereas the limiting species at $z = -1$ would be $H_3B_3^{3-}$.

The first step in our analysis of the experimental curves $z(\log[OH^-])_B$ will be a determination of $\bar{q}$, the average number of vanadium atoms in all complexes except B^{2-}, and $\bar{p}$, the average number of protons bound (in excess to B^{2-}) in these complexes. A description of this calculation will be given in a following section, (in connection with eqns (10), (11) and (12)), but a part of it will be given here as it gives a further proof of the mononuclearity for vanadium at $z = -2$.

As pointed out earlier the complex dominating at $z = -2$, may be written in the form $(B^{2-})_s$. If H^+ is added, a condensation takes place and the average degree of condensation, R^{-1}, increases as H^+ increases. Sillén [30] has shown that R^{-1} may be calculated from the experimental $Z(\log a)_B$-curves and the general formula he gives may be written

$$R = R_0 - \int_{\log a_1}^{\log a} \left(\frac{\partial\ (2+z)}{\partial \log B}\right)_a \mathrm{d} \log a = \frac{1}{s} - \int_{\log a_1}^{\log a} \left(\frac{\partial\ Z}{\partial \log B}\right)_a \mathrm{d} \log a \text{ at constant } B \qquad (9)$$

In this formula $R_0 = \frac{1}{s}$ is an integration constant, the value of which can be determined from the lower limit of integration, s is the number of B^{2-} units in $(B^{2-})_s$, $\log a_1$ the value of $\log a = -\log [OH^-]$ where the integration starts and $(2 + z) = Z$, the average number of H^+ bound per $(B^{2-})_s$.

For obtaining R^{-1} at an arbitrary point on the $z(\log a)_B$-curve the value of s and the integral in (9) must be known. The calculated values for the integral at various $-\log [OH^-]$ obtained from our experimental data are given in Table 2. From this table it is evident that low experimental z-values and high B-values give integrated quantities > 0.5; if $s \geqq 2$ negative values for R^{-1} would then be obtained, according to (9). That must mean that the integrated area is too large to be in agreement with other s-values than $s = 1$.

Again we conclude $s = 1$. The complex with $z = -2$, B^{2-}, is mononuclear.

The mean composition of the polynuclear complexes

To find the species in these vanadate solutions we shall start with the "direct integration method", previously used in the borate study [5], which gives the *mean composition of the complexes formed*. With this integration as

Table 2. Values for $\bar{q}$ and $\bar{p}$ obtained with the "direct integration" method.

-log a	*	**	$\bar{q}$	$\bar{p}$
B = 1.25·10⁻³ M				
4.20	0.01	0.008	1.0	0.7
4.40	0.01	0.008	2.0	1.5
4.60	0.03	0.029	2.0	1.9
4.80	0.05	0.056	1.7	1.7
5.00	0.09	0.106	1.7	1.6
5.20	0.15	0.189	1.7	1.6
5.40	0.12	0.298	1.8	1.6
5.60	0.19	0.425	1.9	1.8
5.80	0.36	0.592	1.9	1.9
6.00	0.40	0.777	1.9	1.9
B = 2.5·10⁻³ M				
4.60	0.07	0.048	2.1	1.5
4.80	0.10	0.086	2.1	1.5
5.00	0.17	0.148	2.2	1.8
5.20	0.24	0.243	2.3	2.0
5.40	0.32	0.368	2.3	2.0
5.60	0.41	0.523	2.4	2.3
5.80	0.48	0.700	2.5	2.5
6.00	0.52	0.880	2.5	2.4
6.10	0.54	0.967	2.5	2.5
B = 10·10⁻³ M				
4.20	0.10	0.085	2.1	1.5
4.40	0.15	0.137	2.2	1.5
4.60	0.22	0.214	2.3	1.7
4.80	0.30	0.321	2.4	2.0
5.00	0.39	0.460	2.5	2.2
5.20	0.47	0.623	2.6	2.4
5.40	0.53	0.801	2.7	2.6
5.50	0.56	0.896	2.8	2.7
5.60	0.58	0.991	2.9	2.7
B = 20·10⁻³ M				
4.00	0.08	0.082	2.1	1.3
4.20	0.13	0.124	2.1	1.3
4.40	0.19	0.185	2.2	1.4
4.60	0.26	0.271	2.3	1.7
4.80	0.36	0.392	2.5	1.9
5.00	0.44	0.640	2.6	2.2
5.20	0.50	0.700	2.7	2.4
5.40	0.56	0.890	2.8	2.5
5.60	0.60	1.080	2.9	2.7
B = 40.0·10⁻³ M				
3.00	0.02	0.017	2.0	1.2
3.20	0.04	0.030	2.1	1.3
3.40	0.06	0.048	2.1	1.2
3.60	0.08	0.075	2.0	1.1
3.80	0.11	0.100	2.1	1.3
4.00	0.16	0.160	2.0	1.2
4.20	0.22	0.233	2.1	1.4
4.40	0.29	0.333	2.2	1.6
4.60	0.38	0.463	2.4	1.9
4.80	0.47	0.620	2.5	2.3
5.00	0.50	0.706	2.6	2.4
5.20	0.56	0.889	2.8	2.7
5.40	0.60	1.079	2.9	2.8
5.60	0.63	1.274	3.0	2.9

$$* \int \left(\frac{\partial Z}{\partial \log B}\right)_a \mathrm{d} \log a \qquad ** \int \left[Z + \left(\frac{\partial Z}{\partial \log B}\right)_a\right] \mathrm{d} \log a$$

the basis we shall try to find the *formulas of the complexes;* the final step in the analysis will be a confirmation of the complexes with one or two *projection maps*.

In the foregoing we have shown that there is a single monomeric complex, B^{2-}, at $z = -2$, ($Z = 0$), and thus polynuclear complexes must be formed when Z increases (see Fig. 2). For high Z values there is a mixture of mononuclear and polynuclear complexes. If direct measurements on $b = [B^{2-}]$ were possible it should be very easy to obtain

$$\bar{q} = \frac{B-b}{BR-b} \qquad (10) \qquad \text{and} \qquad \bar{p} = \frac{BZ}{BR-b} \qquad (11)$$

In these formulas B is the total concentration of vanadium and R is the reciprocal of the average degree of condensation of B^{2-}; so, $(BR-b)$ is the total molar concentration of all species except B^{2-}.

However, even in absence of direct measurements on b, this quantity may be calculated. General expressions for this calculation have been given by Sillén [30] and his formulas will be used here for getting R and b. The formula for getting R has already been given in eqn. (9) and the formula for calculating b is

$$\log \frac{B}{b} = \int_{\log a_1}^{\log a} \left[Z + \left(\frac{\partial Z}{\partial \log B} \right)_a \right] \mathrm{d} \log a \qquad \text{at constant } B \qquad (12)$$

Log a_1 is the log a value where the integration starts, B the total concentration of vanadium and $Z = (z + 2)$, the average number of H^+ bound to B^{2-}, the dominating species at $z = -2$; at $a = a_1$, $b \approx B$ and $\log B/b \approx 0$.

The integration has been carried out numerically from the experimental Z (log a)-curves at a given value of B. $(\partial Z/\partial \log B)_a$ was obtained as the average of the quotients $(\Delta Z/\Delta \log B)_a$ from the plot Z (log B)$_a$ for two neighbouring intervals.

In Table 2 a part of our result from the integration is given, and in Fig 3 $\bar{q}$ and $\bar{p}$ for some concentrations have been plotted against $-\log [OH^-]$. It is indicated by this integration that complexes are formed with $\bar{q}$ ranging bet-

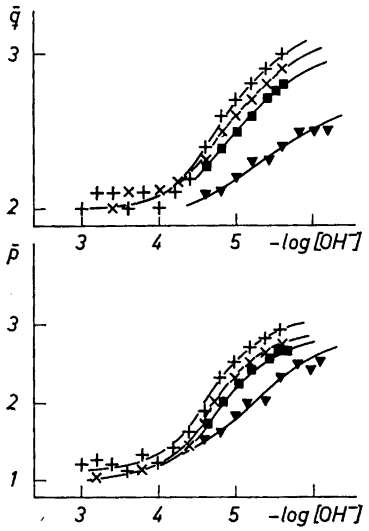


Fig. 3. Analysis by "direct integration"; $\bar{q}$ and $\bar{p}$ plotted against $-\log[OH^-]$. + = 0.040 M; × = 0.020 M; ■ = 0.010 M and ▲ = 0.0025 M.

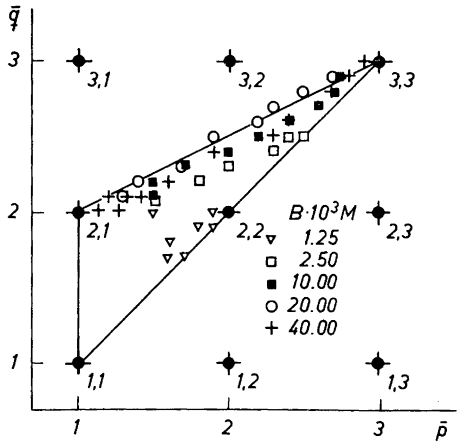


Fig. 4. $\bar{q}$ *versus* $\bar{p}$; note that all points fall within the triangle between (2,1), (3,3) and (1,1).

ween 2.0 and 3.0 and $\bar{p}$ between 1.0 and 3.0. The plot $\bar{q}$ against $\bar{p}$ for $B = 0.040$, 0.020, 0.010 M in Fig. 4 gives a nearly straight line between the sets $(\bar{q}, \bar{p}) = (2,1)$ and (3,3), indicating that the main complexes for these concentrations are HB_2^{3-} and $H_3B_3^{3-}$.

At lower concentrations, $B = 0.0025$ and 0.0012_5 M, the $(\bar{q}\ \bar{p})$ points deviate markedly from this line, thus indicating the formation of some additional complex or complexes at lower B. If one tries to explain these deviations with the formation of a single complex, $\bar{q}$ for this complex must be <3; thus the species may be a monomer $H_{p'}B$ or a dimer $H_{p'}B_2$; p' must be <2 and p'' <3 as the deviations are found at low Z-values and Z is ranging between 0.00 and 1.00. Then only the complexes HB^- and $H_2B_2^{2-}$ remain and of these two HB^- seems to be the more likely one as all data, $\bar{q}$ $(\bar{p})$ in that case fall within the triangle formed by the $(\bar{q}\ \bar{p},)$-coordinates, (3,3); (2,1) and (1,1) corresponding to the composition of the complexes $H_3B_3^{3-}$, HB_2^{3-} and HB^-.

If the two complexes HB_2^{3-} and $H_3B_3^{3-}$ only were formed (10) and (11) may be written

$$\bar{q} = \frac{2\,[HB_2] + 3\,[H_3B_3]}{[HB_2] + [H_3B_3]} = \frac{2 + 3\beta_{33}\beta_{1\,2}^{-1}a^2b}{1 + \beta_{33}\beta_{1\,2}^{-1}a^2b} \qquad (13)$$

$$\bar{p} = \frac{[HB_2] + 3\,[H_3B_3]}{[HB_2] + [H_3B_3]} = \frac{1 + 3\beta_{33}\beta_{1\,2}^{-1}a^2b}{1 + \beta_{33}\beta_{1\,2}a^2b} \qquad (14)$$

or in a normalized form

$$\bar{q} = \frac{2 + 3\,w}{1 + w} \qquad (15a) \qquad\qquad \bar{p} = \frac{1 + 3\,w}{1 + w} \qquad (15b)$$

where $w = \beta_{33}\beta_{1\,2}^{\cdot}a^2b$ (15c)

With (15a) and (15b) it is possible to construct two theoretical curves $\bar{q}$ (log w) and $\bar{p}$ (log w) and compare them with the experimental $\bar{q}$ (log a^2b)- and $\bar{p}$ (log a^2b)-curves. If the assumption is right the experimental $\bar{q}$ and $\bar{p}$ values plotted against log a^2b must give two single curves with the same shape as the calculated $\bar{q}$ (log w)- and $\bar{p}$ (log w)-curves. In Fig. 5 experimental and calculated curves are compared and the fit must be considered satisfactory (except for $B = 2.5.10^{-3}$ M). The difference in coordinates between log a^2b and log w in the position of best fit gives the ratio $\beta_{33}\beta_{1\,2}^{-1}$:

log w − log a^2b = log β_{33} − log β_{12} = -7.40 ± 0.20. (The final constants give log $\beta_{33}\beta_{1\,2}^{-1} = -7.24 \pm 0.10$).

Thus this treatment has indicated the species $H_3B_3^{3-}$ and HB_2^{3-} and given an approximate relationship between their formation constants. For low B values indications were found for a complex HB^- or $H_2B_2^{2-}$.

"Core and links" treatment

In the first instance only the data for $B = 0.0025$—0.020 M and for Z from 0.45 to 1.00 will be discussed. In this range the $Z(\log a)_B$-curves form within

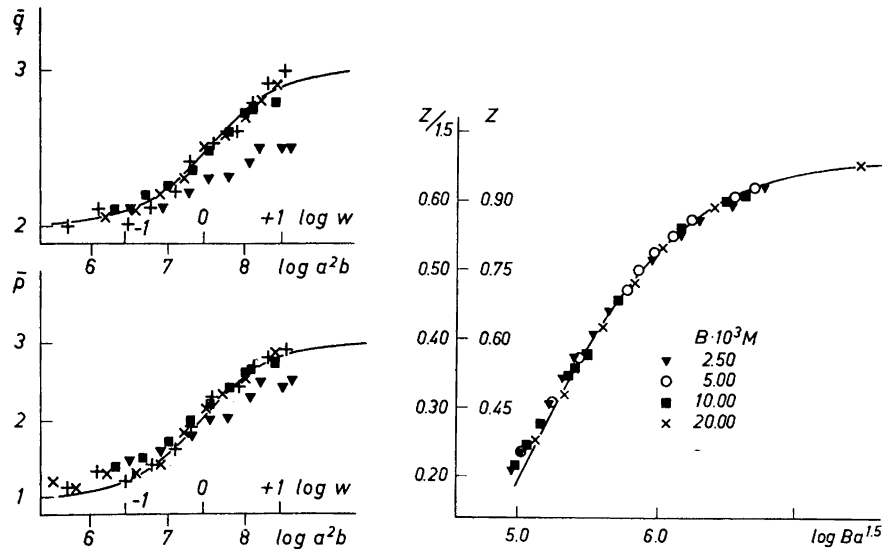


Fig. 5. $\bar{q}$ and $\bar{p}$ plotted against log a^2b. Full curve calculated assuming only HB_2^{3-} and $H_3B_3^{3-}$ (log $\beta_{33}\beta_{12}^{-1} = -7.40 \pm 0.20$) + = 0.040 M; × = 0.020 M; ■ = 0.010 M and ▲ = 0.0025 M.

Fig. 6. Z or $y = 1.5^{-1}\ Z$ as a function of $x = \log B + 1.5 \log a$; Data for $B = 0.0025$ to 0.020 M, and for Z from 0.45 to 1.00 are given. Full curve calculated assuming the single complex $H_3B_3^{3-}$; with log $\beta_{33} = -10.50$.

the limits of experimental error, apparently parallel curves. That should mean that the complex or the complexes formed may be written in the "core + links" form $B(BH_t)_n^{[2+n(2-t)]-}$; t is a constant and n may be an unique integer N or a series of integers such that the product nt is an integer. In ABI[31] it has been shown that

$$t = -\left(\frac{\partial \log B}{\partial \log a}\right)_z \tag{16}$$

From our experimental $Z(\log a)_B$-data in Fig. 2 it is easily found that $t = 1.5$. That should mean that the main species formed have the composition $B(BH_{1.5})_n^{(2+0.5\,n)-}$. If only complexes with that composition are formed, the validity of (16) can be tested, as all experimental points should lie now on a single $y(x)$-curve, with the coordinates

$$x = \log B - 1.5 \log [OH^-] \quad y = 1.5^{-1}\ Z \tag{17}$$

In Fig. 6 the experimental plot of y against x is shown. It is seen that the experimental points according to (16) lie satisfactorily on a single curve and the "core + links" hypothesis may be considered as a good explanation of these data.

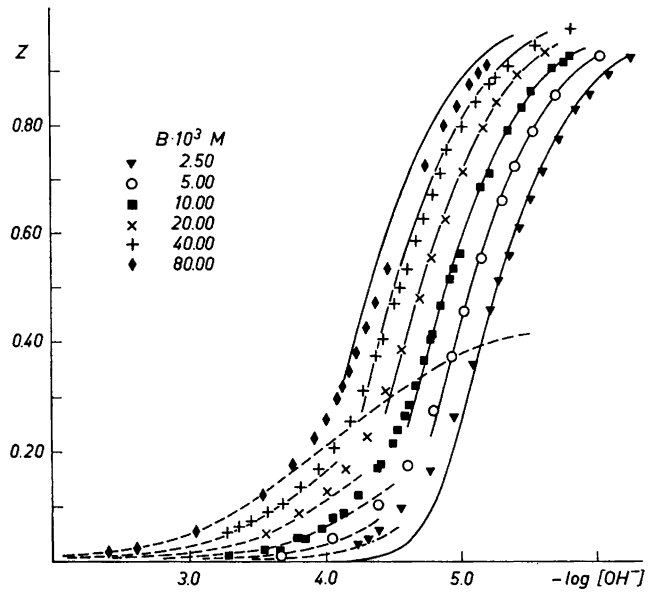


Fig. 7. Data Z (log $[OH^-])_B$. Full curves calculated assuming only $H_3B_3^{3-}$ (log $\beta_{33} = -10.48$). Dotted curves calculated assuming only HB_2^{3-} (log $\beta_{12} = -3.12$).

To decide whether n is a constant or not the curve $y(x)$ was compared with the normalized $y(X)$-curves calculated on the following three assumptions given in ABII [11] page 321:

I no soluble complexes are formed but a precipitate is obtained
II a unique complex $B(A_tB)_N$ is formed in solution
III a series of complexes $B(A_tB)_n$ is formed in solution, with values of n ranging from unity to infinity.

This comparison indicated a single complex, $H_3B_3^{3-}$, with $N = 2$. The normalized $y(x)$-curves used were so constructed that the difference in reading on the X- and log $Ba^{1.5}$-coordinates in the position of best fit gave log β_{33} (see eqns. (24) and (25) in ABII [11]). We found log $\beta_{33} = -10.50 \pm 0.10$.

In order to get further confirmation for that complex and to check the shape of the experimental curves, a normalized $Z(X)_{PQ}$ curve was constructed under the assumption that a single complex H_PB_Q with $P = 3$ and $Q = 3$ was formed. If only one complex is formed, we have

$$B = b + Q\,[H_PB_Q] = b + Q\beta_{PQ}a^Pb^Q$$
$$BZ = P\beta_{PQ}a^P b^Q$$

Elimination of b and rearranging gives:

$$Q\beta_{PQ}B^{Q-1}a^P = w(1-w)^{-Q} = 10^{PX}$$

where $w = QZP^{-1}$. Using w as auxiliary variable it is possible to calculate a normalized $Z(X)_{PQ}$-curve where

$$X = P^{-1} \log w - QP^{-1} \log (1-w) \quad (18a)$$

and $$Z = wPQ^{-1}. \quad (18b)$$

The normalized curve with $Q = 3$ and $P = 3$ was found to fit well with the experimental Z (log $a)_B$-curves (see Fig. 7) for low Z values and the concentrations $B = 0.0025-0.020$ M; the difference in coordinates on the horizontal axis gave $\beta_{33} = -10.48 \pm 0.1$

$$\log a - X = -P^{-1} \log \beta_{PQ}Q - (Q-1)P^{-1} \log B = -\tfrac{1}{3} \log 3\beta_{33} - \tfrac{2}{3} \log B \quad (19)$$

The main product is thus $H_3B_3^{3-}$, which, however, does not suffice to explain the data in the range for $B = 0.0025-0.080$ M and for Z from 0.00 up to 0.45.

In the direct integration we found that $\bar{q}$ and $\bar{p}$ approach the limiting values $\bar{q} = 2$ and $\bar{p} = 1$ when $Z \to 0$. From that we may conclude that the polynuclear complex first formed must be HB_2^{3-}.

With the aid of (18a) and (18b) a normalized $Z(X)_{PQ}$ curve was constructed for a single complex H_PB_Q with Q = 2 and $P = 1$ and compared with the experimental Z(log $[OH^-])_B$-data at low Z-values. An acceptable fit (see Fig. 7) was obtained for the four highest vanadium concentrations, $B = 0.080-0.010$ M, and the difference in reading on the horizontal axis gave, for all concentrations log $\beta_{12} = -3.12 \pm 0.1$.

Confirmation of HB_2^{3-} and $H_3B_3^{3-}$ with a normalized graph log B (log $u)_Z$

In order to get a final confirmation for the formation of the complexes HB_2^{3-} and $H_3B_3^{3-}$, a normalized graph [31a] for the three species, B^{2-}, HB_2^{3-} and $H_3B_3^{3-}$ was constructed and compared with the experimental data.
Reactions:

$$2B^{2-} + H^+ \rightleftharpoons HB_2^{3-}; [HB_2^{3-}] = {}^*\beta_{12}[H^+][B^{2-}]^2 = \beta_{12}ab^2$$
$$3B^{2-} + 3H^+ \rightleftharpoons H_3B_3^{3-}; [H_3B_3^{3-}] = {}^*\beta_{33}[H^+]^3[B^{2-}]^3 = \beta_{33}a^3b^3$$

The condition for the concentrations gives

$$B = b + 2\beta_{12}ab^2 + 3\beta_{33}a^3b^3 \quad (20)$$

$$BZ = \beta_{12}ab^2 + 3\beta_{33}a^3b^3 \quad (21)$$

We wish to transform these expressions to the normalized form

$$Bu = v(1 + 2v + 3uv^2) \quad (22), \qquad BZu = v(v + 3uv^2) \quad (23)$$

and this is achieved by the substitutions

$$u = \beta_{33}\beta_{12}^{-2}a\,; v = \beta_{12}ab\,; B = B\beta_{12}^{3}\beta_{33}^{-1} \quad (24)$$

Elimination of u from (22) and (23) gives

$$u = (Z + v(2Z-1))(1-Z)^{-1}(3v^2)^{-1}$$

and $B = 3v^3(1 + v)\ (Z + v(2Z-1))^{-1}$

With these formulas it is possible to calculate $\log B$ as a function of Z and $\log u$.

The calculated $\log B(\log u)_Z$ graph was compared with the experimental $\log B(\log a)_Z$-data and in the position of best fit the difference in reading along the coordinate axes on the two graphs gives the constants β_{12} and β_{33}

$$\log B - \log B = 3 \log \beta_{12} - \log \beta_{33} \tag{25}$$

$$\log u - \log a = \log \beta_{33} - 2 \log \beta_{12} \tag{26}$$

and $\log \beta_{12} = -3.12 \pm 0.05 = \log \beta_{33} = -10.42 \pm 0.05$.

The dotted curves in Fig. 8 are calculated with these constants. It is seen from this figure, that the agreement is satisfactory. However, small deviations at $B = 0.00125$ M, $B = 0.00062_5$ M and low z-values were found.

Deviations caused by additional complex

It remains now to explain the deviations from the dotted curve in Fig. 8. Such low total concentrations as 0.00125 and 0.00062_5 M can usually be studied with high accuracy only in very favorable cases. Now in our case measurements on low vanadium concentrations can be made in a very sensitive pH-range, and the deviations cannot be due only to experimental error but must be caused also by the formation of some additional complex.

In our treatment of the experimental data with the direct integration method the main part of the data could be explained with HB_2^{3-} and $H_3B_3^{3-}$ but deviations were found for low B and if a single additional complex was formed this could indicate either HB^- or $H_2B_2^{2-}$. These two possibilities have been tested with the aid of normalized graphs $\log B(\log a)_{Z,l}$, and it was found that only the combination with the species B^{2-}, HB^-, HB_2^{3-} and $H_3B_3^{3-}$ gave normalized graphs, which fitted satisfactorily with the experimental data (see Fig. 8). The equations valid for the construction of this normalized graph are

$$Bu = v(1 + lu + 2v + 3uv^2)$$
$$BZu = v(lu + v + 3uv^2)$$

where the same substitutions as in (22) and (23) has been used and

$$l = \beta_1\beta_{12}^2\beta_{33}^{-1} \tag{27}$$

Elimination of u gives

$$u = (Z + v(2Z-1))(1-Z)^{-1}(3v^2 + l)^{-1} \tag{28}$$

and $B = v(1 + v)\ (3v^2 + l)\ (Z + v(2Z-1))^{-1}$ (29)

From (28) and (29) two $\log B\ (\log u)_Z$ graphs with different values of l, $l = 0.005$ and $l = 0.010$, were constructed and compared with the experimental graph. From the calculated graph which fitted best with the experimental

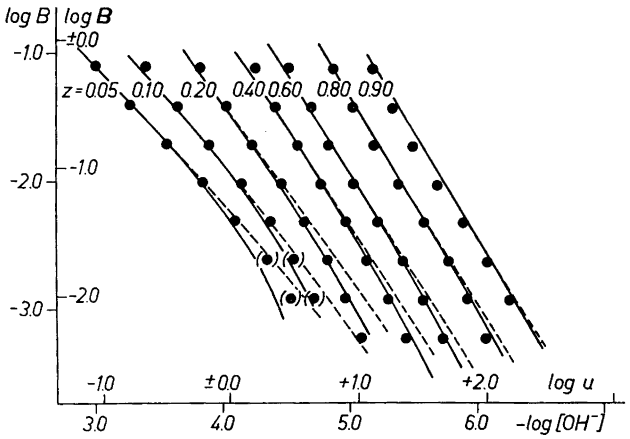


Fig. 8. Projection maps experimental data log $B(\log[OH^-])z$ and normalized curves log $B(\log u)z$ calculated for a) HB_2^{3-} and $H_3B_3^{3-}$ (dotted curve) b) HB^-, HB_2^{3-} and $H_3B_3^{3-}$ assumming $\beta_{11}\beta_{12}^2\beta_{33}^{-1} = 0.01$ (full curve). The position of best fit gave log $\beta_1 = -6.04 \pm 0.08$, log $\beta_{12} = -3.19 \pm 0.05$ and log $\beta_{33} = -10.42 \pm 0.05$.

data, the full curve in Fig. 8, we obtained the constants β_{12} and β_{33} from (25) and (26)

$$\log \beta_{12} = -3.19 \pm 0.05; \quad \log \beta_{33} = -10.42 \pm 0.05$$

and the constant β_1 from eqn. (27) and the value of l giving the best fit ($l = 0.01$).

$$\log \beta_1 = -2 \pm 0.03 - 4.04 \pm 0.05 = -6.04 \pm 0.08$$

Final sets of equilibrium constants

Our potentiometric measurements give a set of data (B,Z,a) for concentrations $0.080\ \mathrm{M} < B < 0.00062_5\ \mathrm{M}$, which may be explained most simply by a successive condensation of B^{2-} to HB_2^{3-} and $H_3B_3^{3-}$. Low concentrations indicated also a mononuclear species HB^-. No evidence has been found for the existence of higher polyions e.g. tetranuclear species.

The equilibrium constants obtained in this work have been deduced according to the methods developed in this institute and are summarized in Table 3.

Table 3.

The constants deduced by:	log β_1	log β_{12}	log β_{33}
Curve fitting $1.5^{-1}Z(\log Ba^{1.5})$			-10.50 ± 0.1
$Z(X)_{PQ}$		-3.12 ± 0.1	-10.48 ± 0.1
Normalized graph log B (log u)$_{z,l}$, $l = 0$		-3.19 ± 0.05	-10.42 ± 0.05
Normalized graph log $B(\log u)_{z,l}$	-6.04 ± 0.08	-3.19 ± 0.05	-10.42 ± 0.05
"Best values" of Nils Ingri	-6.04 ± 0.08	-3.19 ± 0.05	-10.42 ± 0.05

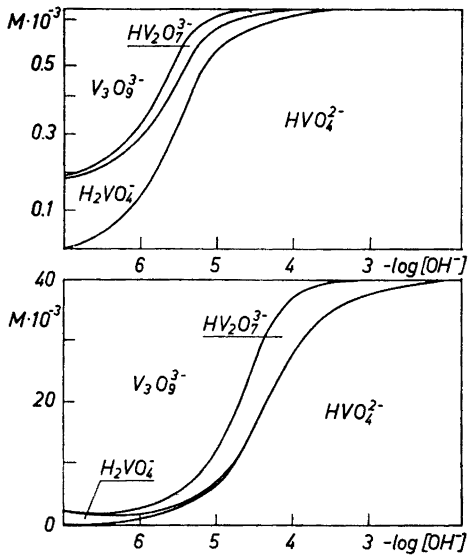


Fig. 9. Distribution of complexes as a function of $-\log[OH^-]$ for the total vanadium concentrations $B = 0.0062_5$ M and $B = 0.040$ M. The formulas have been written with the "phosphate" nomenclature.

The constants obtained in Felipe Brito's calculation [1a] (see the note on page 1971) are summarized in Table 4.

Table 4.

$\log \beta_1$	$\log \beta_{12}$	$\log \beta_{33}$	Method for deducing the constant
-5.97 ± 0.04	-3.16 ± 0.03	-10.42 ± 0.03	normalized map $\log B(\log u)z$
-5.97 ± 0.04	-3.16 ± 0.03	-10.42 ± 0.03	„ „ $\log BZ(\log Bu^{1.5})z$
-5.95 ± 0.06	-3.17 ± 0.04	-10.41 ± 0.03	„ „ $\log BZ(\log Bu)z$
-5.96 ± 0.06	-3.17 ± 0.04	-10.42 ± 0.03	„ „ $\log BZ(\log B(1-Z)u)z$
-5.97 ± 0.05	-3.17 ± 0.04	-10.42 ± 0.03	"Best values" of Felipe Brito.

After we had finished our calculations we discussed the methods used and have agreed on the following set of constants.

$$\log \beta_1 = -6.0 \pm 0.1; \log \beta_{12} = -3.18 \pm 0.05; \log \beta_{33} = -10.42 \pm 0.04$$

Fig. 9 shows the distribution of the various species as a function of $\log [OH^-]$ for the total concentrations $B = 0.00062_5$ M and 0.040 M.

Possible structures of the complexes

Now one may ask if the ions found can be given a reasonable structural interpretation. There are many different methods for evaluating the structure of complexes in aqueous solution (X-ray diffraction of solutions, thermodynamic considerations, various types of spectra *etc.*) but very few of them have been used for studying vanadate(V) solutions.

From a structure-entropy consideration, however, La Salle and Cobble [42] have shown that VO_2^+ rather than $V(OH)_4^+$ is the more probable form of vanadium(V) in very strong acid solution.

Siebert [41] has studied strong alkaline vanadate(V) solutions, using Raman spectra, and from similarities between spectra obtained and spectra of PO_4^{3-} solutions he concluded that the vanadate ion in these solutions is tetrahedral, VO_4^{3-}.

For solutions of intermediate pH this kind of data is lacking. In this case one may guess from what is known on the solid state and assume that vanadium(V) forms the same type of ions or units or least has the same coordination number in solutions as in the solid.

Previous X-ray studies of crystalline vanadium(V) compounds prepared from water solutions indicate that the coordination number of vanadium(V) may be 4,5, or 6.

In solid V_2O_5 investigated by Byström, Brotzen and Wilhelmi (1950) [32], vanadium is surrounded by five oxygen atoms forming a distorted trigonal bipyramid. The bipyramids are coupled by sharing two edges giving infinite chains. These chains are linked laterally to sheets. The lateral linking is established by the attraction between a vanadium atom and an oxygen in two different chains. Thus the coordination around vanadium is between 5 and 6. From water solution V_2O_5 is precipitated at $pH \approx 1.6$. The same type of V-O-sheets have also been found in the mineral hewettite $CaV_6O_{16}(H_2O)_9$ (Barnes, Qurashi [33]).

Evans [34] has pointed out that it may be reasonable to expect the same type of bipyramidal chains, modified to give vanadium a distorted octahedral coordination, also in the solid decavanadates, crystallizing at $pH \approx 6.5$, $(NH_4)_6V_{10}O_{28}(H_2O)_6$ (Lindqvist [2]) $K_6V_{10}O_{28}(H_2O)_9$, $K_6V_{10}O_{28}(H_2O)_{10}$, $Ca_3V_{10}O_{28}(H_2O)_{16}$ (pascoite), $K_2Mg_2V_{10}O_{28}(H_2O)_{16}$ (hummerite) and $K_2Zn_2V_{10}O_{28}(H_2O)_{16}$ (Evans, Mrose and Marvin [3]).

KV_3O_8 belongs to this group also and has been investigated with X-rays by Evans and Block [34]. According to Evans [34] this vanadate contains bipyramidal chains "folded in a herringbone fashion".

At higher pH, 6—8, colorless "metavanadates" may be crystallised and among then KVO_3 (Evans and Block [35], 1954, Petràsovà, Madar and Hanic [36] 1958) and $KVO_3(H_2O)$ (Christ, Clark and Evans [37] 1954) have been investigated by X-ray analysis. In $KVO_3(H_2O)$, the units are trigonal bipyramids which share edges forming a chain. In the structure two V-O-distances are shorter than the other and Hanic [38] has pointed out that this should indicate the existence of VO_2^+ groups in the crystal.

In KVO_3 which crystallizes together with $KVO_3(H_2O)$ one would expect the same trigonal staggered chain but here vanadium is four-coordinated and the tetrahedra share corners forming a string similar to diopside. It is easy to derive the diopside chain from the staggered trigonal bipyramid by a simple stretching process. In fact it may be possible also to convert $KVO_3(H_2O)$ to KVO_3 by grinding (Evans [35]). Diopside structures have been found also for $NaVO_3$ (Sörum [39] 1943) and NH_4VO_3 (Lukesh [40] 1950).

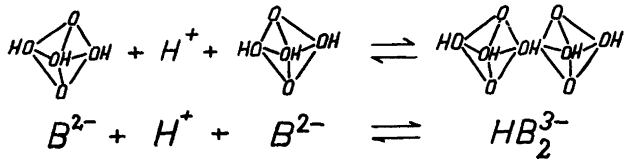


Fig. 10.

From the most alkaline solutions "orthovanadates" may be crystallized, and tetrahedral VO_4^- groups have been established in a number of vanadium compounds especially minerals ($BiVO_4$ [43], (Pb, Zn, Cu)(OH)(VO_4) [44], $Pb_3(VO_4)_2$ [45], $ScVO_4$ [46], and others).

From the configurations in the solid vanadate(V) crystals investigated with X-rays it seems reasonable to propose, for the polynuclear complexes in solution, structures where vanadium either is four- or five-coordinated.

Assuming four-coordination, the vanadium species $H_3B_3^{3-}$ may be given the formula $V_3O_9^{3-}$; structurally that means three VO_4 tetrahedra sharing two corners forming a 6-membered ring. With VO_4-tetrahedra, however, it is difficult to understand the formation of HB_2^{3-}.

On the other hand assuming vanadium to be five-coordinated it is possible to give a mechanism for formation of both HB_2^{3-} and $H_3B_3^{3-}$. In this case the first step in the condensation reaction may be written (Fig. 10)

$$B^{2-} + H^+ + B^{2-} \rightleftharpoons HB_2^{3-}$$

$$VO_2(OH)_3^{2-} + H^+ + VO_2(OH)_3^{2-} \rightleftharpoons (VO_2)_2(OH)_5^{3-} + H_2O$$

and for the reaction (Fig. 11) when HB_2^{3-} reacts with H^+ and B^{2-} forming the trimer $H_3B_3^{3-}$

$$HB_2^{3-} + 2H^+ + B^{2-} \rightleftharpoons H_3B_3^{3-}$$

$$(VO_2)_2(OH)_5^{3-} + 2H^+ + VO_2(OH)_3^{2-} \rightleftharpoons (VO_2)_3(OH)_6^{3-} + 2H_2O$$

Instead of a ring structure $H_3B_3^{3-}$ may also be a chain, coupled as in $KVO_3(H_2O)$, but in this case it is difficult to explain why higher complexes e.g. four — or five-nuclear complexes, have not been detected in our solutions.

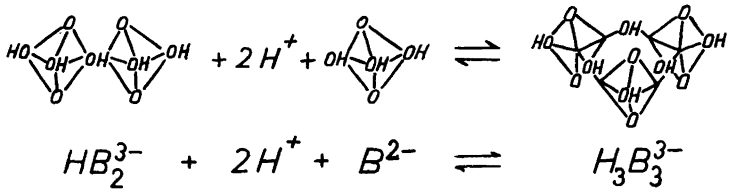


Fig. 11.

Added in proof: Recently K. F. Jahr and L. Schoepp *(Z. Naturforsch. 14 b* (1959) 467, 468) presented the results from an investigation of water solutions of metavanadate using three different cryoscopic methods. They used much higher concentrations than that one used in this work ($C \approx 0.140-0.196$ moles $NaVO_3$ per kg) and concluded that the solutions contained the tetrameric ion, $V_4O_{12}^{4-}$. In addition to that they also found that thallium(I)metavanadate is dissolved in tertbutylchloride giving the compound $(C_6H_9)_4V_4O_{12}$. Whatever the reason for the discrepancy between cryoscopic measurements of Schwarzenbach and Jahr on one side and our emf work on the other side may be, our data certainly cannot be explained by a mechanism where $V_4O_{12}^{4-}$ is the main product.

The authors are indebted to Professor Lars Gunnar Sillén for his great interest and for the many valuable discussions in connection with the preparation of this paper.

We also wish to thank Dr Georg Lundgren for advice and discussions during the work, Mr. Per Jerkeman for his help during the preliminary titrations and Mr. Frans Ericson for practical aid in many ways.

One of us (F. B.) is also indebted to the Spanish Foreign Office for an interchange grant with *Svenska institutet för kulturellt utbyte med utlandet* as well as to *Juan March* Foundation.

Thanks are also due to Mr. David Martin for correcting the English text.

This work forms a part of a program supported by *Statens naturvetenskapliga forskningsråd.*

REFERENCES

1. Rossotti, F. J. C. and Rossotti, H. *Acta Chem. Scand.* **10** (1956) 957 (Part I).
1a. Brito, F. and Ingri, N. *Anales fis. y quim. (Madrid);* Part V *In print.*
2. Lindqvist, I. *Private communication* to F. J. C. Rossotti and H. Rossotti.
3. Evans, H. T., Mrose, M. E. and Marvin, R. *Am. Mineralogist* **40** (1955) 314.
4. Newman, L. and Quinlan, K. P. *J. Am. Chem. Soc.* **81** (1959) 547.
5. Ingri, N., Lagerström, G., Frydman, M. and Sillén, L. G. *Acta Chem. Scand.* **11** (1957) 1034 (Part II).
6. Lagerström G. *Acta Chem. Scand.* **13** (1959) 722 (Part III).
7. Ingri, N. *Acta Chem. Scand.* **13** (1959) 758 (Part IV).
8. Rammelsberg, C. F. *Pogg. Ann.* **98** (1856) 249.
9. Roscoe, H. E. *Phil. Trans.* **158** (1868) 1.
10. Düllberg, P. *Z. physik. Chem.* **45** (1903) 129.
11. Sillén, L. G. *Acta Chem. Scand.* **8** (1954) 318 (AB II).
12. Bjerrum, J., Schwarzenbach, G. and Sillén, L. G. *Stability constants, Part II Inorganic ligands, The Chemical Society Special Publication* No 7. 1958.
13. Britton, H. T. S. and Robinson, R. A. *J. Chem. Soc.* **1930** 1261.
14. Ducret, L. P. *Ann. Chim. (France)* **6** (1951) 705.
15. Britton, H. T. S. and Robinson, R. A. *J. Chem. Soc.* **1932** 1955.
16. Britton, H. T. S. and Robinson, R. A. *J. Chem. Soc.* **1930** 2328.
17. Newman, L., La Fleur, W. J., Brousaides, F. J. and Ross, A. M. *J. Am. Chem. Soc.* **80** (1958) 4491.
18. Jander, G. and Jahr, K. F. *Z. anorg. Chem.* **211** (1933) 49; **212** (1933) 1.
19. Souchay, P. *Bull. soc. chim. France.* **18** (1951) 932.
20. Trujillo, R. and Beltrán, J. *Anales fis. y quim. (Madrid)* **47 B** (1951) 619.
21. Lefebvre, J. *J. Chim. Phys.* **1957** 657.
22. Russel, R. U. and Salmon, J. E. *J. Chem. Soc.* **1958** 4708.
23. Schwarzenbach, G. and Parissakis, G. *Helv. Chim. Acta* **41** (1958) 2425.
24. Souchay, P. *Bull. soc. chim. France* **14** (1947) 914.
25. Souchay, P. and Carpéni, G. *Bull. soc. chim. France* **13** (1946) 160.
26. Trujillo, R. and Tejera, E. *Anales fis. y quim. (Madrid)* **50B** (1954) 399.

27. Hazel, J. F., McNabb, W. M. and Santini Jr. R. *J. Phys. Chem.* **57** (1953) 681.
28. Robinson, R. A. and Sinclair, D. A. *J. Chem. Soc.* **1934** 642.
29. Biedermann, G. and Sillén, L. G. *Acta Chem. Scand.* **10** (1956) 1011.
30. Sillén, L. G. *Mimeographed lecture sheets: Studies on chemical equilibria* (MIT, 1957).
31. Sillén, L. G., *Acta Chem. Scand.* **8** (1954) 299 (AB I).
31a. Sillén, L. G., *Acta Chem. Scand.* **10** (1956) 803.
32. Byström, A., Wilhelmi, K. A. and Brotzen, O. *Acta Chem. Scand.* **4** (1950) 1119.
33. Barnes, W. H. and Qurashi, M. M. *Am. Mineralogist* **37** (1952) 407.
34. Evans, H. T. Jr. and Block, S. Communication in "*Symposium on structure and properties of heteropoly anions*" *Am. Chem. Soc.* Sept. 17. 1956.
35. Evans, H. T. Jr. and Block, S. *Am. Mineralogist* **39** (1954) 327.
36. Petrásová, M., Madar, J. and Hanic, F. *Chem. zvesti* **12** (1958) 410.
37. Christ, C. L., Clark, J. R. and Evans, H. T. Jr. *Acta Cryst.* **7** (1954) 801.
38. Hanic, F. *Chem. zvesti* **12** (1958) 579.
39. Sörum, H. *Kgl. Norske Videnskab. Selskab. Forh.* **16** (1943) 39.
40. Lukesh, J. S. *Acta Cryst.* **3** (1950) 476.
41. Siebert, H. *Z anorg. u. allgem. Chem.* **275** (1954) 225.
42. La Salle, M. J. and Cobble, J. W. *J. Phys. Chem.* **59** (1955) 519.
43. Qurashi, M. M. and Barnes, W. H. *Am. Mineralogist* **38** (1953) 489.
44. Bachmann, H. G. *Acta Cryst.* **6** (1953) 102.
45. Bachmann, H. G. *Neues Jahrb. Mineral.* **1953** 209–223.
46. Wallau E. O. and Shul, C. G. *Phys. Rev.* **73** (1948) 830.

Received July 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 1997–2008

The Viscosity of Degrading Polymer Solutions

THOR A. BAK and KATE BAK

Universitetets Fysisk-Kemiske Institut, Copenhagen, Denmark

The viscosity of a polymer solution is calculated as a function of time for several simplified degradation models, assuming the validity of Staudinger's rule. It is shown how the theoretical results may be compared with measurements obtained using an Ostwald viscometer, which only give a sliding time average of the viscosity, and finally the application of the present very crude theories to enzymatic reactions is discussed. The theoretical results obtained are compared with the experiments of Andersen and Graae on the enzymatic hydrolysis of hyaluronic acid.

In his classical work on the degradation of high polymers W. Kuhn [1] derived an approximate expression for the molecular weight distribution in a sample which before degradation had been monodisperse and of virtually infinite molecular weight. The derivation was based on statistical considerations, and the results are only valid for samples with an average molecular weight small compared to that of the initial sample.

His results have been rederived several times. Sakurada and Okamura [2] used a method similar to Kuhn as did Montroll and Simha [3]. The latter authors, however, generalized the results to be valid for polymers with finite initial molecular weight. Simha [4] and Sillén [5] derived similar results, using a kinetic model which is essentially identical with the first model to be discussed in this paper.

Hultin [6] has used the results of Sillén to derive an approximate expression for the excess viscosity of a solution of a degrading polymer as a function of time. In this derivation is used a limiting process in which it is implicitly assumed that a long time t has elapsed since the start of the experiment. The result obtained, namely that the excess viscosity is proportional to t^{-1}, is therefore meaningless in the limit $t \sim 0$.

It is the aim of this paper to derive formulae for the viscosity for the model discussed by Sillén and for a few other models which may be relevant in connection with the work on enzymatic degradation of high polymers. To do so we shall make use of a different limiting process and obtain results which are correct for all times, in particular for $t = 0$. One of the formulae we obtain may be found in Silléns work too, but there it is stated that it is only an

approximation. We shall show that the result can be derived without approximations save for the trivial one of assuming that the molecular weight of the initial sample is virtually infinite. Finally we want to discuss the applicability of the present very crude theory to systems where the degradation is caused by enzymes.

GENERAL ASSUMPTIONS

The usual model of a degrading polymer is a chain where each link has the same probability of breaking per unit time. Furthermore this probability is independent of the number of bonds in the molecule. It is believed that this is a fairly realistic picture, and therefore we shall first consider this model, but since there is experimental evidence that some polymers, *e. g.* hyaluronic acid [7] may have a preference for breaking in the middle when degraded enzymatically, we also investigate a crude model where the molecules split exclusively in the middle. In this model we can, because of its simplicity, introduce that the probability of splitting the central bond depends on the chain length.

Throughout the paper we shall assume the validity of Staudinger's rule [8], *viz.*

$$\eta_n = k\ c_n M_n^2 \tag{1}$$

in which η_n is the increase in viscosity caused by a solute of molar concentration c_n and molecular weight M_n, and k is a constant. The general validity of this rule may well be questioned, and it is well to bear in mind that the following considerations apply only to polymers which follow the Staudinger rule. In the case of hyaluronic acid this has been shown experimentally [9].

Furthermore we shall assume that for a solution of several species the excess viscosity is additive, *viz.*

$$\eta = \sum \eta_n = k \sum c_n M_n^2 \tag{2}$$

Our last general assumptions are that the degradation is such that the end-product is a repeated unit in the macromolecule which is the starting material, although not necessarily the smallest repeated unit, and that the initial high polymer is monodisperse. We shall indicate below, however, how this last assumption could be dispensed with.

Let the weight of the repeated unit be M. We then have $M_n = nM$, and

$$\eta(t) = k \sum_n c_n(t)(nM)^2 = k' \sum_n n^2 c_n(t) \tag{3}$$

so that, except for a constant factor, $\eta(t)$ is the second moment of the molecular weight distribution.

Following Andersen and Graae [10] we shall denote the reduced excess viscosity $\eta(t)/\eta\ (t = 0)$ by $y(t)$. We then have $y = 1$ for $t = 0$ and, loosely speaking, $1-y$ is a degree of advancement.

MODELS OF DEGRADATION

We shall now study a few simple models. In each case we start by stating the additional assumptions we make for the particular model.

Model 1

Assumption: All bonds between repeated units split with equal probability (w) per unit of time.

Notation: c_k is the concentration of molecules of molecular weight kM. NM is the molecular weight of the initial substance. $k-1$ is the number of bonds in the molecule with concentration c_k.

The equations for the time-dependence of the concentrations are thus:

$$\begin{aligned}
\dot{c}_N &= -w(N-1)c_N \\
&\cdots \\
\dot{c}_k &= 2w\sum_{i=k+1}^{N} c_i - (k-1)wc_k \qquad (4) \\
&\cdots \\
\dot{c}_1 &= 2w\sum_{i=2}^{N} c_i
\end{aligned}$$

By straight-forward integration we get for $c_k(0) = \delta_{kN}$ (that is, $c_k = 1$ for $k = N$, and otherwise zero)

$$\begin{aligned}
c_N &= e^{-(N-1)wt} \\
c_{N-1} &= 2e^{-(N-2)wt} - 2e^{-(N-1)wt} \\
c_{N-2} &= 3e^{-(N-3)wt} - 4e^{-(N-2)wt} + e^{-(N-1)wt} \\
&\cdots \qquad (5) \\
c_2 &= (N-1)e^{-wt} - 2(N-2)e^{-2wt} + (N-3)e^{-3wt} \\
c_1 &= N - 2(N-1)e^{-wt} + (N-2)e^{-2wt}
\end{aligned}$$

These expressions we insert in

$$\eta = k'\sum_n n^2 c_n$$

and get

$$\eta = k' \left[N + 2N \sum_{n=1}^{N-1} e^{-nwt} - 2 \sum_{n=1}^{N-1} n e^{-nwt} \right]$$

or

$$y = \frac{1}{N^2} \left[N + 2N \sum_{n=1}^{N-1} e^{-nwt} - 2 \sum_{n=1}^{N-1} n e^{-nwt} \right] \tag{6}$$

which is the final expression for $y(t)$.

It is easily verified that these expressions for the excess viscosity are identical with the one derived by Sillén: (our notation)

$$\eta = k' \left\{ \frac{1 + e^{-wt}}{1 - e^{-wt}} - \frac{2e^{-wt}(1 - e^{-Nwt})}{N(1-e^{-wt})^2} \right\}$$

The expression for $y(t)$ can be simplified considerably by letting $N \to \infty$, but this limiting process can be carried out in different ways.

Sillén argued that for $N \to \infty$, one will have

$$\eta \sim \frac{1 + e^{-wt}}{1 - e^{-wt}} = \coth \frac{wt}{2}$$

since the last term on the right hand side in the expression for η contains N^{-1}. This limiting process corresponds to increasing the size of the molecule, keeping the size of the repeated unit constant. This leads to an infinite viscosity at $t = 0$, whereas the measured viscosity of course is finite, but at the same time the unnormalized probability of breaking some bond goes to infinity as $(N-1)w$. Therefore the viscosity calculated using this approximation is infinite at $t = 0$, but its rate of change is infinite also, and so the expression $\eta \sim \coth wt/2$ may be a useful approximation at some later time.

The reason for the inconsistency in the result derived above is that one cannot let N go to infinity separately without changing M and w. What is required is to go from the discrete model to a continuous model, *i. e.*, a model where the molecule consists of infinitely many repeated units with infinitely small mass. We let $N \to \infty$ and at the same time $M \to 0$ so that NM stays constant. During this limiting process the number of bonds in a molecule goes to infinity too, but since Nw is an observable quantity practically equal to the rate constant in the first order expression for the disappearance of the initial polymer, we must let $w \to 0$ such that Nw stays constant ($= \alpha$).

We therefore have

$$y = \lim_{\substack{N \to \infty \\ w \to 0}} \frac{1}{N^2} \left[N + 2N \sum_{n=1}^{N-1} e^{-nwt} - 2 \sum_{n=1}^{N-1} n e^{-nwt} \right.$$

$$= \frac{2(\alpha t - 1 + e^{-\alpha t})}{\alpha^2 t^2} \tag{7}$$

Table 1. Θ as a function of y (eqn. 7).

y	0.5	0.4	0.3	0.2	0.1
0.5 +	0.0000	0.3247	0.7101	1.1796	1.7720
0.0 +	2.5569	3.6733	5.4486	8.8732	18.9443

It is easily verified that $y(0) = 1$ as it should be. $\dot{y}(0) = -\alpha/3$ which can be used in fitting the expression to experimental data. For $\alpha t \gg 1$ we have $y \sim t^{-1}$ which is the result obtained by Hultin using the other limiting process.

When one wants to compare the theoretical result with experiments it is convenient to introduce a dimensionless quantity $\Theta = \alpha t$. In terms of this we have

$$y = \frac{2(\Theta - 1 + e^{-\Theta})}{\Theta^2} \tag{8}$$

Table 1 is a tabulation of $\Theta(y)$ which can be compared with $t(y)$ which one gets from experiments and thereby give a determination of α. The data given below for hyaluronic acid do not fit this expression.

So far we have only considered the case where the initial polymer is monodisperse. Although one can in general approximate this fairly well experimentally, it would be interesting to study the case where one starts with an arbitrary initial distribution. We plan to return to this problem in a later publication, but we shall here briefly indicate how, in our mind, this problem best could be attacked. Eqn (4) is rewritten as

$$\dot{c}_k = 2Nw \sum_{i=k+1}^{N} c_i \frac{1}{N} - N^{-1}(k-1)\, Nwc_k$$

and we see that in the limit $N \to \infty$, $w \to 0$, $Nw \to \alpha$ we can introduce the continuous variable $\xi = k/N$ such that $c = c\,(\xi, t)$ satisfies the integral equation

$$\frac{\partial c}{\partial t} = 2\alpha \int_{\xi}^{1} c d\xi - \alpha \xi c \tag{9}$$

which can be rewritten as

$$\frac{\partial^2 c}{\partial \xi \partial t} + \alpha \xi \frac{\partial c}{\partial \xi} + 3\alpha c = 0 \tag{10}$$

after differentiation with respect to ξ. To see the influence of an initial polydispersity this equation is solved subject to the initial condition $c(\xi, 0) = f(\xi)$ where $f(\xi)$ is the molecular weight distribution at time zero.

The model discussed above is reasonable when we consider, for instance, a thermal degradation of the macromolecule, since Pelzer has shown [11] that

for a linear chain the probability of breaking a bond by a unimolecular mechanism is to a first approximation independent of the position of the bond in the molecule and of the molecular weight. When we consider a catalyzed decomposition it may no longer be a reasonable assumption. In particular when we consider an enzymatic decomposition it is in all probability a poor approximation to assume that all bonds break with equal probability, since the enzyme itself is a rather bulky molecule which has to be fitted onto a certain pattern in the degrading macromolecule.

As mentioned above [7] there is experimental evidence that some polymers break preferentially to give very large fragments. We shall now investigate a model where this feature has been further simplified so that we consider molecules which split exclusively in the middle.

Model 2

Assumption: The molecules split only in the middle.
Notation: c_k is the concentration of the molecules with molecular weight $NM2^{-k}$
w_k is the probability that the k'th molecule will break.

2A. (trivial case) $w^k = w$ (all k)
We have

$$\dot{c}_n = - wc_n + 2wc_{n-1} \tag{11}$$

It is convenient to use the generating function:

$$G(x,t) = \sum_n c_n(t)x^n \tag{12}$$

since evidently $y = G(\frac{1}{4},t)$. Multiplying the original differential equation with powers of x and summing we get

$$\frac{\partial G}{\partial t} = - w(1 - 2x)G \tag{13}$$

i. e., $G = e^{-w(1-2x)t}$

$$y = e^{-\frac{1}{2}wt} \tag{14}$$

This case which is in itself without any interest shows the method we shall use to get y directly from the kinetic equations in the more complicated cases.

We shall now let w_k depend on k. For an enzymatic decomposition it is reasonable to assume that this dependence is such that w_k decreases when k increases, since supposedly the enzyme is one suited for decomposition of the original macromolecule. By introducing this in the model we hope to be able to account, for instance, for the rapid initial decrease in viscosity which is

characteristic of the degradation of hyaluronic acid. The initial decrease during which the excess viscosity drops to about one third of its initial value is followed by a very slow decrease.

The simplest dependence is $w_k = w\ 2^{-k}$ (w being a constant) so that w_k is proportional to the molecular weight of the molecule to which it belongs. This model therefore resembles model 1 somewhat, only here all the probability of breaking a bond is located at one particular bond.

2B. Assumption: $w_k = w\ 2^{-k}$ (w constant)

This gives the following equation for c_k:

$$\dot{c}_k = -\ w\ 2^{-k}c_k + 2w\ 2^{-k+1}c_{k-1} \tag{15}$$

From this we get the equation for the generating function:

$$\frac{\partial}{\partial t}\ G(x,t) = -\ w(1 - 2x)G\left(\frac{x}{2},t\right) \tag{16}$$

To solve this we use the Laplace transformation, introducing

$$g(x,s) = \int_0^\infty e^{-st}G(x,t)dt$$

We have ther.

$$s\ g(x,s) - 1 = -\ w(1 - 2x)g\left(\frac{x}{2},s\right) \tag{17}$$

and making a power series expansion of g

$$g(x,\ s) = \sum_{n=0}^{\infty} S_n(s)x^n \tag{18}$$

we get by comparing terms

$$S_0 = \frac{1}{w + s}$$

. . .

. . .

$$S_n = \frac{w}{2^{n-2}\left(s + \frac{w}{2^n}\right)} S_{n-1} \tag{19}$$

. . .

. . .

Transforming back we obtain

$$y = \sum_{n=0}^{\infty} A_n e^{-wt/2^n} \tag{20}$$

$$\text{with } A_0 = \frac{1}{3} - \frac{1}{3 \cdot 7} + \frac{1}{3 \cdot 7 \cdot 15} - \frac{1}{3 \cdot 7 \cdot 15 \cdot 31} + \cdots \tag{21}$$

$$\text{and } A_n = 2^{(n^2-n)/2} \prod_{k=1}^{n} \frac{1}{2^k - 1} A_0 \tag{22}$$

Since $A_{n+1}/A_n \sim 1/2$, the series for y converges for all fixed t. It is convenient to consider y as a function of $\Theta = wt$. We have

$$\left[\frac{dy}{d\Theta}\right]_{\Theta=0} = -\sum 2^{-n} A_n = -\frac{1}{2} \tag{23}$$

which can be used in fitting the theory to experimental data. Because of the rapid convergence of the above series it is easy to calculate a sufficient number of points.

It is interesting to note that this complicated expression gives values for $y(\Theta)$ which almost coincide with those given by the expression derived for model 2A, *viz.* $y = \exp(-\frac{1}{2}\Theta)$. The slopes for $\Theta = 0$ are the same, and in the range $1 < y < 0.35$ the difference $y_B - y_A$ is only 1—2 %. For smaller y this difference increases rapidly, being about 20 % for $y \sim 0.2$. This shows clearly how accurately one must measure in order to be able to decide between two models of this kind. Furthermore it shows that the last mentioned theory does not give any abrupt change in y as we might have hoped.

If we want to keep the idea of molecules breaking in the middle only and obtain the above mentioned abrupt change in viscosity we must change the dependence of w_k on k rather drastically. The only simple relationship we have been able to find which gives a reasonable result is one where w_k has a constant value for all molecules above a certain size and another constant value for molecules with smaller molecular weight. If we consider an enzymatic process, this corresponds to an enzyme which has a selective catalytic effect on large, resp. small molecules.

Model 3

Assumptions:
1. Molecules break in the middle only
2. For molecules with a molecular weight $> M^*$ the rate-constant is w_1, for molecules with molecular weight $\leq M^*$ the rate-constant is $\overline{w_2}$.

Notation:

$w_2 = \frac{1}{2}\overline{w_2}$

c_k is the concentration of molecules with molecular weight $NM2^{-k}$

3A. $M^* = \frac{N}{2} M$

$$\begin{aligned} \dot{c}_0 &= -w_1 c_0 \\ \dot{c}_1 &= -\overline{w_2} c_1 + 2w_1 c_0 \\ \dot{c}_2 &= -\overline{w_2} c_2 + 2\overline{w_2} c_1 \\ &\quad etc. \end{aligned} \tag{24}$$

We solve the first equation to get $c_0 = e^{-w_1 t}$ and use this to get an equation for the generating function

$$\frac{\partial G}{\partial t} + \overline{w_2}(1 - 2x)G = -(w_1 - \overline{w_2})(1 - 2x)e^{-w_1 t} \tag{25}$$

from which we get

$$G = \frac{1}{w_1 - (1-2x)\overline{w_2}}\left[(w_1 - \overline{w_2})(1-2x)e^{-w_1 t} + 2w_1 x e^{-(1-2x)\overline{w_2} t}\right] \tag{26}$$

and

$$y = \frac{w_1 - 2w_2}{2(w_1 - w_2)} e^{-w_1 t} + \frac{w_1}{2(w_1 - w_2)} e^{-w_2 t} \tag{27}$$

3B. $M^* = \frac{N}{4} M$

Using a similar technique we get:

$$y = (a_0 + a_1 t)e^{-w_1 t} + b e^{-w_2 t} \tag{28}$$

with

$$\begin{aligned} a_0 &= \frac{w_1 - 2w_2}{2(w_1 - w_2)} + \frac{w_1(w_1 - 2w_2)}{4(w_1 - w_2)^2} \\ a_1 &= \frac{w_1(w_1 - 2w_2)}{4(w_1 - w_2)} \\ b &= 1 - a_0 \end{aligned} \tag{29}$$

It is easy to see that the general form is

$$y = \left[\sum_{n=0}^{k} a_n t^n\right] e^{-w_1 t} + b e^{-w_2 t} \tag{30}$$

for $M^* = NM2^{-k-1}$. The most important general feature of these equations is that b is smaller the higher n is. If in these models we put $w_2 << w_1$, *i. e.*, if the selectivity of the enzyme is pronounced, we get a sharp change in y. For model 3B this change occurs at $y \gtrsim \frac{1}{4}$, which is fairly close to what one can

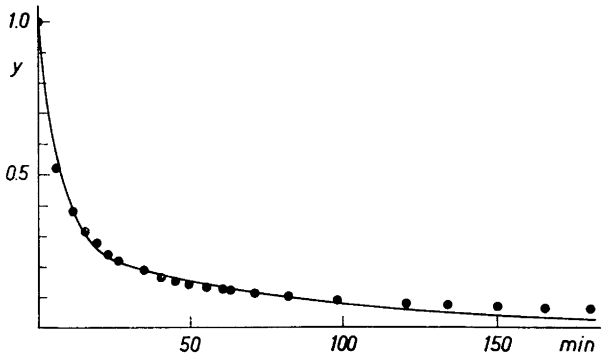


Fig. 1. Relative excess viscosity y as a function of time. The theoretical curve is calculated from eqn. 28, and the points are the experimental results of Andersen and Graae.

observe experimentally for hyaluronic acid. Fig. 1 shows the shape of the curve using $w_1 = 0.2019$ and $w_2 = 0.0130$ and the experimental points of Andersen and Graae [10]. The values for w_1 and w_2 have been calculated from the experimental data using an iterative least square procedure as described by Bond [15]. The agreement is not nearly as good as is the agreement with the empirical formula suggested by Andersen and Graae, but the expression used here has the advantage that it is derived from a specific model.

COMPARISON WITH EXPERIMENTAL DATA

Usually the viscosity of the polymer solution is measured with an Ostwald viscometer, while the degradation is going on, and in that case if the measurement takes 2τ seconds, the measured "viscosity" η is related to the true viscosity y by the equation

$$\eta^{-1}(t) = \frac{1}{2\tau} \int_{-\tau}^{+\tau} y^{-1}(t + t')\mathrm{d}t' \tag{31}$$

This equation is obtained by expressing the length of the capillary in the viscometer in terms of η and y and equating the two expressions. If τ is constant, it is easy to find y from the given function [12] $\eta(t)$. But unfortunately τ is not constant, it is proportional to η, so that the equation reads

$$\eta^{-1}(t) = \frac{1}{2k\eta} \int_{-k\eta}^{+k\eta} y^{-1}(t + t')\mathrm{d}t' \tag{32}$$

We have not been able to solve this equation, but by finding η using different expressions for y we have been able to estimate the error one makes in using η for y. In the experiments published by Andersen and Graae the error (for

$y \sim 0.52$, $t \sim 6$ min.) is approximately 2 % for the most reasonable shapes of $y = y(t)$. For $t \to 0$ the error becomes very large. Even within the measurable range the error can amount to about 10 %.

If experimental data have to be corrected by some iterative procedure using the above integral equation, they have to be extremely accurate in order that one may draw any conclusions whatsoever from the corrected data, and we believe that no such data have been published so far.

If one could measure the viscosity in such a way that each measurement took the same time, or if the measurements were almost instantaneous such as for instance is the case in the Couette viscometer, the correction would be much easier, and in the latter case actually superfluous.

As mentioned above, we have tried to fit the expression derived for model 3B to the uncorrected data given by Andersen and Graae; Fig. 1 shows the agreement obtained. Not too much importance should be attached to the experimental points for small t (where the correction is large) nor to the values for large t (where transglucosidation may be the dominating reaction).

It is easy to make a better fit by adding an extra term or by letting the pre-exponential factors be parameters which can be varied to get a better fit, but these additions would not be explicable in terms of a molecular mechanism.

APPLICATION OF THE THEORY TO CATALYZED PROCESSES

So far we have assumed that the degradation can be described in terms of monomolecular rate constants w_k. When we consider a thermal degradation this seems very reasonable, but it is not at all clear that the same considerations apply to a reaction catalyzed for instance by an enzyme.

In order to see to what extent it may still be a good approximation we shall have to consider the detailed mechanism of the enzymatic degradation. We consider the simplest possible mechanism:

$P_n + E_1 \rightleftharpoons E_2$ (rate constants k_{12} and k_{21})
$E_2 \to P_m + P_l + E_1$ (rate constant k_{23})

where P_n is a polymer with n basic units ($m + l = n$)
E_1 is the enzyme
E_2 is a molecule of composition E_1P_n
Using steady state kinetics [13] we have readily:

$$[E]\frac{dt}{d[P_n]} = \frac{1}{k_{12}}\left(1 + \frac{k_{21}}{k_{23}}\right)\frac{1}{[P_n]} + \frac{1}{k_{23}} \tag{33}$$

letting $[P_n]$ denote the concentration of P_n and [E] denote the concentration of $E_1 + E_2$.

If k_{23} is large, so that the last term on the right hand side is small, we have a first order degradation of P_n. Furthermore, since in this case the concentration of E_2 is low, only very small amounts of the enzyme is bound in the substrate-complex, and the different first order degradations of P-molecules are therefore effectively independent. In that case the previous considerations

therefore are valid, and a "local stationarity" exists in which all the separate first order reactions are in steady states, but not in steady states with respect to each other.

If k_{23} is small so that the term k_{23}^{-1} cannot be neglected, one will usually find that the first term is negligibly small [14], and that the concentration of E_2 is comparatively high. In that case, therefore, the degradation of the macromolecule must be considered as a large number of zero order reactions competing to form complexes with the enzyme, and thereby degrading P-molecules. In this case, as in the rare case where both terms will have to be considered, the previous considerations are not valid.

We are indebted to professor J. A. Christiansen for encouragement and advice and to Mr. A. E. Nielsen for pointing out an error in the first draft of the manuscript. K. B. gratefully acknowledges financial support from *Det teknisk-videnskabelige Forskningsraad.*

REFERENCES

1. Kuhn, W. *Ber.* **63** (1930) 1503.
2. Sakurada, I. and Okamura, S. *Z. physik. Chem.* **A 187** (1940) 289.
3. Montroll, E. W. and Simha, R. *J. Chem. Phys.* **8** (1940) 721.
4. Simha, R. *J. Appl. Phys.* **12** (1941) 569.
5. Sillén, L. G. *Svensk Kem. Tidskr.* **55** (1943) 221.
6. Hultin, E. *Svensk Kem. Tidskr.* **58** (1946) 281.
7. Jensen, C. E. *Studier over Hyaluronsyren,* Copenhagen 1957.
8. See for instance Kruyt, H. R. (ed.) *Colloid Science* II, Amsterdam 1949, p. 109.
9. Jensen, C. E. *Acta Chem. Scand.* **8** (1954) 937.
10. Andersen, S. O. and Graae, J. *Acta Chem. Scand.* **9** (1955) 1431.
11. Pelzer, H. *Z. Elektrochem.* **39** (1933) 608.
12. van der Pol, B. and Bremmer, H. *Operational Calculus,* Cambridge 1950, p. 293 ff.
13. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.
14. For a discussion of this mechanism see: Christiansen, J. A. *Acta Chem. Scand.* **11** (1957) 660.
15. Bond, W. N. *Probability and Random Errors,* London 1935, p. 112.

Received August 3, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2009–2022

The Crystal and Molecular Structure of 2,2′,2″-Triamino-triethyl-amine-Ni(II)-di-thiocyanate

SVEND ERIK RASMUSSEN

Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark *

The crystal structure of 2,2',2''-triamino-triethylamine-Ni(II)-dithiocyanate (Ni tren $(SCN)_2$) was determined from its three-dimensional Patterson function. Superposition methods were applied using both the nickel atom and the two sulphur atoms as searcher atoms. The structure was refined by a least squares analysis, employing three-dimensional data.

The crystal is built up from Ni tren$(SCN)_2$ units. The four nitrogen atoms from the amine and the two nitrogen atoms from the thiocyanate groups form a distorted octahedron around the nickel atom, the thiocyanate groups being in *cis* positions. A full account is given in the text of axial lengths, atomic coordinates, temperature factors, bond lengths and bond angles, and reliability index.

The polyamine 2,2',2''-triamino-triethylamine, usually abbreviated as "tren", was introduced into complex chemistry in 1925 by Mann and Pope [1], who prepared Pt(II)tren I_2 and Pt(IV)tren Cl_2 Cl. They pointed out that the four nitrogen atoms of the amine could hardly form a planar complex with the divalent platinum atom, and they concluded that the Pt(IV) complex probably was of octahedral configuration with the two chlorine atoms occupying *cis* positions.

In later publications [2,3] Mann and Pope described a number of nickel-tren complexes, especially Ni tren$(SCN)_2$. They were of the opinion that the nickel atom was tetracoordinated in this compound. In Ref.[3] a crystal-morphological investigation, carried out by Notham is mentioned. He found that Ni tren$(SCN)_2$ is orthorhombic, with the axial ratios: $a:b:c = 0.736:1:1.167$. In 1935 Cox and Webster [4] confirmed by an X-ray crystallographic investigation that Ni tren$(SCN)_2$ belongs to the orthorhombic system. The size of the unit cell was given as: $a = 10.79$, $b = 14.69$, $c = 8.59$ presumably KX units. The axial ratios are: $a:b:c = 0.736 : 1 : \frac{1.172}{2}$. The space group was found to be $P2_12_12_1$.

* Present address: Chemical Institute, University of Aarhus, Aarhus, Denmark.

The density was measured as 1.55 g/cm^3. Hence there are four Ni tren$(SCN)_2$ units in the unit cell. According to the space group these units must be asymmetric.

It was not possible at that time to solve the structure. The authors pointed out that two alternatives were possible: Either a tetrahedral structure or a *cis*-octahedral structure.

In the same year Jaeger and Beintema [5] determined the unit cell and space group of tren$(HCl)_3$. This compound crystallizes in the cubic system with the space group $P2_13$. Consequently it is highly probable that the tren molecule in this crystal has the symmetry C_3 which means that the four nitrogen atoms are at the corners of a tetrahedron.

Some authors, *e.g.* Reihlen [6], have taken the compound Ni tren$(SCN)_2$ as a proof of the existence of tetrahedral nickel complexes. Asmussen [7] concluded from magnetic measurements that Ni tren-complexes were probably of tetrahedral configuration.

At the discussion of the conference on coordination chemistry in Copenhagen in 1953 [8] Ni tren$(SCN)_2$ was mentioned as an example of a tetrahedral nickel-complex. When the investigation reported here was started, no X-ray evidence had ever been reported of any tetrahedral nickel-complex. As the Ni tren$(SCN)_2$ in my opinion would be the most likely of any known nickel-compound to have a tetrahedral structure, I decided to solve its crystal structure in order to get an unambigous answer to an often debated problem.

A preliminary communication was presented at the conference of coordination chemistry, Rome 1957. It appeared in print in 1958 [9].

EXPERIMENTAL

Professor Schwarzenbach, Zürich, and Professor Basolo, Northwestern University, Illinois, kindly supplied me with samples of tren$(HCl)_3$. The nickel complex was prepared in the following way:

0.01 mole tren$(HCl)_3$ was dissolved in water with 0.03 moles of NaOH. The solution was heated until boiling. A hot solution containing 0.01 mole of $Ni(SCN)_2$ was added dropwise. (No systematic experiments were carried out in order to determine the best possible conditions with respect to the concentrations of the solutions, *etc.*) From the hot solution Ni tren$(SCN)_2$ crystallizes at cooling. It is only slightly soluble in cold water. It was purified by recrystallization from water. In the microanalysis laboratory of the Chemistry Department of the University of Copenhagen the following results were found: S 19.86, C 29.30, N 26.85, H 5.40. Calc. for $NiS_2C_8N_6H_{18}$: S 19.97, C 29.92, N 26.18, H 5.65.

Analysis for nickel was carried out by Ole Bostrup: Calc. 18.28, found 18.11.

The crystals are blue and different habits may be observed; both needles and spheroids. A needle-shaped crystal, 0.5 mm long and approximately 0.1 mm × 0.1 mm square, was mounted on a Weissenberg-goniometer and orientated along the needle axis which proved to be the *c*-axis of Cox and Webster [4]. Equi-inclination diagrams were taken using Cu-radiation. $hk0-hk3$ reflexions were registered. Another crystal was orientated along the *b*-axis. It was 0.2 mm long, rather irregularly shaped and it was less than 0.04 mm thick. The reflexions $h0l-h6l$ were registered with Cu-radiation. Multiple film technique was employed. Intensities of X-ray reflexions were estimated visually using an intensity scale constructed by exposing different parts of a film to an X-ray reflexion from the crystal for regularly increasing intervals of time. 711 independent spectra were measured; 1 399 are accessible with Cu-radiation with the cameras employed. The intensities were converted into relative F^2 values, using Cochran's chart [10]. An approximately absolute scale was established using Wilson's [11] statistical

method, which also yields an average temperature factor, found to be equal to 2.3 Å². No correction for absorption was applied. Guinier powder photographs were taken to obtain accurate lattice constants. The space group given by Cox and Webster was confirmed. All numerical crystallographic data are collected below.

THE SOLUTION OF THE STRUCTURE

In the space group $P2_12_12_1(D_2^4)$, No. 19, a general point is fourfold and its symmetry is 1. As there are four units of Ni tren$(SCN)_2$ in the unit cell, each atom must be in a general position. If the origin is taken as halfway between the three pairs of non intersecting screw axes the coordinates of equivalent positions are: x,y,z; $1/2-x, \bar{y}$, $1/2+z$; $1/2+x$, $1/2-y, \bar{z}$; $\bar{x}$, $1/2+y$, $1/2-z$.

The x,y coordinates of the nickel atom were easily found from a Patterson projection along the c-axis, but the positions of the sulphur atoms could not be determined with the same certainty.

The nickel atom is not heavy enough to dominate the phases of a sufficient number of reflexions to permit determination of the structure. Therefore the Patterson-Harker cuts were calculated (Fig. 1, a, b, c) in order to obtain more information about the lighter atoms. The x,y coordinates of the nickel atom found by the Patterson projection were confirmed by the Harker cuts and the z-coordinate was also determined. The determination of the coordinates of the sulphur atoms was, however, not quite straightforward. There are three high peaks in the Patterson function at 0, 1/2, 1/2; 1/2, 1/2, 1/2; and at 1/2, 0, 0. The two first ones are about four times the height expected for an S-S peak, while the peak at 1/2, 0, 0 has exactly the height of an S-S peak. The three peaks might be caused by one or more atoms in one or more of the following non-equivalent positions: 1/4, 1/4, 0; 1/4, 1/4, 1/2; 1/2, 1/4, 0; 1/2, 1/4, 1/2. The Patterson projection is of no use in this special case in distinguishing between the x-coordinates 0.250 and 0.500 by aid of the vectors between these two points and the nickel atom, because the x-coordinate of nickel is 0.375.

It is well known from the work of Buerger [12] that crystal coordinates cannot be determined unambiguosly from Harker cuts. The ambiguities mentioned above are, however, of a rather special type, as they are connected with the fact that the peak at 0, 1/2, 1/2 is common for two Harker cuts and the peak at 1/2, 1/2, 1/2 is common for three Harker cuts.

The general ambiguities which arise in the determination of crystal coordinates from a Harker cut belonging to a twofold screw axis, may be stated in the following way:

Coordinate transformations, such as $x' = x \pm 1/2$; $y' = y$; $y' = y \pm 1/2$; $x' = x$ and $x' = x \pm 1/2$; $y' = y \pm 1/2$ cause no changes in the Patterson function. It causes, however, a fundamental difference, whether different atoms are referred to the same origin or to different origins. For each atom that is recognized in the Harker cut there are four possible choices of origin for its crystal coordinates. Once an origin has been chosen for one atom, all other atoms must be referred to the same origin to give a correct structure.

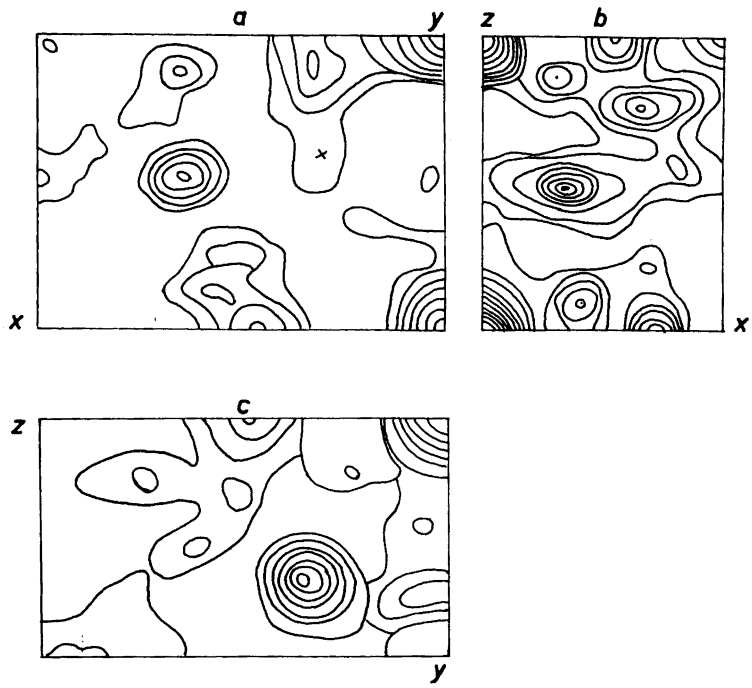


Fig. 1. The three Patterson Harker cuts.

In order to make the correct choice for all atoms it is necessary to have other informations than those obtainable from the Harker cut.

In the present investigation the Harker cuts were severely masked by non-Harker peaks and consequently they gave no information of the positions of the lighter atoms.

The benefit of sharpening the Patterson projection in order to obtain greater resolution was considered to be somewhat doubtful. It was considered to be safer to operate with artificial point atoms on "the other side" of the Fourier transform, and the Harker-Kasper [13] inequalities were applied to the $hk0$ reflexions.

The coordinate transformations stated above influence the signs of certain classes of $hk0$ structure factors. Once an origin is chosen, the signs of these structure factors are determined. On the other hand, a choice of sign for a structure factor determines the origin.

In the projection of $P2_12_12_1$ on a pinacoid it is convenient to take the origin in a screw axis. There are four non-translational equivalent screw axes in a unit cell, and the transformations mentioned above move the origin from one screw axis to another. The structure factors of the types: $h = 2n + 1$; $k = 2n+1$ and $h+k = 2n + 1$, change signs at certain of these transformations, and that means that the sign of one structure factor of each of these two classes of reflexions may be chosen arbitrarily.

The first Harker-Kasper inequality,

$$2|U(hk0)|^2 \leq 1 + U(2h2k0),$$

gave one sign. (840). The inequality,

$$4|U(hk0)|^2 \leq [1 + U(2h00)]\ [1 + U(02k0)],$$

gave a few other signs. These relations only give signs for $h = 2n$, $k = 2n$. With these signs and the two signs which could be chosen abitrarily, a large number of signs was obtained by application of the relations,

$$[U(hk0) \pm U(h'k'0)]^2 \leq [1 \pm U(h+h',\ k+k',\ 0)]\ [1 \pm U(h-h',\ k-k',\ 0)].$$

The nomenclature applied is that of Gillis [14].

In all 66 signs were obtained by these methods. 14 signs were later proved to be wrong.

The Fourier projection calculated with the signs obtained from the inequalities is given in Fig. 2. In the projections shown the origin is taken in a c screw axis. No refinement was obtained, because the projection was not interpreted correctly. It is not very much different from the correct final projection (Fig. 3), and the value of the Harker-Kasper method should be judged from a comparison between these two projections, and not from the fact that the structure was finally solved in another way.

A full three-dimensional Patterson was calculated. The a and b axes were divided in 60ths and the c axis in 30ths. The term $F(000)^2$ obtained by Wilsons method was included in the calculations. If the height of a peak may be taken as proportional to its volume, the height of a single Patterson peak with the scale applied here is given as $H_{ij} = 0.35 \cdot z_i z_j$, where z_i and z_j are the atomic numbers of atoms i and j. On this scale we should expect peaks in the following order: 3 Ni-Ni (218), 8 Ni-S (140), 10 S-S (90), 24 Ni-N (62), 32 Ni-C (52), 48 S-N (39), 64 S-C (34), the rest of the peaks are so low that they disappear in the general background. The Patterson function of $P2_12_12_1$ has the symmetry P_{mmm}. Consequently it is only necessary to compute it within the space defined by $\frac{a}{2}, \frac{b}{2}, \frac{c}{2}$. All peaks must appear within this volume. If the volume of a peak is approximately 1 Å^3, only about 140 spherical peaks could be "packed" into the octant of the Patterson space without overlapping. It is very unlikely that a close "packing" of peaks should ever occur, but even if it were the case, the number of peaks here is so great that overlapping cannot be avoided.

The nickel-nickel vectors had already been recognized in the Harker cuts and the following coordinates were chosen for the nickel atom given in 60ths of the coordinate axes with the origin halfway between the three screw axes: $22\frac{1}{2}$, 25, 5; $\overline{7\frac{1}{2}}$, 5, $\overline{5}$; $7\frac{1}{2}$, $\overline{25}$, $\overline{25}$; $37\frac{1}{2}$, $\overline{5}$, 25.

The structure was solved by what has been called "image seeking" or "super-position" methods. Buerger [15] has given a very profound analysis of

the existing methods of extracting a crystal structure from its vector diagram. The principle of these methods is an extensive use of the symmetry of the Patterson space. If the coordinates of one atom, a, may be fixed, *e.g.* x_a, y_a, z_a, the following vectors to another atom, *e.g.* b, must exist. These are: x_b-x_a, y_b-y_a, z_b-z_a; $1/2 - (x_b + x_a)$, $(y_b + y_a)$, $1/2 + (z_b-z_a)$; $1/2 + (x_b-x_a)$, $1/2 - (y_b + y_a)$ $(z_b + z_a)$; $x_b + x_a$, $1/2 + (y_b-y_a)$, $1/2 - (z_a+z_b)$, with the symmetry of the space group considered here. By shifting the origin of the Patterson function to the four equivalent points of the searcher atom, a, the vectors to the atom, b, are all gathered in the point x_b, y_b, z_b. Different methods may be used to recognize the interatomic vectors. Robertson and Beevers [16] and McLachlan [17] added the numbers of the transcribed Patterson functions, McLachlan [18] and Buerger [15] suggested a multiplication of the vectors, but the closest approach to the electron density is the minimum function of Buerger [15]. Buerger realized that the smallest of symmetry related Patterson peaks would have the greatest chance of being a genuine single peak, and therefore its value should be close to being proportional to the electron density of the crystal. A few examples of the application of the minimum function will be given. Graphical methods were first applied to give a rough idea of the whereabouts of the atoms, but more accurate results were obtained by studying the numerical values of the Patterson function. First the sulphur atoms were sought, using nickel as the searcher atom. One of the positions which could be deduced from the Harker cuts, was confirmed. In 60ths of the coordinate axes its position was found to be 15, $\bar{1}6$, 0 on the basis of the following vectors with their weights given in parentheses,
$7\frac{1}{2}$, 19, 5(191); $22\frac{1}{2}$, 21, 5(146); $7\frac{1}{2}$, 9, 25(170); $22\frac{1}{2}$, 11, 25(196).

The effective atomic number of nickel towards Cu-radiation is 25. The height of a nickel vector is, therefore, according to the relation given above; $H_{ij} = 0.35 \cdot 25 \cdot z_j$.

The minimum of the four peaks given above is 146. The atomic number, z_j, of the atom which yields this vector with the nickel atom is therefore $z \leq \frac{146}{0.35 \cdot 25} = 16.7$. Thus it seems highly probable that the atom found is a sulphur atom. The other sulphur atom was placed in $26\frac{1}{2}$, $8\frac{1}{2}$, $\bar{1}\bar{1}$ on the basis of the following vectors with the nickel atom: 19, $26\frac{1}{2}$, 14(169); 4, $16\frac{1}{2}$, 16(146); 11, $13\frac{1}{2}$, 24(152); 26, $3\frac{1}{2}$, 6(161). Minimum 146, atomic number 16.7. This close correspondence between the atomic numbers of the two sulphur atoms must be purely accidental and is by no means characteristic of the accuracy of the method. The correctness of the coordinates may be checked by the vectors between the two non-symmetry related sulphur atoms, $11\frac{1}{2}$, $24\frac{1}{2}$, 11(103); $18\frac{1}{2}$, $5\frac{1}{2}$, 19(110); $11\frac{1}{2}$, $7\frac{1}{2}$, 19(132); $18\frac{1}{2}$, 22, 11(122). As the minimum here is 103, the atomic number is $z \leq \frac{103}{0.35 \cdot 16} = 18.3$.

It was not possible to determine the positions of the C and N atoms from the nickel vectors alone, as there were more vectors of appropriate height than there were atoms. Because of this, the vectors between the S and the C and N atoms were also employed, and in that way a number of positions suggested by the nickel atom could be ruled out. In Buerger's [15] formulation

it would be: "The image seeking polyhedron comprised of the four nickel atoms and the eight sulphur atoms roved over the Patterson function finding images of itself, whereby the appropriately weighted minimum funtion was mapped out". When applying the minimum function due regard must be taken to the uncertainty of the values of the Patterson function. With the errors inherent in the measurement of intensities, non-convergence of series *etc.* an indeterminacy of about 30 % must be allowed for. This means that atomic numbers as low as 4—4 1/2 must be accepted for C and N atoms, although if more than two or three of the twelve vectors indicated such a low atomic number, the position was not accepted as an atomic site. A few examples will be given of the best and of the worst of the results:

C_1 : 31, 25, $\bar{1}\bar{1}$,

Ni-C vectors: $8\frac{1}{2}$, 0, 16 (106); $21\frac{1}{2}$, 20, 6 (146); $23\frac{1}{2}$, 10, 14 (76); $6\frac{1}{2}$, 30, 24 (61).

$$z \leq \frac{61}{0.35 \cdot 25} = 7.$$

S-C vectors: 16, 9, 41 (73); 14, 11, 41 (55); 14, 39, 11 (53); 16, 41, 11, (80); $5\frac{1}{2}$, $16\frac{1}{2}$, 0 (64); $25\frac{1}{2}$, $3\frac{1}{2}$, 22 (75); $2\frac{1}{2}$, $13\frac{1}{2}$, 8 (45); $27\frac{1}{2}$, $33\frac{1}{2}$ $33\frac{1}{2}$, 30 (80),

$$z \leq \frac{45}{0.35 \cdot 16} = 8.8$$

C_6: 36, 20, 7.

Ni-C vectors: $13\frac{1}{2}$, 5, 2 (75); $16\frac{1}{2}$, 15, 12 (40); $1\frac{1}{2}$, 25, 18 (98); $28\frac{1}{2}$, 15, 28 (146),

$$z \leq \frac{40}{0.35 \cdot 25} = 4.6$$

S-C vectors: 21, 4, 23 (25); 9, 6, 23 (82); 9, 26, 7 (56); 21, 24, 7 (82); $9\frac{1}{2}$, $11\frac{1}{2}$, 18 (80); $20\frac{1}{2}$, $1\frac{1}{2}$, 4 (25); $2\frac{1}{2}$, $18\frac{1}{2}$, 26 (50); $27\frac{1}{2}$, $28\frac{1}{2}$, 12 (95),

$$z \leq \frac{25}{0.35 \cdot 16} = 4.5$$

N_6: $11\frac{1}{2}$, 25, 5.

Ni-N vectors: 11, 0, 0 (256); 19, 20, 10 (188); 26, 30, 20 (115); 4, 10, 30 (148);

$$z \leq \frac{115}{0.35 \cdot 25} = 13.$$

S-N vectors: $3\frac{1}{2}$, 9, 25 (65); $26\frac{1}{2}$, 11, 25 (58); $26\frac{1}{2}$, 21, 5(98); $3\frac{1}{2}$, 19, 5 (82); 15, $16\frac{1}{2}$, 16 (26); 15, $3\frac{1}{2}$, 6 (66); 22, $13\frac{1}{2}$, 24 (120); 15, $26\frac{1}{2}$, 14 (43),

$$z \leq \frac{26}{0.35 \cdot 16} = 5.$$

By a systematic application of this modification of the minimum function, a satisfactory solution of the structure was obtained.

A refinement of the structure was started by difference projections along the *c*-axis. By two successive difference projections the *R*-index decreased from 29 % to 21.7 % without application of a temperature factor. At that

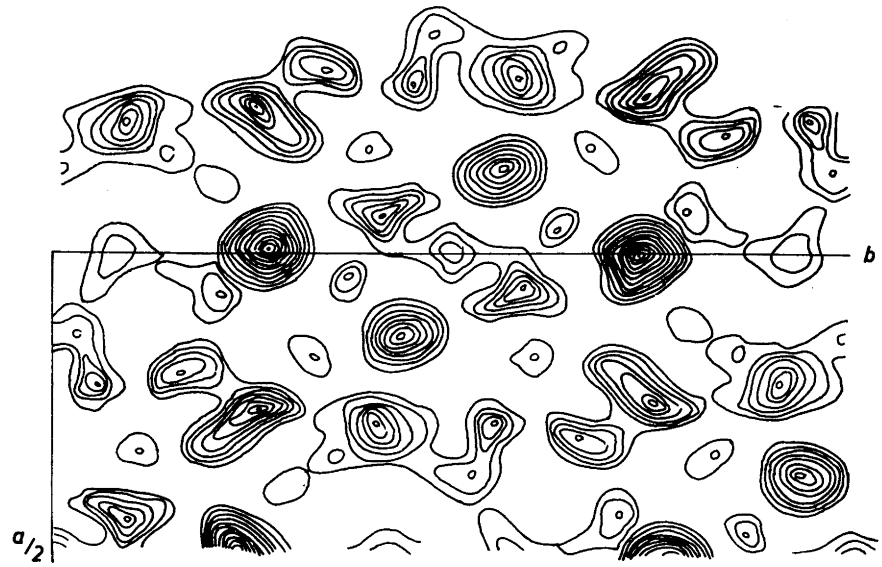


Fig. 2. Fourier projection along the c-axis calculated with signs obtained from Harker-Kasper inequalities.

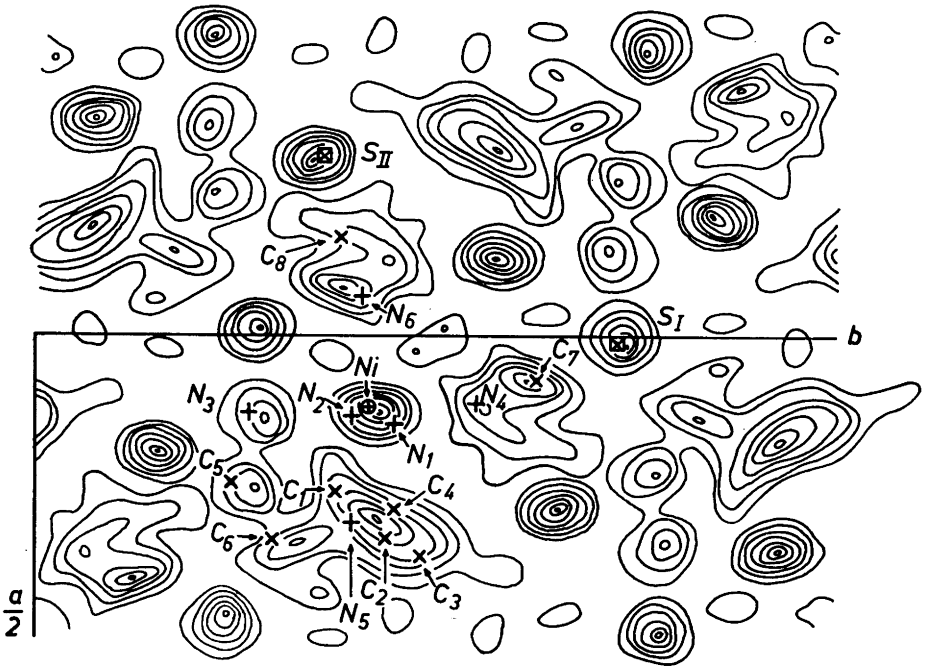


Fig. 3. Final Fourier projection. The atomic positions obtained from the least squares analysis are marked for one molecule.

stage a least squares analysis was carried out using the method of Sayre [19]. Miss Dolores Leagus carried out the work of programming and machine computation.

Two runs were made. One using only 711 observed F values, the other including 181 non observed F values, which were within the $\sin\Theta$ range of the observed F values. The weights of the F values were taken as proportional to their multiplicity (Geller and Abrahams [20], 1958).

The scattering factors used were the following: for Ni the values of Thomas and Umeda [21] corrected for dispersion for Cu-radiation using $\Delta f = -3.1$. For S the values of Tomiie and Stam [22] and for C and N those of Freeman [23].

The final coordinates and temperature factors, as well as the coordinate changes used for the final calculations are stated below. The results are those obtained using observed F values only. The reliability index $R = \frac{\Sigma||F_o| - |F_c||}{\Sigma|F_o|}$ is 13.6 % for these computations. The calculations which included non-observed intensities, gave only slightly different results. The former results were chosen because a re-examination of the Weissenberg-diagrams revealed that 26 observable, although weak intensities were overlooked in the first measurements.

Three strong intensities were evidently affected by extinction, as the calculated F values were 30—40 % higher than the observed values. There are no grave discrepancies between observed and calculated F values. The calculated values of the non-observed intensities are all small, except the ones mentioned above, which could be found on the Weissenberg diagrams. Because of the individual agreements between observed and calculated F values the structure must be essentially correct.

DISCUSSION OF THE STRUCTURE

For convenience all pertinent data of the crystal structure are collected here.

Crystal system: Orthorhombic.

Unit cell from Guinier powder diagrams with Cu$K\alpha$-radiation:

$$a = 10.82_1 \text{ Å}, \qquad b = 14.71_9 \text{ Å}, \qquad c = 8.62_0 \text{ Å}.$$

Space group: $P2_12_12_1$ No. 19, (D_2^4).

Chemical formula: $NiS_2C_8N_6H_{18}$. Four units in the unit cell.

Origin halfway between screw axes. The coordinates and temperature factors obtained are stated below.

Final coordinates and temperature factors, and parameter changes applied for the last least squares calculations.

	x	y	z	B	Δx	Δy	Δz	ΔB
C_8	0.081087	0.385216	0.129809	2.2577	−0.000147	−0.000232	0.000131	−0.1455
C_7	0.326618	0.630510	0.037545	1.3770	0.000197	−0.000196	0.000300	−0.0259
C_6	0.603609	0.305235	0.113750	1.0891	0.000308	0.000082	−0.000466	0.0492
C_5	0.506174	0.246387	0.196275	3.4090	0.000299	0.000324	0.000104	−0.1347
C_4	0.547679	0.453238	0.350924	2.5217	0.000726	−0.000234	0.000249	−0.4579
C_3	0.610642	0.479480	0.196238	2.4667	0.000434	−0.000349	−0.000410	−0.0513
C_2	0.600520	0.439341	0.918240	2.5288	0.000126	0.000631	0.000899	0.0042
C_1	0.518879	0.376199	0.796445	2.2823	0.000398	0.000193	−0.000042	−0.1470
N_6	0.186183	0.410673	0.104150	3.4070	0.000347	0.000842	0.000219	0.0025
N_5	0.566400	0.397124	0.074243	2.5477	0.000130	0.000486	−0.000148	−0.2428
N_4	0.367356	0.557289	0.037369	3.6104	0.000153	0.000034	0.000189	0.1573
N_3	0.381603	0.267628	0.132519	3.0013	−0.000198	−0.000113	0.000187	−0.0831
N_2	0.388276	0.394525	0.837901	3.2958	−0.000287	−0.000158	0.000233	−0.0667
N_1	0.407727	0.450679	0.329351	3.0380	−0.000362	0.000116	−0.000407	−0.1101
S_{II}	0.442430	0.139506	0.818595	2.6136	0.000021	0.000030	−0.000040	−0.0187
S_I	0.242260	0.272368	0.510750	3.5559	−0.000001	−0.000033	−0.000024	0.1195
Ni	0.372013	0.417211	0.085269	1.4411	−0.000009	0.000093	0.000082	−0.0197

Realiability index: $R = \dfrac{\Sigma||F_o| - |F_c||}{\Sigma|F_o|} = 13.6\ \%$ for 711 independent measured structure factors.

Bond lengths and bond angles:

N_5-C_2	= 1.527 Å (tren)	$<C_2-N_5-C_3$	= 99°55'
C_2-C_1	= 1.657 Å "	$<C_6-N_5-C_2$	= 120°14'
C_1-N_2	= 1.482 Å "	$<C_6-N_5-C_3$	= 115°28'
N_5-C_3	= 1.675 Å "	$<C_5-C_6-N_5$	= 115°19'
C_3-C_4	= 1.574 Å "	$<N_3-C_5-C_6$	= 108°21'
C_4-N_1	= 1.525 Å "	$<N_1-C_4-C_3$	= 108°48'
N_5-C_6	= 1.452 Å "	$<N_2-C_1-C_2$	= 103°42'
C_6-C_5	= 1.539 Å "	$<C_4-C_3-N_5$	= 102°35'
C_5-N_3	= 1.489 Å "	$<C_1-C_2-N_5$	= 99°59'
N_4-C_7	= 1.164 Å (thiocyanate)	$<N_4-C_7-S_I$	= 170°6'
C_7-S_I	= 1.629 Å "	$<N_6-C_8-S_{II}$	= 172°48'
N_6-C_8	= 1.218 Å "	$<N_1-Ni-N_2$	= 152°29'
C_8-S_{II}	= 1.607 Å "	$<N_5-Ni-N_6$	= 168°51'
$Ni-N_1$	= 2.195 Å	$<N_3-Ni-N_4$	= 178°1'
$Ni-N_2$	= 2.166 Å	$<Ni-N_6-C_8$	= 163°28'
$Ni-N_3$	= 2.242 Å	$<Ni-N_4-C_7$	= 156°21'
$Ni-N_4$	= 2.104 Å		
$Ni-N_5$	= 2.127 Å		
$Ni-N_6$	= 2.020 Å		

The accuracy of this structure determination may be more difficult to assert than its correctness. During the last refinement the R-index only decreased from 13.8 % to 13.6 %. The coordinate changes used for this calculation might be used as a criterion for the underterminacy of the bond lengths. The nickel atom was moved 0.0014 Å for the last refinement, the two sulphur atoms 0.0005 Å, and the light atoms somewhat more. The arithmetic

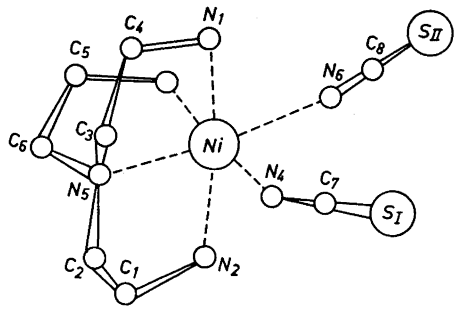


Fig. 4. Parallel projection of the Ni tren molecule.

mean of their displacements is 0.006 Å. Hence one might believe that bond lengths were determined with an accuracy of 0.01 Å.

There are, however, several reasons for viewing this low figure with some scepticism. In the first place, no correction has been applied for either extinction or absorption. Secondly, the effect of 18 hydrogen atoms has been neglected. They are almost bound to affect some of the intensities as they after all carry 10 % of the scattering electrons, although they can hardly ever, all of them, scatter in phase. Thirdly, isotropic temperature factors have been used, although it is certain that the heat oscillations are anisotropic.

A more empirical viewpoint will be taken on the accuracy of the results.

The two thiocyanate groups will be taken as completely equivalent with respect to bond lengths and bond angles. Consequently we shall take the mean values of the two sets of interatomic distances as standard values: N—C = 1.19 ± 0.03 Å, C—S = 1.62 ± 0.01 Å, < N—C—S = 171°.

This means that the N—C distance may be as much as 0.06 Å in error, and the C—S distance 0.02 Å in error. For comparison the following results will be quoted:

Compound	N—C	C—S	<N—C—S	Ni—N—C—S	Ref.
$Nien_2(NCS)_2$	1.20	1.66	175°	2.14	24
$Ni(NH_3)_4(NCS)_2$	1.20	1.61	180°	2.07	25
$Ni[CS(NH_2)_2]_2(NCS)_2$	1.16	1.60	180°	1.99	26
AgSCN	1.186 ± 0.086	1.636 ± 0.029	~180°		27
$C_2H_4(SCN)_2$	1.18	1.63	172°		28
NH_4SCN	1.24 ± 0.02	1.58 ± 0.02	180°		29
$NH_4[Cr(SCN)_4(NH_3)_2]$	1.14 ± 0.03	1.80 ± 0.03	180°		30

The results are all from X-ray investigations.

Beard and Dailey [31] find by microwave spectroscopy on HNCS the following results:

N—C = 1.218 Å, C—S = 1.557 Å, N—H = 1.01 Å, < H—N—C = 136°, assuming that the N—C—S group is linear.

The following conclusion may tentatively be drawn from these figures.

The bond lengths and angles of the thiocyanate group in complexes, in which it is considered as being predominantly ionically bond, falls within the limits: N—C = 1.19 ± 0.03 Å, C—S = 1.63 ± 0.03 Å, < N—C—S = 175±5°. By the complex formation the N—C bond is apparently shortened and the C—S bond is stretched with respect to the bonds of the SCN^- ion of the NH_4SCN lattice. In the Reinecke ion $[Cr(SCN)_4(NH_3)_2]^-$ the N—C bond is the shortest and the C—S bond the longest of any known distances of the thiocyanate group. This is probably connected with the fact that the Reinecke salt exhibits an absorption spectrum of the electron transfer type.

This survey indicates that the indeterminacy of the bond lengths of the thiocyanate group obtained in this paper is probably ± 0.03 Å and at most 0.06 Å.

It is difficult to say whether the bond lengths of the tren molecule are determined with the same accuracy or not.

It is established with certainty that the three N—C—C—N rings are non-planar. In the free tren molecule the three rings are probably equivalent as the molecule has almost certainly the symmetry C_3. With this symmetry it is impossible to fix the four nitrogen atoms at the four corners of an octahedron. The octahedral structure becomes possible when *one* of the rings changes its conformation into one, which is symmetrical with the original one. In Fig. 4 the rings $N_5—C_6—C_5—N_3$ and $N_5—C_2—C_1—N_2$ have identical conformations. One ring can be made to cover the other by rotating the molecule around an axis passing through the N_5 atom, and the nickel atom. But none of these two rings can be made to cover the $N_5—C_3—C_4—N_1$ ring, as this ring in enantiomorphous with the two other ones. Some of the distances of the tren molecule are anomalous. This may be connected with the fact that the molecule is severely strained in the complex. By forming an octahedral complex, only one ring can avoid strain. The distances of the ring $N_5—C_6—C_5—N_3$ are quite normal. In the ring of which the conformation is different from the two other ones, the nitrogen-carbon distance, $N_5—C_3$, is rather high: 1.68 Å. The distance $C_2—C_1$ is also higher than the usual single bond distance. It is at present difficult to say, whether these abnormal distances are really caused by steric strain, or are the results of inadequate measurements, assumptions and computations. By comparison with the accuracy of the determination of the dimensions of the thiocyanate groups, one might be inclined to believe that the apparent effects of the strain are real. By looking through "Interatomic Distances" [32] one finds that C—C distances above 1.60 Å often are reported for strained molecules.

The nickel-nitrogen distances appear to exhibit the following systematics:

Nickel-primary amine nitrogen:	2.20 ± 0.04 Å
Nickel-tertiary amine nitrogen:	2.13 ± 0.04 Å
Nickel-thiocyanate nitrogen:	2.06 ± 0.04 Å

The octahedron of the six nitrogen atoms has no more than triclinic symmetry. The deviations from the regular octahedron are made clear by giving the angles between *trans*-nitrogen atoms and the nickel atom:

$$< N_1NiN_2 = 152°, \quad < N_5NiN_6 = 168°, \quad < N_3NiN_4 = 178°.$$

It is still an open question whether X-ray diffraction methods at present are able to yield determinations of bond lengths of very high precision. Two examples will be quoted which may throw some doubt on the validity of "established" bond lengths. Lynton and Cox [33] and Rowe and Post [34] independently determined the structure of thianthrene and refined the structure by essentially equivalent mathematical methods. Visual estimations of intensities were used in both determinations. Many of the C—C distances of the two determinations agree within 0.01—0.02 Å, but three of the distances deviate with 0.04 Å. In both cases a standard deviation of 0.012 Å is quoted.

The detailed structure of the glycine zwitter ion is another case for discussion. Hahn and Buerger [35] determined the structure of diglycine hydrochloride using Cu-radiation, and measured intensities with a Geiger counter. Marsh [36] redetermined the structure of glycine itself, using Mo-radiation, and visual estimation of intensities. Hahn and Buerger find the following distances for the glycine molecule: N—C = 1.52 ± 0.01 Å, C—C = 1.48 ± 0.01 Å. Marsh reports that N—C = 1.474 Å, and C—C = 1.523 Å. It is as yet an open question whether these discrepancies are real and are caused by the different packing in the different crystals, or whether they are fictitious, and caused by the different ways of measuring intensities or in the different ways of refining the structure. Hahn and Buerger employed isotropic temperature factors, while Marsh used anisotropic B factors.

Because of the non-planarity of the N—C—C—N rings, the Ni tren$(SCN)_2$ molecule is asymmetric and should exist in two enantiomorphous forms. The space group $P2_12_12_1$ permits a separation of left-handed and right-handed crystals simply by handpicking. In that way it should be possible to make optically active solutions of the complex. The non-planar conformation of N—C—C—N rings seems to occur generally in complexes. An interesting consequence of this conformation is the possibility of stereoisomers of *trans*-bis-ethylene-diamine complexes.

At the same time as the structure of Ni tren$(SCN)_2$ was established, Venanzi [37] reported that Powell had proved by X-ray methods that $[(C_6H_5)_3P]_2NiCl_2$ has a tetrahedral structure. In a preliminary publication Gill, Nyholm and Pauling [38] have presented X-ray evidence for a tetrahedral $NiCl_4^{--}$ ion in the compound $[(C_6H_5)_4As]_2NiCl_4$.

It is at present very difficult to say, which factors are decisive for the stereochemistry of nickel complexes.

An investigation has been started on tren$(HCl)_3$ in order to obtain accurate details of the stereochemistry of the non-complexed tren molecule.

While the present paper was in preparation, a note on the structure of Ni tren$(SCN)_2$ by Hall and Woulfe [39] appeared. I have been in correspondence with the authors. The two independent solutions of the structure appear to be essentially in agreement.

When the structure of Hall and Woulfe has been refined, a comparison of the results will be published.

A complete table of observed and calculated F values and a more complete account of the present work will be published in a forthcoming monograph in Danish, with an English summary [40].

Acknowledgements. I am greatly indebted to Prof. Dr. A. Tovborg Jensen for the hospitality he has shown me by allowing me to work in his laboratory at the Royal Veterinary and Agricultural College, where part of the experimental work was performed.

I also wish to thank Prof. Dr. Bodil Jerslev of the Danish School of Pharmacy for the loan of a Weissenberg honiometer.

The *Carlsberg Foundation* is thanked for providing part of the equipment used in the investigations, and economic support from the *Ole Rømer Foundation* is gratefully acknowledged. The Fourier summations were performed by aid of a Hägg-Laurent computer built by Frank[41].

Last, but not least, I wish to express my sincere thanks to Miss Dolores Leagus of the *Bell Telephone Company* for planning and carrying out the lengthy machine computations.

REFERENCES

1. Mann, F. G. and Pope, W. J. *Proc. Roy. Soc. London A.* **109** (1925) 444.
2. Pope, W. J. and Mann, F. G. *Chem. & Ind. London* **44** (1925) 834.
3. Mann, F. G. and Pope, W. J. *J. Chem. Soc.* **1926** 482.
4. Cox, E. G. and Webster, K. C. *Z. Krist. A.* **92** (1935) 478.
5. Jaeger, F. M. and Beintema, I. *Proc. Acad Sci. Amsterdam* **38** (1935) 243.
6. Reihlen, H. *Ann.* **448** (1927) 314.
7. Asmussen, R. W. *Magnetokemiske Undersøgelser over uorganiske Komplexforbindelser*, Copenhagen 1944, p. 222.
8. Proceedings of the Symposium on Co-Ordination Chemistry, Copenhagen 1953, p. 72.
9. Rasmussen, S. E. *J. Inorg. & Nuclear Chem.* **8** (1958) 441.
10. Cochran, W. *J. Sci. Instr.* **25** (1949) 253.
11. Wilson, A. J. C. *Nature* **150** (1942) 152.
12. Buerger, M. J. *J. Appl. Phys.* **17** (1946) 579.
13. Harker, D. and Kasper, J. S. *Acta Cryst.* **1** (1948) 70.
14. Gillis, J. *Acta Cryst.* **1** (1948) 174.
15. Buerger, M. J. *Acta Cryct.* **4** (1951) 531.
16. Beevers, C. A. and Robertson, J. H. *Acta Cryst.* **3** (1950) 164.
17. McLachlan, D. *Univ. Utah Eng. Expt. Sta. Bull.* **1950** 50.
18. McLachlan, D. *Proc. Natl. Acad. Sci. U.S.* **37** (1951) 115.
19. Friedlander, P. H., Love, W. and Sayre, D. *Acta Cryst.* **8** (1955) 732.
20. Abrahams, S. C. and Geller, S. *Acta Cryst.* **11** (1958) 437.
21. Thomas, L. H. and Umeda, K. *J. Chem. Phys.* **26** (1957) 253.
22. Tomiie, Y. and Stam, C. H. *Acta Cryst.* **11** (1958) 126.
23. Freeman, A. J. *Acta Cryst.* **12** (1959) 261.
24. Lingafelter, E. C., Brown, *Private communications.*
25. Yukhno, E. K. and Porai-Koshits, M. A. *Kristallografiya* **2** (1957) 239.
26. Nardelli, M., Braibanti, A. and Fava, F. *Gazz. chim. ital.* **87** (1957) 1209.
27. Lindqvist, I. *Acta Cryst.* **10** (1957) 29.
28. Bringeland, R. and Foss, O. *Acta Chem. Scand.* **12** (1958) 79.
29. Zdanov, G. S. and Zvonkova, Z. V. *Comm. au XIII Congres Intern. de Chim. pure et appl.* **1953** 175.
30. Saito, Y., Takeuchi, Y. and Pepinsky, R. *Z. Krist.* **106** (1955) 476.
31. Beard, C. I. and Dailey, B. P. *J. Chem. Phys.* **18** (1950) 1438.
32. *Interatomic Distances*, London 1958.
33. Lynton, H. and Cox, E. G. *J. Chem. Soc.* **1956** 4886.
34. Rowe, I. and Post, B. *Acta Cryst.* **11** (1958) 372.
35. Hahn, T. and Buerger, M. J. *Z. Krist.* **108** (1957) 419.
36. Marsh, R. E. *Acta Cryst.* **11** (1958) 654.
37. Venanzi, L. M. *J. Inorg. & Nuclear Chem.* **8** (1958) 137.
38. Gill, N. S., Nyholm, R. S. and Pauling, P. *Nature* **182** (1958) 168.
39. Hall, D. and Woulfe, M. D. *Proc. Chem. Soc.* **1958** 346.
40. Rasmussen, S. E. *Om krystal- og molekylstrukturen of 2, 2',2''-triaminotriethylamin-nikkel(II)-dithiocyanat.* Copenhagen, 1960.
41. Frank, V. *J.Sci. Instr.* **34** (1957) 210.

Received August 24, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2023–2035

The Use of High Speed Digital Computers for the Least Squares Calculation of Complexity Constants *

JAMES C. SULLIVAN, JAN RYDBERG** and WILLIAM F. MILLER

Chemistry Division and Applied Mathematics Division, Argonne National Laboratory, Lemont, Illinois, U.S.A.

Some general aspects are given of the calculation of stability constants for mononuclear complexes by means of a weighted least squares technique. Computor (the IBM 704, and the GEORGE) programs are developed for the treatment of solvent extraction and potentiometric titration data. The results of the use of these programs are compared in a number of examples with results of earlier (usually graphical) calculations.

Despite the extensive amount of research that has been conducted during the past twenty years the problems encountered in the study of the association between cations and complexing ligands, in aqueous systems, are far from completely resolved. Most of the data in the literature has been interpreted on the basis of the model, originally suggested by N. Bjerrum [1], that the formation of complexes always appears to occur in step-wise fashion with the stabilities of the various species characterized by a series of mass action constants.

If the free energy data of complex ion systems are to be correlated with other properties of the constituents of such systems (as for example the radii of the cations) it is imperative that the original data be summarized in a manner efficient for comparative purposes.

Approximation techniques have been generally employed for the calculation of association constants [2,3]. The increasing availability of high speed digital computers makes it feasible to use a standard objective treatment, *e.g.*, the least squares adjustment, in the calculation of such constants. Computations involved in such an adjustment become difficult when a desk type of calculator is used both because of the large amount of data generally accumulated in the study of a complex ion system and because of the fact that the normal equations are often illconditioned.

* Based on work performed under the auspices of the U. S. Atomic Energy Commission.

** On leave of absence from the University of Stockholm, and the Research Institute of National Defense, Stockholm, Sweden.

Along with the calculation of the best values for the association constants, the digital computer calculation makes it feasible to calculate the standard deviation to be assigned to each constant (based on the over-all fit of the calculated curve to the experimental points) as well as to provide some measure of the consistency of the model used (based on the same criteria) without recourse to *a priori* assumptions concerning the specific number of entities involved.

In the present communication we will present an outline of the methods that have been used to express the relevant equations in forms suitable for the digital computer computations and present the results obtained for a few selected systems.

1. GENERAL ASPECTS OF THE CALCULATION OF SUCCESSIVE CONSTANTS OF MONONUCLEAR COMPLEXES BY A WEIGHTED LEAST SQUARES TECHNIQUE

Basic equations and definitions

[M] = concentration of uncomplexed central ion or molecule
[A] = concentration of unbound ligand
[MA_n] = concentration of the nth complex

$$M + A = MA,\ M + 2A = MA_2, \ldots,\ M + nA = MA_n \tag{1}$$

The equilibrium constant for the nth complex is

$$\beta_n = \frac{[MA_n]}{[M][A]^n} = \prod_1^n k_n \tag{2}$$

where the k's are constants for the formation of the individual complexes formed in a stepwise manner. The total central ion concentration C_M is

$$C_M = [M] + \sum_{n=1}^{N} [MA_n] \tag{3}$$

and the total ligand concentration C_A is

$$C_A = [A] + \sum_{n=1}^{N} n\ [MA_n] \tag{4}$$

where N is the maximum number of ligands bound per metal atom. The ligand number, $\bar{n}$, is defined [2] as the average number of ligands combined with each central atom

$$\bar{n} = \frac{C_A - [A]}{C_M} = \frac{\sum_{n=1}^{N} n\ \beta_n [A]^n}{\sum_{n=0}^{N} \beta_n\ [A]^n} \tag{5}$$

The degree of formation [2] of a given complex, MA_C, is

$$\alpha_C = \frac{[MA_C]}{C_M} = \frac{\beta_C[A]^C}{\sum_{n=0}^{N} \beta_n [A]^n} \quad (6)$$

Various combinations of the above basic equations have been used for the estimation of association constants [3]. Underlying eqn. 2 is the basic assumption that the ratio of the activity coefficients of the various species involved in the equilibrium are constant. A test of the validity of the model specified by eqn. 2 is not included in this investigation.

Some general aspects of the calculations

When, as in our examples below, either a potentiometric titration or a solvent extraction procedure is used to investigate the formation of complex ions, the particular equations that are relevant for the specific procedure can be expressed in terms of a power series. The parameters of this power series (which are to be determined by a least squares procedure) are constants of the type defined in eqn. 2 or related to that type of constant by an elementary transformation. On the basis of the model we are using, negative parameters and those whose standard deviation spans zero will be rejected, that is, we shall determine the best set of parameters in the least squares sense subject to the restriction that each parameter be positive, in fact, that each parameter minus one standard deviation be positive.

An outline of the least squares analysis used as a basis for the computer programing is given in Appendix I.

The equations that we are to subject to the least squares adjustment generally merit the term of opprobrium, "illconditioned" [4]. One can understand how a condition sufficient to bring about this state of the equations arises from a consideration of the physical aspects of the problem.

An experiment to determine association constants involves the variation of the concentration of the complexing ligand under consideration over as wide a range possible concomitant with the conditions of observational feasibility and invariance of activity coefficient ratios. The usual result is the variation of such a concentration (or independent variable) over many orders of magnitude with a much smaller range covered by the measured property (or dependent variable). Such a disparity in the range of the dependent and independent variables is sufficient to bring about ill-conditioned systems.

From the computational viewpoint this ill-conditioning means that there are a large number of nearly correct solutions to the equations. The existence of this wide variety of nearly correct solutions leads to difficulties in obtaining numerical values for the parameters. Although it is feasible to compute a measure of the degree of conditioning of the equations to be solved [4,5] since we are primarily concerned with the parameters obtained, and their standard deviation, we have not performed any such calculations in the course of this investigation.

The capacity of the digital computers is generally large enough so that the effect of rounding off errors on the matrix inversion is minimal for the computations to be described. This tends to eliminate one of the sources of difficulty encountered when the matrix inversion technique is used for the solution of the normal equations.

Prior to any computer calculation, the data is (of course) studied graphically to determine: (a) the highest power of the independent variable in the series that should be considered (*i.e.* N); (b) that a smooth curve can indeed be drawn through the experimental points.

After the calculation has been completed the computed curve is plotted and the scatter of the experimental points around this curve is examined to determine if there are systematic deviations, *i.e.*, a large number of consecutive points which lie on one side of the curve. In addition, since a log-log scale (with its attendant distortion) is generally most convenient for a graphical representation of the type of data considered here, a point by point comparison between experimental and calculated values is carried out to insure the adequacy of the functional form used in the computation.

Weighting procedures

In the analysis outlined in the Appendix it has been explicity assumed that only the measured distribution coefficients (in the case of solvent extraction experiments) and the measured free ligand concentration (for the potentiometric titration investigations) have associated random errors. The validity of this assumption is securely based on the experimental procedures used in these investigations. It is also apparent from considerations of the experimental procedures that the individual observations have different variances over the range of free ligand concentrations studied. This necessitates weighting the individual measurements.

With the foregoing in mind an estimate of the weight of an individua observation in experiments of the type to be considered here is most reasonably obtained by detailed considerations of the specific investigation. For example, in the case of the Np(IV)—HSO_4^- system [6], since the measured distribution was always adjusted to approximately unity and replication indicated the same precision at all points, the use of $1/y$ as a weighting parameter is reasonable.

For other distribution studies the initial approximation of the weights may be obtained from the measured standard deviation at free ligand concentrations for which replicate measurements were made or by considering the error to be feared in the distribution coefficient caused by the error in the analysis of each phase. For studies in which a radiometric assay procedure is used this latter quantity is readily estimated using standard formulas [7]. Caution must be exercised in assigning weights based on the variance from replicate measurements unless the number of measurements at each point is large. When only a few sets of duplicate measurements are available consideration should be given to using an average percentage uncertainty as first approximation to the individual weights.

For potentiometric titration experiments similar considerations may be used as the basis for initial weight approximations. For example in the absence of replicate experiments the type of precision to be expected in an emf measurement might be used as the basis of assigning weights to an individual observation. To avoid prolixity, further discussion of this point will be deferred until the individual examples are considered.

In the calculations to be described use has been made of the errors of external consistency [8] for the computation of the standard derivations of the parameters and as a test of the validity of the hypothesis under consideration. This approach is a reasonable one to use if we consider the type of data that is to be treated. For example, it is easy to visualize a solvent extraction experiment with systematic errors in a certain region of free ligand concentration. Errors of this type would never be observed by replication and consequently weights based on the apparent precision of measurements at a point in this region would not be realistic for the calculation of the weights of the parameters. Therefore the square of the actual deviation between experimental and calculated points is a logical scale to use for the calculation of the standard deviations of the parameters.

In addition, the reasonableness of an arbitrary assignment of weights to individual observations can be justified by the fact that if one is fitting with the correct functional form and has assigned the correct weights, then the mean value of $\chi^2/k-1 = 1$ *.

Computer programs

Three separate programs were used in the course of this study, two of them being specifically developed for this investigation.

The digital computer "George", developed at Argonne National Laboratory [9], has available a program for the least squares calculation of the a_n in the polynomial

$$y = \sum_{0}^{N} a_n x^n \tag{7}$$

The input data for this program are y, x and the weight of y. This program is suitable for the computation of the β_n in the polynomials used by either Leden [10] or Olerup [11] for graphical estimations of the parameters.

$$\frac{C_M-[M]}{[M]\,[A]} = \sum_{n=1}^{N} \beta_n\,[A]^{n-1} \text{ (Leden)} \tag{7a}$$

$$\frac{C_M}{[M]} = \sum_{n=0}^{N} \beta_n\,[A]^n \text{ (Olerup)} \tag{7b}$$

As illustrated by the treatment of the Np(IV)$-HSO_4^-$ data, the form of the input is altered depending on the type of measurements made; *cf.* Ref.[12].

* See Appendix for a definition of this quantity.

A program was developed for the IBM 704 for use in solvent extraction investigations. This procedure calculates the best (in the least squares sense) a_n in the polynomial

$$yx^N = z = \sum_0^N a_n x^n \tag{8}$$

The input data are x, y, and estimated errors in the determination of the concentrations in both phases from which weights are calculated [12]. This program can, *e.g.*, be used for data that can be summarized by eqn. 6. The development of the program in the specific form used in the present investigation emphasizes the underlying philosophy of machine computations, *i.e.*, the minimization of transformations applied to the original experimental data prior to computer input.

The $\bar{n}$ relation of Bjerrum [2] (eqn. 5) can be rearranged

$$\sum_{n=0}^{N} (\bar{n}-n)\,\beta_n\,[\mathrm{A}]^n = \sum_{n=0}^{N} (C_\mathrm{A}-[\mathrm{A}] - n\,C_\mathrm{M})\,\beta_n\,[\mathrm{A}]^n = 0 \tag{9}$$

A program was developed for the IBM 704 to calculate the best values for the β_n from the equation:

$$0 = \sum_0^N (y-x-nz)\;\beta_n x^n \tag{9a}$$

The input data are $y = C_\mathrm{A}$, $x = [\mathrm{A}]$ and $z = C_\mathrm{M}$ as well as the estimated standard deviation that occurs in the measurement of x.

Output data that are common to all three programs includes: the values of the parameters with their standard deviation (based on external consistency); the value of $S_{min.}$ *, calculated values of the function, using the derived constants, for the original experimental x values.

2. EXAMPLES OF THE TREATMENT OF SOLVENT EXTRACTION DATA

The System Np(IV)-HSO_4^- [6]. The use of theonyltrifluoroacetone (TTA) as a chelating agent for the extraction of cations from an aqueous into an organic phase has been used by a number of investigators [13] to obtain data that may be interpreted in terms of ionic association. The assumptions upon which this interpretation is based have been clearly stated [13a] and will be used in the following treatment. The partition ratio of the concentration of Np(IV) in the organic phase to the total concentration of Np(IV) in the aqueous phase, Q, under conditions of constant acidity and ionic strength and corrected to unit activity of TTA in the organic phase can be expressed as,

$$Q = Q_0 / \sum_0^N \beta_n\,[\mathrm{HSO_4^-}]^n \tag{10}$$

where $\beta_0 = 1$, and $N = 2$.

* See Appendix.

Simple transformations lead to

$$y = a_0 + a_1 [HSO_4^-] + a_2 [HSO_4^-]^2 \quad (11)$$

where $y = 1/Q$, $a_0 = 1/Q_0$ (Q_0 is the normalized distribution coefficient of Np(IV) in the absence of HSO_4^-), $a_1 = \beta_1/Q_0$, $a_2 = \beta_2/Q_0$. This puts the original data in a form suitable for the least squares calculation of the a_n using the computer program based on eqn. 7.

The weight of the individual observation was taken as $1/y$ as previously indicated. In this case a test of the internal consistency of the data is made possible by comparison of the calculated value of Q_0 with the independent experimental determination of this quantity. The relevant output data are

Table 1. Parameters describing the system Np(IV)-HSO_4^-, 25°C, μ = 2.00, $[H^+]$ =2.00.

	Q_0	k_1	k_2
This work	$(2.94 \pm .23) \times 10^4$	118 ± 14	16 ± 2
Original [6]	$(2.84 \pm .04) \times 10^4$	135 ± 14	16 ± 2

summarized in Table 1, along with the results of the original computations made by the limiting value method of Leden [10]. The uncertainties listed in the above are standard deviations as are all such uncertainties stated in this work.

The values for the parameters obtained by the least squares adjustment are in reasonable agreement with the original graphical estimates. It is of interest to note that the original estimate of the errors (%) in the parameters k_1 and k_2 are of the same order of magnitude as those determined by the more objective computation described here. However the estimation of the uncertainty in Q_0, based on the experiments summarized in Table 2 of the original investigation, is seen to be significantly smaller than the standard error based on eqn. 8, Appendix. This emphasizes the logic of the choice of external consistency as a basis for weighting the parameters determined by the least squares adjustment.

Pu(IV)-acetylacetone. A large number of solvent extraction investigations have been conducted for the purpose of determining association constants between metal ions and organic ligands [14]. The assumptions basic to such an interpretation have been outlined [15] and are implicitly accepted in the following treatment.

We will consider the least squares adjustment of the data presented for the Pu(IV)-acetylacetone system [3i] as an example of the utility of digital computers in the interpretation of such distribution results. Considering only uncharged species in the organic phase, and under conditions of constant ionic strength from considerations similar to those previously discussed, we may write eqn. 8 as

$$y = [Aa]^4/Q = a_0 + a_1 [Aa] + a_2 [Aa]^2 + a_3 [Aa]^3 + a_4 [Aa]^4 \quad (12)$$

where Q is the measured partition ratio of the concentration of $PuAa_4$ in the organic phase to all species of Pu in the aqueous phase, [Aa] is the free

Table 2. Parameters for the system Pu(IV)-acetylacetone, $\mu = 0.1$, 25°C.

Computational	$-\log a_0$	$-\log a_1$	$-\log a_2$	$-\log a_3$	$-\log a_4$
Least squares		26.22 ± 0.11		8.45 ± 0.06	2.54 ± 0.07
Two parameter *	36.4 ± 1.3	25.5 ± 0.8	16.2 ± 0.7	8.5 ± 0.4	2.44 ± 0.20
Ligand number *	36.0 ± 1.3	25.0 ± 0.8	16.9 ± 0.7	8.4 ± 0.4	2.50 ± 0.20
Limiting value *	36.6 ± 1.0	25.8 ± 0.8	16.2 ± 0.7	8.6 ± 0.4	2.56 ± 0.20

* A description of the approximation techniques as used is given in Ref. [3i]

acetylacetone ligand concentration in the aqueous phase, $a_4 = 1/\lambda_4$ where λ_4 is the distribution coefficient of $PuAa_4$, $a_3 = \beta_3/\lambda_4\beta_4$, $a_2 = \beta_2/\lambda_4\beta_4$, $a_1 = \beta_1/\lambda_4\beta_4$, and $a_0 = 1/\lambda_4\beta_4$. From the published data one may estimate a weight for the original observations based on a 20 % standard error in the measured partition ratio Q. This type of estimate is obtained from a consideration of the results of replicate experiments since the analytical scheme does not readily afford a basis for choosing weights based on the uncertainties to be expected in the determination of Q.

The relevant output data is summarized in Table 2 along with the graphical estimates of the parameters computed in the original publication.

The most interesting aspect of the results obtained is that the least squares treatment of the data allows a complete description of the results using only three parameters. Attempts to force a fit to the data in terms of five parameters, *i.e.*, in terms of the *a priori* assumption that the formation of the complex ions $PuAa^{+3}$, $PuAa_2^{+2}$, $PuAa_3^{+}$, $PuAa_4$, proceeds in a regular manner, always resulted in values for the parameters a_0 and a_2 which were inconsistent with the specified model. This example emphasizes one of the most useful features of the computational method under consideration that it provides a description of the results in a manner consistent only with the precision of the observations.

Chemical bias coupled with a highly ill-conditioned system made possible the results obtained originally by the limiting value and ligand number methods. The results obtained with the two parameter method are a reflection of the assumptions used in that approach and emphasize the limitations imposed by Dyrssen and Sillen in their development of this technique [3b], *i.e.*, that the equations developed are exact only for the cases where one or two species are formed.

From the chemical viewpoint this result implies that, at equilibrium, in the experiments considered, the aqueous phase concentration of the species $PuAa_2^{2+}$ was not large enough to have any observable influence on the measured distribution coefficient. The data are adequate to provide reliable values of the distribution coefficient, the product k_2k_3 and k_4. Any further information adduced form these results must be viewed with the reservation accorded postulates of unknown scientific validity.

3. POTENTIOMETRIC TITRATION DATA

Eqn. 9 is the basis for computation of complexity constants in systems where the total metal, ligand and free ligand concentrations are the measured quantities. Data from the pioneering work of J. Bjerrum [2] will be used to demonstrate applications of this computer program.

The Systems Ag(I)—NH_3 and Cu(II)—NH_3. The results obtained for the first mentioned system with an estimated weight of an individual observation corresponding to a standard deviation of 0.7 % in the free ligand concentration (yielding $\chi^2/k-1=1.00$) are presented in Table 3. For comparison we also include the values obtained from the computations in the original paper as well as a set of values for the association constants computed by Irving and Rossotti [3c]. The later investigators made their computations by rearranging what is essentially eqn. 9 into a form linear in β_1 and β_2 and performing an unweighted least squares computation. The results obtained in this example emphasize a number of important aspects of the present investigation.

Table 3. Association constants for the system Ag(I)-NH_3, μ = 2.0 (NH_4NO_3), t = 30°C.

	log k_1	log k_2
Bjerrum [2]	3.20	3.83
This work	3.181 ± 0.008	3.848 ± 0.008
Irving and Rossotti [3c]	3.19	3.84

(1) When experiments of high precision (as in this case) are available a weighted least squares treatment provides the best summary of such data. This is demonstrated by the fact that the precision of the original data justifies the use of one more significant figure than was originally presented. Another question is if the third decimal figure is of any significance because of systematic errors; however, this will not be discussed here.

(2) When graphical adjustments are properly carried out they will provide values for the constants consistent with the results obtained by the analytical methods being discussed here.

(3) The values given by the third treatment tabularized points out a pitfall for the unwary. The maximum amount of informations consistent with the precision of the experiments is not obtained unless the least squares treatment of the data is carried out in a proper manner.

It is instructuve to consider a second example from the same work [2] in order to point out difficulties that may arise when using the present computer program. It is obvious that if any two rows or columns of the matrix **W** (eqn. 5, Appendix) are nearly multiples of one another a situation will exist that makes for near indeterminancy with the consequent difficulties in a computation of the parameters. Another more subtle point that will be troublesome can be shown by the following example.

For the system Cu—NH_3 calculations based on all the observations failed to yield significant parameters. Inspection of the data will show that at the point with $\bar{n} = 4.002$ there is a change in the value of χ by approximately a factor of 10. The introduction of this number into the matrix **W** may req-

Table 4. Association costants for the system Cu(II)-NH_3, $\mu = 2.0$ (NH_4NO_3), t = 30°C.

	log k_1	log k_2	log k_3	log k_4
Bjerrum [2]	4.19	3.50	2.88	2.19
This work	4.134 ± 0.004	3.528 ± 0.008	2.87 ± 0.01	2.15 ± 0.02

uire a range of numerical values exceeding the capacity of the computor. The use of other techniques, such as orthogonal polynomials is under investigation in an attempt to extend the computations over the complete range of the variables. A computation excluding this point gives the results summarized in Table 4. A weight proportional to a standard deviation in the free ligand concentration of 0.72 % will yield a value of $\chi^2/k-1 = 1.00$ for this system.

The conclusions to be drawn from these results are self-evident in the light of our previous discussion.

CONCLUSIONS

If quantitative comparisons between the association constants of complex ion systems are to be made in terms of electrostatic effects or any other of the fundamental properties of these systems such as suggested by J. Bjerrum [2] it is necessary not only that precise data be available but also that such data is summarized in the most efficient possible manner. Such a summary is provided by the least squares adjustment of experimental observations. It should be emphasized that the least squares adjustment of data for the calculation of complexity constants is readily feasible at the present because of the development of high speed computers. The concepts involved have been well known for over a hundred years.

Attention should be directed to the limitations on the information that is provided by such an adjustment. This particular method of treating the data can only compensate for the ubiquitous random errors which plague the experimentalist. The use of this method cannot compensate for inadequate data.

However, when properly applied, the least squares treatment does provide the best summary of experimental observations. We hope that the availability of the programs for the IBM 704 will be of assistance to investigators who do desire to use this treatment of their data.

APPENDIX

Presented below, in outline form, is the analysis upon which the computer programs we have described are based. Consider the equation

$$y = \sum_{n=0}^{N} a_n x^n \tag{1}$$

where the dependent variable is subject to random errors. The weighted least squares method requires that a set of a_n be determined which minimizes

$$S = \sum_{i=1}^{I} w_i \left(\sum_{0}^{N} a_n x_i^n - y_i \right)^2 \tag{2}$$

The choice of

$$w_i = \frac{1}{\sigma_i^2} \tag{3}$$

where σ_i^2 is the variance of the measurement y_i, corresponds to the Chi-squared minimum method *.

The normal equations resulting from the minimization of S with respect to variations in the a_n are:

$$\begin{bmatrix} \sum_i w_i & \sum_i w_i x_i & \dots & \sum_i w_i x_i^N \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \sum_i w_i x_i^N & \sum_i w_i x_i^{N+1} & & \sum_i w_i x_i^{2N} \end{bmatrix} \begin{bmatrix} a_0 \\ a_1 \\ \cdot \\ \cdot \\ \cdot \\ a_N \end{bmatrix} = \begin{bmatrix} \sum_i w_i y_i \\ \sum_i w_i y_i x_i \\ \cdot \\ \cdot \\ \cdot \\ \sum_i w_i y_i x_i^N \end{bmatrix} \tag{4}$$

or in matrix notation $\mathbf{WA} = \mathbf{Y}$. (5)

Eqn. 5 is solved by inverting $\mathbf{W}$ and multiplying this inverse matrix into the vector $\mathbf{Y}$. Thus

$$\mathbf{A} = \mathbf{W}^{-1}\,\mathbf{Y} \tag{6}$$

yields the best values of the parameters $a_0, a_1, \dots, a_N$. The minimum value of S, that is the right hand side of eqn. 2 using the best values of the $\{a_n\}$, is then calculated, and the standard deviations of the parameters are calculated by external consistency *via*

$$\sigma_{a_i}{}^2 = \mathbf{W}_{ii}^{-1} \frac{S_{\min}}{k-1}, \tag{7}$$

where $\mathbf{W}_{ii}^{-1}$ is the ith diagonal element of the inverse of $\mathbf{W}$, and k is the number of degrees of freedom for the system (4)**. In this formulation the number of degrees for the system is $[I-(N+1)]$***. Furthermore the sample values of $S_{\min}$ for a system of k degrees of freedom have the Chi-squared distribution for $k-1$ degrees of freedom****. Thus we should expect $S_{\min}$ to have the χ^2 distribution for $(I-N)$ degrees of freedom and eqn. 7 becomes

$$\sigma_{a_i}{}^2 = \mathbf{W}_{ii}^{-1} \frac{S_{\min}}{I-N} \tag{8}$$

The consistency of the resulting parameters $\{a_n\}$, the measurements $\{y_i\}$, and the estimated errors of measurement $\{\sigma_i\}$ is tested by comparing the value of $S_{\min}$ with the expected value of χ^2 for $(I-N)$ degrees of freedom.

---

* Harald Cramér, *Mathematical Methods of Statistics*, Princeton University Press, Princeton 1946, § 18.1 and § 30.3.

** Dumond and Cohen, *Rev. Modern Phys.* **25** (1953) 691.

*** Harald Cramer, *Mathematical Methods of Statistics*, § 29.2, p. 379 ff.

**** *Ibid.*, § 29.3, p. 381 ff.

With the following modifications, eqn. 9, as applied to the titration data, can be given the same treatment.

Let

$$U = \sum_{n=0}^{N} (y - x - nx)\, \beta_n x^n \tag{9}$$

In the titration experiment, one should have $U = 0$. One can determine that set of $\{\beta_n\}$ which, for a given set of $\{x_i, y_i, z_i\}$, makes U nearest to zero in the least squares sense by minimizing

$$S = \sum_{i=1}^{I} w_i\ U^2(x_i,\ y_i\ z_i) \tag{10}$$

with respect to variation of the parameters $\{\beta_n\}$. The weights, w_i, should be taken as

$$w_i = \frac{1}{\delta U^2} \tag{11}$$

where δU is the variation in U for fluctuations in the variables (x, y, z). If most of the variation in U is due to variation in x, then

$$\delta U = \frac{\partial U}{\partial x}\ \sigma_x \tag{12}$$

Since one needs to know the β_n in order to calculate $\partial U/\partial x$, an iterative procedure was employed here. First a set of approximate β's was calculated for $w_i = 1$. Using these approximate β's, the weights *via* eqnns. 12 and 11 were calculated. Then an improved set of β's are calculated. Generally three such iterations yield a consistent solution.

Acknowledgement. The authors wish to express their appreciation to Dr. J. C. Hindman whose advice and criticism have enhanced the development of this investigation. In addition we wish to thank the personnel of the Applied Mathematics Division, in particular the programmers, Mrs. M. Hanson, Mrs. S. Steidl and Mr. W. Greenhow, for their technical assistance.

REFERENCES

1. Bjerrum, N. Kgl. *Kgl. Danske Videnskab Selskab. Skrifter, naturvidenskab. math. Afdel.* (1) **12** (1925).
2. Bjerrum, J. *Metal Ammine Formation in Aqueous Solution*, P. Hasse and Son, Copenhagen 1941.
3. For a review of the standard techniques: (a) Sullivan, J. C. and Hindman, J. C. *J. Am. Chem. Soc.* **74** (1952) 6091.
   Other methods are discussed by: (b) Dyrssen, D. and Sillén, L. G. *Acta Chem. Scand.* **7** (1953) 663.
   (c) Irving, H. and Rossotti, H. S. *J. Chem. Soc.* **1953** 3397.
   (d) Block, B. P. and McIntyre, G. H. Jr. *J. Am. Chem. Soc.* **75** (1953) 5667.
   (e) Rossotti, F. J. C. and Rossotti, H. S. *Acta Chem. Scand.* **9** (1955) 1166.

(f) Irving, H., Rossotti, F. J. C. and Williams, R. J. P. *J. Chem. Soc.* **1955** 1906.
(g) Gryder, J. W. *J. Am. Chem. Soc.* **77** (1955) 6196.
(h) Hearon, J. Z. and Gilbert, J. B. *Ibid* **77** (1955) 2594.
(i) Rydberg, J. *Arkiv Kemi* **9** (1956) 109.
(j) Colleter, J.-C. and Romain, P. *Bull. soc. chim. France* **1958** 858.
4. Turing, A. M. *Quart. J. Mech. & Applied Math.* **1** (1948) 287.
5. Box, G. E. P. and Hunter, J. S. *Biometrika* **41** (1954) 190.
6. Sullivan, J. C. and Hindman, J. C. *J. Am. Chem. Soc.* **76** (1954) 5931.
7. Friedlander, G. and Kennedy, T. W. *Nuclear & Radiochemistry*, J. Wiley & Sons, New York 1955.
8. Deming, W. E. *Statistical Adjustment of Data*, J. Wiley & Sons Inc., New York 1943
9. *J. Assn. Computing Machinery* **3** (1956) 45.
10. Leden, I. *Z. physik. Chem.* **A 188** (1941) 160.
11. Olerup, H., Diss. Lund 1944.
12. Rydberg, J., and Sullivan, J. C. *Acta Chem. Scand.* **13** (1959) 2057.
13. For examples see:
(a) Connick, R. E. and McVey, W. H. *J. Am. Chem. Soc.* **71** (1949) 3182.
(b) Connick, R. E. and Reas, W. H. *J. Am. Chem. Soc.* **73** (1951) 1171.
(c) Betts, R. H. and Leigh, R. *Can. J. Research* **B 28** (1950) 514.
(d) Zebroski, E. J., Alter, H. W. and Heumann, F. K. *J. Am. Chem. Soc.* **73** (1951) 5946.
(e) Day, R. A., Jr. and Powers, R. M. *J. Am. Chem. Soc* **76** (1954) 3895.
14. For a comprehensive list of references in this field see:
(a) Rydberg, J. *Svensk Kem. Tidskr.* **67** (1955) 499.
(b) Dyrssən, D. *Svensk Kem. Tidskr.* **68** (1956) 212.
15. Rydberg, J. *Acta Chem. Scand.* **4** (1956) 1503.

Received August 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2036–2038

Preparation of a New Zinc-rich Insulin Compound

K. MARCKER*

Roskilde Medical Company, Roskilde, Denmark

It is shown, that crystalline insulin, when treated with alcoholic solutions of zinc chloride in concentrations up to 10 % w/w in the limit combines with two moles of Zn and five moles of $ZnCl_2$. Thus the stoichiometric composition of the compound is insulin,2Zn,5$ZnCl_2$. The compounds with $MgCl_2$, $CaCl_2$, $NiCl_2$, $MnCl_2$, $CdCl_2$, and $CoCl_2$ have been prepared in a similar way. Their compositions are approximately: (Insulin, 1/2 Zn, 1/2 Mg); (Insulin, 1/2 Zn, 1 Ca); (Insulin, 1/2 Zn, 1 1/2 Ni) $NiCl_2$; (Insulin, 1/3 Zn, 1 2/3 Mn)6 $MnCl_2$; (Insulin, 1/3 Zn, 1 2/3 Cd)3 $CdCl_2$; (Insulin, 2 Co)5$CoCl_2$.

For a long time it has been known that zinc combines with insulin forming a zinc-insulin complex. Hallas-Møller *et al.*[1] have shown that the zinc content in crystalline insulin under certain conditions rises to a figure of about two moles per Sanger unit or even higher. It is the purpose of this paper to describe the preparation of a new zinc-rich crystalline insulin compound which in chemical composition differs from those previously described.

The following technique is used in preparing the zinc-rich insulin: 0.5 g of crystalline insulin (0.8 % Zn) is suspended in solutions containing various amounts of dry $ZnCl_2$ in 50 ml of absolute alcohol. The suspension is shaken overnight and filtered the following day on a Büchner funnel. The insulin is washed twice with 20 ml of absolute alcohol and once with 30 ml of dry ether. It is then dried in air, and finally the zinc-content is determined by titration with EDTA and Eriochromblack T as indicator. The results are shown in Fig. 1. By gradually increasing the concentration of $ZnCl_2$ in the suspension it is found that the zinc-content in insulin at concentrations higher than 10 % remained constant at a value of about 7.7—7.8 %. Assuming a molecular weight of 6 000 (a Sanger unit = 5 778 + 6 % which is the water content of dry insulin) this figure corresponds fairly closely with seven zinc atoms per molecule of insulin. The zinc-rich insulin contains chloride and the content of chloride is then determined according to Volhard. The results of these analyses are that five atoms of the total zinc-content in insulin are present

* Present address: Københavns Universitets Fysisk-Kemiske Institut, Copenhagen, Denmark.

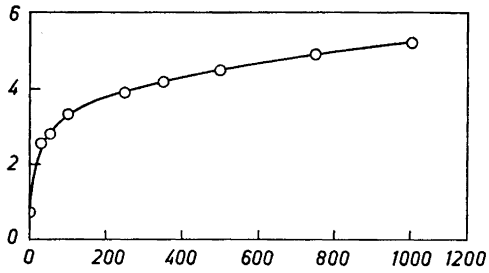


Fig. 1. The reaction between insulin and $ZnCl_2$. Ordinate: moles of Zn per unit of 6 000. Abscissa: mg $ZnCl_2$ in 50 ml of absolute alcohol.

as the chloride. The stoichiometry of the zinc-rich insulin compound is thus as follows: Insulin,2Zn,5$ZnCl_2$.

In order to evaluate the influence of the crystal size of insulin on the zinc uptake from the solution, two different insulin preparations with a crystal diameter of about 10 μ and 20 μ, respectively, were prepared and subjected to the procedure described above. The results were:

Crystal diameter appr. 10 μ	Crystal diameter appr. 20 μ
% Zn in insulin 7.79 =	% Zn in insulin 7.42 =
7.15 moles per unit of 6 000	6.81 moles per unit of 6 000

Although the zinc contents of the two preparations are not identical it is evident that the crystal size is relatively unimportant for the zinc uptake.

A few other zinc salts have been tried. These are $Zn(OOCCH_3)_2$, $ZnBr_2$ and ZnI_2. The results expressed in stoichiometric formulas are: (Insulin, 2Zn) $4ZnBr_2$; (Insulin, 2Zn)$3ZnI_2$ and for $Zn(OOCCH_3)_2$, (Insulin, 2Zn). These results might be explained by assumption of steric hindrances on the sites of binding between the insulin and the metal salt.

By suspending the zinc-rich insulin in absolute alcohol a small amount of the $ZnCl_2$ diffuses out of the crystal until an equilibrium has been attained. By suspending the zinc-rich insulin in water, however, all the $ZnCl_2$ rapidly

Table 1. The reaction between insulin and metal salts. The zinc content in untreated insulin is 0.8 % = 0.73 atoms per unit of 6 000.

Metal chloride	% metal in insulin	moles of metal per unit of 6 000	moles of Zn per unit of 6 000	Proposed stoichiometric formula
$MgCl_2$	0.23 [a]	0.57	0.62	(insulin,$\frac{1}{2}$Zn,$\frac{1}{2}$ Mg)
$CaCl_2$	0.65 [a]	0.97	0.55	(insulin,$\frac{1}{2}$Zn,1 Ca)
$NiCl_2$	2.60	2.65	0.51	(insulin,$\frac{1}{2}$Zn,1$\frac{1}{2}$Ni)1$NiCl_2$
$MnCl_2$	6.80	7.42	0.34	(insulin,$\frac{1}{3}$Zn,1$\frac{2}{3}$Mn)6$MnCl_2$
$CdCl_2$ [b]	9.24	4.93	0.29	(insulin,$\frac{1}{3}$Zn,1$\frac{2}{3}$Cd)3$CdCl_2$
$CoCl_2$	6.70	6.81	<0.1	(insulin,2Co)5$CoCl_2$

[a] These preparations contain no chloride.

[b] $CdCl_2$ is only sparingly soluble in absolute alcohol. The figure quoted is thus probably not the final value.

diffuses out, and the zinc content of the insulin decreases to a value of about 2 moles per Sanger unit. This effect shows that the binding between $ZnCl_2$ and insulin is unstable in aqueous solution.

The reaction between insulin and other metal chlorides has also been investigated. The metal salts used are $NiCl_2$, $CdCl_2$, $MgCl_2$, $MnCl_2$, and $CoCl_2$. The results are shown in Table 1.

As indicated in Table 1 the incorporation of metal chloride into insulin replaces zinc from the insulin. This phenomenon proved to have a remarkable effect on the crystal structure of insulin. When the preparations of insulin contain more than 1/3 mole Zn per unit of 6 000 it is still crystalline, but containing less than this amount the crystal structure is deteriorated. A more detailed report on the above mentioned effects including an X-ray crystallographic investigation on insulin preparations containing varying amounts of zinc will be published elsewhere.

I am gratefully indebted to Dr. med. H. Christensen, Roskilde Medical Company, for his guidance and valuable suggestions and to Miss Tove Ahrens for excellent technical assistance.

REFERENCE

1. Hallas-Møller, K., Petersen, K. and Schlichtkrull, J. *Ugeskrift for Læger* **113** (1951) 1761.

Received August 13, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2039–2046

The Position of the Primary Amino Group in the Steroidal Alkaloid Solanocapsine

PER M. BOLL* and HANS A. LILLEVIK

The Kedzie Chemical Laboratory, Michigan State University, East Lansing, Michigan, U.S.A.

Even under conditions preventing hydrolysis of a possible glycosidic linkage in the steroidal alkaloid solanocapsine, the compound could not be isolated as a glycoside. Data suggesting that the compound has a 3α-amino group instead of the 3α-hydroxyl group usually present in *Solanum* alkaloids are presented. Based on the behavior of acetyl derivatives the hydroxyl group of solanocapsine hitherto considered unreactive is found reactive.

This work was started with the purpose of isolating the antibacterial principle from *Solanum pseudocapsicum*. *In vitro* activity against *Mycobacterium tuberculosis* of root extracts had been reported previously [1]. During the isolation work the antibacterial principle was also found to be present in leaves and stems and was identified as the alkaloid solanocapsine [2].

In 1936 Barger and Fraenkel-Conrat [3] reported the isolation of solanocapsine from *S. pseudocapsicum* and assigned the empirical formula $C_{26}H_{44}N_2O_2$ or $C_{25}H_{42}N_2O_2$ to the compound. Furthermore, the two workers obtained an amorphous alkaloid, solanocapsidine, which was found to have the formula $C_{26}H_{42}N_2O_4$. No alkaloidal glycoside was isolated.

In 1945 Rochelmeyer *et al.*[4] suggested a structural formula for solanocapsine with 27 carbon atoms. The formula was proposed without any experimental evidence.

Later, Schlittler and Uehlinger [5] found that the empirical formula $C_{27}H_{46}N_2O_2$, H_2O was more in agreement with elementary analyses of solanocapsine. By this formula solanocapsine can be classified together with other 27 carbon atom *Solanum* alkaloids (other being solanidine, tomatidine, solasodine *etc.*).

Solanocapsine is the only alkaloid from the genus *Solanum* which has not been isolated as a glycoside. So far, both Barger and Fraenkel-Conrat and Schlittler and Uehlinger have isolated the alkaloid from dried leaf material and have not been able to isolate a glycoside, although the former authors

* Present adress: Royal Danish School of Pharmacy, Chemical Laboratory B, Copenhagen, Denmark.

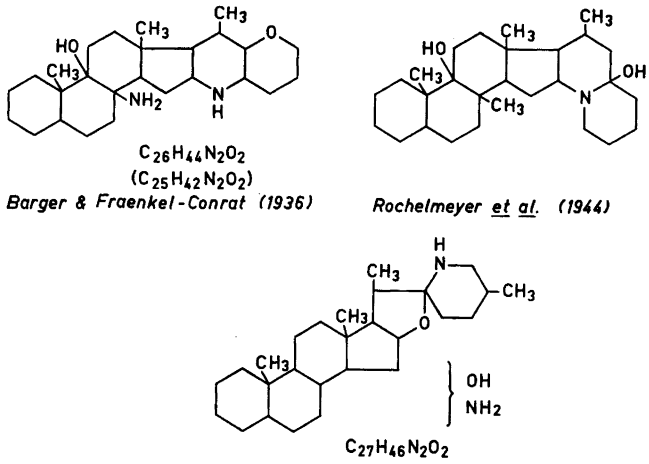


Fig. 1.

obtained reactions for carbohydrates on crude alkaloid preparations, an observation we have confirmed. Therefore, the possibility of an extraordinary labile glycosidic bond being acted upon enzymatically during drying of the plant or during isolation was carefully examined. Enzyme inactivation by placing the plants, immediately after harvesting, in absolute ethanol or in boiling water and using weak acids in the isolation procedure did not yield a glycoside. It is regarded that solanocapsine is present in the plant as a free alkaloid.

It has been stated that *Solanum pseudocapsicum* besides solanocapsine contains an amorphous alkaloid solanocapsidine [3], solanine [6], and solanidine [6]. During the isolation and purification of solanocapsine an amorphous, resinous material was often obtained; but it was always possible, as Schlittler and Uehlinger also observed, by repeated precipitation from aqueous ethanol to convert this material to crystalline solanocapsine. By paper chromatography only one alkaloid was observed, although it has to be mentioned that Schreiber [7] also by paper chromatography has detected another minor alkaloid.

The elementary analysis of solanocapsine was in agreement with the empirical formula proposed by Schlittler and Uehlinger. Since both Barger and Fraenkel-Conrat and Schlittler and Uehlinger have isolated 3′-methyl*cyclo*pentenophenanthrene and 2-ethyl-5-methylpyridine after selenium dehydrogenation of solanocapsine, we have assumed that solanocapsine has a solasodan nucleus and have mainly been interested in the position of the functional groups.

The two above mentioned groups have found that solanocapsine contains a primary amino group, a secondary amino group and a hydroxyl group. The evidence for two amino groups came from methylation of the nitrogen atoms with formic acid and formaldehyde, which gave a trimethyl derivative $C_{30}H_{52}N_2O_2$. This observation was confirmed by the present investigation.

Further evidence for the existence of the two amino groups was given by Schlittler and Uehlinger by reacting solanocapsine with nitrous acid. They observed that by reacting with two equivalents of nitric acid, a neutral, unsaturated nitroso derivative $C_{27}H_{42}N_2O_3$ resulted. In this study the same was observed and a Van Slyke amino nitrogen determination indicated that one mole of nitrogen was liberated per mole of solanocapsine, from which can be concluded that a primary amino group is present. The ultraviolet spectrum of the obtained nitroso derivative showed a peak at 346 mμ and another of

Table 1.

Compound	Maxima	
Nitroso derivative of solanocapsine	346 mμ (log E 2.05)	240 mμ (log E 3.89)
Di(*cyclo*hexylmethyl)-N-nitrosamine [8]	355 mμ (log E 1.95)	240 mμ (log E 3.90)
1-Nitrosopiperidine [8]	350 mμ (log E 2.00)	235 mμ (log E 4.25)
N-Nitrososolasodine [9]	370 mμ (log E 1.98)	234.5 mμ (log E 3.78)

low intensity at 240 mμ. In Table 1 this spectrum is compared with spectra of three nitrosamines. Solanocapsine therefore contains a secondary amino group.

Solanocapsine does not give any precipitate with digitonin contrary to the other *Solanum* alkaloids and is, therefore, not considered to have a 3β-hydroxyl group. It can not have a 3α-hydroxyl group, since it would have been possible to dehydrate such a group. Barger and Fraenkel-Conrat maintain that they could eliminate the hydroxyl group with boiling alkali, but neither Schlittler and Uehlinger nor our investigation could repeat it. On the other hand, the nitroso derivative of solanocapsine gives a precipitate with digitonin. Therefore, it seems plausible to conclude that the primary amino group is placed in the 3-position.

Not only 3β-hydroxy steroids (with 3-OH and 10-CH_3 *cis* to each other) are precipitated as digitonides, but several 3β-amino steroids give precipitates contrary to the corresponding 3α-amino steroids. It is, therefore, assumed that the amino group in solanocapsine is in the 3α-position. An inversion of configuration must have happened during the deamination. Mills [10] described deamination of aminodecalins with nitrous acid and found it to be a true stereospecific reaction. Equatorial amino groups afforded alcohols of same configuration, while axial amino groups reacted by inversion of configuration. That is in agreement with the concept that the 3β-hydroxy group in steroids with the same stereochemical configuration as cholesterol is equatorial and, therefore, thermodynamically more stable.

Hydrogenation of solanocapsine at room temperature and about 3 atm pressure with reduced platinum oxide catalyst gave a derivative, which did not show any melting point depression mixed with solanocapsine, but the

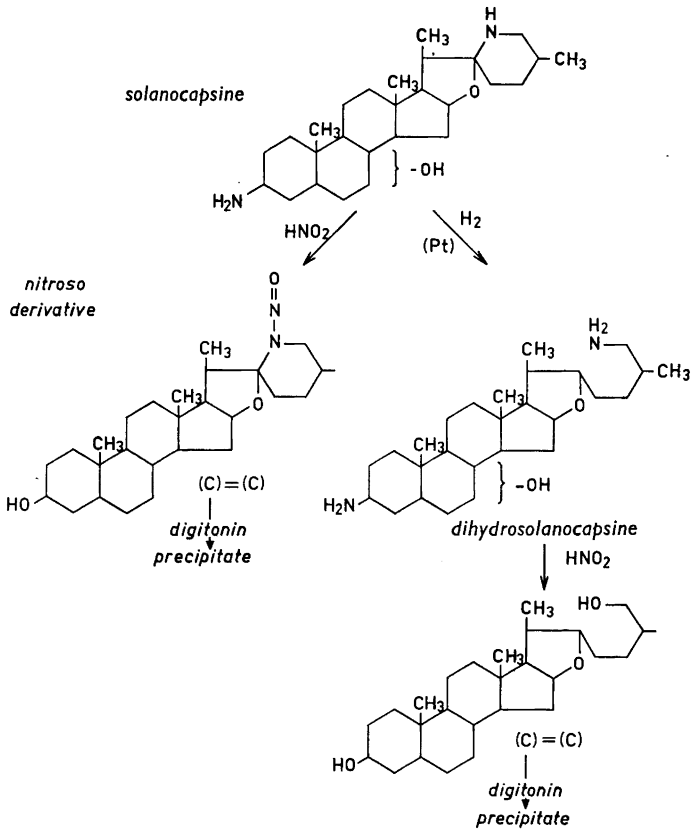


Fig. 2.

specific rotation was changed. A Van Slyke amino nitrogen analysis of the dihydro compound showed that two moles of nitrogen were liberated per mole of compound and that the resulting compound did not give the ultraviolet absorption characteristic for a nitrosamine. It seems safe to conclude that an opening of the piperidine ring has occurred. This is contrary to the opening of the oxide ring in solasodine during hydrogenation.

Schlittler and Uehlinger prepared an amorphous acetyl derivative of solanocapsine, considered by them to be N,N-diacetylsolanocapsine. Several attempts to prepare a diacetate after their procedure never gave the exact elementary analysis expected for a diacetylsolanocapsine. But the compound is not a N,N-diacetylsolanocapsine, but rather an O,N-diacetylsolanocapsine. The reasons for such a conclusion are the following: 1) The derivative was soluble in dilute acetic acid indicating that a basic group probably is present. 2) The infrared spectrum of the derivative showed the characteristic absorption bands for an O-acetyl group and a secondary amide.

Barger and Fraenkel-Conrat acetylated solanocapsine by refluxing with acetic anhydride for 5 h and obtained an amorphous acetate. On the basis of the elementary analysis it was considered to be a diacetate. But it seems that the authors based their calculation on the wrong empirical formula. When recalculated on the basis of a solanocapsine with 27 carbon atoms, the analysis is more in agreement with a triacetate. The acetate prepared in this investigation after their procedure gave an elementary analysis which is in agreement with a triacetate and acetyl group analysis also indicated three acetyl groups. The infrared spectrum showed the bands characteristic for an O-acetyl group and a secondary amide. Furthermore, the intensity of the 6.06 μ band was considerably higher than that of the 6.45 μ band. Since a tertiary amide ($CH_3CO-NRR'$) only shows one characteristic absorption band at 6.06 μ, it is concluded that the third acetyl group is attached to the secondary amino group. The derivative is considered to be O,N,N'-triacetylsolanocapsine.

Barger's as well as Schlittler's group considered the hydroxyl group to be a tertiary or an unreactive hydroxyl group. The conclusion was based on the acetyl derivatives. This investigation has shown that the hydroxyl group can be acetylated, but not tosylated.

EXPERIMENTAL

Isolation. Solanocapsine was isolated and purified by the method of Schlittler and Uehlinger [3]. The plants were grown in the fields belonging to the Department of Horticulture, harvested late fall, and dried in a forced draft oven at 60°. As an example of obtained yields: 500 g of ground leaves gave 8.0 g crude solanocapsine dihydrochloride (1.6 %).

Attemps to isolate an alkaloidal glycoside. Two methods of enzyme inactivation were utilized: 1) Submersion of the harvested plants in absolute ethanol and 2) immediate freezing in dry ice and storage in a deep-freeze until the day of isolation when they were boiled with water.

1) The roots and tops of 15 fresh plants were immediately separated and each part covered with absolute ethanol and stored until time for isolation. A total of 1 400 g of dehydrated tops were macerated in a Waring blender with 14 l of 2 % acetic acid and allowed to stand for 48 h with occasional stirring. The supernatant was removed by filtration and the plant pulp was similarly re-extracted with 5 l of 2 % acetic acid for 24 h and filtered. The combined filtrates were made distinctly alkaline with ammonia and heated to about 50°. After standing overnight the supernatant was decanted and the precipitate collected on a filter. After drying at room temperature the precipitate weighed 0.76 g. The dark brown precipitate was refluxed five times with 100-ml portions of methanol, each time for an hour. 61 mg of residue was obtained. The roots (350 g) were treated in the same manner as described for the tops. After removal of the methanol 20 mg of green residue remained.

2) For the second part of the experiment 13 plants were harvested, immediately placed in dry ice and preserved in the deep-freeze. At the time of glycoside extraction the plants were placed in boiling water for 10 min and then isolation procedure continued in the same manner as above. 128 mg of residue was obtained.

After recrystallization from ethanol the three residues isolated all melted in the broad range of 175—195°C, which suggested impurities. Chromatography in three different solvents showed that the three residues all gave same R_F-value as solanocapsine, when the spots were developed with Dragendorff reagent. If the compounds were chromatographed in 1-butanol-ethanol-water (80:20:20) and the spots developed with aniline hydrogen phthalate, only the compound isolated from the plant tops and the compound isolated from the frozen plants showed spots of D-glucose.

Solanocapsine. To a solution of 500 mg solanocapsine dihydrochloride in 10 ml ethanol-water (1:1) was under stirring added dilute ammonium hydroxide. The yield of crude solanocapsine was 370 mg. After recrystallization from ethanol-water (1:1) it crystallized as platelets which melted with decomposition at 215—216° (lit.[5] m. p. 216— 217°, lit.[3] m. p. 222°); $[\alpha]_D^{25}$ +25 (methanol). The m. p. was usually lower. Only in one case was it possible to reproduce the given m. p. (Found: N 6.20, 6.29; H_2O 3.98; m.wt. 456, 481 (Rast); 482 (neutr. equiv.). Calc. for $C_{27}H_{46}N_2O_2$, H_2O: N 6.24; H_2O 4.01; m.wt. 448.7.)

A Van Slyke amino nitrogen determination gave evolution of 0.99 mole of nitrogen.

No unsaturation and no absorption in the ultraviolet part of the spectrum was observed.

To a solution of 10 mg solanocapsine in 10 ml of ethanol was added 10 ml of a 1 % ethanolic solution of digitonin. The solution remained clear, even after 10 h of standing.

No consumption of periodic acid was observed, even after 10 h. The oxidation was attempted at 3 different hydrogen ion concentrations.

Solanocapsine dihydrochloride. 200 mg of crude solanocapsine dihydrochloride was recrystallized twice from water. The yield was 105 mg and the substance did not melt below 300° (lit.[5] m. p. 300°, lit.[3] m. p. 324°). (Found: C 62.27; H 9.40; N 5.59; H_2O 3.34. Calc. for $C_{27}H_{46}N_2O_2$, 2 HCl, H_2O: C 62.17; H 9.66; N 5.37; H_2O 3.36.)

Solanocapsine picrate. The picrate was prepared as described by Schlittler and Uehlinger. The derivative melted at 198—199° (lit.[3] m. p. 200—201°).

Solanocapsine oxalate. Again the procedure of Schlittler and Uehlinger was followed; m. p. 287—289° (lit.[5] m. p. 288—289°).

Trimethylsolanocapsine. The derivative was prepared following the procedure of Schlittler and Uehlinger. Recrystallized from absolute methanol the compound melted at 208° (lit.[5] m. p. 209°). (Found: N 5.86, 5.72. Calc. for $C_{30}H_{52}N_2O_2$: N 5.93.)

Nitroso derivative. The derivative was prepared according to Schlittler and Uehlinger. After recrystallization from absolute methanol the derivative melted at 194—196° (lit.[3] m. p. 194°, lit.[2] 200°). Microhydrogenation (PtO_2): 0.92 double bond.

Digitonide of nitroso derivative. To a solution of 70 mg of the nitroso derivative in 20 ml ethanol was added 20 ml of a 1 % ethanolic digitonin solution. After standing overnight the copious precipitate was filtered, washed with water and dried to give 45 mg of digitonide. The derivative was washed with 5 ml of ethanol and dried. (Found: N 1.82; 1.90. Calc. for $C_{83}H_{134}N_2O_{32}$: N 1.68.)

Dihydrosolanocapsine. A mixture of 200 mg solanocapsine and 200 mg of platinum oxide in 10 ml of glacial acetic acid and 5 ml of ethanol was shaken under 3 atm pressure in an Adams hydrogen apparatus. After filtration of the catalyst the solvent was removed by distillation under diminished pressure. The residue was dissolved in ethanol-water (1:1) and dilute ammonium hydroxide was added to precipitate 170 mg of compound. It was recrystallized from ethanol-water (1:1), melted at 210—212°, and a mixed melting point with solanocapsine did not show any depression. $[\alpha]_D^{25}$ +40 (methanol). Determination of active "H": Found: 0.42 (room temp.), 0.84 (100°). Calc. for 2 active "H": 0.44, for 4 active "H": 0.89.

A Van Slyke amino nitrogen determination gave evolution of 1.97 moles of nitrogen.

Diacetylsolanocapsine. The derivative was prepared using the procedure of Schlittler and Uehlinger. 150 mg solanocapsine gave 150 mg of a gelatinous acetate. The acetylated alkaloid was chromatographed on 8.7 g of alumina. Eluate fractions of 20 ml were collected. The composition of the fractions is given in Table 2.

Fractions 10—13 were combined and re-precipitated three times from methanol-ethyl ether (1:5). It was not possible to obtain a crystalline product. The amorphous derivative after drying melted at 198—200° (lit.[3] m. p. 193—196°). The compound is soluble in dilute acetic acid. (Found: C 69.90; H 9.84; CH_3CO 1.3 eq, 1.6 eq. Calc. for $C_{31}H_{50}N_2O_4$, H_2O: C 70.77; H 9.62; CH_3CO 2 eq.)

Triacetylsolanocapsine. Barger and Fraenkel-Conrat's procedure was followed. A mixture of 500 mg solanocapsine and 10 ml of acetic anhydride was refluxed in an oil bath for 5 h. The solvent was removed under diminished pressure and the brown, oily residue dissolved in ethanol. Water was added to slight turbidity and the compound was allowed to crystallize. Thus a yield of 400 mg of product melting at 140—150° was obtained. The compound was amorphous and even repeated solution in hot benzene-ligroin

Table 2.

Fraction	Eluant	Residue
1— 3	benzene	9 mg brown oil, soluble in ether
4— 5	benzene + 5 % chloroform	7 mg colorless oil, soluble in ether
6— 7	benzene + 10 % chloroform	9 mg colorless oil, soluble in ether
8— 9	benzene + 25 % chloroform	5 mg colorless oil, soluble in ether
10—13	chloroform + 1 % methanol	79 mg white foam, insoluble in ether
14—16	chloroform + 2 % methanol	6 mg greenish oil, insoluble in ether

did not change it to a crystalline compound. The melting point of the purified compound was 150—155° (lit.[2] m. p. 150—160°).

A further attempt was made to convert the derivative into a crystalline compound by column chromatography. A solution of 83 mg of acetylated solanocapsine in 5 ml benzene was chromatographed on 9 g of alumina and eluate fractions of 20 ml were collected. The composition of the fractions is shown in Table 3.

Table 3.

Fraction	Eluant	Residue
1— 3	benzene	5 mg brownish oil, soluble in ether
4— 5	benzene + 5 % chloroform	4 mg colorless oil, soluble in ether
6— 7	benzene + 10 % chloroform	6 mg colorless oil, soluble in ether
8— 9	benzene + 25 % chloroform	4 mg colorless oil, soluble in ether
10—13	chloroform + 1 % methanol	56 mg white foam, insoluble in ether
14—16	chloroform + 2 % methanol	5 mg greenish oil, insoluble in ether

Fractions 10—13 were combined and after evaporation of solvent gave an amorphous product still melting at 150—155°. The derivative was insoluble in dilute acetic acid. (Found: C 71.61; H 9.49; CH_3CO 2.8 eq. Calc. for $C_{33}H_{52}N_2O_5$: C 71.17, H 9.42; CH_3CO 3 eq.)

Attempted tosylation. A solution of 100 mg solanocapsine in 2.5 ml of pyridine was treated with 100 mg of *p*-toluene sulfonyl chloride. After standing 12 h, 5 ml of water was added and the resulting precipitate was washed with much water. The product was recrystallized from ethanol-water (1:1). Only solanocapsine was recovered as evidenced by mixed melting point and infrared spectrum.

Attempted dehydration. By acid catalysis: A solution of 50 mg of solanocapsine in 15 ml concentrated hydrochloric acid and 35 ml ethanol was refluxed for 10 h. After 50 ml of water was added, the solution was made distinctly ammoniacal and then extracted four times with 100-ml portions of ethyl ether. The combined fractions were dried over anhydrous sodium sulfate and evaporated to dryness. The residue was recrystallized from ethanol-water (1:1). Only solanocapsine was recovered as evidenced by mixed melting point and infrared spectrum. *By base catalysis:* Dehydration in alkaline solution was attempted using the procedure of Barger and Fraenkel-Conrat. The product obtained was found by mixed melting point and infrared spectrum to be solanocapsine. The product gave a condensation product with acetone, which is contrary to the compound thus obtained by Barger and Fraenkel-Conrat.

This investigation was supported in part by research grant E-227 from the *National Institutes of Health.* We wish to thank Professors J. L. Faierley and J. C. Speck of this laboratory for valuable advice.

REFERENCES

1. Frisbey, A., Roberts, J. M., Jennings, J. C., Gottshall, R. Y. and Lucas, E. H. *Mich. Agr. Expt. Sta. Quart. Bull.* **35** (1953) 392.
2. Boll, P. M., Lillevik, H. A., Gottshall, R. Y. and Lucas, E. H. *Antibiotics Ann.* **1955–1956** 255.
3. Barger, G. and Fraenkel-Conrat, H. L. *J. Chem. Soc.* **1936** 1537.
4. Rochelmeyer, H., Stützel, H. and Chen, H. *Arch. Pharm.* **282** (1944) 92.
5. Schlittler, E. and Uehlinger, H. *Helv. Chim. Acta* **35** (1952) 2034.
6. Muenscher, W. C. *Poisonous Plants of the United States*, The Macmillan Company, New York 1951, p. 210.
7. Schreiber, K. *Personal communication.*
8. Goldberg, M. V. and Kirchsteiner, H. *Helv. Chim. Acta* **26** (1943) 289.
9. Briggs, L. H., Harvey, W. E., Locker, R. H., McGillivray, W. A. and Seelye, R. N. *J. Chem. Soc.* **1950** 3013.
10. Mills, J. A. *J. Chem. Soc.* **1953** 260.

Received July 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2047–2056

Changes in Molecular Weight of Acid Mucopolysaccharides in Connective Tissue Due to Hormone Treatment, Dehydration and Age

EIGILL HVIDBERG and C. E. JENSEN († 6/11—1959)

Department of Pharmacology, and Institute of Physical Chemistry, University of Copenhagen, Copenhagen, Denmark

Acid mucopolysaccharides have been isolated from animal skin and characterized by means of viscosimetry, chromatography and metachromasia. It is shown that the molecular weight of these substances decreases with age. A depolymerization of these substances apparently also takes place during severe dehydration and under the influence of cortisone, while oestradiol treatment results in a polymerization. It is concluded that the size of the hyaluronic acid molecule is an important factor in the waterbinding mechanism of connective tissue.

It is generally accepted that in the connective tissue there is a close relationship between the amount and physical state of the acid mucopolysaccharides on one side and the water content on the other side. Oestrogenic treatment has for some time been known to increase the amount of mucopolysaccharides as well as the content of water in the skin (Taylor and Sprunt [1], and Schmidt [2]), and severe dehydration causes a minor decrease in the hexosamine content in the skin of mice and rats, (Hvidberg [3,4]). Further it has been observed (Boas and Foley [5], Hvidberg [4]) that both the hexosamine and the water content of the connective tissue decrease during growth in rats. From these and other investigations the amount of the acid mucopolysaccharides seems to be important for the waterbinding mechanism of connective tissue. However, we also found it worth while to study the possible rôle played by the degree of polymerization of the mucopolysaccharides. We therefore have determined the molecular weights of these substances isolated from animals of different ages and after different treatments, in order to provide some information on the waterbinding mechanism of connective tissue.

MATERIALS AND METHODS

As experimental animals were used white male mice, 7—8 weeks old, weighing about 25 g, and white female rats of different ages, namely: 2 days, 10 days, 26 days, 6 weeks, about 3 months, and about 6 months old. In the experiments with mice the groups contained about 40 animals, and in the experiments with rats the groups contained from about 60 to about 25 animals depending on the age. The animals were kept under constant environmental conditions with free access to commercial food-stuff and also to water, except the group of dehydrated mice.

Groups of mice treated in different ways: 1. Control group. 2. Group deprived of water for 7 days. 3. Group treated with oestradiol monobenzoate (Ovex ® Leo), 10 μg in 0.1 ml of vegetable oil, injected subcutaneously in the occipital region on the 1st and the 3rd day; killed on the 7th day. 4. Group treated with cortisone acetate (Organon ®) 1 mg in 0.1 ml (diluted with physiological saline) given intraperitoneally daily from the 1st to the 15 th day, and killed just after.

Only skin from the distal part of the back was used. After depilation the skin and underlaying connective tissue were removed, freeze-dried, and defatted carefully according to the method described by Hvidberg [3]. Then all the tissue pieces from each group were pooled.

Isolation processes. In order to isolate mucopolysaccharides from the dry and defatted tissue the following methods were employed. It appeared necessary to use different techniques depending upon the origin of skin. *Mice:* The minced skin was stored under acetone for a fortnight in order to denature proteins thus rendering these substances insoluble (Schütte and Greiling [6], Jensen [7]). Then the acetone was removed by soaking in distilled water for 2 h. The broth was then treated with acetic acid for 2 days in order to remove iron, and then the broth was neutralised with potassium hydroxide to pH 8—9. The broth was filtered througth 8 layers of gauze, and dialysed against running tap water for three days. To the dialysate were added 3 volumes of absolute alcohol. The precipitate was partly stringy, and partly flocculent. After rinsing with alcohol and ether a white powder was obtained. *Rats*: The above method did not appear to be successful regarding isolation of mucopolysaccharides from rat skin, as the tissue would not disintegrate. Therefore we employed enzyme digestion using a crude extract from pancreas (Pancreatin ® Novo). In agreement with Gardell [8] we found that crystalline trypsin and chymotrypsin were less effective than the pancreatic enzymes. The pH was adjusted to 7—8 and the digestion lasted for three days at room temperature. The broth was then filtered through gauze. The protein split products were precipitated with 10 % trichloroacetic acid after the method of Lundquist [9]. After neutralisation and dialysis the mucopolysaccharides were obtained and rinsed as described above. During the isolation processes care was taken that the temperature did not exceed room temperature and the pH was less than 9. This was done as both high temperature and alkaline reaction involve the danger of degrading effects of mucopolysaccharides (Snellman [10]).

The isolated substances were tested for metachromasia by preparing dry smears. These were fixed in basic lead acetate, and stained with a 0.05 % solution of toluidine blue (Toluidinblau nach Hoyer Riedel-de Haën A. G., Hannover, Germany) at pH 6.5, beforé and after incubation with hyaluronidases. Testicular hyaluronidase was prepared by Leo Pharmaceutical Products, Copenhangen and bacterial hyaluronidase was prepared by dr. V. Faber, the Danish State Serum Institute.

Viscosimetric investigations were performed in the viscosimeter described by Dalgård-Mikkelsen and Kvorning [11].

Chromatographic investigations were undertaken after the isolated substances had been hydrolysed with 2.0 N HCl for 15 h at 100°C in sealed ampullae. Samples containing 10 mg/ml were placed on Whatman filter paper No. 1 in a volume of 20 μl, and run in 2,6-lutidine:water (65:35) for 24 h using the descending type of chromatography. Standard solutions (10 mg/ml) of D-glucosamine hydrochloride (Nutritional Biochemical Corporation) and D-galactosamine hydrochloride (Mann Research Laboratories) were placed on the paper in a volumn of 5 μl each. For the development was used a 0.1 % ninhydrin solution in acetone. For publication the chromatogrammes, were copied in drawings as shown in Fig. 4.

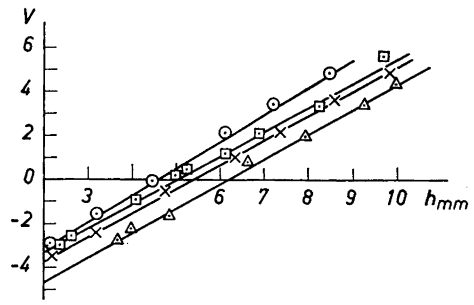


Fig. 1. Osmotic pressure of isolated mucopolysaccharides from skin of mice. The graph shows the relation between height of the air column (abscissae) and the rate of movement of the upper miniscus of the air column (ordinates) in micrometer divisions in 10 min. The values for the equilibrium length are given in Table 1.

⊙ — ⊙ Control (untreated animals)
△ — △ Dehydrated animals
× — × Animals treated with cortisone
⊡ — ⊡ Animals treated with oestradiol

The molecular weight was calculated from osmotic pressure measurements, which were performed by the micro-osmometer described by Christiansen and Jensen [12]. The semipermeable membranes were prepared from a pyroxylin solution (Parlodion "Mallinckrodt") containing 4 g in 100 ml of an alcohol-ether mixture (75:25); to this mixture were added 5 ml of ethylene glycol freshly distilled *in vacuo*. The membranes were dried for about one hour.

RESULTS

From the following reasons we conclude that the substances isolated from the skin of the experimental animals really are mucopolysaccharides, more or less contaminated with impurities. The powders were 1) white or nearly white, 2) highly hygroscopic, and 3) easily soluble in water. 4) The aqueous solutions were viscid, and 5) the viscosity decreased considerably upon incubation with both hyaluronidases. 6) Further more, the substances were strongly metachromatic with toluidineblue, this metachromasia being highly sensitive to testicular hyaluronidase and partly sensitive to the streptococcal enzyme. 7) Chromatography of the hydrolysates showed spots corresponding to authentic glucosamine and galactosamine. 8) Although being not a proof the calculat-

Table 1. The molecular weight of mucopolysaccharides isolated from skin of mice, calculated from the measurement of the osmotic pressure (w = weight of the material in mg per ml, and h = equilibrium length in cm) M means the molecular weight. 1 and 2 refer to two seperate experiments with the same material.

Treatment	w 1	w 2	h 1	h 2	$M \times 10^{-3}$ 1	$M \times 10^{-3}$ 2	$M \times 10^{-3}$ mean	± % of control group	Hexosamine and Water contents * Hex. mean	Hex. sem	Water mean	Water sem
Control	7.02	5.80	0.54	0.46	321	320	321	—	521	± 9	316	± 8
Dehydrated	4.48	4.92	0.55	0.62	201	196	198	−40	481	± 8	211	± 5
Oestradiol	9.01	9.40	0.48	0.50	463	464	464	+44	1 065	±24	540	±15
Cortisone	5.04	5.50	0.51	0.54	251	244	247	−20	528	± 9	261	± 7

* The figures of these columnes are taken from completely equal series published by Hvidberg and Schmidt [16], and Hvidberg [3]. The content of hexosamine is in mg, and the content of water is in g, both per 100 g dry, defatted tissue. sem = Standard error of the mean.

Table 2. The molecular weight (M) of mucopolysaccharides isolated from skin of rats of different ages, calculated from the measurement of the osmotic pressure (w = weight of material in mg per ml, and h = equilibrium length in cm).

Age	w	h	$M \times 10^{-3}$	Hexosamine and Water contents *			
				Hex.		Water	
				mean	sem	mean	sem
2 days	7.58	0.61	266	676	±14	571	±7
10 days	6.01	0.62	239	540	±6	400	±2
26 days	5.59	0.62	219	538	±7	318	±2
6 weeks	5.88	0.74	196	488	±7	272	±4
3 months	5.68	0.84	167	410	±6	228	±2
6 months	6.68	1.15	143	342	±6	218	±2

* The figures of these columnes are taken from an other investigation using equal groups of rats (Hvidberg [4]). Hexosamine and water contents are given in mg and g, respectively, per 100 g dry, defatted tissue. sem = Standard error of the mean.

ed molecular weights do not exclude the anticipation that the substances are mucopolysaccharides.

As seen from Table 1, which shows the molecular weights of substances isolated from mice skin, the molecular weight is considerably lower, when the mice had been dehydrated for seven days. Also cortisone treatment seems to cause a marked fall in the molecular weight, while the oestrogenic treatment causes a considerable increase. According to our experience on the relationship between substrate concentration and osmotic pressure (Jensen [7], Jensen and Marcker [13]), the differences between the molecular weights are likely still greater than the figures apparently indicate. An example of the osmotic measurements are given in Fig. 1.

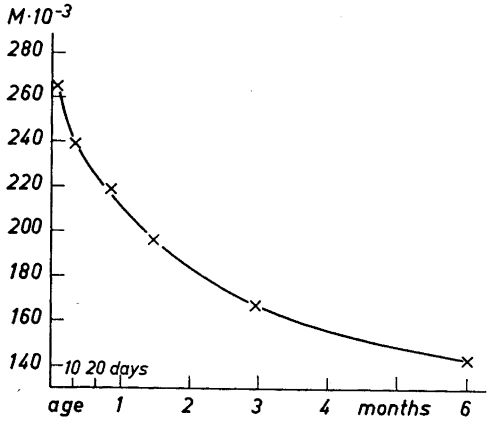


Fig. 2. The relation between age (abscissae) and molecular weight (ordinates) of mucopolysaccharides isolated from skin of rats of different ages.

Table 3. Metachromasia of the isolated mucopolysaccharides from skin of mice and rats.
Intensities: + + + + : very strong reaction
+ + + : strong reaction
+ + : medium reaction
+ : weak reaction
O : no or practically no reaction
The quoted intensities of the metachromatic reactions are based only on the subjective impressions of the authors and involves no exact measurements.

Animals	Group	Intensity of metachromasia		
		Genuine metachromasia	After treatment with bacterial hyaluronidase	After further treatment with testicular hyaluronidase
Mice	Control	+ + +	+	O
—	Dehydrated	+ +	+	O
—	Oestradiol	+ + + +	+	O
—	Cortisone	+ (+ + ?)	+	O
Rats	2 days old	+ + +	+	O
—	10 days old	+ + +	+	O
—	26 days old	+ + +	+	O
—	6 weeks old	+ + +	+ +	O
—	3 months old	+ + +	+ +	O
—	6 months old	+ + +	+ +	O

Table 2 gives the results of the osmotic measurements and the calculated molecular weights of the substances isolated from skin of rats of different ages. It is obvious from Table 2 and from Fig. 2, that the molecular weight decreases with age. The decrease is greatest during the first weeks of life.

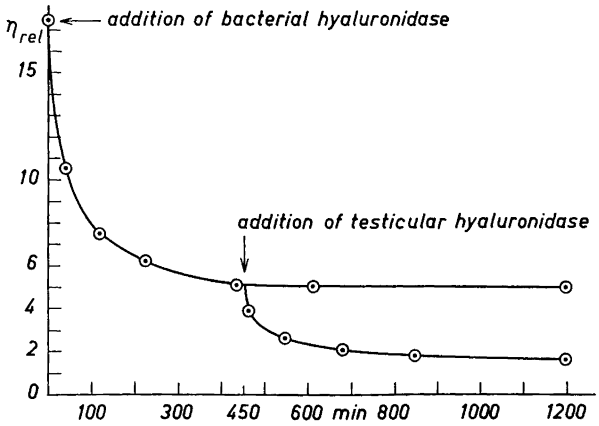


Fig. 3. Degradation of isolated mucopolysaccharides from skin of control mice, after enzymatic incubation, shown by the fall in viscosity. Abscissae: Times after incubation. Ordinates: Relative viscosities.

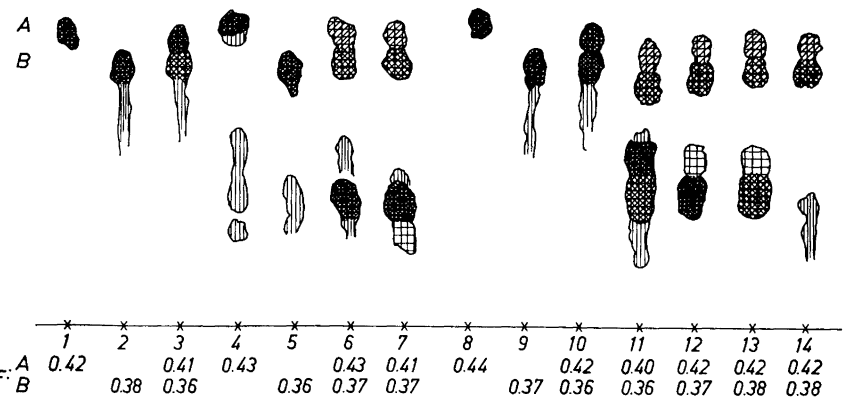


Fig. 4. Drawing of paper chromatogrammes of hydrolyzed mucopolysaccharides isolated isolated from skin of rats and mice, showing the spots of glucosamine (A) and galactosamine (B). Further the R_F-values are given. Other spots are impurities, probably hydrolysates of protein.

1. Authentic glucosamine
2. Authentic galactosamine
3. Authentic glucosamine and galactosamine
4. Hydrolysate of hyaluronic acid (from human umbilical cords)
5. Hydrolysate of chondroitin sulphuric acids (from ox nasal septa)
6. 2 days old rats
7. 10 days old rats
8. As No. 1
9. As No. 2
10. As No. 3
11. 26 days old rats
12. 3 months old rats
13. 6 months old rats
14. Control mice (untreated animals)

From Table 2 it is further seen that hexosamine as well as water content also decrease with age (compare Hvidberg [4]).

Table 3 shows the metachromasia of the isolated substances. We have to point out that the quoted intensities are based only on subjective impressions and involve no exact measurements. The most remarkable observations are that the intensities of the metachromatic reactions in the dehydrated and the cortisone treated animals are weaker than in the normal and especially than in the oestradiol treated mice. As seen the intensities do not appear to change with age. Further it is seen that practically all metachromasia is abolished by treatment with testicular hyaluronidase. When the substances were incubated with streptococcal hyaluronidase an obvious decrease in intensity is seen, but this decrease is not so marked in the older groups of rats.

Fig. 3 gives an example of the viscosimetric investigation showing the degradation of the isolated substance following incubations partly with testicular, partly with streptococcal hyaluronidases. The curves indicate that the preparation contains both hyaluronic acid and chondroitin sulphuric acids.

The chromatographic investigations are shown in Fig. 4 which gives a drawing of the paper chromatogrammes. From this study it might be concluded that the isolated powders contain substances which upon hydrolysis yielded both glucosamine and galactosamine. Perhaps the investigation indicates that the ratio between hyaluronic acid and chondroitin sulphuric acids decreases slightly with increasing age. Unfortunately, we had not so great amounts of the substances which allowed us to perform chromatographic investigations on dehydrated and hormone treated mice and on the 6 weeks old rats. As references were used, besides authentic glucosamine and galatosamine, hydrolysates of hyaluronic acid prepared from human umbilical cords according to Jensen [14] and chondroitin sulphuric acid isolated from ox nasal septa according to Blix and Snellman [15]. As seen most preparations are contaminated with impurities, probably protein split products.

DISCUSSION

As we think it most valuable to know the physico-chemical behaviour of the acid mucopolysaccharides in the state in which they occur in the living connective tissue, we have made use of very mild isolation procedures. However, we are well aware, indeed, that this demand is not obtainable, the substances being always altered during the isolation procedures. But we think that the experiments in this study with the isolated material, help us to give a fairly good picture of the real conditions. The quantitative extraction of the acid mucopolysaccharides is a matter of considerable difficulty, and considering the mild methods here used, we are of the opinion, that only a relative small part of the total amount of the mucopolysaccharides has been isolated at all. This might be considered as a source of error too.

As shown in the experiments with mice the molecular weight of the mucopolysaccharides decreases after intense cortisone treatment. At the same time the relative amount of these substances is not altered as judged from the hexosamine determinations (Hvidberg and Schmidt [16]), but the content of water in the connective tissue is significantly lower. Also in dehydrated mice the molecular weight was found to be considerably lower than in normal animals. According to Hvidberg [3] the relative hexosamine content was somewhat smaller in the dehydrated mice, and naturally the water content of the connective tissue was markedly reduced. On the other hand the molecular weight of the mucopolysaccharides from the oestradiol treated animals was much higher than in normal animals; besides, this treatment nearly doubled the relative amount of mucopolysaccharides, and caused a considerable increase in the water content, (*cf.* Schmidt [2], Hvidberg [3]).

These observations are also interesting when compared to the findings in the metachromatic studies, because the metachromasia of the mucopolysaccharide is closely connected to the degree of polymerization. But it is important to stress once again that we are well aware that these observations are based only on subjective impressions. We have found that the metachromatic intensity is weaker in the cortisone treated animals. Histochemical studies performed by several investigators, *e.g.* Asboe-Hansen [17], have demonstrated that

cortisone treatment decreases the metachromasia of the connective tissue ground substance. This seems to agree with our findings. Dehydrated animals also showed a weaker metachromasia (Table 3). The experiments on these two groups (cortisone treated and dehydrated animals) suggest that a depolymerization of the mucopolysaccharides is followed by a decrease in the metachromasia, and this is, as mentioned above, in agreement with the opinion of most investigators.

In the experiments with rats it is shown that the molecular weight of the mucopolysaccharides is decreasing with age. During the same period a decrease in the water and in the hexosamine content is observed (Table 2, Hvidberg [4]). As may be inferred from the above experiments with hormone treated and dehydrated mice, it is not directly obvious that only the hydrophilic hyaluronic acid is depolymerized with increasing age. The findings with toluidine blue and with chromatography seem to indicate a minor increase in the ratio between chondroitin sulphuric acids and hyaluronic acid. This point of view is supported by the general observation that hyaluronic acid is preferably found in foetal connective tissues (Slack [18]). Furthermore, in experiments on pig skin, Meyer *et al.*[19] found that the ratio of chondroitin sulphate to hyaluronic acid was 1.25 in grown up animals, while this ratio was only 0.2 in the skin of pig embryos.

The fact that connective tissue, containing hyaluronic acid, really binds great amounts of water, is easily explainable when considering the physicochemical properties of hyaluronic acid. This molecule is threadlike (highly asymmetric, Jensen and Carlsen [20]), very large, and carries a great many negative charges (one per disaccharide unit). As it contains many OH-groups there are great possibilities for establishing hydrogen bondings, resulting in a threedimensional network. Thus it is obvious that this mucopolysaccharide is capable of forming a highly hydrophilic gel. It must be anticipated that this gel can bind great amounts of water even when present in low concentrations, as the more asymmetrical the colloidal particles are, the lower is the minimum concentration of the colloid at which gelation occurs (Jirgensons and Straumanis [21]) Although not a proof, it is likely that the water of the connective tissue (also skin) is found in a gel-like structure as no water escapes from the tissue, when it is punctured (Schmidt [2], Gibian [22]). According to our experience hyaluronic acid must be associated with protein in order to form a typical gel (see below).

Thus the ground substance of the connective tissue in which the structural elements are imbedded is a hydrophilic gel. Most probably this gel consists of a protein-hyaluronate complex (Bensley [23], Meyer [24], and Day [25]). The waterbinding mechanism of the connective tissue is most likely closely related to the gel-structure of the ground substance, but this mechanism depends on several, partly unknown, factors. The gel-like structure of the ground substance can be broken and the viscosity destroyed if the hyaluronic acid is depolymerized, (Jensen and Vilstrup [26] and a review by Schmidt [2]). It is a question if this rapid procedure influences the waterbinding mechanism, but from our investigations and the above considerations it might be suggested, that the waterbinding in the gel of the ground substance partly depends on the size of the hyaluronic acid molecules. This theory, already touched by

other investigators (see Altschuler and Angevine [27]), is supported by our findings, that there is a connection between the decrease in the molecular weight and the water content (cortisone treated animals), and between the increase in molecular weight and the water content (oestradiol treated animals). Furthermore it was observed, that when the water had been drawn from the connective tissue (the dehydration experiments), the molecular weight was found to be smaller, this also indicating that the waterbinding mechanism is altered (secondarily?). Finally the relation between the decrease in the molecular weight and the decrease in the water content of the connective tissue during growth also supports the hypothesis here forwarded. It must, however, be added, that the drop in the molecular weight of the isolated mucopolysaccharides during growth might partly be caused by alterations in the ratio between the two types of acid mucopolysaccharides in the connective tissue. As is known chondroitin sulphuric acids have smaller molecular weights than hyaluronic acid and do not have nearly as strong hygroscopic properties as this substance (Jensen [7]).

Acknowledgements. A part of this investigation was carried out in The Laboratory for Investigation of the Pharmacology of Connective Tissue with a grant from the *Danish National Association against Pheumatic Deseases.*

We are very indebted to professor J. A. Christiansen, Ph. D. and to professor K. O. Møller, M. D. for constant interest and advices.

We are grateful to Mrs. Inger Andreassen for technical assistance.

We want to thank Dr. V. Faber, the Danish State Serum Institute, Copenhagen, for preparing the streptococcal enzyme.

We are grateful to Novo Therapeutisk Laboratorium for generous supply of Pancreatin, to Leo Pharmaceutical Products, Copenhagen, for donations of Ovex and testicular hyaluronidase, and to Alfred Benzon A/S for the supply of cortisone acetate, Organon.

REFERENCES

1. Taylor, H. and Sprunt, D. *J. Exptl. Med.* **78** (1943) 91.
2. Schmidt, A. *Studier over Spredningsreaktionen i Bindevæv*, Thesis. Copenhagen 1958.
3. Hvidberg, E. *Acta Pharmacol. Toxicol.* **16** (1959) 28.
4. Hvidberg, E. *Acta Pharmacol. Toxicol.* **16** (1959) 55.
5. Boas, N. and Foley, J. *Proc. Soc. Exptl. Biol. Med.* **86** (1954) 690.
6. Schütte, E. and Greiling, H. *Z. physiol. Chem. Hoppe-Seyler* **302** (1955) 55.
7. Jensen, C. E. *Studier over Hyaluronsyre.* Thesis. Arnold Busck, Copenhagen 1957.
8. Gardell, S. *Arkiv. Kemi* **4** (1952) 449.
9. Lundquist, F. *Acta Physiol. Scand.* **17** (1949) 44.
10. Snellman, O. In Turnbridge. R. E. ed. *Connective Tissue Symposium*, Blackwell, Oxford 1957, p. 97.
11 Dalgaard-Mikkelsen, S. and Kvorning, S. A. *Acta Pharmacol. Toxicol.* **4** (1948) 169.
12. Christiansen, J. A. and Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 1247.
13. Jensen, C. E. and Marcker, K. *Acta Chem. Scand.* **12** (1958) 855.
14. Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 603.
15. Blix, G. and Snellman, O. *Arkiv Kemi, Mineral. Geol.* **19A** (1945) No. 32.
16. Hvidberg, E. and Schmidt, A. *Acta Pharmacol. Toxicol.* **15** (1959) 293.
17. Asboe-Hansen, G. *Bull. histol. appl. et tech. microscop.* **27** (1950) 5.
18. Slack, H. G. B. *Am. J. Med.* **26** (1959) 113.
19. Meyer, K., Davidson, E., Linker, A. and Hoffman, Ph. *Biochim. et Biophys. Acta.* **21** (1956) 506.
20. Jensen, C. E. and Carlsen, F. *Acta Chem. Scand.* **8** (1954) 1357.

21. Jirgensons, B. and Straumanis, M. E. *Colloid Chemistry*. Pergamon Press. Ltd. London 1954.
22. Gibian, H. *Mucopolysaccharide und Mucopolysaccharidasen*. Franz Deuticke, Wien 1959, p. 122.
23. Bensley, S. *Anat. Record* **60** (1934) 93.
24. Meyer, K. *Physiol. Revs.* **27** (1947) 335.
25. Day, T. *J. Physiol.* **117** (1952) 1.
26. Jensen, C. E. and Vilstrup, Th. *Acta Chem. Scand.* **7** (1953) 1128.
27. Altschuler, C. H. and Angevine, D. M. In Asboe-Hansen G. ed. *Connective Tissue in Health and Desease*, Munksgaard, Copenhagen 1954, p. 178.

Received August 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2057–2069

Least Squares Computer Calculations of Equilibrium Constants from Solvent Extraction Data *

JAN RYDBERG** and JAMES C. SULLIVAN

Chemistry Division, Argonne National Laboratory, Lemont, Illinois, U.S.A.

The principle of weighted least squares was used for calculating equilibrium constants from solvent extraction data. Two different programs for digital electronic computers were developed, depending on the type of complexes investigated. With these programs, the equilibrium constants in the systems Th(IV)-acetylacetone-benzene-0.01 M $NaClO_4$, and Zr(IV)-F^- (and $-HSO_4^-$)-thenoyltrifluoroacetone-benzene-2.0 M $HClO_4$, were calculated. The results are discussed and compared with the equilibrium constants originally computed by graphical methods.

The methods used for calculation of equilibrium constants from solvent extraction data have been generally graphical in nature (*e.g.* Connick and McVey [1], Rydberg [2,3], Dyrssen and Sillén [4], and Irving, Rossotti and Williams [5]) These methods do not give an objective measure of precision of the constants and may lead to the calculation of more parameters than are justified by the data. A more accurate procedure is to treat the data according to the principle of least squares which leads to the best values and maximum number of parameters together with their standard deviations.

The application of high speed digital computers to least squares calculations of complexity constants has been outlined in a recent paper (Sullivan, Rydberg and Miller [6]). In that study, solvent extraction was discussed only briefly. The purpose of the present paper is to give a fuller account of the application of these methods to solvent extraction data.

SOLVENT EXTRACTION EQUATIONS

Here we shall consider only the case when mononuclear complexes are formed between a metal ion $M^{\nu+}$ and negatively charged anions A^{r-} and B^{s-},

* Based on work performed under the auspices of the U. S. Atomic Energy Commission.

** On leave of absence from the University of Stockholm, and the Research Institute of National Defense, Stockholm, Sweden.

and where only uncharged complexes MA_vB_p, where $v = (v-ps)/r$, are extracted into the organic solvent. If Q is the partition ratio of all species of M between the organic and aqueous phase, Q can be expressed in terms of concconcentrations of the free ligands, $[A^{r-}]$ and $[B^{s-}]$, as

$$Q = \frac{\sum_{0}^{P'} \lambda_{vp}\beta_{vp}[A^{r-}]^v\,[B^{s-}]^p}{\sum_{n=0}^{N}\sum_{p=1}^{P} \beta_{np}[A^{r-}]^n\,[B^{s-}]^p} \tag{1}$$

where

$$\lambda_{vp} = \frac{[MA_vB_p]_{org}}{[MA_vB_p]_{aq}} \tag{2}$$

and

$$\beta_{np} = \frac{[MA_nB_p]}{[M][A]^n[B]^p} \tag{3}$$

In eqn. 1, $P' \leq P$. For simplicity, charges are here omitted, and index "org" refers to the organic phase, while index "aq" or no index refers to the aqueous phase. Brackets indicate concentrations, and it is understood that the equations refer only to experimental conditions, where the activity factors of all species may reasonably be assumed constant; *cf.* Refs.[7,8]

Few solvent extraction studies of the formation of mixed complexes MA_nB_p have been carried out.[3,5] Instead the purpose has generally been to study the formation of complexes either with an inorganic or with an organic ligand, *cf. e.g.* Ref.[9] The conditions are therefore usually chosen so that no mixed complexes are formed, and so that only species of one of the ligands (say A) are extracted into the organic solvent. Under these circumstances eqn. 1 can be reduced to

$$Q = \frac{\lambda_v\beta_v[A]^v}{1 + \sum_{1}^{N} \beta_n[A]^n + \sum_{1}^{P} \beta_p[B]^p} \tag{4}$$

It is always possible to test if eqn. 1 or 4 is valid, *i.e.* if mixed or only simple complexes are formed, by running experiments at various [A] for constant values of [B]; *cf.* Ref.[10]

Here we shall consider only the cases of eqn. 4 when complexes with one type of ligand can be neglected compared to complexes with the other kind of ligand, *i.e.* when $\Sigma\beta_n[A]^n$ is either much greater or much smaller than $\Sigma\beta_p[B]^p$. The experimental conditions can usually be chosen to fit these mathematical conditions. For example, if complexes with an organic ligand A are being studied, B is chosen to be ClO_4^- which is known to have a negligible tendency to form complexes. Or if, for example, an inorganic ligand B is being studied, which does not form extractable complexes with M, the conditions are chosen so that in the absence of B the partition curve log Q *versus*

log [A] has the slope v, which means that complexes with A in the aqueous phase can be neglected, *i.e.* $\Sigma\beta_n[A]^n \ll 1$.

Thus eqn. 4 can be written (choosing $r = 1$),

$$\text{for } \sum \beta_p[B]^p \ll 1, \qquad \frac{[A]^v}{Q} = \frac{1}{\lambda_v\beta_v}\sum_0^N \beta_n[A]^n \tag{5a}$$

Introducing the abbreviations $x = [A]$, $y = 1/Q$, and $a_n = \beta_n/\lambda_v\beta_v$, eqn. 5a can be written

$$y \cdot x^N = \sum_0^N a_n x^n \tag{6a}$$

Similarly, eqn. 4 can be written ($r = s = 1$),

$$\text{for } \sum \beta_n[A]^n \ll 1, \qquad \frac{[A]^v}{Q} = \frac{1}{\lambda_v\beta_v}\sum_0^P \beta_p[B]^p \tag{5b}$$

Introducing the abbreviations $x = [B]$, $z = [A]^v/Q$ and $a_p = \beta_p/\lambda_v\beta_v$, eqn. 5b can be written

$$z = \sum_0^P a_p x^p \tag{6b}$$

We shall for simplicity refer to eqns 5a and 5b as to the equations representing investigations on "extractable" and "non-extractable" complexes, respectively.

Eqns. 6a and 6b are solved by the methods of weighted least squares as outlined in a previous paper [6]. The equations for the "non-extractable" complexes will be further discussed in a later section.

THE WEIGHT OF THE DATA

The weight ω_i for an individual observation i of unit weight is (*cf.*, *e.g.*, Ref.[13])

$$\omega_i = 1/\sigma_i^2 \tag{7}$$

where here a measure of the variance σ_i^2 is to be obtained from a consideration of the errors σ_i encountered in the experimental work. If the weight of all points is changed by multiplying the variances by a constant factor, this does not change the values of the computed parameters nor their standard deviations (*i.e.* $a_n \pm \sigma_{a_n}$, where σ_{a_n} is computed by external consistency), but changes the value of $S_{\min}$, where

$$S_{\min} = \Sigma\omega_i \; V_i^2 \tag{8}$$

V_i is the difference between the measured value of the function and that calculated with the best set of parameters at that point. $S_{\min}$ has the same statistical distribution as χ^2 (see, *e.g.*, Deming [13] or Fisher [14]) with k degrees of freedom; as the weight is defined above, we can put $S_{\min} = \chi^2$. Here, k is the number of observations (L) minus the number of parameters minus 1,

i.e. $k = L-N-1$. When the mathematical model (here eqn. 5) and the weights are consistent with the experimental data, the mean value of χ^2/k will be equal to 1, with a range of $\pm$ 0.5 at the 70 % probability level (*cf.* Ref.[14]).

TYPES OF ERRORS IN SOLVENT EXTRACTION

We will obtain estimates of the weights by considering the random errors encountered in the experimental work. It is assumed that systematic errors are minimized by appropriate methods.

Only three measures of random errors will be considered, namely errors in Q caused by errors in the counting when radiometric methods are used, errors in Q caused by the measuring technique when other methods are used, and errors in Q caused by errors in the determination of the free ligand concentration.

Errors in Q due to radiometric counting, $\sigma_Q(\mathrm{I})$. In radiometric measurements, the partition ratio Q is determined from the specific radioactivities (*i.e.*, counts per min, cpm, for a fixed amount of sample) of the organic and aqueous phases, I_{org} and I_{aq}, respectively; *i.e.*, $Q = I_{org}/I_{aq}$, provided the measuring conditions are equal for the samples from both phases. The corresponding standard deviations σ_{org} and σ_{aq} are obtained from the number of counts measured; if the time of mesurement is t, $\sigma = \sqrt{I/t}$ (*cf.*, *e.g.*, Ref.[11]). Thus we can define

$$\sigma_Q(I) = Q \sqrt{\left(\frac{\sigma_{org}}{I_{org}}\right)^2 + \left(\frac{\sigma_{aq}}{I_{aq}}\right)^2} \tag{9}$$

Constant percentage error in Q, $\sigma_Q(\%)$. When Q is not directly calculated from radioactivity measurements or when the analyses are carried out in so many different steps that it becomes hard to separate the individual errors involved, the error may be estimated from the spread of Q values at constant ligand concentration. This error will be given in P % of the Q value; thus the percentage standard error is

$$\sigma_Q(\%) = PQ \tag{10}$$

Error in Q caused by error in the ligand concentration, $\sigma_Q(A)$ *and* $\sigma_Q(B)$. The equations for the least squares treatment were developed on the basis that [A] and [B] were free of error [6,12]. However, this is not always the case, though these errors can often be neglected compared to the error in the Q measurement. In order to make possible an approximate test of the influence of an error in the free ligand concentration, this error has to be attributed to Q as the present computor program is set up. It is possible to do so, if Q([A]) and ([B]) are known. At the present, we shall only consider the influence of an error σ_A in [A].

A graphical function Q([A]) is obtained from the smooth line through the points of a log Q plot against log [A]. It has been shown [2] that this line can be represented by

$$\frac{\mathrm{d}\ \log\ Q}{\mathrm{d}\ \log[\mathrm{A}]} = \nu - \bar{n} \tag{11}$$

where $\bar{n}$ is the mean ligand number. This equation may be rearranged to give

$$\mathrm{d}\,Q = 2.303\ Q\ (\nu - \bar{n})\ \mathrm{d}\ \log[\mathrm{A}] \qquad (12)$$

If the standard deviation in log [A] is called $\sigma_{\log \mathrm{A}}$, this equation can also be written

$$\sigma_Q(\mathrm{A}) \approx 2.3\ Q\ (\nu - \bar{n})\ \sigma_{\log \mathrm{A}} \qquad (13)$$

provided σ_A is small compared to [A].*

Generally (in practice) $\sigma_Q(\mathrm{A})$ is $\gg \sigma_Q(\mathrm{B})$. When the error in [B] has to be computed, equations similar to those above can be derived for $\sigma_Q(\mathrm{B})$.

COMPUTER PROCEDURE

Two computers of the Applied Mathematics Division of this laboratory were used: the IBM 704 and the GEORGE. While the 704 program was only designed for the so-called "extractable complexes" where $\nu = N$ (eqn. 6a), the GEORGE program is set up to handle both "extractable complexes" and "non-extractable complexes" because of the simpler mathematical form of eqn. 6b. The input and output data of these programs are given in Table 1. Both computors use a floating point decimal system.

When the 704 program is used for non-radiometric data, $I_\mathrm{aq} \pm \sigma_\mathrm{aq}$ is replaced by $1 \pm \sigma_Q/Q$, and $I_\mathrm{org} \pm \sigma_\mathrm{org}$ is replaced by $Q \pm 0$.

The programs are so arranged that when negative parameter values are obtained, they are automatically omitted and the least squares computations are rerun withouth them; however, the computations yielding the negative parameters are always printed out.

The time at the 704 is about 20 seconds for feeding in the program and data cards and carrying out the computations for a regular program, while the printing out may take from less than a minute to several minutes depending on the length of the problem (number of repetitive runs). The GEORGE program, which uses punched tape, is slightly slower than the 704 program.

INVESTIGATION OF AN EXTRACTABLE COMPLEX **

As an example of the use of the least squares procedure on equations 5a and 6a, the Th(IV)-acetylacetone-benzene-0.01 M $NaClO_4$ system (Rydberg [2]) was chosen (see Fig. 1).

Selection of errors. The procedure used in the experimental paper gives directly I_org and I_aq. The standard deviations σ_org and σ_aq are given for some of the measurements; the rest could be obtained from unpublished primary data. From these standard deviations, $\sigma_Q(I)$ can be calculated accord-

* The logarithmic error $\sigma_{\log K}$ of a constant $K \pm \sigma_K$ is equal to $\sigma_K/2.303K$ only for small values of σ_K/K; *e.g.* for $\sigma_K/K = 0.067$, the error in the computed $\sigma_{\log K}$ is 3 %; *cf. e. g.* Ref.[13]

** See definition p. 2059, and eqns 5a and 6a.

Table 1. Input and output data of the IBM 704 and GEORGE programs.

IBM 704, program 495/CHM 104: Equation $x^N I_{aq}/I_{org} = \sum_0^N a_n x^n$.	
Input	Output
$I_{aq} \pm \sigma_{aq}$	x (given x, and $10^{-c_1} \geq x \geq 10^{-c_2}$
$I_{org} \pm \sigma_{org}$	$I_{aq}/I_{org} = y$
x (free ligand concentration, $10^0 > x > 10^{-10}$)	$a_n \pm \sigma_{a_n}$
N (≤ 8)	$\bar{y}$ (y computed with given equation and a_n values above)
L (number of experimental points)	$\sigma_{\bar{y}}$
Θ_n (indicating a_n parameters to be omitted)	χ^2/k
c_1 and c_2 (integer values between 1 and 10 for $c_1 < c_2$)	
GEORGE, program S-3-128: Equation $z = \sum_0^P a_p x^p$	
Input	Output
z (or z' or z'' according to eqn. 18)	$\sum_0^{2P} \omega_i x_i^p = W$, and W^{-1}
ω_z (or corresponding ω_z' and ω_z'')	$\sum_0^P a_p x^p$
x (free ligand concentration)	a_n and σ_{a_n}
P (≤ 10)	$S_{min} = \sum_0^P \omega_i (a_p x_i^p - z_i)^2$
Θ_n (parameters to be omitted)	
L (number of experimental points)	

ing to eqn. 9 (this is not necessary to do in practice when the program is run on the 704 computer; see Table 1).

A constant percentage error was also tested. From the spread of points with approximately identical x_i values (see Fig. 1), a standard deviation of $\pm$ 0.046 log units was estimated. This corresponds approximately to a 10 % error in Q. Thus according to eqn. 10, $\sigma_Q(\%) = 0.10Q$.

The [A] values are calculated from rather accurately known data, except for the pH value; the pH error was assumed [2] to be $\pm$ 0.02. Because all error

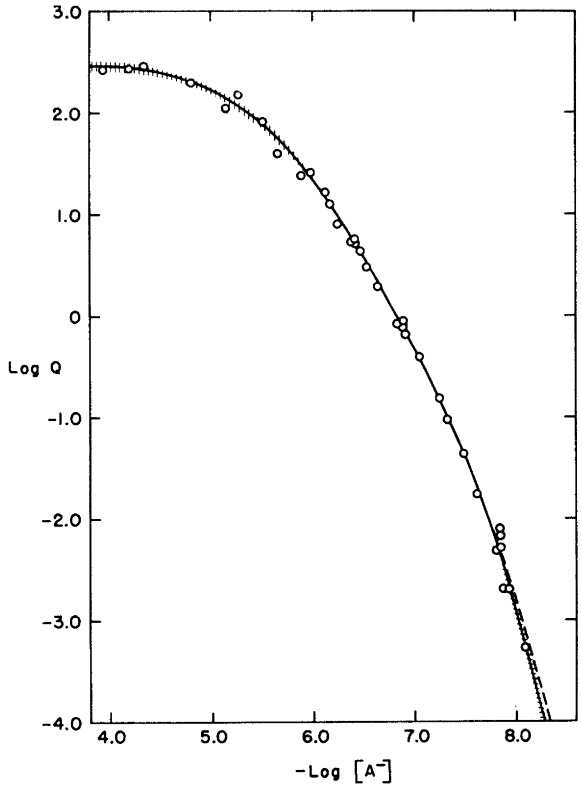


Fig. 1. The Th(IV)-acetylacetone-benzene-0.01 M $NaClO_4$ system. Log Q as a function of $-\log[A^-]$ computed with parameters obtained for a $\sigma_Q(I,A)$ error (solid line) and a $\sigma_Q(\%)$ error (broken line). The shaded area indicates the precision of the computed curve.

in [A] can be attributed to this pH error, we can write $\sigma_{\log A} = \sigma_{pH} = 0.02$. Introducing this into eqn. 13 gives

$$\sigma_Q(A) \approx 0.5\ Q\ (\nu - \bar{n}) \tag{14}$$

The value $\nu - \bar{n}$ is obtained from eqn. 11.

It shall be assumed that $\sigma_Q(A)$ occurs only in combination with the usually much larger $\sigma_Q(I)$. The combined standard deviation in Q is then

$$\sigma_Q(I,A) = \sqrt{(\sigma_Q(I))^2 + (\sigma_Q(A))^2} \tag{15}$$

These standard deviations will be used below to assign weights to the data.

Results of the χ^2 test. When the data were run on the IBM 704 computer, the a_1 values obtained were negative and had standard deviations around or in excess of 100 %. Since negative parameters are of no significance as the equations are derived in this paper, a_1 had to be discarded. Only four para-

Table 2. Computation of χ^2/k for various kinds of errors and various weighing factors in the thorium acetylacetonate system; the left part contains σ_Q (I,A) errors and the right part σ_Q (%) errors.

σ_Q (I)	0.5	1.0	2.0	3.0	4.0	1 σ_Q (%) = est. 10 % error	
σ_Q (A)	χ^2/k	χ^2/k	χ^2/k	χ^2/k	χ^2/k	σ_Q (%)	χ^2/k
0	22.4	5.73	1.43	0.62	0.36	0.5	20.6
0.125	17.9	5.32	1.40	0.63	0.36	1.0	5.14
0.25	12.2	4.82	1.33	0.61	0.35	1.5	2.28
0.50	6.84	3.03	1.12	0.56	0.33	2.0	1.28
0.75	4.61	2.19	0.91	0.50	0.31	2.5	0.82
1.00	3.46	1.69	0.76	0.44	0.28	3.0	0.57

meters (a_0, a_2, a_3 and a_4) were therefore considered in the following. We shall thus assume that eqn. 6a with these four parameters is the correct mathematical model for our experimental data.

The χ^2/k values obtained in the various runs are given in Table 2. The table reveals that the σ_Q(I) value of 1 assumed on the basis of the number of radioactivity counts is too small (*i.e.* χ^2/k is too big, 5.73). The weight should rather correspond to 2 or 3 times σ_Q(I) (*i.e.* $\omega_i = 1/(2 \text{ or } 3 \text{ times } \sigma_Q(\text{I}))^2$), somewhat depending on the correct size of σ_Q(A); a consistent combination is found for 2"σ_Q(I)" and 0.75"σ_Q(A)". These values seem also reasonable from an examination of the original paper; while the pH error may be slightly overestimated, probably the I_{org} and I_{aq} values involve some other errors than the counting errors only; *cf.* Ref.[1]

From Table 2 it is also seen that, if a constant percentage error was justified, it should have been 2.5"σ_Q(%)" rather than 1"σ_Q(%)", where 1σ_Q(%) corresponds to an estimated 10 % error in Q. This can be interpreted as an average 25 % error in the primary Q values, corresponding to $\pm$ 0.11 log units. A reinspection of the points in Fig. 1 does not make such a poor precision in Q improbable.

The equilibrium constants. The parameter values obtained for the various kinds of errors and with different weighing factors are given in Table 3. It is indicated in the table that the parameters and their precisions are unchanged if the errors (here σ_Q(I) and σ_Q(%)) are multiplied by a constant weighing factor. However, there is no constant factor relating the various σ_Q(I,A) errors to each other (see eqn. 15); therefore $a_n \pm \sigma_{a_n}$ varies with these errors, though within a rather limited range. The overlapping of the a_n values illustrates that this is an illconditioned system.

The parameters obtained for the 2"σ_Q(I)"—0.75"σ_Q(A)" combination are given in Table 4, together with the equilibrium constants calculated from the relations

$$k_n = a_n/a_{n-1} \tag{16}$$

and

$$\lambda_\nu = 1/a_\nu \tag{17}$$

Table 3. Parameter values ($a_0 10^{29}$, $a_2 10^{13}$, $a_3 10^7$ and $a_4 10^2$) and χ^2/k (within parenthesis) obtained for the thorium-acetylacetone system when various kinds of standard deviations are multiplied by a constant weighing factor. The upper group corresponds to a counting error only, and the lower group to a percentage error only (parameter values independent of weighing factor), while the twelve central groups represent mixed counting and ligand errors (eqn 15).

σ_Q(A) \ σ_Q(I)	0.5	1.0	2.0	4.0
0	.....	0.510 ± 0.098 0.181 ± 0.006 0.252 ± 0.022 0.344 ± 0.060 (1.43 for 2″ σ_Q (I)″)	.....	
0.125	0.500 ± 0.095 0.181 ± 0.007 0.253 ± 0.022 0.346 ± 0.053 (17.9)	0.508 ± 0.096 0.181 ± 0.007 0.253 ± 0.022 0.345 ± 0.057 (5.32)	0.509 ± 0.097 0.181 ± 0.006 0.253 ± 0.022 0.344 ± 0.059 (1.40)	0.509 ± 0.098 0.181 ± 0.006 0.252 ± 0.022 0.344 ± 0.060 (0.36)
0.50	0.418 ± 0.092 0.183 ± 0.009 0.250 ± 0.024 0.347 ± 0.038 (6.84)	0.474 ± 0.093 0.181 ± 0.008 0.252 ± 0.023 0.347 ± 0.045 (3.03)	0.500 ± 0.095 0.181 ± 0.007 0.253 ± 0.022 0.346 ± 0.053 (1.12)	0.507 ± 0.097 0.181 ± 0.007 0.252 ± 0.022 0.345 ± 0.058 (0.33)
1.0	0.366 ± 0.093 0.187 ± 0.011 0.240 ± 0.025 0.342 ± 0.034 (3.46)	0.417 ± 0.092 0.183 ± 0.009 0.249 ± 0.024 0.347 ± 0.037 (1.69)	0.474 ± 0.092 0.181 ± 0.008 0.253 ± 0.023 0.347 ± 0.045 (0.76)	0.500 ± 0.095 0.181 ± 0.007 0.253 ± 0.022 0.346 ± 0.053 (0.28)
σ_Q (%)	.....	0.366 ± 0.070 0.180 ± 0.018 0.259 ± 0.045 0.330 ± 0.047 (1.28 for 20 % error)	.....	

Table 4 also contains the primary constants β_n and ψ_n given in the original paper, where $\psi = \beta_{4-n}/\beta_4$.

The least squares method shows that the experimental data are not accurate enough to provide two separate equilibrium constants k_1 and k_2, which dominate in the lowest Q region. Thus it is not possible from the experimental data to prove the existence of the first complex, MA^{3+}, while the results support the presence of all the other complexes (MA_2^{2+}, MA_3^{+} and MA_4). The computations of separate k_1 and k_2 values in the original paper has been somewhat biased, because of the reasonable chemical assumption that all stepwise complexes should exist. The k_1 and k_2 values of the ligand number and limiting value methods are therefore given within parenthesis in Table 4.

Table 4. Parameters and equilibrium constants for the thorium acetylacetonate system.

Least square method		Ligand number method [2]		Limiting value method [2]	
Primary parameters	Equilibrium constants	Primary parameters	Equilibrium constants	Primary parameters	Equilibrium constants
$a_0\ 10^{30} = 4.89 \pm 0.93$	$\log k_1k_2 = 15.57 \pm 0.08$	$\beta_1\ 10^{-8} = 1.42$	$(\log k_1 = 8.15)$	$\psi_1\ 10^5 = 1.0 \pm 0.1$	$(\log k_1 = 7.82 \pm 0.16)$
		$\beta_2\ 10^{-15} = 3.80$	$(\log k_2 = 7.43)$	$\psi_2\ 10^{12} = 5.4 \pm 0.3$	$(\log k_2 = 7.73 \pm 0.09)$
$a_2\ 10^{14} = 1.81 \pm 0.07$	$\log k_3 = 6.15 \pm 0.04$	$\beta_3\ 10^{-21} = 9.83$	$\log k_3 = 6.41$	$\psi_3\ 10^{19} = 1.0 \pm 0.2$	$\log k_3 = 6.27 \pm 0.05$
$a_3\ 10^8 = 2.53 \pm 0.22$	$\log k_4 = 5.14 \pm 0.07$	$\beta_4\ 10^{-26} = 8.70$	$\log k_4 = 4.95$	$\psi_4\ 10^{27} = 1.5 \pm 0.5$	$\log k_4 = 5.00 \pm 0.04$
$a_4\ 10^3 = 3.41 \pm 0.45$	$\log \lambda_4$* $= 2.48 \pm 0.06$	$\lambda_4 = 330 \pm 30$	$\log \lambda_4 = 2.52 \pm 0.04$	$\lambda_4 = 330 \pm 30$	$\log \lambda_4 = 2.52 \pm 0.04$

* The λ_4 value is corrected in the same manner as in ref.[2] because of the measuring technique.

From Table 4 it is seen that if only the numerical values of the constants are considered (*e.g.* comparing log k_1k_2 of the least squares method with the log k_1 + log k_2 given with the other methods), the values of the different methods agree well.

Fig. 1 shows the primary data and two curves passing through the points. The solid curve is computed with parameters obtained with the σ_Q(I,A) error, and the dashed curve is computed with the parameters of the σ_Q(%) error (parameters found in Table 3). The shaded band indicates the standard deviations in the computed Q values due to the σ_{a_n} error. Approximately the same precision is obtained with the computations based on the σ_Q(%) error, as expected from the approximately identical σ_{a_n}/a_n values (see Table 3) for the two kinds or errors.

INVESTIGATION OF NON-EXTRACTABLE COMPLEXES *

The complex formation between Zr(IV) and F^- and HSO_4^- in 2 M H^+ are chosen as examples (Connick and McVey [1]) of investigations of inorganic systems, *i.e.* where $\Sigma\beta_n[A]^n \ll 1$ in eqn. 4.

Development of suitable equations. In this case eqns 5b and 6b are valid, because complexes formed between Zr(IV) and ligands other than F^- and HSO_4^- in the aqueous phase are neglected [1]. The organic phase (benzene) is supposed to contain only the organic ligand thenoyltrifluoroacetone (HTTA), and its extractable $Zr(TTA)_4$ complex. It can be found that at constant hydrogen ion concentration, $[TTA^-]$ = const. $[HTTA]_{org}$. Eqn. 5b can be written

$$z' = \left(\frac{[HTTA]^4}{Q}\right)_{[H]} = \frac{1}{Q'} = \frac{1}{Q'_0}\sum_{p=0}^{P}\beta_p[B]^p, \qquad (18a)$$

where $[B] = [F^-]$ or $[HSO_4^-]$. Q' is sometimes called the normalized distribution ratio. Since $\beta_0 = 1$, it follows that at $[B] = 0$, $Q' = Q'_0$, *i.e.* Q'_0 is the normalized distribution ratio in the absence of any complexing inorganic ligand. Eqn. 18a can easily be rearranged to

$$z'' = \frac{Q'_0 - Q'}{Q'\,[B]} = \beta_1 + \beta_2[B] + \beta_3[B]^2 + \ldots \qquad (18b)$$

which shall be used below in the F^- case.

Preparing the data for the computer; selection of errors. Eqns 18a and 18b can be solved by the least squares method using the GEORGE program. The parameters computed for z' will be $a_p = \beta_p/Q'_0$, and for the z'' data $a_p = \beta_{p+1}$ for $0 \leq p \leq P$. In both cases it is assumed that only z contains errors. If the standard deviations corresponding to z' and z'' are called σ' and σ'', it can readily be shown that

* See definition p. 2059, and eqns 5b and 6b.

$$\sigma' = P/Q' \tag{19a}$$

$$\sigma'' = \sqrt{2}\left(\frac{PQ_0'}{Q'\,x}\right) \tag{19b}$$

where P is the percentage error in Q' and Q_0' (assumed to be identical). With these standard deviations the weights are computed according to eqn. 7 for known P values.

The standard deviations in Q can be estimated from the experimental data to be about 4.2 % (using the 5 points on the line in Fig. 1 and in Table 1 of Ref.[1]). Thus we have $P = 0.042$.

Results on the Zr(IV)-HSO_4^--system. This system was tested according to eqns 18a and 19a. For $P > 3$, the higher parameters turned out negative or with standard errors over 100 %; thus it could be established that only three complexes between Zr(IV) and HSO_4^- had to be considered.

Using the primary data and the equations to compute Q' from them (eqn. 17 in Ref.[1])* four positive parameters were obtained, from which the three constants β_1, β_2 and β_3 were calculated. With the 4.2 % standard deviation, χ^2/k is 1.00, demonstrating consistency with the estimates from the primary data.

The equilibrium constants k_1, k_2 and k_3 are given in Table 5, together with Connick and McVey's original results. The agreement is found to be quite good.

Results on the Zr(IV)-F^--system. This system was investigated using eqns 18b and 19b. (The Q_0' value was taken from Table III in Ref.[1]) With the data run on GEORGE, three positive parameters β_1, β_2 and β_3 were obtain-

* There seems to be some inconsistency between the primary data and E_0' in Table III of Ref.[1]

Table 5. Equilibrium constants and their precision for the Zr(IV)-HSO_4^- and -F^- complexes investigated by extraction of the $Zr(TTA)_4$ complex into benzene [1]. Constant ionic media: 2 M $HClO_4$ at 25°C.

Ligand	Equilibrium constants			Reference
	k_1	k_2	k_3	
HSO_4^-	460	53 ± 4 *	$1^{+1}_{-0.5}$ *	Connick and McVey
»	431 ± 30 ***	54 ± 7 ***	0.83 ± 0.43 ***	This work
F^-	$(6.3 \pm 0.6)\ 10^5$**	$(2.1 \pm 0.2)\ 10^4$**	$(6.7 \pm 2.0)\ 10^2$**	Connick and McVey
»	$(6.30 \pm 0.52)\ 10^5$	$(1.94 \pm 0.22)\ 10^4$	$(7.5 \pm 1.1)\ 10^2$	This work

* Standard deviation in k_2 is estimated [1] to 8 %, and in k_3 to plus or minus a factor of 2

** Standard deviations in k_1 and k_2 is estimated to 10 %, and in k_3 to 30 %.[1]

*** Computed with E_c^0 from E_c, γ and $(HK)_b$ according to eqn. 17 in Ref.[1]

ed. In order to obtain $\chi^2/k = 1.00$, the standard deviation in Q' and Q_0' has to be 6.3 %. Thus, in this case, the original estimate of 4.2 % was somewhat too small.

The equilibrium constants are given in Table 5; they are seen to agree well with those given in the original paper. Particularly impressive is the good graphical estimates of standard deviations in the equilibrium constants made by the original authors.

Using the equilibrium constants computed with the least squares methods, Q' can be calculated as a function of the free ligand concentration, and the curves thus obtained may be compared with the corresponding curves in the original paper. The curves for both the F^- and HSO_4^- systems were found to coincide over the investigated range with the curves given in the original paper.

Acknowledgement. We wish to express our appreciation to Dr. W. Miller, Mr. W. Greenhow and Mrs. M. Hanson of the Applied Mathematics Division, Drs. J. C. Hindman and A. Zielen of the Chemistry Division, for the valuable assistance rendered to us in this investigation.

REFERENCES

1. Connick, R. E. and McVey, W. H. *J. Am. Chem. Soc.* **71** (1949) 3182.
2. Rydberg, J. *Acta Chem. Scand.* **4** (1950) 1503.
3. Rydberg, J. *Arkiv Kemi* **8** (1955) 101, 113; *Rec. trav. chim.* **75** (1955) 207.
4. Dyrssen, D. and Sillén, L. G. *Acta Chem. Scand.* **7** (1953) 663.
5. Irving, H., Rossotti, F. J. C. and Williams, R. J. P. *J. Chem. Soc.* **1955** 1906.
6. Sullivan, J. C., Rydberg, J. and Miller, W. *Acta Chem. Scand.* **13** (1959) 2023.
7. Glueckauf, E., McKay, H. A. C. and Mathieson, A. R. *Trans. Faraday Soc.* **47** (1951) 437.
8. McKay, H. A. C. *Trans. Faraday Soc.* **48** (1952) 1103.
9. Morrison, G. H. and Freiser, H. *Solvent Extraction in Analytical Chemistry*, J. Wiley and Sons, New York 1957.
10. Sullivan, J. C. and Hindman, J. C. *J. Am. Chem. Soc.* **76** (1954) 5931.
11. Friedlander, G. and Kennedy, T. W. *Nuclear and Radiochemistry*, J. Wiley and Sons, New York 1955.
12. Rydberg, J. *Acta Chem. Scand.* **14** (1960). *In print.*
13. Deming, W. E. *Statistical Adjustment of Data*, J. Wiley and Sons, New York 1948.
14. Fisher, R. A. *Statistical Methods for Research Workers*, Oliver & Boyd, Edinburg 1954 (12th Edition).

Received August 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2070–2074

Synthesis of Uracil-2, 4, 5, 6-^{14}C with High Specific Activity

Studies in Microsynthesis I

GÖSTA EHRENSVÄRD and JAN LILJEKVIST

Institute of Biochemistry, University of Lund, Lund, Sweden

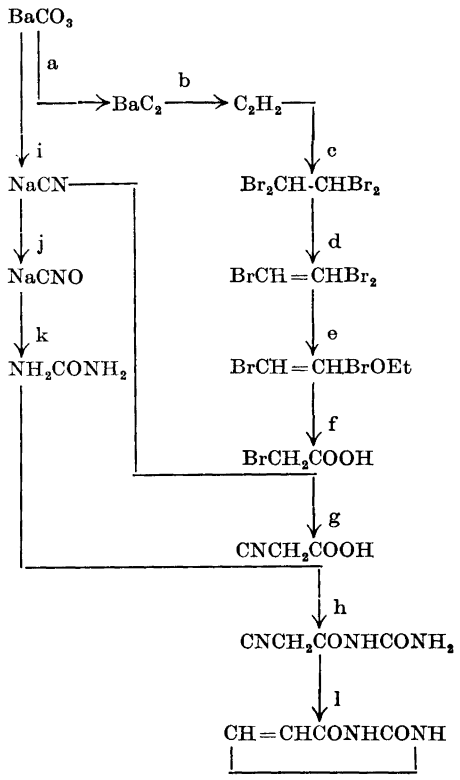
A method for preparing 2, 4, 5, 6-^{14}C-uracil in small scale from $BaCO_3$-^{14}C of highest possible specific activity is presented. The synthesis is carried out in the 100 μmole range giving a total yield of 10 % of 2, 4, 5, 6-^{14}C-uracil the activity of wich being unchanged from that of the starting material. The technique used may find applications in other fields of organic micro-synthesis.

In the course of an investigation on the chemical and biochemical effects of nuclear decay of some atom species incorporated into the molecular structure of organic compounds it was found necessary to develop procedures for multistep synthesis in the 25—250 μmole range. A special problem has been to synthesize uracil and thymine with a maximal ^{14}C activity (round 100 μC/μmole) in order to study the consequences of the decay of 5-^{131}I-uracil, and the overall effects of the $^{14}C \rightarrow {}^{14}N$ transition [1]. The starting material has been barium carbonate with an activity of 24.75 μC/μmole and the synthesis achieved according to the scheme in Table 1.

Each of the above conventional steps (for references see Table 1) has been modified to enable work in the 100 μmole range. The present procedure, as described below, gives a total yield of uracil from barium carbonate of 10 % with no dilution of the ^{14}C-activity. Of the different single steps the preparation of barium carbide has been considerably simplified, enabling the reduction of the barium carbonate with metallic barium to be carried out without the presence of helium [2]. Step *f*, the hydrolysis of 1,2-dibromovinyl-ethyl-ether to bromoacetic acid [5,6] was considerably difficult to run in small scale until a suitable closed micro vessel was employed. Step *l* involving the hydrogenation of cyanoacetylurea to uracil was found to give very poor yield as carried out according to the original procedure of Rupe *et al* [16]. In acetate buffer of pH 4.15, however, the yield was consistently 45 %.

As regards the technical aspect of the present work the aim has been to keep the number of transfers as low as possible. Reaction $c \rightarrow l$ are thus carried

Table 1.



Reaction steps	References
a	2
b	3
c	3
d	4
e	5, 6
f	5, 6
g	7, 8
h	9, 10
i	11, 12
j	13, 14, 15
k	15
l	16

out in the same reaction vessel, the construction of which enabling amount of solutions of around 500 μl to be effectively closed off, thus avoiding procedures involving heating under reflux. With regard to the latter, we have found that any reflux procedure in the 100 μmole range especially in the case of a two-phase system, involve considerable losses, and should be replaced with closed space procedures. The reaction vessel employed (see Fig. 1) could act as a micro-autoclave, standing a pressure up to 3 atm., as a microhydrogenation vessel, and as a centrifuge tube.

EXPERIMENTAL

Barium carbide-^{14}C. 49.3 mg barium carbonate (250 μmole, spec. act. 24.75 μC/μmole) was weighed on a small aluminum foil (weight about 50 mg) and a lump of ether washed barium metal, corresponding to the double theoretical amount was added. The foil was wrapped around the reactants to form a small pellet, which was put in a 4 ml Pyrex test tube. The tube was heated with a burner to red heat. The reaction was complete in a few seconds and the tube was allowed to cool. The tip containing the reaction mixture was broken off and crushed to a fine powder in a diamond mortar. This powder was used directly for preparing acetylene.

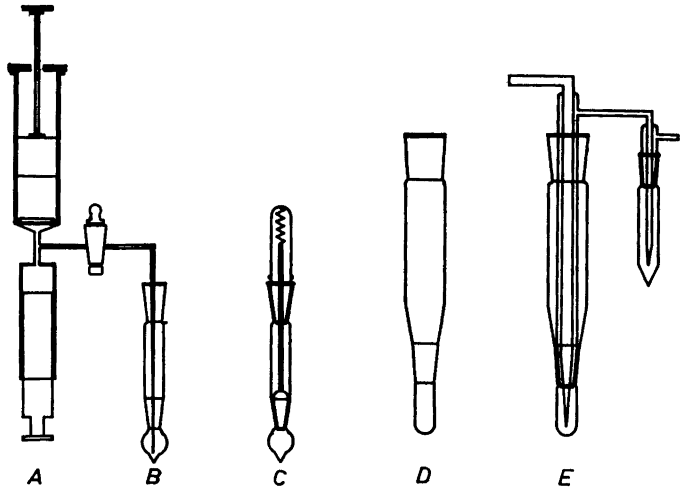


Fig. 1. Gas generator and reaction vessel for small-scale synthesis.

The gas generator A consist of two connected ordinary all-glass syringes, one holding 25 ml, the other 10 ml. In the bottom part of the upper, larger syringe a close-fitting porcellain filter plate is placed, covered with a filter paper disc, cut to exact size. Lubricants were avoided; the stopcock of the assembly was of non-lubricated Teflon-type. The arrangement permits a quantitative generation of acetylene from barium carbide in a closed system, see text.

The receiver tube B fulfills a multiple function as reaction vessel, autoclave and centrifuge tube. The bottom compartment with a volyme of 1 000 μl can be closed off by fitting a stopper into the lower joint, the shaft of the stopper extending 1 cm above the top opening of the vessel. Stopper and joint could be of standard B5- or B7-type, carefully ground. The upper joint of the vessel, a B 10, is closed with a ground stopper, containing a spring housed in the top part, thus enabling the exertion of a pressure upon the shaft of the lower stopper. The tube and its upper stopper are held together with a clip for spherical joints. By this means the small 1 000 μl compartment could act as a micro-autoclave standing a pressure up to 3 atm. Hydrogenation is likewise easy to carry out by connection the vessel to a hydrogen tank *via* an arrangement enabling the flushing out of air. Centrifugation could be carried out in a swing-out head centrifuge at 1 000 $\times$ *g*.

Sodium cyanide-^{14}C was prepared from barium carbonate by a modification of the procedure of Jeanes [11]. After 2.5 hours heating of an intimate mixture of barium carbonate 39.4 mg, metallic sodium 40 mg and zink powder 200 mg in a porcelain combustion boat housed in a quartz tube in ammonia atmosphere, the contents are worked up as follows. The porcelain boat was transferred to a distillation unit, 5 ml 20 % perchloric acid added and 10 ml of distillate collected in 240 μl 1 N sodium hydroxide in a receiver vessel. The solution was now divided into two equal parts and transferred to two vessels of the type shown in Fig. 1 D. The alkaline solution was titrated with 0.1 N silver nitrate solution to a faint turbidity thus enabling a quantitative estimation of the yield of cyanide. Then 250 mg sodium hydrogen carbonate was added and the silver cyanide was precipitated by addition of an equal amount of 1 N silver nitrate solution as titrated before. The silver cyanide was centrifuged off into the smaller compartment of the vessel. After washing with water and acetone, twice with each, the dry silver cyanide samples could be stored for further working up to sodium cyanide.

The conversion of silver cyanide into sodium cyanide was accomplished by inserting into the lower joint a unit (see Fig. 1 E) enabling the sweeping out of hydrogen cyanide with nitrogen. 400 μl 1 N hydrochloric acid was added above the now closed lower joint and the stream of nitrogen accordingly adjusted. After a momentary lifting of the cone,

the hydrochloric acid was allowed to react with silver cyanide. The hydrogen cyanide swept out is absorbed in 120 μl 1 N sodium hydroxide thus yielding a concentrated solution of sodium cyanide, to be immediately utilized whether for making cyanoacetic acid or urea. The time for sweeping out the hydrogen cyanide could be kept as low as 15 min by occasional gentle heating of the reaction mixture. A yield of more than 90 % of concentrated sodium cyanide solution from barium carbonate is obtained.

Urea-^{14}C. The concentrated sodium cyanide solution (120 μl) prepared as above was transferred to a 4 ml centrifuge tube containing freshly prepared copper hydroxide (precipitated from 13.10 mg cryst. copper sulphate). The mixture was cooled in an ice bath and the cyanide was oxidized to cyanate by the dropwise addition of a potassium permanganate solution (12.18 mg in 400 μl of water), excess being destroyed with hydrogen peroxide. After the manganese dioxide formed was separated off by centrifugation and washed three times with water the supernatants were combined and transferred to reaction vessel C (see Fig. 1) and evaporated to round 200 μl *in vacuo*. After addition of 13.2 mg ammonium sulphate and 50 μl concentrated aqueous ammonia the tube was immediately closed off as shown in Fig. 1 C and heated under gentle shaking at 70° for 40 min. The tube was allowed to cool, opened and the contents evaporated to dryness *in vacuo*. The dry residue was extracted 4 times with *n*-butanol with shaking at 70° for 20 min each time. The extracts were evaporated *in vacuo* to dryness at 50°. The dry urea was taken up in 50 μl of water immediately before its further conversion to cyanoacetyl-urea (see below).

Bromoacetic acid- 1,2-^{14}C from barium carbide-^{14}C. The smaller syringe of the plunger arrangement (see Fig. 1, A, B) was separately filled with 4 ml of 1 N hydrochloric acid and attached to the T-tube, carefully avoiding any entrance of the acid into the system. The stopcock should at this stage be open. Freshly prepared barium carbide from 49.3 mg of barium carbonate was carefully deposited on the filter paper at the bottom part of the 25 ml syringe. The plunger was put in place as tightly to the bottom as possible. After this the stopcock is closed, the small vessel B is charged with 100 μl of bromine and 100 μl of water and is attached to the rest of the assembly, so that the capillary outlet safely dips under the surface of the bromine. The plunger of the acid-filled syringe is now slowly pressed up allowing not more acid than to moisten the filter paper disc into the 25 ml syringe, thus starting the reaction. When the gas evolution has started the plunger of the lower syringe is manipulated downwards thereby sucking back most of the acid from the upper system. Excess pressure caused by occasional friction of the plunger is to be avoided. The stopcock is now cautiously opened enabling the gas to pass through the bromine at round 1 bubble per second. Conveniently the upper plunger could now be pressed down with a micrometer screw. When all gas is driven out of the system a small amount of acid is again allowed into the upper compartment — the stopcock being closed at this stage — and the procedure being repeated until no more gas is evolved.

The reaction vessel D, now containing *1, 1, 2, 2, -tetrabromoethane* plus excess bromine and water is centrifuged for a few minutes enabling all droplets of the reaction procedure to collect in the tip. 100 μl of concentrated formic acid is added [17], the mixture gently heated at the water bath to 60° until decolorization, cooled to 10° and recentrifuged. The water solution above the tetrabromoethane droplet is carefully sucked away with a capillary pipette as complete as possible; the remainder of water is removed by storing the open tube over phosphorus pentoxide in a desiccator over night at ordinary pressure.

To the reaction vessel, now containing around 15 μl of dry 1,1,2,2,-tetrabromoethane, 150 μl of a sodium ethoxide solution (50 mg of sodium per 1 000 μl of ethanol) is added, corresponding to 315 μ-atomes of sodium. The bottom part of the reaction vessel is then closed with the stopper arrangement (see Fig. 1) and the content heated to 60° in a small oilbath under shaking (a "Microid flask shaker" is used throughout). After 1 h the vessel is allowed to cool off, is opened and 300 μl of water added. After centrifugation for 10 min a droplet slightly colored, has separated out, representing mainly *1,2-dibromovinyl-ethyl ether*. A check should be made that actually all oil is separated from the alcohol-water supernatant by adding around 50 μl of additional water. In case of cloudiness the vessel should be recentrifuged. The supernatant alcohol-water solution is now removed as complete as possible with a capillary pipette, 300 μl of water added, and the vessel closed again and heated in an oilbath under shaking at 110° for 1 h. At this stage the dibromovinyl-ethyl ether has become completely hydrolysed; no cloudiness should appear upon cooling of the content of the tube. This solution represents *bromoacetic acid-1,2-^{14}C*.

Uracil-2,4,5,6-^{14}C from bromoacetic acid-1,2,-^{14}C. The acid solution containing the bromoacetic acid-1,2-^{14}C is now titrated under gentle shaking with 1 N sodium hydroxide, using an Agla micro-syringe with a fine glass capillary entering the solution, thymol blue being used as an indicator. As an average of 20 experiments 120 μl of 1 N sodium hydroxide solution is required for neutralization. 120 μl of the concentrated sodium cyanide solution prepared as above is now added, the vessel closed and heated for 20 min in an oilbath at 100°. After cooling, the vessel is opened, 150 μl of 1 N hydrochloric acid added and the content evaporated to dryness. This procedure is carried out by attaching the vessel *via* the upper joint to a B 10 male joint, connected by a flexible arrangement to a water aspirator pump. During the evaporation procedure the vessel is vigorously shaken and heated to 30°; at sufficient shaking frequency splashing is prevented. A pilot test of this procedure is recommended. To the dry residue, now representing *cyanoacetic acid-1,2,3-^{14}C* plus salts 15 μl of a water solution of urea prepared as above is added with a micro pipett followed by the addition of 500 μl of acetic anhydride. The solids are suspended in the anhydride and the mixture now heated to 60° for 20 min under gentle shaking; the vessel could at this stage be open. After cooling down the content is evaporated to dryness, as previously described, yet at 50°. The dry residue representing *cyanoacetylurea*, plus salts, is dissolved in 500 μl of sodium acetate buffer pH 4.15 ionic strength 0.1 and approximately 20 mg of Raney nickel (calculated dry weight) is added as a thick paste from an ordinary alcoholic suspension. The upper joint of the reaction vessel is safely connected to a hydrogen tank *via* an arrangement enabling the flushing out of air by intermittent suction and application of hydrogen. The subsequent hydrogenation of cyanoacetyl urea is carried out at a hydrogen pressure of 2.5 atm under vigorous shaking at 65° during 1 h. After release of the hydrogen pressure the reaction vessel is centrifuged and the supernatant is withdrawn with a micro pipett from the catalyst, the latter washed once with 100 μl of water and again centrifuged. The combined solutions are chromatographed on a 23 cm wide strip of Whatman 3 MM paper, the solvent system being methylethylketone-acetone-water-formic acid (100 %) 80:4:12:2 (Reio [17]). R_F for uracil in this system is 0.25; for localization of the compound the paper was photographed after drying in UV-light. After repeated elution of the uracil-containing paper region with a solution of acetone-1 N hydrochloric acid 4:1, totally 40 ml, the evaporated eluate was rechromatographed and again eluted in the same way. Careful evaporation to dryness gives a residue of *uracil-2,4,5,6-^{14}C*, the purity of which was checked by its UV-absorption curve. Yield of uracil from barium carbonate was 10 % with a specific activity of around 100 μC per μmole.

REFERENCES

1. Ehrensvärd, G., Liljekvist, J., Mosbach, K. and Fritzson, P. *Acta Chem. Scand.* **12** (1958) 485.
2. Cheronis, N. D. *Micro and Semimicro Methods*, in Weisberger *Technique of Organic Chemistry*, Vol. VI, 3966 Interscience New York 1954.
3. Sabanejeff, A. *Anal. Chem.* **178** (1875) 113.
4. Lemoult, M. *Bull soc. chim. France*, Sér. 3 **33** (1905) 194.
5. Crompton, H. and Vanderstichele, P. L. *J. Chem. Soc.* **117** (1920) 691.
6. *cf Beilsteins Handbuch der Organischen Chemie*, 1. Erg.w. **1**, p. 377.
7. Fiquet, E. *Bull. soc. chim. France*, Sér. 3 **25** (1901) 593.
8. Fritzson, P. *Scand. J. Clin & Lab. Invest.* **4** (1952) 375; *Chem. Abstr.* **47** (1953) 9917 d.
9. Traube, W. *Ber.* **33** (1900) 1380.
10. Baum, F. *Ber.* **41** (1908) 530.
11. Jeanes, J. K. *Science* **118** (1953) 719.
12. Prelog, V., Urech, H. J., Bothner-By, A. A. and Wuersch, J. *Helv. Chim. Acta* **38** (1955) 1106.
13. Smith, L. H., Yates, P. *J. Am. Chem. Soc.* **76** (1954) 6080.
14. Gall, H. and Lehman, G. *Ber.* **61** (1928) 670.
15. Haley, E. E. and Lambooy, J. P. *J. Am. Chem. Soc.* **76** (1954) 2926.
16. Rupe, H., Metzger, A. and Vogler, H. *Helv. Chim. Acta* **8** (1925) 850.
17. Reio, L. *J. Chromatography* **1** (1958) 338.

Received August 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2075–2081

Multiplicity in the Interpretation of Linear Kinetics *

ERIK JØRGENSEN and JANNIK BJERRUM

The Royal Danish School of Pharmacy, and Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark

Spectrophotometric investigations of multicomponent linear kinetics may lead to several possible interpretations when the spectra of the intermediates are unknown. This is illustrated for an investigation by Schläfer *et al.*[1] on the acidic aquation of the tris(ethylenediamine) to the *cis*-diaquobis(ethylenediamine)chromium(III) ion *via* an unknown intermediate.

Schläfer *et al.* consider only one of the two existing possibilities, *viz.* the one according to which the intermediate should exist in dominating amounts, and have approximately the same spectrum as the luteo ion. However, we are able to show that the other existing possibility, according to which the intermediate is found only in relatively small amounts, is the more probable in view of our results [2] on the aquation of the corresponding hexammine chromium ion and the requirements which the experiments place on the spectrum of the intermediate. This interpretation is further substantiated by Schläfer *et al.*'s experiments [3] on the racemization of the optically active luteo ion.

In our opinion the intermediate which is formed in acidic solution is simply an aquopentammine ion, $Cr\,en_2(enH)H_2O^{++++}$, stabilized by the uptake of a hydrogen ion on the displaced amino group.

It has long been recognized that a system of first order partial reactions leads to a rate expression which is linear in exponential functions [4]. Several authors [5-7] have discussed such systems and one of the present authors has introduced non-orthogonal matrices for the treatment of data additively composed of the properties of the various species [8].

The experimental data may be analyzed into a number of exponential functions, but the fixing of the proper sequence of the exponential coefficients requires additional information.

In a kinetic system of n components the time-dependence of the total property $U = \tilde{\boldsymbol{u}}\,\boldsymbol{x}$, where $\boldsymbol{u}$ is the property vector and $\boldsymbol{x}$ a concentration vector, can be described by

$$U = \tilde{\boldsymbol{u}}\lambda\{\exp(-\lambda_1 t),\exp(-\lambda_2 t), \ldots,\exp(-\lambda_{n-1} t),1\} \qquad (1)$$

* Presented in part at the International Conference on Coordination Chemistry, London 1959.

where $\boldsymbol{u}_\lambda$ is the coefficient vector. The exponential coefficients λ_i may be arranged in several different ways in relation to the reaction paths involved. Each arrangement leads to a requirement for the properties of the species, unique only for consecutive, irreversible reactions. In this case $(n-1)!$ sets of properties, $\boldsymbol{u}$, form the possibilities, and as there is only one proper sequence a choice must be made. The relationship between the property vector, $\boldsymbol{u}$, and the coefficient vector, $\boldsymbol{u}_\lambda$, is given by the previously derived expression [8]

$$\boldsymbol{u} = (x_1^0)^{-1}\widetilde{P}\boldsymbol{u}_\lambda \tag{2}$$

where x_1^0 is the initial concentration of the first component, and P a matrix constructed for the purpose.

The occasion for this paper is a discussion by Schläfer and Kling [1] on the properties of an intermediate shown to be formed as the first reaction product in the acidic hydrolysis of the tris(ethylenediamine)chromium(III) ion. Their discussion is based on the assumption that the spectrum of the intermediate which according to their calculations is formed in relatively high concentrations (up to $\sim$ 80 %) is very close to the spectrum of the luteo ion. For this reason they are forced to make improbable conclusions regarding the constitution of the intermediate. We have therefore reconsidered Schläfer's measurements with a view to an alternative explanation.

According to Schläfer and Kling, the family of spectral curves shows common isosbestic points and the liberation of one ethylenediamine molecule from the tris(ethylenediamine) chromium ion to be the first distinct step. The values of the rate constant calculated with this assumption show, however, a marked dependence on the progression of the aquation. Therefore, the reaction mechanism is proposed to be the following:

$$\mathrm{A} \xrightarrow{k_1} \mathrm{X} \xrightarrow{k_2} \mathrm{B}$$

where the consecutive rate constants are denoted by k_1 and k_2. A stands for the tris(ethylenediamine)chromium ion, and B for the *cis*-diaquobis(ethylenediamine) chromium ion, while X is the unknown intermediate.

Instead of the proper expression for the dependence of the optical density, E_t, on time, t, Schläfer and Kling use a pseudo-zero order rate law as a working hypothesis. In our opinion this is an incorrect simplification. Therefore, we have found it necessary to recalculate their data in terms of exponential functions. We have attempted to get the best representation of the data using (1) in the form

$$E_t = a_0 + a_1\exp(-k_1t) + a_2\exp(-k_2t) \tag{3}$$

where the coefficients a_0, a_1 and a_2 are constants for each wavelength.

Table 1 contains in the first column the time in hours. In the second column are given Schläfer and Kling's experimental values for the optical densities, E_t, with the corresponding uncertainties. In the third column are given the values calculated from Schläfer's linear representations and the deviations from the experimental results. Similarly, the fourth column contains the values calculated from our linear combinations of exponential functions. It is seen

that our theoretical formulae with only one significant figure in the rate constants fit the experimental material at least as well as Schläfer and Kling's linear approximations. As the reaction proceeds, a further aquation of the reaction products takes place. Consequently, we have disregarded all points past 63 h as being influenced by this further aquation.

It is now possible to calculate the spectrum of the intermediate by means of the numerical values of a_0, a_1 and a_2 in (3). Depending upon the choice of k_1 and k_2 between the rate constants, 0.06 and 0.008 h^{-1}, we get two different

Table 1. Optical density, E_t, of 0.01 M tris(ethylenediamine) chromium(III) ion in 0.1 M $HClO_4$ as a function of time at 30°C. Experimental values and values calculated from Schläfer's linear representation (S) and from our sets of exponential functions (J).

	(S)		(J)
340 mμ:	$0.507-0.001959t$		$0.174-0.351\ \exp(-0.008t)$ $-0.018\ \exp(-0.06t)$
Hours	experimental	calculated(S)	calculated(J)
0	0.509 (±0.001)	0.507 (−0.002)	0.507 (−0.002)
3	0.501 (±0.001)	0.501 (0.000)	0.502 (+0.001)
6	0.494 (±0.001)	0.495 (+0.001)	0.496 (+0.002)
9	0.490 (±0.001)	0.489 (−0.001)	0.490 (0.000)
15	0.478 (±0.001)	0.478 (0.000)	0.478 (0.000)
20	0.468 (±0.001)	0.468 (0.000)	0.468 (0.000)
23	0.462 (±0.001)	0.462 (0.000)	0.461 (−0.001)
27	0.452 (±0.001)	0.454 (+0.002)	0.453 (+0.001)
33	0.441 (±0.001)	0.442 (+0.001)	0.441 (0.000)
37	0.433 (±0.001)	0.435 (+0.002)	0.433 (0.000)
41	0.425 (±0.001)	0.427 (+0.002)	0.425 (0.000)
45	0.418 (±0.001)	0.419 (+0.001)	0.418 (0.000)
48	0.411 (±0.002)	0.413 (+0.002)	0.412 (+0.001)
51	0.406 (±0.001)	0.407 (+0.001)	0.407 (+0.001)
54	0.402 (±0.001)	0.401 (−0.001)	0.401 (−0.001)
58	0.393 (±0.002)	0.393 (0.000)	0.394 (+0.001)
63	0.384 (±0.002)	0.384 (0.000)	0.386 (+0.002)
	(S)		(J)
390 mμ:	$0.139+0.000715^{*}t$		$0.261-0.124\ \exp(-0.008t)$
0	0.139 (±0.001)	0.139 (0.000)	0.137 (−0.002)
3	0.140 (±0.001)	0.141 (+0.001)	0.140 (0.000)
6	0.143 (±0.001)	0.143 (0.000)	0.143 (0.000)
9	0.145 (±0.001)	0.145 (0.000)	0.146 (+0.001)
15	0.149 (±0.001)	0.150 (+0.001)	0.151 (+0.002)
20	0.153 (±0.001)	0.153 (0.000)	0.155 (+0.002)
23	0.157 (±0.001)	0.155 (−0.002)	0.158 (+0.001)
27	0.161 (±0.001)	0.158 (−0.003)	0.161 (0.000)
33	0.166 (±0.001)	0.163 (−0.003)	0.166 (0.000)
37	0.168 (±0.001)	0.165 (−0.003)	0.169 (+0.001)
41	0.172 (±0.001)	0.168 (−0.004)	0.172 (0.000)
45	0.174 (±0.001)	0.171 (−0.003)	0.174 (0.000)
48	0.178 (±0.002)	0.173 (−0.005)	0.177 (−0.001)
51	0.179 (±0.001)	0.175 (−0.004)	0.179 (0.000)
54	0.182 (±0.002)	0.178 (−0.004)	0.180 (−0.002)
58	0.184 (±0.001)	0.180 (−0.004)	0.183 (−0.001)
63	0.188 (±0.001)	0.184 (−0.004)	0.186 (−0.002)

* This value is not calculated by Schläfer. It is estimated by means of the two other slopes.

Table 1. (Continued)

440 mμ:	(S) $0.633-0.002212t$	(J) $0.251+0.406\exp(-0.008t)$ $-0.026\exp(-0.06t)$	
0	0.631 (±0.001)	0.633 (+0.002)	0.631 (0.000)
3	0.625 (±0.001)	0.626 (+0.001)	0.626 (+0.001)
6	0.619 (±0.002)	0.620 (+0.001)	0.620 (+0.001)
9	0.616 (±0.002)	0.613 (−0.003)	0.614 (−0.002)
15	0.602 (±0.002)	0.600 (−0.002)	0.601 (−0.001)
20	0.590 (±0.002)	0.589 (−0.001)	0.589 (−0.001)
23	0.582 (±0.001)	0.582 (0.000)	0.582 (0.000)
27	0.572 (±0.002)	0.573 (+0.001)	0.573 (+0.001)
33	0.560 (±0.001)	0.560 (0.000)	0.559 (−0.001)
37	0.551 (±0.002)	0.551 (0.000)	0.550 (−0.001)
41	0.543 (±0.001)	0.542 (−0.001)	0.541 (−0.002)
45	0.533 (±0.002)	0.533 (0.000)	0.533 (0.000)
48	0.524 (±0.002)	0.527 (+0.003)	0.526 (+0.002)
51	0.520 (±0.002)	0.520 (0.000)	0.520 (0.000)
54	0.513 (±0.002)	0.514 (+0.001)	0.514 (+0.001)
58	0.506 (±0.003)	0.505 (−0.001)	0.505 (−0.001)
63	0.495 (±0.002)	0.494 (−0.001)	0.496 (+0.001)

spectra by insertion in (2), which takes on a very simple form in the present case. It corresponds to the second example of our previous paper [8].

With the terms of eqn (3) the molar extinction coefficients, ε, are the following:

$$\varepsilon_A = 100(a_0 + a_1 + a_2)$$
$$\varepsilon_B = 100a_0$$
$$\varepsilon_X = 100(a_0 + a_2(k_1-k_2)/k_1)$$

The calculated values of ε_x at the three wavelengths examined by Schläfer and Kling are given in Table 2 and plotted in Fig. 1, which also gives the spectra of the tris(ethylenediamine) ion (A) and the *cis*-diaquobis (ethylenediamine)chromium ion (B). If the suggestion of Schläfer and Kling, *viz.* $k_1 > k_2$ is correct, one would expect a spectrum of the intermediate nearly identical with that of the luteo ion. However, if on the other hand $k_1 < k_2$, nothing need to be said *a priori* about the absorption spectrum of the intermediate. In the latter case the intermediate compound also accumulates to a much smaller extent so the requirement is less rigorous. $k_1 = 0.06$ and $k_2 = 0.008$ h^{-1} leads to a maximum amount of 73 % of the intermediate while the other sequence only involves 10 %.

Table 2. Molar extinction coefficients for the tris(ethylenediamine) chromium ion (A), the cis-diaquobis(ethylenediamine) chromium ion (B) and for the unknown intermediate (X).

wavelength		340 mμ	390 mμ	440 mμ
ε_A		50.7	13.7	63.1
ε_X	$k_1 > k_2$	47.8	15.4	60.3
	$k_1 < k_2$	29.1	26.1	42.0
ε_B		17.4	26.1	25.1

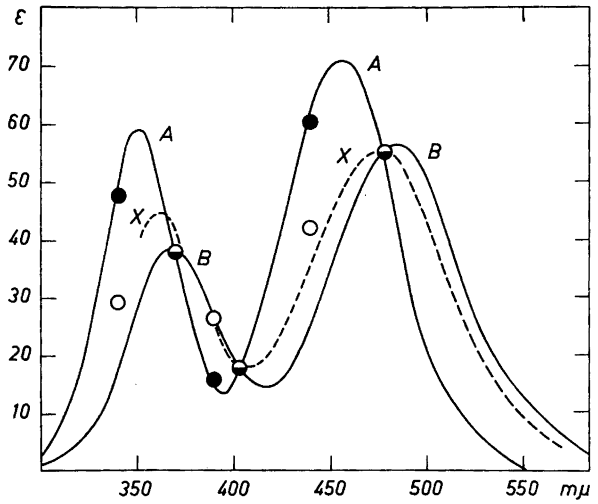


Fig. 1. The spectra of the tris(ethylenediamine) and the *cis*-diaquobis (ethylenediamine) chromium(III) ions (curves A and B) in 0.1 M $HClO_4$ according to Schläfer and Kling[1]. The dashed curve is an estimate of the spectrum of the intermediate complex (X) based on the similarity with the ammine complexes in Fig. 2.

○ — calculated molar extinction coefficient of X in the case $k_1 < k_2$.
● — calculated molar extinction coefficient of X in the case $k_1 > k_2$.

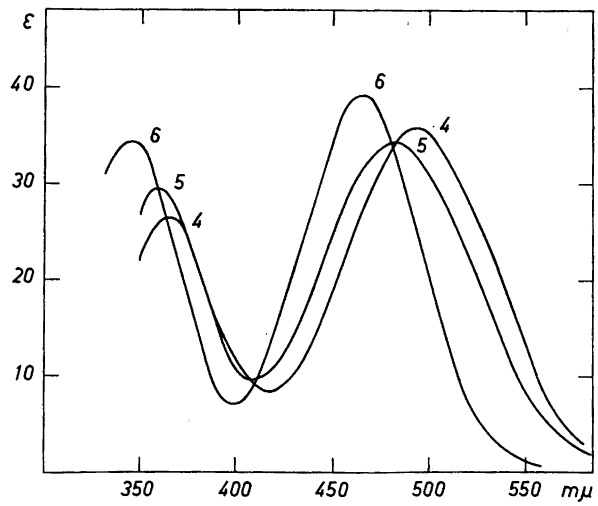


Fig. 2. The spectra of the hexammine, aquopentammine and diaquotetrammine chromium(III) ions (curves 6, 5 and 4) in acidic solution according to Jørgensen and Bjerrum [2].

In our investigation of the aquation of chromium(III) ammines [2] we found at 40°C for the rate constants of the hexammine and aquopentammine the values 0.0051 and 0.085 h^{-1}, respectively. Properly corrected for the difference in temperature (a conversion factor of 0.25) the values directly comparable with the present rate constants, 0.006 and 0.08 h^{-1}, are 0.0013 and 0.021 h^{-1}, respectively.

It is interesting to note that the tris(ethylenediamine) complex reacts more rapidly than the corresponding hexammine ion. The activation energies are 24.3 and 26.0 kcal, respectively. This suggests a considerable tension in the chelate ring, and makes it probable that the first step is simply an opening of the ring followed by the uptake of a proton. Professor R. G. Inskeep * has shown that the rate of aquation of the tris(ethylenediamine)chromium ion is practically independent of the hydrogen ion concentration in the range from 0.1 to 1 M $HClO_4$. Furthermore it is remarkable that the hexammine chromium(III) ion aquates 16 times slower than the aquopentammine chromium(III) ion, and most probably this evidence can be taken as a support for the case $k_1 < k_2$. It is, however, still more indicative to compare the requirements placed on the spectrum of the intermediate with the spectrum actually found in the ammonia system. The spectra of the hexammine (6), aquopentammine (5) and diaquotetrammine (4) chromium ions are shown in Fig. 2. The shape and the position of the spectra of the hexammine and the tetrammine chromium ions are well known to be very similar to those of the tris(ethylenediamine) and bis(ethylenediamine) chromium ions. This argument applied to the spectrum of the pentammine chromium ion would predict a spectrum for the corresponding complex in the ethylenediamine system as outlined in Fig. 1 (the dashed curve). This curve is seen to fit quite well to the points calculated for the sequence of rate constants assumed by us. At the wavelength, 390 mμ, one of the exponential terms in (3) vanishes (see Table 1). Here, X and B seem to have an isosbestic point (see Table 2).

The existence of the pentammine as intermediate is further proved by Schläfer and Seidel's [3] investigations on the racemization of the active luteo ion. The rate-determining constant, 0.00814 h^{-1}, is equal to the slow one found in the hydrolysis reaction, and the peculiar irregularity in the beginning of the experiment points towards two steps with reaction probabilities of approximately 1 h^{-1}. In Schläfer's interpretation, one of these fast reactions must be due to the racemization of the luteo ion, and the slow rate-determining step is the racemization of the never isolated intermediate through aquation. Our interpretation places the slow racemization on the aquation of the luteo ion, while the partially hydrolyzed species racemize fast. This is in agreement with the fact that the luteo ion can only racemize through intramolecular rearrangement while the lower complexes have the possibility of racemization through water exchange.

Thus it seems to us that it has been possible unequivocally to correlate the experimental facts with the assumption that the first reaction product in the hydrolysis of the tris(ethylenediamine) chromium ion in acidic solution

* Guest at Chemistry Department A on sabbatical leave from University of Vermont, Vermont, U.S.A.

is an aquopentammine ion, Cr $en_2(enH)H_2O^{++++}$, stabilized by the uptake of a hydrogen ion on the displaced amino group.

REFERENCES

1. Schläfer, H. L. and Kling, O. *Z. physik. Chem.* **16** (1958) 14.
2. Jørgensen, E. and Bjerrum, J. *Acta Chem. Scand.* **12** (1958) 1047.
3. Schläfer, H. L. and Seidel, H. *Z. physik. Chem.* **16** (1958) 1.
4. Skrabal, A. *Homogenkinetik*, Verlag von Theodor Steinkopff, Leipzig 1941, p. 190.
5. Matsen, F. A. and Franklin, F. J. *J. Am. Chem. Soc.* **72** (1950) 3337.
6. Frost, A. A. and Pearson, R. G. *Kinetics and Mechanisms*, John Wiley and Sons, Inc., New York, 1956, p. 160.
7. Berman, M. and Schoenfeld, R. *J. Appl. Phys.* **27** (1956) 1361.
8. Jørgensen, E. *Acta Chem. Scand.* **12** (1958) 1057.

Received September 7, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2082–2088

Determination of Oxygen in Silver-Copper and Gold-Silver-Copper Alloys by Hydrogen Reduction

EINAR JACOBSEN*

Central Institute for Industrial Research, Blindern, Norway

A simple apparatus for determination of oxygen in silver and gold is described. The method is based upon the determination of the water formed by reduction of the oxide in the sample with hydrogen gas. In a 1 g sample oxygen contents from 0.0001 to 0.1 % can be determined within 30 min. The apparatus has been used at the Central Institute for Industrial Research, Blindern, Norway, for more than a year with excellent results.

Oxygen in metals is usually determined by the vacuum-fusion method. Since silver is volatile at the temperatures required in this method and because of the cost of such elaborate systems, a more simple method would be desirable. In certain non-ferrous metals, oxygen can be determined by a method based upon reduction with hydrogen. In this method the sample is heated in hydrogen, which reacts with any oxygen present in the sample to form water. The water-vapour formed is either absorbed and weighed [1,2] or condensed in a trap and subsequently vaporized in an otherwise evacuated system [3-7]. The determination of oxide in the sample has also been performed by measuring the hydrogen gas consumed in the above reaction [8].

In the present work samples containing only a few ppm of oxygen were to be analysed, and an extremely accurate apparatus had to be constructed. The present communication describes an apparatus based upon hydrogen reduction, by which as little as 1 ppm of oxygen can be determined within 30 min. The apparatus is somewhat similar in construction to those by Baker [6] and Nitsche [7]. It is modified in several respects, however, in order to increase the accuracy and speed of the analysis.

* Present address: Kjemisk institutt A, Universitetet i Oslo, Blindern, Norway.

DESCRIPTION OF APPARATUS

A schematic diagram of the apparatus is shown in Fig. 1. Hydrogen is taken from a cylinder and purified by passing through a Deoxo catalytic purifier (Baker platinum division, London) (A in Fig. 1) in which oxygen is reduced to water. It is then dried in a 800 ml flask (C) containing phosphorus pentoxide. 800 ml of hydrogen is sufficient for one day's analyses and this amount can be dried completely overnight in the flask. A mercury valve (B) between the Deoxo purifier and the reservoir prevents air from entering the apparatus during the filling of the reservoir.

The combustion unit consists of a clear silica tube (D) with a B 19 male joint in one end. The joint fits to a female joint in the apparatus and is held securely in place by a spring clamp. The quartz tube (14 mm bore and an overall length of 40 cm) is heated by means of a small furnace, consisting of a Mullite tube wound with 0.9 mm Kanthal A-1 wire (E).

The temperature was controlled by means of a Variac and measured with with a chromel-alumel thermocouple (F) placed in the constricted end of the Mullite tube. By appropriate adjustment of the Variac, any desired tempera ture between 20 and 1 000°C could be obtained in less than 5 min. The silica

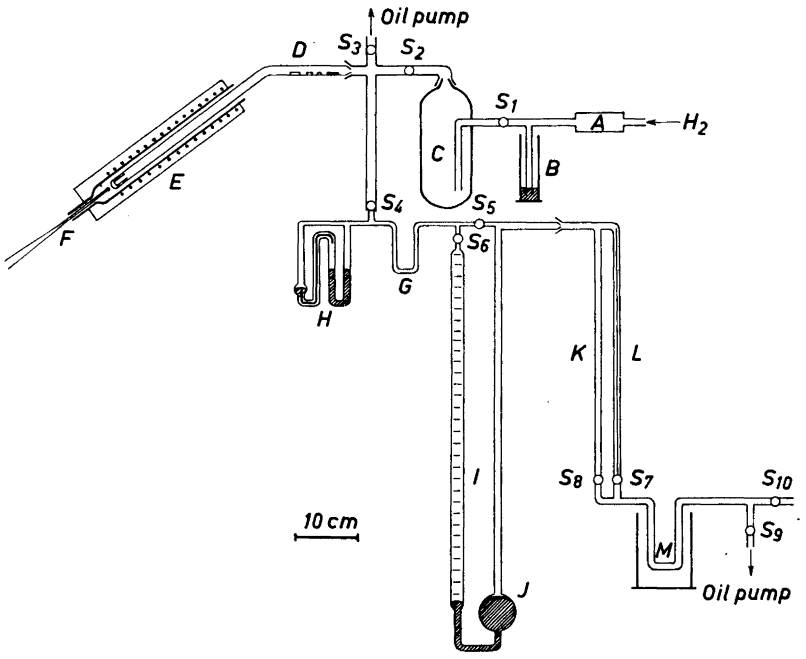


Fig. 1. Schematic diagram of apparatus for determination of oxygen in metals by hydrogen reduction. A Deoxo catalytic purifier, B mercury valve, C 800 ml flask containing phosphorus pentoxide, D combustion tube, E furnace, F thermocouple, G water trap, H mercury manometer, I 100 ml buret, J mercury reservoir, K 8 mm bore tube, L 0.5 mm bore tube, M water trap, $S_1 - S_{10}$ stopcocks.

tube was bent at an angle in order to reduce heat radiation to the joint. The specimens to be analysed were placed in the horizontal part of the combustion tube, in front of a small magnet. The lower end of the tube contained a crucible of sintered alumina.

A U-formed tube (G) with 4 mm bore served as a water trap. It was cooled by a mixture of solid carbon dioxide and acetone. The trap was sealed to a small mercury manometer (H) and a buret (I). The manometer consisted of a 1 mm capillary sealed to 8 mm bore tubes. The capillary and the attached U-tube of the manometer was filled with redistilled mercury. When evacuated, the mercury stem in the upper part of the capillary is broken, and the pressure is recorded as the difference in height of the mercury columns in the U-tube. The buret had a capacity of 100 ml and was graduated into 0.2 ml. The lower end of the buret communicated with a mercury reservoir (J) with a capacity of approximately 120 ml.

The pump train consisted of two 40 cm long tubes (K and L) with 8 mm and 0.5 mm bore, respectively. Between the tubes and the oil pump was placed a U-formed trap immersed in solid carbon dioxide/acetone mixture. The purpose of this trap was to prevent moisture from the air to enter the apparatus, and also to prevent mercury vapour from diffusing to the oil pump, which is connected at S_9.

The entire apparatus, with exception of the combustion tube, was made of Pyrex glass. The stopcocks S_2, S_3, and S_4 were of the hollow plug type with 4 to 5 mm hole through the plug. The rest of the stopcocks had an oblique bore of 4—5 mm through a compact plug. Stopcocks and glass joints were lubricated with a silicone base lubricant. The apparatus could be maintained at a vacuum of 0.01 mm Hg for several days.

PROCEDURE

The specimens to be analysed were cleaned by filing with a clean file, or scraping with a sharp knife in order to remove surface oxide, and then degreased by means of benzene. The samples were then placed in the horizontal part of the combustion tube. Powdered samples were wrapped in a tin foil of known oxygen content.

The apparatus is evacuated through S_9 by a Hyvac oil pump, capable of exhausting the system to 0.01 mm Hg. S_9 is then closed and pure hydrogen admitted to the system through S_2. The apparatus is evacuated once more, and pure hydrogen again admitted until the pressure is 40—60 mm Hg, as indicated by the mercury manometer. A thermos bottle containing the cooling mixture is added to the water trap (G) and the furnace pushed over the inclined part of the combustion tube. After heating the sample at 900°C for 20 min tap S_7 is opened, and excess hydrogen is slowly removed through the capillary by pumping. After 5 min, S_8 is opened, and the system is evacuated for a few more minutes. Stopcocks S_4, S_5, and S_6 are then closed, the cooling mixture removed from the trap and the water vapour allowed to vaporize. The pressure of the water vapour is recorded on the manometer, using a low power microscope or a cathetometer.

Normally a blank run shows less than 0.2 mm Hg. If the blank exceeds this value, the whole procedure is repeated until successive blanks agree to within 0.1 mm Hg. The water vapour is removed through the pump train.

Hydrogen is again admitted to a pressure of 40—70 mm Hg, the cooling mixture added to the trap, and one of the specimens is pushed into the hot zone of the combustion tube by means of the magnet. After heating for 20 minutes, excess hydrogen is slowly pumped out through the capillary. 5 minutes afterwards tap S_8 is opened and the apparatus exhausted for a few more minutes. Stopcocks S_5, S_7, S_8, and S_9 are then closed, and S_{10} opened. Stopcock S_7 is then opened slowly, letting air through the capillary, and forcing the mercury in the reservoir to fill the buret. The last trances of hydrogen are then pumped out through S_3. Stopcocks S_3, S_4, and S_7 are closed, the cooling mixture removed and the water allowed to vaporize. The pressure and temperature of the vapour are recorded.

Since water vapour at room temperature deviates appreciably from ideal gas behaviour for pressures exceeding 10 mm Hg, useful vapour pressures are confined to lower values. Whenever a vapour pressure above or near 10 mm Hg is recorded, the air pressure in the mercury reservoir is reduced by opening S_9 and S_7 of the pump train. The mercury in the buret falls correspondingly, thus increasing the volume of the water vapour. By appropriate adjustment of the pressure in the pump train, any desired volume of the water vapour at its corresponding pressure can be obtained.

Before proceeding with the next sample, the apparatus is exhausted and a new blank determined. If the blank is unchanged compared to the previous check, this indicates that the last specimen has been completely reduced and that no leaks in the apparatus have occurred.

CALIBRATIONS AND CALCULATIONS

The total volume enclosed by stopcocks S_4, S_5, S_6 (*i.e.* water trap, manometer and associated tubings) was calibrated by introducing small volumes of air and recording their pressures at a number of different readings of the buret. The apparatus was also calibrated by reducing known amounts of lead oxide following the above procedure. Both methods gave concordant results, *i.e.* 14.2 ml. The buret had a capacity of 100 ml and, consequently, the volume in which the water vaporizes could be adjusted to any desired value between 14.2 and 114 ml.

If the total volume of the water vapour is V ml, the pressure recorded on the manometer P mm Hg and the room temperature t°C, the weight W_0 of the water formed by the reduction can be calculated from the equation:

$$W_0 = \frac{P}{760} \cdot \frac{V}{1 + \gamma t} \cdot \frac{18.016}{22414} \text{ g } H_2O$$

The corresponding weight of oxygen (W) in the sample is:

$$W = 9.39 \times 10^{-7} \times \frac{P \cdot V}{1 + \gamma t} \text{ g } O_2$$

By means of a cathetometer the water vapour pressure can be measured to ± 0.1 mm Hg. From the above equation it is evident that a pressure of 0.1 mm Hg in a volume of 14.2 ml corresponds to 0.00128 mg oxygen, which is the smallest amount of oxygen that can be detected with the apparatus described. The volume in which the water vaporizes can be increased to a maximum value of 114 ml. A water vapour pressure of 10 mm Hg in this volume corresponds to 0.996 mg oxygen. By using a sample weighing 1 g and adjusting the volume appropriately, the apparatus will cover the entire range from 0.00013 to 0.1 % of oxygen. The determination of oxygen contents lower than 0.00013 % might be performed by increasing the sample weight, or by decreasing the volume of the water trap and associated tubings.

The blank was found to be independent of the initial hydrogen pressure, indicating that the hydrogen gas was completely dry and oxygen-free. At furnace temperatures below 850°C, the blank usually showed 1×10^{-6} g oxygen. At temperatures above 850°C, however, the blank increased with increasing temperature and time of heating. This is probably due to diffusion of oxygen form the atmosphere through the hot zone of the quartz tube. Consequently, the diameter of the tube was chosen as small as possible and the same temperature and time of reaction were employed in both the blank and analysis.

In order to speed up the reduction of the oxides, the specimens were heated to a temperature slightly above the melting point. Metals with high melting

Table 1. Determination of oxygen in lead and copper.

Oxide taken mg	Oxygen equivalent mg	Reaction temperature °C	Reaction time min	Oxygen found mg	Error %
Lead oxide					
9.95	0.713	650	5	0.642	−10.0
9.90	0.710	700	10	0.660	− 7.0
9.75	0.698	700	10	0.654	− 6.3
12.30	0.882	700	15	0.845	− 4.2
9.40	0.674	650	20	0.674	0
9.65	0.692	650	25	0.698	+ 0.9
12.75	0.914	650	30	0.901	− 1.4
9.22	0.661	650	20	0.646	− 2.3
4.45	0.319	650	20	0.320	0
8.36	0.599	650	20	0.606	+ 1.1
Cupric oxide					
3.15	0.634	900	10	0.600	− 5.4
3.56	0.716	980	10	0.681	− 4.9
2.45	0.493	980	20	0.494	+ 0.2
3.69	0.742	950	25	0.742	0
3.31	0.666	950	30	0.654	− 1.8
2.25	0.453	900	20	0.452	− 0.2
3.79	0.762	950	20	0.747	− 2.0
3.12	0.628	950	20	0.638	+ 1.6

The samples were wrapped in tinfoil of known oxygen content. All values are corrected for the blank.

Table 2. Oxygen contents of silver-copper and gold-silver-copper alloys

Sample	Weight, g	Oxygen found mg	Oxygen found %
83 % Ag, 17 % Cu	2.0320	0.105	0.0051
	2.3930	0.129	0.0054
	1.4582	0.072	0.0049
	1.0212	0.053	0.0052
	3.0520	0.156	0.0051
			Mean 0.00514 ± 0.00024 %
92.5 % Ag 7.5 % Cu	3.8205	0.078	0.0020
	4.0502	0.087	0.0021
	2.4350	0.049	0.0020
	4.5900	0.105	0.0023
			Mean 0.0021 ± 0.0002 %
83 % Ag, 17 % Cu (Vacuum melted)	3.0880	0.0012	0.00004
	2.8605	0.0012	0.00004
58.4 % Au 20.8 % Ag 20.8 % Cu	1.0219	0.015	0.0015
	2.3500	0.033	0.0014
	1.4350	0.023	0.0016
			Mean 0.0015 ± 0.0001 %
58.5 % Au 16.6 % Cu 24.9 % Ag	1.7650	0.015	0.0009
	1.3700	0.012	0.0009
	3.8450	0.031	0.0008
			Mean 0.0009 ± 0.0001 %

points (copper for instance) were added to a previously deoxidized melt of tin. In this way the reduction temperature could be kept below 900°C, thus taking advantage of a low blank value. Surface oxides in copper and copper-silver alloys are fairly rapidly reduced even at a temperature of about 400°C. This is important when analysing samples having a very rough surface, as the surface oxide could be removed by heating in a hydrogen atmosphere at 400°C prior to the determination of oxygen in the bulk of the sample. According to Baker [6], cleaning with oxidising acids should be avoided as they are liable to leave an oxygen film on the surface.

RESULTS

To test the accuracy and precision of the method, samples of lead oxide (Analar C.P. product of yellow PbO) and cupric oxide (Merck C.P. product of CuO) were analysed by the procedure outlined above. The reduction temperatures were 650 and 950°C for the lead and copper samples, respectively. From the results in Table 1 it appears that only 90 % of the oxygen content is extracted during the first 5 min. A heating time of 20 min, however, was found sufficient for a quantitative reduction of oxygen in the samples. This is in contrast with the results by Nitsche [7] who claims that heating for 5 min at 900°C is sufficient for quantitative reduction of oxygen in copper.

A series of determinations oxygen in silver-copper and gold-copper-silver alloys were then performed. As shown in Table 2, the oxygen content found in different samples of the same alloy, showed a deviation of $\pm$ 2 ppm from the mean value. Considering the small samples and a non-uniform distribution of oxygen in the metal, the results must be considered satisfactory.

The apparatus described is obviously applicable to all metals in which the oxide is reduced by hydrogen below 1 000°C. Volatile constituents in the samples will vaporize and condense in the cool part of the combustion tube. Metallic mirrors of silver and lead were observed, but apparently they did not influence the results of analysis. This was verified by adding samples of known oxygen content to the melt. As stated by Baker [6] errors due to volatile constituents, as zinc for instance, may be minimised by using a large bath of tin in the combustion tube. Metallic mirrors may sometimes be of analytical interest. Thus, even minute amounts of cadmium in gold was detected as a black deposit well separated from those of less volatile constituents as for instance silver.

Acknowledgement. The author wishes to thank Central Institute for Industrial Research, Blindern, Norway, for permission to publish this paper.

REFERENCES

1. Oberhoffer, P. *Metall u. Erz* **15** (1918) 33.
2. Worner, H. W. *J. Inst. Metals* **66** (1940) 131.
3. Jander, W. and Krieger, A. *Z. anorg. u. allgem. Chem.* **232** (1937) 57.
4. Grosheim-Krysko, K. W., Hofmann, W. and Hanemann, H. *Metallkunde* **36** (1944) 91.
5. Barteld, K. and Hofmann, W. *Erzbergbau u. Metallhüttenw.* **5** (1952) 102.
6. Baker, W. A. *Metallurgia* **40** (1949) 188.
7. Nitsche, E. and Waibel, F. *Wiss. Veröffentl. Siemens-Werken* **15** (1936) 87.
8. Funston, E. S. and Reed, S. A. *Anal. Chem.* **23** (1951) 190.

Received August 12, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2089–2100

Crystal Structures of the Compounds Dimethylammonium Bromide-Bromine (2:1) and Dimethylammonium Chloride-Iodine (2:1)

K. O. STRØMME

Universitetets Kjemiske institutt, Blindern-Oslo, Norway

The crystal structures of the compounds (2:1) dimethylammonium bromide-bromine $[(CH_3)_2NH_2Br]_2 \cdot Br_2$ and (2:1) dimethylammonium chloride-iodine $[(CH_3)_2NH_2Cl]_2 \cdot I_2$ have been determined by X-ray methods. The compounds are isomorphous, the space group is $C2$ and the lattice parameters are $a = 20.00$ Å; $b = 6.01$ Å; $c = 5.38$ Å; $\beta = 94.6°$ and $a = 20.43$ Å; $b = 5.88$ Å; $c = 5.45$ Å; $\beta = 94.3°$, respectively.

The halogen atoms are arranged in nearly linear chains, *viz.* Br—Br—Br—Br and Cl—I—I—Cl (the halogen-halogen-halogen angle $\approx 173°$.) In these chains the distance between the two central atoms is a little longer than those in the free halogen molecules (obs. Br—Br dist. = 2.42 Å and obs. I—I = 2.74 Å). The bonds from the central atoms to the nearer of the terminal halogen atoms appear to be moderately strong and the distances intermediate between those to be expected for covalent and van der Waals values.

Each terminal halogen atom is connected to three nitrogen atoms. Two of these halogen-nitrogen distances are similar to the halogen-nitrogen distances found in ammonium halides, but the third halogen-nitrogen distance is appreciably shorter (N—Br = 3.05 Å, 3.49 Å, 3.50 Å; N—Cl = 3.05 Å, 3.27 Å, 3.28 Å). This short bond lies along one of the nitrogen's tetrahedral directions, approximately. The angle between this direction and the line joining halogen-halogen is about 95°.

The structures of salts formed by ammonium cations and polyhalide anions are of considerable interest. Among the substances already investigated are several containing the trihalide ion to which a negative charge is attached.

The present report deals with the crystal structures of the 2:1 addition compounds formed by dimethylammonium bromide and chloride with bromine and iodine, respectively. In these compounds a negative charge should be associated with half the number of halogen atoms present in the compound under consideration.

EXPERIMENTAL

The compounds $[(CH_3)_2NH.HBr]_2.Br_2$ or $[(CH_3)_2NH.HCl].I_2$ are formed by addition of diluted bromine (or iodine) to a solution containing the dimethylammonium bromide (or chloride) in chloroform.[1] Good crystals may be obtained by careful cooling of a saturated solution or by slow evaporation of the chloroform. The compounds may also be obtained by adding diluted bromine (or iodo monochloride) to the free base in chloroform.[2] The halogen thus reacts with the methyl groups giving halogen substituted amine and hydrobromic (or hydrochloric) acid which in turn neutralizes the free base. The salt then combines with bromine (or iodine, liberated from iodomonochloride) in the given ratio.

The active halogen was determined volumetrically by titration against a standardized solution of thiosulphate.

The bromine compound is orange in colour and the iodine compound reddish brown. Their decomposition points are 73 and 100°C, respectively.

The crystals are instable and were kept in thin-walled boron lithium capillaries when under exposure to X-rays.

Weissenberg and oscillation photographs were taken with Cu-*K* radiation and rotation about the *b* and *c* axes. Using the multiple film technique two series of Weissenberg photographs were obtained for each compound with exposure times from 1 to 100 h. The intensities were estimated visually and corrected for Lorentz- and polarization effects. No correction for absorption — or secondary extinction effects — was applied.

For the bromine compound 143 *h0l* and 88 *hk*0 reflexions are theoretically attainable with Cu-*Ka* radiation. Actually 119 and 81 were observed, respectively. For the compound containing iodine there are 143 theoretical, against 123 exptl. *h0l* reflexions. A correction of the form $\exp.(-B \sin^2 \Theta/\lambda^2)$ was applied to the calculated structure factors The *B* factors were found by inspection to be:

	B (*h0l*) (Å^{-2})	*B* (*hk*0) (Å^{-2})
Bromine compound	4.90	2.50
Iodine "	4.80	2.50

The atomic scattering factors employed in the calculation of the structure factors are taken from the *Internationale Tabellen* [3].

CRYSTAL DATA

The crystals of the two species are isomorphous with monoclinic unit cells. The crystals form flat needles, the needle axes coinciding with the *b* axes. According to the observed X-ray extinctions the three *C*-centered space groups *Cm*, *C*2 and *C*2/*m* are possible. The latter is the only one in which centers of symmetry are present. For reasons mentioned below, however, it turned out that *C*2 was the correct space group. It gives rise to only one centrosymmetric projection, that along [010]. The unit cell dimensions are as follows:

	a	*b*	*c*	β
$[(CH_3)_2NH_2Br]_2 \cdot Br_2$	20.00 Å;	6.01 Å;	5.38 Å;	94.6
$[(CH_3)_2NH_2Cl]_2 \cdot I_2$	20.43 Å;	5.88 Å;	5.45 Å;	94.3

With densities found by the flotation method (2.15 g/cm^3 and 2.18 g/cm^3) this leads to 2 molecules of $[(CH_3)_2NH_2Br]_2 \cdot Br_2$ or $[(CH_3)_2NH_2Cl]_2 \cdot I_2$ in the unit cell.

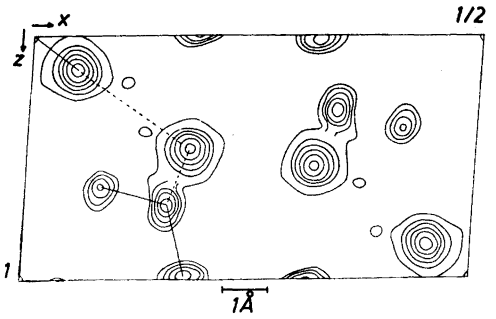


Fig. 1. The electron density projection along the *b* axis of dimethylammonium bromide-bromine (2:1).

Contours for bromine : 4, 12, 20, 28..... el. Å^{-2}
For nitrogen, carbon. *etc.* : 4, 5, 6, 7, 8, el. Å^{-2}.

DETERMINATION OF THE STRUCTURES

Trial and error computations of the parameters of the independent halogen atoms, starting with the bromine compound, led to results which strongly indicated that the plane group for the projection along the *b* axes is *p*2. This made the space group *Cm* improbable. A Patterson projection along [010] turned out to be in full agreement with these findings and the next step was the computation of the corresponding electron density projection. Starting with the *x* and *z* coordinates of the bromine atoms already derived and neglecting the lighter atoms, a first Fourier map was obtained which, beside the peaks corresponding to the bromines, also contains lower peaks due to carbon and nitrogen atoms. After four refinements no further phase shifts occurred. The final Fourier map is reproduced in Fig. 1. The reliability factor for this projection is $R = 0.12$.

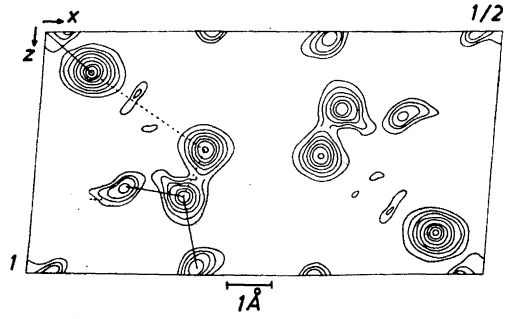


Fig. 2. The electron density projection along the *b*-axis of dimethylammonium chloride-iodine (2:1).

Contours for iodine : 4, 13, 23, 33 .....el. Å^{-2}.
For chlorine : 4, 7, 10, 13, 16 .....el. Å^{-2}.
For nitrogen, carbon *etc.* : as in Fig. 1.

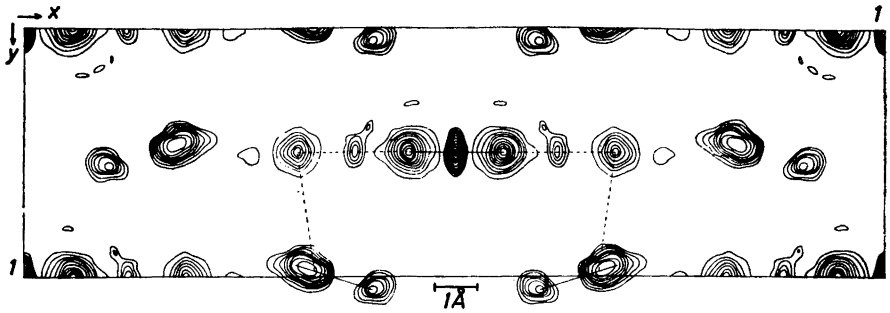


Fig. 3. The electron density projection along the *c* axis of dimethylammonium chloride-iodine (2:1).

Contours for chlorine : 4, 8, 12, 16 .....el. Å^{-2}.
For iodine, nitrogen, carbon *etc.* as in Fig. 2.

Hoping that a further refinement of the x and z parameters should be possible by a difference synthesis, a Fourier chart was worked out subtracting the contributions from the bromine atoms. The map thus obtained did not reveal any electron density gradients in the positions of the removed bromine atoms which could serve as a basis for further refinement. In the case of the light atoms the x and z values obtained from this synthesis have been regarded as the more reliable and chosen as the final ones.

The corresponding [010] projection of the isomorphous compound containing iodine was worked out next. Due to the high scattering power of the iodine atom relative to the other atoms approximate values of its x and z coordinates could easily be determined by trial and error methods. The Fourier map after three refinements is reproduced in Fig. 2. The R factor was found to be 0.16. The larger value of the R factor in this case is probably caused chiefly by more pronounced absorption effects. A difference synthesis led to slight changes in the coordinates of the light atoms but not of the heavy ones.

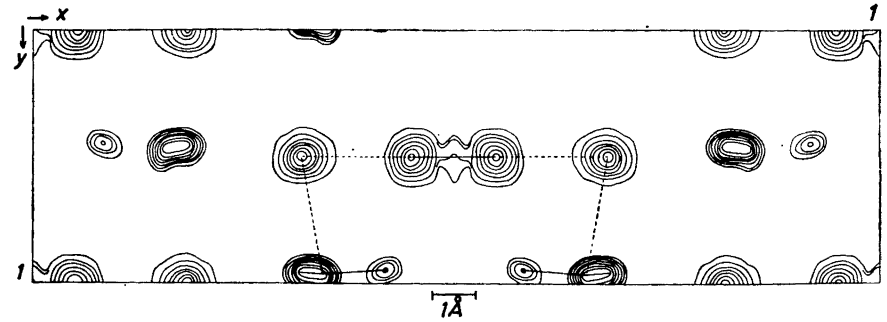


Fig. 4. The electron density projections along the *c* axis of dimethylammonium bromide-bromine (2:1).

Contours as in Fig. 1.

It will be noted that the space group $C2/m$ would have required the four amine groups to be situated in m with the molecular mirror planes coinciding with those of the space group, thus bringing about one double peak for the two amine carbons in the [010] projection. In Figs. 1 and 2 three peaks for the amine group appear. This finding is not compatible with the above requirement, and $C2/m$ is thus ruled out in favour of $C2$, which has a general 4-fold position and therefore imposes no restriction on the position of the amine group.

The y parameters of the independent heavy atoms were determined from Patterson projections along the c axes starting with the iodine compound. It turned out that these parameters in the first step might be set equal to zero, thereby satisfying a condition for a 4-fold position in the centrosymmetric plane group cmm, although the correct plane group is the noncentrosymmetric cm. According to this finding the phase angles were not expected to deviate much from 0 and 180°, the deviations being dependent on the contributions from the light atoms.

Values of the y parameters of the amine groups were obtained by a trial and error computation using the correct expressions for the structure factors, the x parameters obtained from the [010] projection and data for the amine group already known [4]. The contributions from the light and heavy atoms were then included in the calculation of the phase angles and a Fourier synthesis based on the plane group cm was carried out. After three refinements the electron density map reproduced in Fig. 3 was obtained. The R factor was found equal to 0.14. The x parameters found for the halogen atoms in the two projections agree to within 0.001 of the axial lengths.

In the case of the bromine compound the y parameters of the nitrogen atom was arbitrarily put equal to $\frac{1}{2}$(or 0). The corresponding y parameters of the two independent carbon atoms belonging to the same amine group were then calculated using a model before a Fourier refinement process was started. The electron density map after three refinements is shown in Fig. 4. The value of the R factor is 0.12. The x parameters found for the halogen atoms in the two projections agree to within 0.001 of the axial length.

Tabelle 1. Atomic coordinates in fractions of the cell edges. Origin at a 2-fold axis.

	A $[(CH_3)_2NH{\cdot}HCl]_2{\cdot}I_2$; B $[(CH_3)_2NHHBr]_2{\cdot}Br_2$.			
	Atom	x	y	z
A	I	0.055	0.000	0.162
	Cl	0.186	0.001	0.493
	N	0.166	0.480	0.688
	C_1	0.099	0.551	0.672
	C_2	0.188	0.456	0.952
B	Br_1	0.050	0.000	0.143
	Br_2	0.180	−0.002	0.473
	N	0.157	0.467	0.714
	C_1	0.083	0.449	0.630
	C_2	0.183	0.459	0.983

Table 2. $|F_{obs}|$-, $|F_{calc}|$- and α_{calc}-values. The F-values represent one fourth of the absolute values.

	Compound containing bromine			Compound containing iodine		
$h\ k\ l$	$\|F_{obs}\|$	$\|F_{calc}\|$	α,°	$\|F_{obs}\|$	$\|F_{calc}\|$	α,°
2 0 0	3.0	2.2	0	14.9	23.5	0
4 0 0	4.1	3.3	180	< 1.6	0.8	0
6 0 0	17.9	17.8	0	5.4	6.7	180
8 0 0	36.9	41.4	180	40.6	47.0	180
10 0 0	13.4	12.7	180	19.2	23.6	180
12 0 0	< 1.8	0.1	180	7.3	11.2	180
14 0 0	15.3	14.4	180	4.2	4.6	180
16 0 0	7.6	7.6	0	15.7	15.5	0
18 0 0	6.1	5.5	0	10.5	11.1	0
20 0 0	< 2.2	0.7	0	7.1	5.7	0
22 0 0	6.6	6.2	0	5.7	6.4	0
24 0 0	< 1.7	0.1	180	5.0	5.3	180
0 0 1	9.3	11.3	0	10.3	9.8	0
2 0 1	23.7	19.7	0	8.8	8.2	0
4 0 1	4.8	4.9	180	19.4	30.2	180
6 0 1	5.9	38.3	180	33.9	46.4	180
8 0 1	3.4	2.0	180	16.1	19.6	180
10 0 1	14.8	14.5	180	14.3	12.8	180
12 0 1	12.3	10.2	180	6.7	9.8	0
14 0 1	16.3	17.6	0	21.8	24.8	0
16 0 1	3.8	4.1	0	10.2	12.8	0
18 0 1	4.5	4.7	0	8.0	9.4	0
20 0 1	7.6	6.4	0	4.7	1.2	0
22 0 1	3.6	2.9	180	7.5	7.9	180
24 0 1	1.4	1.3	180	3.1	5.4	180
2 0 $\bar{1}$	40.5	48.0	0	38.5	43.1	0
4 0 $\bar{1}$	29.3	30.2	0	47.9	46.7	0
6 0 $\bar{1}$	1.9	2.2	180	13.4	17.7	0
8 0 $\bar{1}$	1.6	17.2	0	< 2.4	0.2	0
10 0 $\bar{1}$	11.7	15.2	180	22.3	25.9	180
12 0 $\bar{1}$	19.5	18.4	180	27.5	28.4	180
14 0 $\bar{1}$	4.2	3.0	180	15.2	15.4	180
16 0 $\bar{1}$	12.2	9.8	180	4.7	5.1	180
18 0 $\bar{1}$	3.9	2.7	0	8.7	8.8	0
20 0 $\bar{1}$	7.2	5.9	0	10.4	10.2	0
22 0 $\bar{1}$	< 2.0	0.3	0	5.4	7.4	0
24 0 $\bar{1}$	3.6	3.4	0	3.4	5.6	0
0 0 2	17.7	16.8	0	4.5	5.1	180
2 0 2	31.5	28.1	180	38.2	46.7	180
4 0 2	29.6	29.6	180	30.4	32.1	180
6 0 2	3.2	2.6	0	9.3	10.3	180
8 0 2	20.1	18.4	180	7.7	6.2	180
10 0 2	4.6	5.6	0	22.0	22.8	0
12 0 2	14.3	16.5	0	17.3	20.4	0
14 0 2	4.3	2.1	180	8.2	8.8	0
16 0 2	8.6	8.8	0	11.6	10.6	0
18 0 2	4.9	5.1	0	5.7	6.1	180
20 0 2	4.6	4.6	180	9.2	9.9	180

$h\ k\ l$	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°	$F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°
22 0 2	2.4	0.8	0	4.3	5.1	180
24 0 2	< 1.1	2.2	180			
2 0 $\bar{2}$	7.1	7.3	180	6.2	3.8	0
4 0 $\bar{2}$	27.7	23.2	0	36.2	30.8	0
6 0 $\bar{2}$	33.8	30.3	0	40.8	37.3	0
8 0 $\bar{2}$	4.6	5.6	180	14.1	13.6	0
10 0 $\bar{2}$	11.5	11.4	0	7.6	9.6	0
12 0 $\bar{2}$	4.4	2.6	180	13.7	12.1	180
14 0 $\bar{2}$	15.3	15.3	180	19.8	20.4	180
16 0 $\bar{2}$	2.2	1.4	0	10.1	10.8	180
18 0 $\bar{2}$	6.6	7.7	180	1.0	11.5	180
20 0 $\bar{2}$	4.2	4.0	180	3.8	2.4	0
22 0 $\bar{2}$	4.4	4.4	0	7.3	8.7	0
24 0 $\bar{2}$	< 1.4	0.1	0	3.1	4.6	0
0 0 3	22.5	23.5	0	26.8	29.9	180
2 0 3	15.2	12.8	180	23.5	18.5	180
4 0 3	4.4	1.4	180	11.6	8.6	180
6 0 3	13.1	12.2	180	4.7	6.1	0
8 0 3	7.6	8.9	0	24.7	22.5	0
10 0 3	8.9	9.4	0	19.6	16.1	0
12 0 3	3.1	2.7	0	12.3	17.5	0
14 0 3	8.7	9.1	0	< 2.8	1.2	0
16 0 3	2.5	0.6	180	10.1	11.0	180
18 0 3	3.4	3.2	180	6.3	7.5	180
20 0 3	1.7	0.3	180	5.3	6.3	180
2 0 $\bar{3}$	6.7	9.1	0	16.9	17.6	180
4 0 $\bar{3}$	5.0	5.4	180	< 2.1	5.8	180
6 0 $\bar{3}$	5.2	3.0	0	14.8	12.9	0
8 0 $\bar{3}$	24.1	23.8	0	30.5	28.1	0
10 0 $\bar{3}$	2.8	2.2	0	17.0	16.5	0
12 0 $\bar{3}$	4.7	4.1	0	9.7	9.2	0
14 0 $\bar{3}$	5.4	4.5	0	< 2.7	1.7	180
16 0 $\bar{3}$	9.5	9.8	180	15.1	14.7	180
18 0 $\bar{3}$	3.5	2.8	180	8.2	8.6	180
20 0 $\bar{3}$	< 2.0	0.7	180	3.5	6.0	180
22 0 $\bar{3}$	3.3	3.2	180	4.1	3.0	180
24 0 $\bar{3}$	1.4	1.6	0			
0 0 4	< 2.1	0.5	180	10.9	7.7	180
2 0 4	4.8	4.7	180	< 2.6	0.5	180
4 0 4	7.3	6.3	180	11.2	11.2	0
6 0 4	15.0	14.4	0	26.1	22.9	0
8 0 4	3.7	3.9	0	10.9	6.8	0
10 0 4	2.5	2.4	0	7.2	5.3	0
12 0 4	7.2	6.9	0	< 2.7	2.7	180
14 0 4	3.5	4.1	180	11.2	11.0	180
16 0 4	2.4	1.7	180	6.5	6.0	180
18 0 4	2.0	6.2	180	5.8	5.8	180
2 0 $\bar{4}$	25.1	21.2	180	28.7	27.5	180
4 0 $\bar{4}$	2.6	0.1	180	16.9	14.6	180
6 0 $\bar{4}$	5.4	3.9	0	4.6	3.1	180
8 0 $\bar{4}$	5.3	5.2	180	5.0	1.9	180
10 0 $\bar{4}$	12.5	12.0	0	17.9	17.9	0
12 0 $\bar{4}$	6.3	5.3	0	13.6	13.5	0
14 0 $\bar{4}$	< 2.2	0.9	180	7.6	6.3	0

$h\ k\ l$	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°
16 0 $\bar{4}$	4.4	5.0	0	4.2	6.2	0
18 0 $\bar{4}$	3.4	2.8	180	5.4	6.6	180
20 0 $\bar{4}$	2.9	2.9	180	6.1	7.6	180
22 0 $\bar{4}$	< 0.9	0.0	0	< 1.5	2.6	180
0 0 5	6.8	4.8	180	2.0	1.5	0
2 0 5	2.7	1.4	0	13.9	12.7	0
4 0 5	8.1	8.7	0	14.1	10.6	0
6 0 5	< 2.2	0.1	0	7.4	5.3	0
8 0 5	5.1	4.4	0	4.4	2.8	0
10 0 5	3.7	3.2	0	7.2	6.4	180
12 0 5	3.4	4.1	180	9.4	9.1	180
14 0 5	2.0	1.1	180	5.9	5.4	180
2 0 $\bar{5}$	2.3	1.9	0	1.6	0.4	180
4 0 $\bar{5}$	7.9	8.6	180	12.5	11.3	180
6 0 $\bar{5}$	6.9	6.6	180	16.6	16.4	180
8 0 $\bar{5}$	2.6	1.6	0	7.4	5.5	180
10 0 $\bar{5}$	2.0	2.2	180	5.6	2.1	180
12 0 $\bar{5}$	3.5	3.8	0	6.1	5.9	0
14 0 $\bar{5}$	4.5	5.0	0	10.4	10.3	0
16 0 $\bar{5}$	< 0.8	0.2	0	5.9	4.8	0
18 0 $\bar{5}$				3.5	2.5	0
0 0 6	3.5	3.0	0	12.2	11.8	0
2 0 6	3.5	3.8	0	11.1	6.4	0
4 0 6	< 1.8	0.4	0	2.0	1.7	0
6 0 6	3.8	4.2	0	4.4	0.0	180
8 0 6	1.8	1.3	180	7.6	8.3	180
10 0 6	1.2	2.0	180	5.3	5.4	180
2 0 $\bar{6}$	2.6	3.3	180	5.4	2.8	0
4 0 $\bar{6}$	< 1.8	0.1	0	< 2.4	0.2	180
6 0 $\bar{6}$	3.2	2.3	180	4.4	3.6	180
8 0 $\bar{6}$	3.8	4.6	180	9.9	9.4	180
10 0 $\bar{6}$	1.6	1.3	0	6.0	4.6	180
12 0 $\bar{6}$	1.4	1.0	180	< 1.8	3.1	180
14 0 $\bar{6}$	< 1.1	0.0	0	< 1.6	0.6	0
0 0 7	0.7	0.1	0	1.8	2.2	0
2 0 0	2.6	2.4	0	20.3	23.1	0
4 0 0	4 6	4.6	180	< 1.5	2.0	0
6 0 0	18.5	19.2	0	5.4	7.6	180
8 0 0	39.4	45.1	180	51.5	49.9	180
10 0 0	16.2	14.8	180	25.6	24.5	180
12 0 0	2.3	5.1	180	10.3	18.4	180
14 0 0	20.0	18.2	180	10.1	4.7	180
16 0 0	9.8	11.0	0	20.7	19.1	0
18 0 0	8.2	7.6	0	16.8	14.5	0
20 0 0	< 2.4	1.1	0	10.8	11.3	0
22 0 0	8.2	9.9	0	9.0	6.2	0
24 0 0	2.0	0.2	180	< 2.2	6.2	180
1 1 0	32.2	35.9	3.7	40.5	42.9	358.5
3 1 0	4.9	3.0	232.2	18.3	21.0	357.7
5 1 0	20.4	20.7	359.4	3.9	3.2	22.2
7 1 0	13.6	15.4	181.9	21.8	32.2	178.8
9 1 0	26.8	31.6	181.2	27.3	35.9	182.1
11 1 0	4.8	2.2	166.9	19.6	20.6	181.0
13 1 0	18.9	16.8	178.2	14.4	10.4	181.2

h k l	$\|F_{obs}\|$	$\|F_{calc}\|$	*a*,°	$\|F_{obs}\|$	$\|F_{calc}\|$	*a*,°
15 1 0	4.3	3.4	180.7	16.8	13.2	2.3
17 1 0	16.4	16.5	358.8	22.3	21.5	1.1
19 1 0	1.5	1.3	355.0	10.8	10.2	0.0
21 1 0	5.9	7.3	0.4	9.8	9.7	358.5
23 1 0	4.8	5.8	359.8	< 2.2	1.3	184.0
25 1 0				5.9	6.3	178.5
0 2 0	60.6	61.4	353.9	65.3	62.1	0.8
2 2 0	4.4	2.9	34.4	18.7	20.2	6.7
4 2 0	2.2	2.4	126.5	4.0	3.7	330.4
6 2 0	15.8	1.2	353.0	8.0	7.3	207.3
8 2 0	33.1	36.5	175.7	34.1	41.4	178.2
10 2 0	13.4	12.7	182.7	17.3	21.4	176.2
12 2 0	2.5	2.2	258.6	9.3	10.3	177.9
14 2 0	15.9	15.8	178.1	7.7	3.9	182.9
16 2 0	8.5	9.4	359.2	20.3	17.0	356.3
18 2 0	6.6	6.6	4.1	14.9	12.7	360.0
20 2 0	3.9	1.2	22.0	9.8	10.0	3.3
22 2 0	6.8	8.2	357.7	7.0	5.4	3.2
24 2 0				5.7	5.5	180.0
1 3 0	25.3	25.7	6.8	30.5	30.7	357.9
3 3 0	3.0	4.3	214.8	13.2	20.2	353.4
5 3 0	15.2	13.7	358.0	4.8	2.8	86.9
7 3 0	11.1	11.0	184.1	21.3	23.1	177.8
9 3 0	22.0	23.2	182.5	23.8	26.3	184.7
11 3 0	2.9	0.9	141.5	12.6	14.3	181.3
13 3 0	12.4	12.3	174.9	9.3	8.2	183.7
15 3 0	3.5	2.7	181.4	11.5	10.5	5.5
17 3 0	10.5	12.2	356.5	21.0	14.6	3.8
19 3 0	1.4	0.7	346.6	6.2	8.5	355.8
21 3 0	4.4	5.4	1.2	8.1	7.4	356.7
23 3 0	4.0	4.4	359.2			
0 4 0	35.9	32.7	350.3	35.1	34.5	0.0
2 4 0	2.3	2.4	37.6	15.0	13.1	8.2
4 4 0	2.3	2.0	85.0	6.7	3.1	17.3
6 4 0	10.2	7.5	347.1	4.9	5.0	205.6
8 4 0	21.2	21.8	172.9	26.2	25.9	177.2
10 4 0	8.2	8.0	184.0	13.5	14.2	175.9
12 4 0	2.7	2.2	242.2	7.8	7.3	178.2
14 4 0	9.8	10.5	176.5	< 2.7	2.6	180.0
16 4 0	5.5	6.8	358.5	14.0	12.6	354.7
18 4 0	3.9	4.5	5.7	9.2	9.4	359.6
20 4 0	2.9	0.7	31.9	6.5	6.7	4.8
1 5 0	14.7	14.7	5.0	20.1	17.8	359.3
3 5 0	2.9	3.2	201.4	8.4	6.6	348.3
5 5 0	9.2	7.6	356.2	6.7	2.0	82.1
7 5 0	6.3	6.1	184.0	14.5	14.1	178.2
9 5 0	11.7	13.6	182.6	17.0	16.1	185.4
11 5 0	2.5	0.5	102.6	8.0	7.9	178.7
13 5 0	6.5	6.4	172.2	7.4	5.3	185.6
15 5 0	3.1	1.6	181.2	8.3	5.1	5.3
17 5 0	5.5	6.5	354.5	10.4	8.0	4.9
19 5 0	1.1	0.3	336.7			
0 6 0	16.3	14.7	354.1	20.0	17.4	358.0
2 6 0	< 2.3	1.4	39.5	8.1	6.9	9.2

$h\,k\,l$	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	α,°
4 6 0	< 2.3	1.1	63.3	< 2.7	2.1	354.0
6 6 0	5.3	4.2	344.4	4.4	2.7	221.9
8 6 0	9.6	10.0	171.0	15.3	14.2	176.0
10 6 0	3.8	3.9	184.3	8.5	8.5	178.0
12 6 0	2.0	1.9	221.3	< 2.1	4.4	180.9
14 6 0	5.1	4.9	174.9	< 1.7	1.0	167.9
16 6 0				3.9	7.0	352.2
1 7 0	6.0	6.4	4.4	8.8	9.2	1.7
3 7 0	3.2	1.7	192.9	< 1.9	3.2	357.7
5 7 0	2.6	3.0	354.2	< 1.8	1.0	69.7
7 7 0	2.6	2.6	182.5	6.1	6.5	175.8
9 7 0	4.8	5.7	182.0			

It will be observed from Figs. 3 and 4 that a nitrogen and a carbon atom are overlapping in these projections. The y coordinates of these atoms were found by using standard methods for localization of maxima, the x parameters for these atoms being taken from the [010] projections. Atomic coordinates are listed in Table 1, observed and calculated structure amplitudes including phase angles in Table 2.

DESCRIPTION OF THE STRUCTURE

In Table 3 some calculated bond distances and angles are presented. The listed sums of covalent and ionic radii are based on Pauling's values [5]. Suffixes

Table 3. Bond distances and angles

A $[(CH_3)_2NH{\cdot}HCl]_2{\cdot}I_2$; B $[(CH_3)_2NH \cdot HBr]_2{\cdot}Br_2$.

	Bond	Bond length(Å)	Sum of covalent radii(Å)	Sum of ionic r.(Å) (≈ sum V.d.W.r.)	Angle
A	I—I	2.74	2.66		I—I—Cl 173.4°
	I—Cl	3.11	2.32	3.97	I—Cl—N 94.2°
	Cl—N_1	3.05	1.70	3.29	
	Cl—N_1'	3.28		3.29	
	Cl—N_2	3.27		3.29	
	N—C_1	1.47	1.47		
	N—C_2	1.48	1.47		
	I—N_1	4.51		3.64	
B	Br_1—Br_1	2.42	2.28		Br—Br—Br 173.0°
	Br_1—Br_2	3.03	2.28	3.90	Br—Br—N 95.5°
	Br_2—N_1	3.15	1.84	3.43	
	Br_2—N_1'	3.49		3.43	
	Br_2—N_2	3.50		3.43	
	N—C_1	1.52	1.47		
	N—C_2	1.51	1.47		
	Br_1—N_1	4.57		3.43	

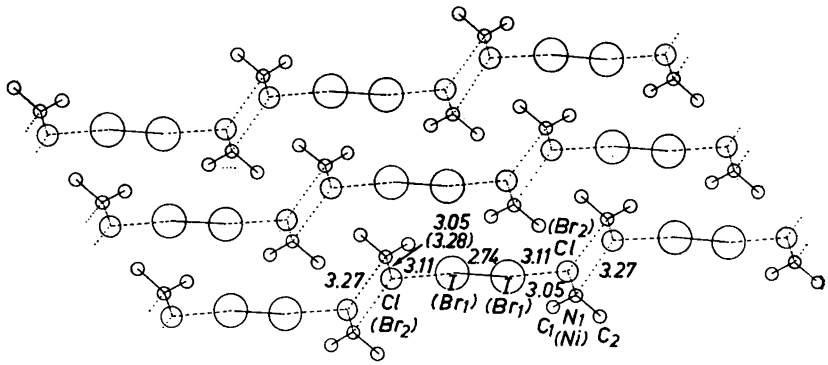


Fig. 5. The structure of dimethylammonium chloride-iodine (2:1) as seen along [010].

attached to the atomic symbols refer to Fig. 5 which shows the structure of dimethylammonium chloride-iodine (2:1) projected along [010].

Standard deviations in bond lengths are as follows:

$$\sigma(\text{I—I}) = 0.003 \text{ Å} \quad ; \quad \sigma = (\text{Br}_1\text{—Br}_1) = 0.004 \text{ Å}$$
$$\sigma(\text{I—Cl}) = 0.008 \text{ Å} \quad ; \quad \sigma = (\text{Br}_1\text{—Br}_2) = 0.004 \text{ Å}$$
$$\sigma(\text{Cl—N}) = 0.021 \text{ Å} \quad ; \quad \sigma = (\text{Br}_2\text{—N}) = 0.020 \text{ Å}$$

A striking feature of these structures is the halogen chains, Cl—I—I—Cl or Br—Br—Br—Br, which show only a slight deviation from linearity, the halogen-halogen-halogen angles being approximately 173°. The bond between the two atoms in the middle of the chain is covalent, although a slight bond elongation is observed in both cases. This change in bond length is due to an interaction between the atoms in the middle of the chain and the adjacent terminal ones. The bond distance between a middle and a terminal halogen atom is nearly equal to the mean of the respective covalent and van der Waals radius sums (*cf.* the structure of caesium tetraiodide)[6]. Moreover, each of the terminal halogen atoms is connected to three nitrogen atoms. It will be noticed from Table 3 that one of the corresponding bond distances (Cl—N_1 or Br—N_1) is considerably shorter than the two others (Cl—N_1' and Cl—N_2 or Br_2—N_1' and Br_2—N_2) which are of practically equal length. Furthermore, the Hal—N_1 bond is directed along one of the tetrahedral bond directions from nitrogen thus indicating the presence of a hydrogen bridge.

The lengths of the two other halogen-nitrogen bonds mentioned above agree well with the nitrogen-halogen distances observed in ammonium halides.

The halogen chain may be interpreted as an addition complex formed by two halogen ions (donors) and one halogen molecule (acceptor).[7]

The author is grateful to Professor O. Hassel for his interest in this work. The investigation has been supported by *The Rockefeller Foundation*.

REFERENCES

1. Norris, J. F. *Am. Chem. J.* **20** (1898) 51.
2. Remsen, I. and Norris, J. F. *Am. Chem. J.* **18** (1896) 91.
3. *Internationale Tabellen zur Bestimmung von Kristallstrukturen*, Vol. II, Gebrüder Borntraeger, Berlin 1935.
4. Allen, P. W. and Sutton, L. E. *Acta Cryst.* **3** (1950) 46.
5. Pauling, L. *The Nature of the Chemical Bond*, Cornell University Press, New York 1948.
6. Havinga, E. E., Boswijk, K. H. and Wiebenga, E. H. *Acta Cryst.* **7** (1954) 487.
7. Hassel, O. *Molecular Physics* **1** (1958) 241.

Received September 4, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2101–2106

Studies Related to Naturally Occurring Acetylene Compounds

XXVII. The Synthesis of a Mixture of Trideca-1:3:11-triene-5:7:9-triyne (II) and *trans* 1-phenylhepta-1:3-diyn-5-ene (V). A Novel Cyclisation Reaction

LARS SKATTEBÖL and NILS ANDREAS SÖRENSEN

SINTEF and Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

A synthesis of (II) is described which unexpectedly also yielded some (V). The cyclisation leading to (V) was confirmed by the isolation of diphenylacetylene(IX) from a similar reaction starting with 1-phenylocta-5:7-dien-2-yne-1:4-diol (VIII). A mechanism for this novel cyclisation reaction is briefly discussed.

A number of aliphatic and aromatic polyacetylenic hydrocarbons have been isolated from higher plants [1]. Among these are a series of straight chain C_{13} compounds, isolated from some members of the family *Compositae:*

trans $CH_3 \cdot CH=CH \cdot C\equiv C \cdot C\equiv C \cdot CH=CH \cdot CH=CH-CH=CH_2$ (Ref.[2])

(I)

$CH_3 \cdot CH=CH \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot CH=CH-CH=CH_2$ (Ref.[3])

(II)

cis and *trans* $CH_3 \cdot CH=CH \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot CH=CH_2$ (Refs.[2,13])

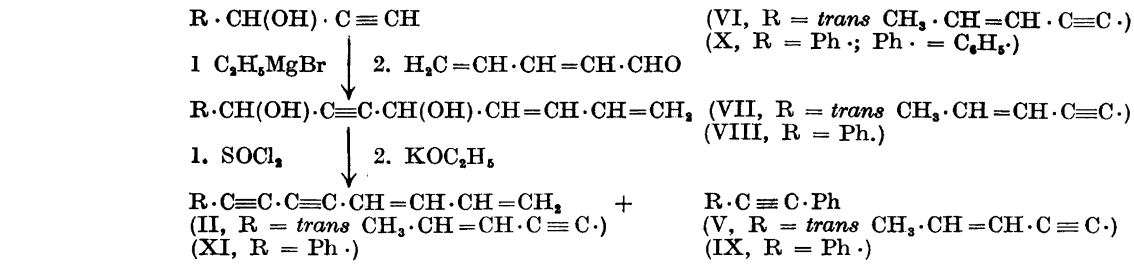
(III)

$CH_3 \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot C\equiv C \cdot CH=CH_2$ (Ref.[4])

(IV)

The structures of (I) [5], (II) [6], (III) [7] and (IV) [7] have been confirmed by synthesis but the preparation of (II) has only been reported since the present work was started. This paper describes a different approach to the synthesis of this compound which resulted in a mixture of (II) and *trans* 1-phenylhepta-1:3-diyn-5-ene (V). The latter also occurs naturally [3], accompanying (II) in a number of *Coreopsis* species.

The following sequence of reactions was expected to yield (II):



2:4-Pentadienal was prepared from 2:3-dihydropyran * according to Woods and Sanders [8], and the aldehyde was shown to be the *trans* isomer by infrared spectroscopy. *trans* Octa-6-ene-1:4-diyn-3-ol (VI) was prepared according to Jones, Skatteböl and Whiting [7]. The Grignard reaction between these compounds gave the corresponding glycol (VII) as a crystalline compound in 27 % yield, m.p. 65—67°. The yield of crude material was considerably higher but losses occurred during the careful purification. The ultraviolet absorption maximum at 2 260 Å showed that no anionotropic rearrangement had taken place, and that structure (VII) was correct. This was confirmed by infrared measurements which also indicated that the double bonds had the *trans* configuration.

The subsequent chlorination with thionyl chloride and dehydrochlorination with potassium ethoxide at —40° gave a small amount of a liquid product, which was purified by chromatography on alumina. The ultraviolet spectrum of the petroleum-ether eluate was practically identical with that of (II) [3,6] as far as the long wavelength maxima were concerned (Fig. 1). The infrared spectrum, however, showed some bands at 1 493, 753 and 687 cm^{-1} which indicated the presence of some aromatic contaminant. The product was distilled under high-vacuum and the distillate gave surprisingly an absorption in ultraviolet light (Fig. 1) similar to that of *trans* 1-phenylhepta-1:3-diyn-5-ene (V) [3]. The infrared spectrum was also very similar to that of (V). It seemed probable that a cyclisation had taken place in the dehydrochlorination step.

Chromatography of the distillation residue eventually gave a liquid which, from ultraviolet and infrared spectra, consisted mainly of (II). Because of the very small amounts available, a serious attempt to separate this mixture was not made. The mixture did not give an adduct with maleic anhydride in benzene and illumination with daylight, in the presence of iodine, produced small hypsochromic shift (25—30 Å) of the ultraviolet absorption maxima. These results together with a band at 716 cm^{-1} in the infrared spectrum are in accordance with a *cis* configuration about the double bond at the 3-position in (II). However, the presence of the *trans* isomer was not excluded, and the mixture consisted probably of these two isomers and some (V). The start-

* We are indebted to Dr. R. D. Smith and E. I. duPont de Nemours and Company for a generous gift of 2:3-dihydropyran.

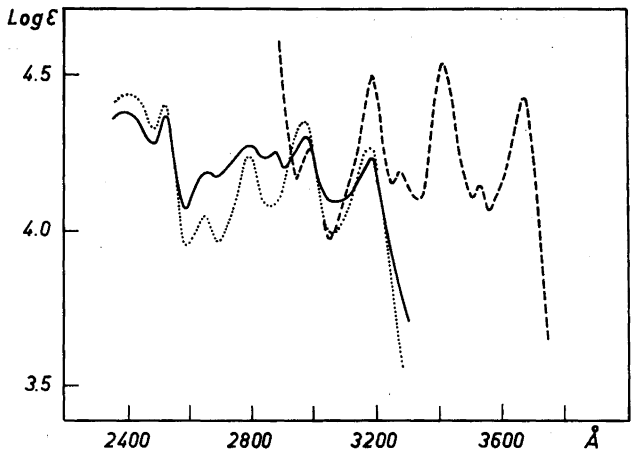


Fig. 1. Ultraviolet absorption in ethanol of:
Trideca-1:3:11-triene-5:7:9-triyne (II) --------
1-Phenylhepta-1:3-diyn-5-ene (V) ————
» » » » , authentic [3] .....

ing material (VI) was definitely the *trans* isomer, and the reactions were not expected to cause any isomerisation of the double bond at the 11-position.

In order to confirm this interesting cyclisation, we wanted to carry out the reactions with 1-phenylocta-5:7-dien-2-yne-1:4-diol (VIII) in the same way as described above for (VII). In this case the anticipated cyclisation product would be diphenylacetylene (IX), a stable crystalline compound, and the isolation of this, and its identity with authentic diphenylacetylene, would provide unambiguous proof for the cyclisation reaction.

The Grignard reaction between 1-phenylprop-2-yn-1-ol (X) and pentadienal was carried out as in the previous case. The glycol (VIII) was obtained in 14 % yield as a pure crystalline compound, m.p. 75—76 . The ultraviolet absorption maximum at 2 245 Å again showed that no anionotropic rearrangement had occurred, and the infrared spectrum was in agreement with a *trans* configuration. Treatment of this with thionyl chloride gave a dichloride which absorbed strongly at 2 705 Å in ultraviolet light. A rearrangement had evidently taken place, with the formation of a dienyne-chromophore, *viz.* —C≡C·CH=CH—CH=CH—CH_2Cl. For comparison,

$$HC{\equiv}C{\cdot}CH{=}CH{\cdot}CH{=}CH{\cdot}CH_2Br$$

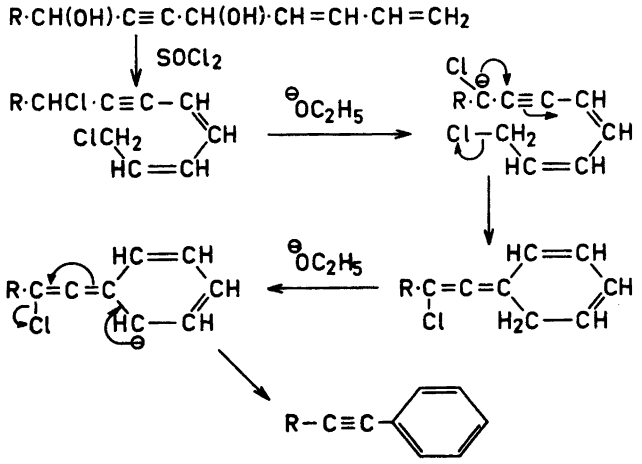
is reported [10] to absorb at 2 720 Å.

The crude dichloride was dehydrohalogenated, and the product chromatographed on alumina. A liquid was obtained by elution with petroleum-ether containing 10—30 % benzene. Its ultraviolet spectrum revealed the presence of a mixture with the longest wavelength maximum at 3 665 Å. This maximum probably belonged to (XI), and a group of maxima at 2 975, 2 865 and 2 770 Å indicated that also some diphenylacetylene was present. The first attempts

to separate diphenylacetylene from the mixture by chromatography were not wholly successful, as only enriched fractions were obtained. However, when a sample was left in air at room temperature for some hours, chromatography gave a small amount of a colourless crystalline substance, m.p. 57°, in all respects identical with an authentic sample of diphenylacetylene [9].

Isolation of the second main product, *viz.* 1-phenylocta-1:3-diyne-5:7-diene (XI), was not attempted as it was expected to be rather tedious and was little interest to this work.

The mechanism of this cyclisation reaction can be visualized in the following way:



The anionotropic rearrangement in the chlorination step would be expected to give a mixture of *cis* and *trans* isomers, by analogy with previous results obtained with similar compounds [11]. The *cis* isomer is sterically favourable for a cyclisation, which would subsequently lead to the formation of a benzene derivative. The scheme given here for the actual dehydrochlorination step is certainly dubious, as the general mechanism for this very fast elimination reaction is not known.

This cyclisation is the more interesting as the two compounds (II) and (V) have been isolated from the same plants, *viz.* some *Coreopsis* species. One cannot exclude the possibility that the benzene derivative (V) is formed by cyclisation of the dienyne system of (II), but it seems more reasonable that they both derive from a common precursor. The resemblance between the two substances $Ph \cdot CH_2C{\equiv}C \cdot$ and

$$CH_2{=}CH \cdot CH{=}CH \cdot C{\equiv}C \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{=}CH \cdot CH_2OCOCH_3$$

isolated [12] from *Carlina vulgaris*, and

$$Ph \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{=}CH \cdot CH_2OCOCH_3$$

isolated [2] from some *Coreopsis* species, is also of considerable interest in this connection.

EXPERIMENTAL

All melting points are uncorrected. The ultraviolet absorption data were obtained in ethanol, unless otherwise stated, using a Beckman, Model DK recording spectrophotometer. A Perkin Elmer instrument, Model 21, was used for the infrared measurements. The carbon and hydrogen determinations were carried out by Alfred Bernhardt, Mülheim, Ruhr, Germany.

trans *Octa-6-ene-1:4-diyn-3-ol(VI)* was prepared in Manchester [7] from hex-4-en-2-yn-1-al and ethynylmagnesium bromide.

Penta-2:4-dienal was prepared according to Wooes and Sanders [8], in 25 % overall yield from 2:3-dihydropyran.

trans *Trideca-1:3:11-triene-6:9-diyne-5:8-diol.* (VII). A solution of *trans* octa-6-ene-1:4-diyn-3-ol (VI, 5.8 g) in tetrahydrofuran (10 ml), was added during 30 min to an ice-cooled solution of ethylmagnesium bromide, prepared from magnesium (2.4 g) in tetrahydrofuran (60 ml). After 1.5 h at room temperature, freshly ditilled pentadienal (1.5 g) in tetrahydrofuran (5 ml) was added with stirring and ice-cooling. The reaction mixture was then left with stirring at room temperature for 4 h and decomposed with a saturated aqueous solution of ammonium chloride. Extraction with ether, and evaporation of the solvents gave a dark-coloured liquid which was purified by chromatography on deactivated alumina (5 % of 10 % acetic acid). The glycol was eluted with ether and rechromatographed under the same conditions. The light yellow liquid obtained, was dissolved in ether-petroleum-ether, and after standing at −25° gave some crystals. Recrystallisation from the same solvent yielded the glycol (970 mg, 27 %), m.p. 65−67° (Found: C 76.7; H 6.8. Calc. for $C_{13}H_{14}O_2$; C 77.20; H 6.98). Ultraviolet absorption: λ_{max} 2 260 Å, ε = 43 000. The infrared spectrum (in nujol): 3 300 (O−H), 2 210 (C≡C), 1 605 (C=C), 948 (C=C−H), 983 and 923 cm^{-1} (C=CH−H).

Trideca-1:3:11-trien-5:7:9-triyne (II) and trans *1-phenylhepta-1:3-diyn-5-ene* (V). The glycol (VII, 880 mg) was dissolved in dry ether (3 ml) and cooled in an ice-bath. Thionyl chloride (1 ml) was added with stirring and the reaction mixture left for 2 h. Ether and excess thionyl chloride were evaporated off under vacuum, and the residue dissolved in 10 ml of dry ether. This solution was added with stirring to potassium ethoxide, prepared from potassium (1.5 g) in dry ethanol (25 ml), and cooled to −40°. The reaction mixture was left while the temperature rose to −10° (30 min). Addition of water and isolation with ether gave a dark-coloured liquid which was purified by chromatography on alumina. With petroleum ether a product was eluted, which after further chromatography was obtained as a light yellow oil with the following absorption maxima in ultraviolet light: 3 665, 3 525, 3 410, 3 275, 3 190 and 2 995 Å (Fig. 1). The infrared spectrum (liq.) showed bands at 752 and 688 cm^{-1}, characteristic of a substituted benzene ring, in addition to bands expected for the aliphatic hydrocarbon (II).

Part of the mixture was high-vacuum distilled, and a light yellow liquid was obtained at 40−45° (bath temperature)/10^{-5} mm. The ultraviolet spectrum resembled that of the naturally occurring substance (V)[3] (Fig. 1). The infrared spectrum (liq.) had the following main absorption maxima: 3 060 (ar. C−H), 2 870 (C−H), 2 190 (C≡C), 1 630 (C=C), 1 600, 1 493, (ar. C=C), 1 445 (−CH_3)), 995, 940, 922 (C=C−H), 753, 687 (ar. C−H), 716 cm^{-1} (C=C−H). Except for the fairly weak band at 716 cm^{-1} the spectrum was almost identical with that of (V).

Isomerisation. Part of the mixture, dissolved in hexane with a few crystals of iodine, was placed in daylight for some hours. The ultraviolet maxima were shifted 25−30 Å to shorter wavelengths.

trans *1-Phenylocta-5:7-diene-2-yne-1:4-diol (VIII)* was prepared in the same way as for (II) above, by condensing 1-phenylprop-2-yne-1-ol (X, 13.2 g) with pentadienal (8.2 g). The reaction time in this experiment was 13 h. Chromatography and recrystallisation from ether-petroleum-ether yielded the glycol, (2.90 g, 14 %), m.p. 75−76°. (Found: C 78.61; H 6.66. Calc. for $C_{14}H_{14}O_2$: C 78.48; H 6.59). Ultraviolet absorption: λmax. 2 245, 2 220 (infl.) Å. ε = 36 000 and 35 300. The infrared spectrum (in $CHCl_3$): 3 360 (−H), 1 615 (C=C), 1 500 (ar C=C), 1 000, 953, 905 (C=C−H), 690 cm^{-1} (ar. C−H).

Diphenylacetylene (IX). The glycol (VIII, 2.0 g) was dissolved in dry ether (6 ml) and cooled in an ice-bath. Thionyl chloride (2 ml) was added with stirring, and the reaction mixture left for 1 h at room temperature. Ether and excess thionyl chloride were then evapo-

rated off under vacuum, and the residue dissolved in dry ether (10 ml). Ultraviolet absorption: $\lambda_{max.}$ 2 705 Å. The solution was added dropwise to a stirred solution of potassium ethoxide, prepared from potassium (2.0 g) in ethanol (25 ml.) and cooled to −40°. The mixture was stirred for 30 min, the bath-temperature being allowed to rise to −10°. Addition of water and isolation with ether gave a viscous liquid which was chromatographed on alumina. A light yellow-coloured liquid was eluted with petroleum-ether 10−20 % benzene. Ultraviolet absorption: $\lambda_{max.}$ 3 665, 2 975, 2 865, 2 770, 2 625, 2 570 Å. The last fraction, presumed to be enriched in diphenyl-acetylene, was evaporated and left at room temperature for some hours. Chromatography yielded a small amount (~ 3 mg) of a crystalline substance, eluted with petroleum-ether, 20 % benzene. The compound was recrystallised from ethanol-water, m.p. 57°. Ultraviolet absorption λmax. 2 960, 2 870, 2 785, 2 715, 2 640 and 2 360 Å. ε = 29 000, 23 000, 33 500, 25 500, 23 000 and 9 500, respectively. Infrared absorption (in CCl_4): 3 100 [ar. C−H). 1 610, 1 505, 1 450 (ar. C=C), 1 405, 1 025, 910 and 687 cm^{-1} (ar. C−H).

Authentic diphenylacetylene [9], m.p. 60°, had the following maxima in ultraviolet light: λmax. 2 960, 2 870, 2 785, 2 715, 2 640 and 2 360 Å. ε = 31 700, 23 600, 38 000, 25 600, 23 100, 8 600, respectively. The two compounds, mixed 1:1, melted at 57−58° and their infrared spectra were identical.

REFERENCES

1. Comprehensive reviews: Bohlmann, F. and Mannhardt, H. J. Acetylenverbindungen im Pflanzenreich, *Fortschritte der Chemie organischer Naturstoffe*, XIV Band, Springer-Verlag, Wien 1959; Bu'lock, J. D. *Quart. Rev.* **10** (1956) 371.
2. Sörensen, J. S. and Sörensen, N. A. *Acta Chem. Scand.* **8** (1954) 1741.
3. Sörensen, J. S. and Sörensen, N. A. *Ibid.* **12** (1958) 756.
4. Sörensen, J. S., Holme, D., Borlaug, E. T. and Sörensen, N. A. *Ibid.* **8** (1954) 1769.
5. Bohlmann, F. and Mannhardt, H. J. *Ber.* **88** (1955) 1330.
6. Bohlmann, F. and Herbst, P. *Ibid.* **91** (1958) 1631.
7. Jones, E. R. H., Skatteböl, L. and Whiting, J. C. *J. Chem. Soc.* **1958** 1054.
8. Woods, G. F. and Sanders, H. *J. Am. Chem. Soc.* **68** (1946) 2483.
9. Smith, J. S. and Falkof, M. M. *Org. Syntheses* **22** (1942) 50.
10. Bohlmann, F. and Viehe, H. G. *Ber.* **87** (1954) 712.
11. Bell, I., Jones, E. R. H. and Whiting, M. C. *J. Chem. Soc.* **1957** 2597.
12. Sörensen, J. S. and Sörensen, N. A. *Acta Chem. Scand.* **8** (1954) 1763.
13. Jones, E. R. H., Thompson, J. M. and Whiting, M. C. *Acta Chem. Scand.* **8** (1954) 1944.

Received August 17, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2107–2113

Spectrophotometric Determination of Formula, Rate of Formation and Stability Constant of the Boron 1,1′-Dianthrimide Complex

F. J. LANGMYHR and O. B. SKAAR

University of Oslo, Chemical Institute A, Blindern, Norway

The reaction between boron and 1,1′dianthrimide has been investigated spectrophotometrically. By means of the method of continuous variation the mole ratio between the reactants was found to be 1:1. The velocity constant for the formation of the complex in 93.8 % sulphuric acid at 71°C was determined to be $k = 1\,600$ $M^{-1} \cdot h^{-1}$. The stability constant in 93.8 % sulphuric acid and at 70°C was found to be $K = 1.4 \times 10^5$ M^{-1}.

A survey of the literature on the determination of boron with 1,1'-dianthrimide shows that neither the formula nor the stability constant of the complex has been determined previously. Mr. Danielsson [1] of Metallografiska Institutet, Stockholm, has informed the authors that his investigations on the composition of the complex indicate the mole ratio 1:1.

In the present investigation the formula, the rate of formation and the stability constant of the complex have been determined spectrophotometrically.

EXPERIMENTAL

Instruments. Extinction measurements were made with a Zeiss spectrophotometer PMQ II, a matched set of 1.000 cm glass cells being used.

Reagents. Boric acid of reagent grade quality was used for the preparation of standard solutions.

The 1,1′-dianthrimide (E. Merck) was recrystallized twice from nitrobenzene before use.

Sulphuric acid, 95–97 %, and other chemicals were of reagent grade quality. The strength of the sulphuric acid was found to be 96.8 %. Precautions were taken to prevent the acid from absorbing water vapour, and to avoid discoloration by contamination with dust.

Ordinary distilled water from an all metal still was employed.

Standard solutions. 0.5715 g of boric acid (H_3BO_3) were weighed out, dissolved in concentrated sulphuric acid and diluted to 1 000 ml with concentrated sulphuric acid. From this standard solution (containing 100 μg B per ml) 50 ml were pipetted into another

1 000 ml volumetric flask and diluted to the mark with concentrated sulphuric acid. From this latter solution (containing 5 μg B per ml) known amounts of boron were taken.

0.0794 g of 1,1'-dianthrimide were dissolved in concentrated sulphuric acid and diluted to 1 000 ml with the same acid.

Glassware. The different solutions were prepared and heat-treated in 50 ml bottles *(Jena Geräteglas)* with ground-in glass stoppers.

Heat treatment. The heat treatment was given in a thermostatically controlled drying oven of standard construction.

ABSORPTION CURVES

Absorption curves were plotted of solutions of the boron 1,1'-dianthrimide complex and of solutions of 1,1'-dianthrimide.

The solutions measured spectrophotometrically were prepared in the following way. 1 ml of boron standard solution (containing 5 μg boron), 1 ml of distilled water, 11 ml of concentrated sulphuric acid and 5 ml of 1,1'-dianthrimide standard solution were pipetted into a 50 ml bottle *. The concentration of sulphuric acid in the final solution was 93.8 %. After mixing the solution was heated for 16 h at 70° ± 2°C. The corresponding blank solution contained 1 ml of distilled water, 12 ml of concentrated sulphuric acid and 5 ml of 1,1'-dianthrimide standard solution. Heating time and temperature were as described for the sample solution.

Fig. 1, Curve 1 gives the absorption curve of the boron 1,1'-dianthrimide complex measured against the blank solution. Curve 2 shows the absorption curve of the 1,1'-dianthrimide blank solution measured against concentrated sulphuric acid.

The boron 1,1'-dianthrimide complex has a distinct maximum at 630 mμ. At lower wavelengths there are other, smaller maxima. The maximum at 630 mμ is suitable for the spectrophotometric determination of boron. At 630 mμ the Beer—Lambert law is followed up to at least 7 μg B per 17.6 ml, the molar extinction coefficient for the system being $\varepsilon = 1.97 \times 10^4$.

DETERMINATION OF FORMULA

Job's [2] method of continuous variation, as modified by Vosburgh and Cooper [3], was employed for elucidating the composition of the complex.

According to Job's method the extinctions are measured for series of solutions in which the sum of the concentrations of boron and 1,1'-dianthrimide is kept constant, while the ratio between the reactants is varied.

The series of solutions needed for the determination of the formula were prepared from the standard solutions of boron and 1,1'-dianthrimide (*vide supra*). Each sample solution was finally measured against a corresponding blank containing the same amount of 1,1'-dianthrimide. The concentration of sulphuric acid in the final solutions was again 93.8 %.

In the amounts of boron taken are included boron present as impurity in the reagents. The constant concentration of boron in the 17.6 ml reagent solutions was found to be 0.19×10^{-5} mole. The sample solutions were heated for 16 h at 70° ± 2°C. On the other hand, the blanks were not heated. By this

* Due to contraction the final volume is not 18.0, but 17.6 ml.

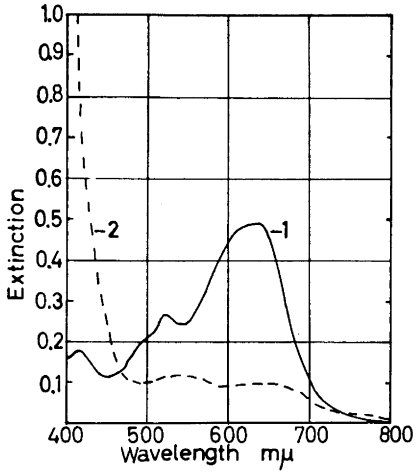


Fig. 1. Curve 1, Absorption curve of the boron 1,1'-dianthrimide complex in 93.8 % sulphuric acid. Curve 2, Absorption curve of 1,1'-dianthrimide in 93.8 % sulphuric acid.

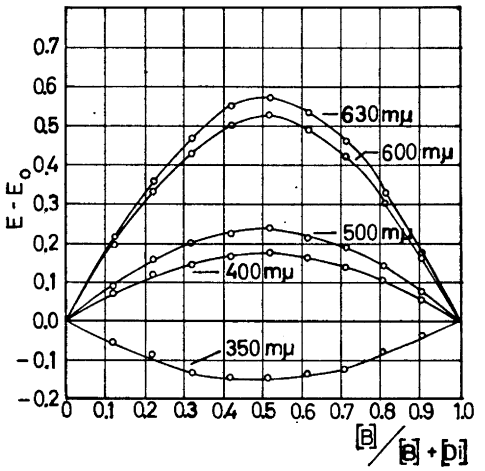


Fig. 2. Curves of continuous variation for mixtures of boron and 1,1'-dianthrimide.

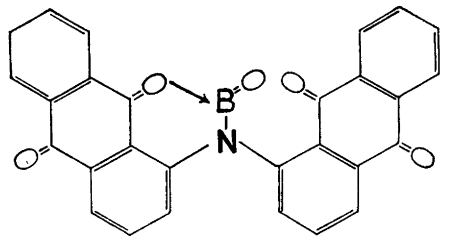
procedure the colour originating from the boron present in the blanks was not developed.

The extinctions were measured at five different wavelengths to guard against the possibility that more than one complex is present. Measurements, in which the sum of concentrations of boron an 1,1'-dianthrimide is varied, have not been carried out.

In Fig. 2 the curves of continuous variation are plotted. It is seen that negative extinction values were obtained for the curve plotted at 350 mμ. At this wavelength the blank solutions absorb more than the corresponding sample solutions.

From Fig. 2 it can be concluded that the mole ratio of boron to 1,1'-dianthrimide is 1:1.

Structure of the complex. Having determined the mole ratio of the complex, it is possible to suggest the following structure of the boron 1,1'-dianthrimide complex in solution.



The structure is in accordance with that given by Grob and Yoe [4] for the complex between boron and 5-benzamido-6'-chloro-1,1'-bis (anthraquinonyl) amine.

DETERMINATION OF RATE OF REACTION

In concentrated sulphuric acid boron (assumed to be present as metaboric acid, HBO_2) and 1,1'-dianthrimide react at a very slow rate. The rate of reaction increases with the temperature, but even at 70°C the colour is developed relatively slowly.

In the present investigation the progress of the reaction between boron and 1,1'-dianthrimide was followed spectrophotometrically. On the basis of these kinetic studies the reaction velocity for the formation of the complex was determined.

The experimental details were as follows: 12 sample solutions were prepared, each consisting of 1 ml of distilled water, 10 ml of concentrated sulphuric acid, 2 ml of boric acid standard solution (corresponding to 10 μg B) and 5 ml of 1,1'-dianthrimide standard solution. In addition a blank solution was prepared, in which the volume of boric acid solution was replaced by 2 ml of concentrated sulphuric acid. All sample solutions were placed in the drying oven at 71° $\pm$0.5°C and heated for different periods of time. After the prefixed time of heating, 2 sample solutions were removed, cooled in the air to room temperature and the extinction was measured against the unheated blank. In the extinctions given in Table 1 for the sample solutions are also included the contribution from the constant amount of boron present as impurity, *viz.* 0.19×10^{-5} mole B per 17.6 ml reagent solution. Corrections were further made for the different amount of free 1,1'-dianthrimide present in sample and blank solutions. The corrected extinction data are given in Table 1.

Table 1. Formation of boron 1,1'-dianthrimide complex in 93.8 % sulphuric acid at 71°C. Extinction (1 000 $\times$ E) for solutions heated for the specified number of hours after the removal of the first pair of sample solutions from drying oven.

Heating time, h	0	2	4	6	18	43
Average E of 2 samples (1)	104	231	326	369	594	720
Blank (not heated) (2)	10	9	8	7	5	3
Increase of E (1−2)	94	222	318	362	589	717

The following, rate-determining mechanism of reaction between metaboric acid (HBO_2) and 1,1'-dianthrimide ($(Anthra)_2NH$)) is suggested:

	$(Anthra)_2NH$ +	$HOBO \rightleftharpoons$	$(Anthra)_2NBO$ +	H_2O
Concentrations at the time $t = 0$	a	b	0	y
Concentrations at the time t	$a - x$	$b - x$	x	y
Concentrations at the time $t = \infty$	$a - z$	$b - z$	z	y

The reaction takes place in concentrated sulphuric acid, and the water formed is taken up by the acid. The concentration of water (y) can then be considered constant.

The following kinetic equation is obtained for the formation of the complex:

$$\frac{dx}{dt} = k(a-x)(b-x) - k''xy \qquad (1)$$

where k and k'' are the velocity constants of the forward and the reverse reaction, respectively.

Further $k''y = k'$ and $K'/y = K$, where K' is the stability constant of the complex. In this investigation y was considered constant at a constant concentration of sulphuric acid, and K was introduced as apparent stability constant. By introducing these values in eqn. 1 and integrating between x_1, t_1 and x_2, t_2 the following expression is obtained for k:

$$k = \frac{2.303 \cdot \log\left[\frac{a+b+1/K+\sqrt{\ }-2x_2}{a+b+1/K-\sqrt{\ }-2x_2} \cdot \frac{a+b+1/K-\sqrt{\ }-2x_1}{a+b+1/K+\sqrt{\ }-2x_1}\right]}{[t_2-t_1]\sqrt{\ }} \qquad (2)$$

The square root sign $\sqrt{\ }$ stands for $\sqrt{(a+b+1/K)^2-4ab}$.

To calculate k from eqn. 2, it is necessary to know K (the apparent stability constant) and the constant impurity of boron originating from the reagents. By means of methods described below approximative data for K and the constant impurity of boron were obtained experimentally, and these data gave a preliminary value of k. This first value of k was employed to adjust the data for K and the constant impurity of boron, and these improved data were again used to correct k. The approximations were carried out until constant values were obtained. The final values were:

$$K = 1.4 \times 10^5 \ \mathrm{M^{-1}}$$
$$k = 1\,600 \ \mathrm{M^{-1} \cdot h^{-1}}$$

These data are valid in 93.8 % sulphuric acid and at 70° and 71°C, respectively.

The constant impurity of boron was found to be 0.19×10^{-5} mole B per 17.6 ml of reagent solution.

The experimental data (Table 1) gave a constant value of k, thus confirming the validity of eqn. 1.

DETERMINATION OF APPARENT STABILITY CONSTANT

The following expression is obtained for the apparent stability constant K of the boron 1,1'-dianthrimide complex:

$$K = \frac{z}{(a-z)(b-z)}$$

For experimental determination of K, the two concentrations in the denominator should preferably be of approximately the same magnitude, and should not differ too much from that of the numerator. These conditions are obtained by choosing small and nearly equal concentrations of the reactants.

The usual equilibrium measurements were not suitable for the determination of the apparent stability constant of the present system. Prolonged heating of solutions of 1,1'-dianthrimide resulted in destruction of considerable amounts of reagent. The following, special procedure was therefore introduced.

A series of sample solutions were prepared according to the formula:

1 ml of distilled water + $(17-v_1-v_2)$ ml of concentrated sulphuric acid $+v_1$ ml of boron standard solution + v_2 ml of 1,1'-dianthrimide standard solution.

In one series of experiments v_1 was 1.2 ml while v_2 was 2, 3.5 and 5 ml. In another series v_1 was 2 and v_2 was 1.5, 2 and 3 ml. Blank solutions were not prepared, but the extinctions due to the amount of 1,1'-dianthrimide not taking part in the reaction were calculated (Specified as Blank in Table 2).

The sample solutions were then heated for 40 h at 70° ± 2°C. By this procedure the solutions were brought near equilibrium without destructing appreciable amounts of 1,1'-dianthrimide. After cooling, the extinctions (E_{40}) were measured against concentrated sulphuric acid. These extinction data gave an approximative value of K (applied for the preliminary determination of k). The final, equilibrium extinction values ($E\infty$) were determined by calculating the difference ($E\infty - E_{40}$). This relatively small correction to the E_{40} data was found by approximation as described above. From the data for $E\infty$ the equilibrium concentration of complex was found with the application of the molar extinction coefficient ($\varepsilon = 1.97 \times 10^4$). Equilibrium concentrations of boron and 1,1'-dianthrimide could then be calculated.

Table 2. Spectrophotometric determination of apparent stability constant of the boron 1,1'-dianthrimide complex. (70°C and 93.8 % sulphuric acid.)

	Extinctions (1 000 × E_{40}) at the concentrations specified					
Concentration of boron/1.05 × 10^{-5} M	3.18	3.18	3.18	5.18	5.18	5.18
Conc. of 1,1'-dianthrimide/1.05 × 10^{-5} M	2.00	3.50	5.00	1.50	2.00	3.00
Average E_{40} for 2 samples (1)	272	406	503	252	330	468
Blank (2) (calculated)	1	3	6	1	1	1
Corrected, average E_{40} values (1 − 2)	271	403	497	251	329	267
$E\infty - E_{40}$	32	36	27	10	15	26
$E\infty$	303	439	524	261	344	493
Calculated apparent stability constant $K/10^5$ M^{-1}	1.54	1.39	1.53	1.35	1.39	1.38

From the data in Table 2 the average value of 1.4×10^5 M^{-1} was obtained for the apparent stability constant of the complex in 93.8 % sulphuric acid.

An approximative value for the constant impurity of boron present in the reagents was found spectrophotometrically. Two identical blank solutions were prepared, and one of the solutions was heated for 40 h at 70°C. The extinction of this solution was measured against the solution not heated and the content of boron was calculated to be 0.16×10^{-5} mole B per 17.6 ml reagent solution. This value was employed for the preliminary calculation of k.

REFERENCES

1. Danielsson, L. *Private communication.*
2. Job, P. *Ann. Chim.* **9** (1928) 113.
3. Vosburgh, W. C. and Cooper, G. R. *J. Am. Chem. Soc.* **63** (1941) 437.
4. Grob, R. L. and Yoe, J. H. *Anal. Chim. Acta* **14** (1956) 253.

Received June 25, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2114–2118

Polarographic Determination of Hydroxylamine, alone and in Comparison with Hydroxamic Acid

TH. ØSTERUD and MILDA PRYTZ

Universitetets Farmasøytiske Institutt, Blindern-Oslo, Norway

Hydroxylamine has been polarographically reduced in ethanol-water solutions of tetraethylammonium iodide. The pH of the supporting electrolyte was increased by addition of small amounts of tetraethylammonium hydroxide.

The pH of the tetraethylammonium iodide solution before addition of hydroxide was 5.4. In this medium the hydroxylamine gave three distinct waves. The first wave which had $E_{½} = -1.44$ *vs* S.C.E. (—1.11 *vs* Hg), disappeared as soon as pH was raised, *i.e.* the buffer-capacity was increased. At pH = 5.6 this wave had disappeared and did not reappear on further increase of pH. In the pH-interval 5.6 to 13.3 the curves showed two well defined waves, the first, which had $E_{½} = -2.1$ *vs* S.C.E. (—1.74 *vs* Hg), being the highest. At pH = 11 it started decreasing, while the height of the second wave having $E_{½} = -2.35$ *vs* S.C.E. (—1.9 *vs* Hg) kept a constant low height. At pH greater than 14 the first wave seemed to coalesce with the second wave, which in this strong alkaline medium suddenly reaches an appreciable height.

The measurements of hydroxamic acid were a corroboration of earlier investigations, as we wanted to test eventual differences as regards the form of the curves and the values of the half wave potentials.

The first wave for the hydroxamic acid shows a half wave potential about 0.2 V lower than for the hydroxylamine, and the second wave about 0.1 V higher. The form of the curves did not differ noticeably.

Inasmuch as hydroxylamine is unstable in alkaline medium we controlled its deterioration at pH = 11.7 to 12.3 by taking a series of diagrams at fixed intervals of half an hour. Already after half an hour a decrease of wave height was unmistakable, and after an hour and a half the wave height had reached a low value which was controlled the next day. It had not changed appreciably over night. Next the reaction velocity was increased by raising the temperature to 60°C and 100°, respectively. A 60°C a wave height of about 1/3 of the original was reached after 30 min, and at 100°C even after a few minutes.

The time effect on solutions of acethydroxamic acid and on solutions of propiohydroxamic acid was also tested at pH = 11 to 11.7; but the polarographic waves of these acids were not influenced by time. The effect of raising the temperature was also tested. No differences in the curves were observed.

These results seem to indicate that eventual hydroxylamine waves need not interfere with polarographic waves of aliphatic hydroxamic acids if the hydroxylamine is decomposed by heating in alkaline solution (pH 12 to 14) before the polarograms are taken.

As our ultimate aim is to determine aliphatic esters polarographically after transformation into hydroxamic acids it is of interest to see if these acids give curves that differ from those of hydroxylamine, inasmuch as the reaction mixture may contain an excess of hydroxylamine.

Some mixtures of hydroxylamine and hydroxamic acid were also polarographed, and the effect of time and heating was tested. The pH was 10 and did not change appreciably during the experiments. The readings were taken at room temperature.

We presume to have established by these experiments that hydroxamic acid may be polarographically determined in a solution containing hydroxylamine, inasmuch as the latter may be destructed by heating, whereas the former is not affected.

EXPERIMENTAL

A recording polarograph from Radiometer, Copenhagen, was employed. As working cell was used an arrangement of Nife cells in series with a rectifier. The usual precautions against oxygen were taken. For investigation of the temperature influence a water bath was used.

The tetraethylammonium iodide for the supporting electrolyte was delivered by Merck, Darmstadt. It had to be recrystallized repeatedly, as it otherwise was apt to give quite

Table 1. Polarographic waves of hydroxylamine and of acethydroxamic acid in relation to pH. Measured at 1/sens. = 50; $c_{aceth.} = 7 \times 10^{-3}$ M; $c_{hydrox.} =$ about 10^{-3} M.

a. Mercury anode

Hydroxylamine			Acethydroxamic acid		
pH	$E^1_{1/2}$	$E^2_{1/2}$	pH	$E^1_{1/2}$	$E_{1/2}$
	(1.11)				
5.4	1.52	1.90	4.8	1.52	1.99
5.6	1.74	1.89			
5.8	1.76	1.92	5.8	1.59	2.04
7.2	1.80	1.96			
7.7	1.76	1.92			
8.8	1.74	1.89	8.8	1.59	2.04
11.0	1.73	1.87	11.0	1.58	2.03
13.3	1.65	1.78	13.3	1.54	2.03

b. S.C.E. anode

Hydroxylamine			Acethydroxamic acid		
pH	$E^1_{1/2}$	$E^2_{1/2}$	pH	$E^1_{1/2}$	$E^2_{1/2}$
	(1.44)				
5.4	2.07	2.35			
5.6	2.03	2.31			
6.8	2.06	2.34			
11.0	2.11	2.46	10.7	None	2.54
12.5	2.11	2.48	12.5	»	2.46
~14.	2.15	?	13	»	2.47

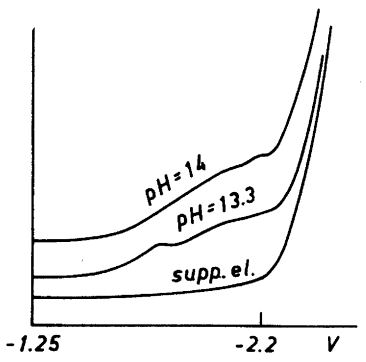
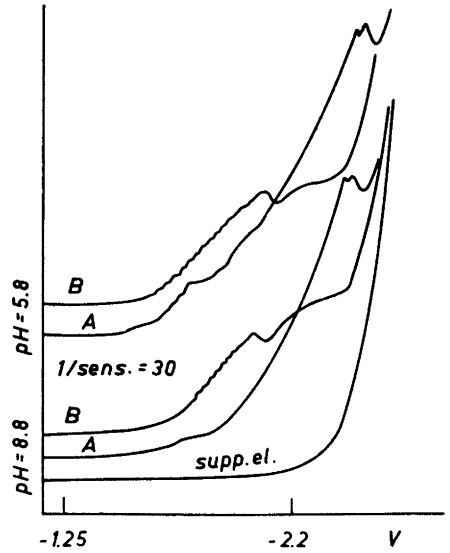


Fig. 1. a. Electroreduction of hydroxylamine at pH 13.3 and 14.



b. Electroreduction of acethydroxamic acid, A, and of hydroxylamine, B, at pH 5.8 and 8.8 1/sens. = 30.

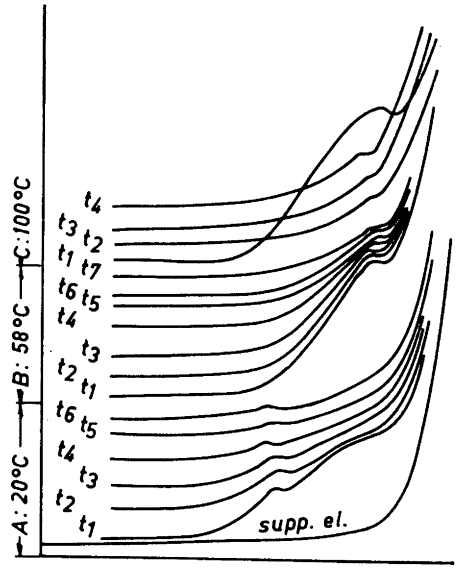


Fig. 2. Reduction of wave height of hydroxylamine with time at different temperatures:
A. at 20°C; $t_1 = 0$; $t_2 = ½$ hour; $t_3 = 1$ hour; $t_4 = 1\ ½$ hour; $t_5 = 2$ hours; $t_6 = 26$ hours
B. at 58°C; $t_1 = 0$; $t_2 = 3$ min.; $t_3 = 5$ min.; $t_4 = 12$ min.; $t_5 = 15$ min.; $t_6 = 18$ min.; $t_7 = 30$ min.
C. at 100°C; $t_1 = 0$; $t_2 = 15$ min; $t_3 = 10$ min.; $t_4 = 20$ min.

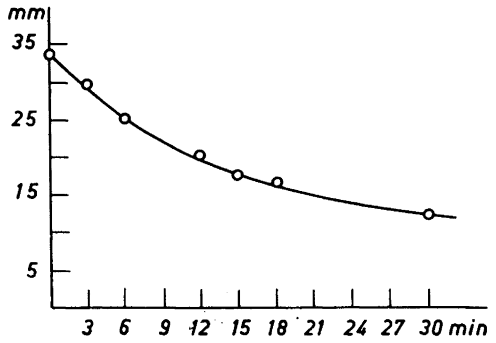


Fig. 3. Destruction of hydroxylamine at 58°C. pH = 12.3 Wave heights as ordinates. Lapse of time as abscissae.

irrelevant maxima and minima. 50 % alcohol proved to be the best solvent for recrystallization. For our previous investigation we used a tetraethylammonium iodide from the British Drughouses; but unfortunately we had very little salt left. Tetrabutylammonium iodide from A. Frederick Smith, Columbus, Ohio, gave straight and good curves without any recrystallization.

As a maximum depressor was used tylose, about 0.005 %.

RESULTS

The polarographic curves for hydroxylamine showed two waves similar to those we had earlier found for hydroxamic acid [1], but with other values for the half wave potentials. These are given for both hydroxylamine and acethydroxamic acid in Table 1.

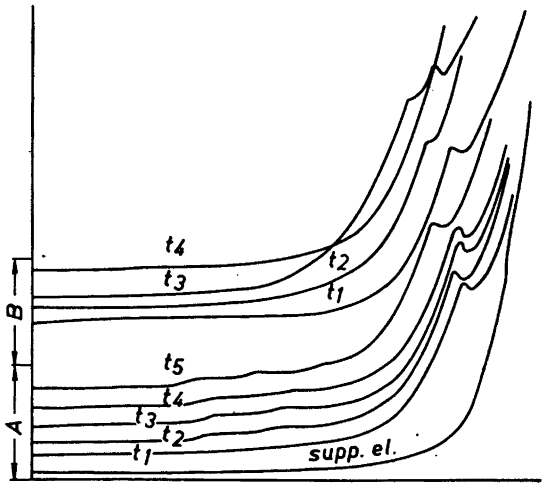


Fig. 4. Electroreduction of acethydroxamic acid at 20°C and at 100°C.
A. at 20°C; $t_1 = 0$; $t_2 = ½$ hour; $t_3 = 1$ hour; $t_4 = 1\ ½$ hour; $t_5 = 50$ hours.
B. at 100°C; $t_1 = 0$; $t_2 = 20$ min.; $t_3 = 50$ min.; t_4 after a lapse of 24 hours.

Some of the curves are given in Fig. 1 a and b.

It appears from Fig. 1 a that the hydroxylamine waves coalesce at pH =14. The first wave can still be seen at pH = 13.3, but at pH = 14 it has disappeared and the second wave has increased highly.

As regards the reduction of the hydroxylamine-waves with time and rise of temperature, Fig. 2 shows that at 20°C the wave height decreases appreciably during one hour and a half, while at 60°C half an hour is sufficient for a corresponding decrease, and at 100°C even a very short time will be enough. The decomposition of hydroxylamine with time at 60°C is shown in Fig. 3.

An inquiry into an eventual time- and temperature-effect for acethydroxamic acid and propiohydroxamic acid was also made. As will be seen from Fig. 4, the polarographic curves remained unaltered with time, even though the temperature was raised.

Mixtures of hydroxylamine and hydroxamic acid were also polarographed; but the polarograms brought no new information.

DISCUSSION

For the hydroxylamine as well as for hydroxamic acids we found that in alkaline solutions (at corresponding pH-intervals) they gave polarographic curves with two distinct waves. The half wave potentials for the hydroxylamine waves lay between those for acethydroxamic acid. At high pH values the hydroxylamine waves seemed to coalesce. The result was a single, rather high wave. A corresponding effect was not found for hydroxamic acids.

On the polarographic waves for hydroxamic acid no time effect was observed even if the test solutions were left standing till next day. The polarographic waves for hydroxylamine in alkaline solutions, on the other hand, decreased perceptibly if the polarographic determination was delayed. Heating accelerated the decrease greatly.

One may conclude as a consequence of these results that esters transformed to hydroxamic acids may be determined polarographically even if the reaction mixture contains some excess of hydroxylamine. All that needs to be done is to heat the mixture on a water bath for a short time before the curves are taken at room temperature.

The authors are indebted to Bjørn Bergmann-Paulsen for the recording of the polarograms.

REFERENCE

1. Prytz, M. and Østerud, Th. *Acta Chem. Scand.* **11** (1957) 1530.

Received June 6, 1959.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2119–2134

Proceedings of the Second Scandinavian Summer Meeting of Biochemistry, Medical Chemistry, Pharmacology and Physiology Turku, Finland, August 27—29, 1959

Papers Read in the Sections of Biochemistry and of Medical Chemistry

The Acid Mucopolysaccharides in Human Arterial Tissue

M. O. Dyrbye

Connective Tissue Research Laboratory University Institute of Medical Anatomy, Copenhagen, Denmark

Biochemical analyses of the mucopolysaccharides have shown:

1) The total hexosamine content of human aortae, pulmonary, coronary and cerebral arteries (intima-media layers) varied from 0.3 % to 0.18 % based upon wet weight of the tissues. The content of esterified sulfates — which is taken as the sulfate of the mucopolysaccharides in ester linkage — varied from 0.075 % to 0.042 %. The cerebral arteries showed the lowest concentration of both hexosamine and sulfate. The molar concentration of hexosamine (liberated by acid hydrolysis) was approximately twice as high as the molar concentration of the esterified sulfate in the genuine arterial tissue [1—3].

2) Aortae with severe calcification showed a significantly lower hexosamine concentration than aortae without calcification. The concentration of esterified sulfate in pulmonary arteries was significantly higher for older than for younger subjects [3]. In cerebral arteries both esterified sulfate and hexosamine were found to be higher for older than for younger individuals [3]. Apart from this no correlation between hexosamine, sulfate and age or degree of arteriosclerosis has been observed.

3) The acid mucopolysaccharides have been extracted from large amounts of pooled human aortic tissue. It was found that about 50 % of the extracted material was chondroitin sulfuric acid-A, 15 % was chondroitin sulfuric acid-B, approximately 15 % was heparitin sulfuric acid and the rest (about 20 %) seemed to be composed of substances related to heparin [4].

4) Mucopolysaccharides isolated from 30 different aortae contained hexosamine, hexuronic acid and sulfate in equimolar amounts. The content of galactosamine was 79 % of the total hexosamine. The galactosamine glucosamine ratio was significantly higher in older than in younger individuals [5].

5) Paper electrophoresis of the above-mentioned 30 samples of isolated mucopolysaccharides showed two fractions which stained metachromatically with toluidine blue. The faster moving averaged about 2/3 of the total isolated material and was assumed to be chondroitin sulfuric acid, while the slower moving compound seemed to be a mixture of different mucopolysaccharides [6,7].

6) In vitro experiments on human aortae by means of ^{35}S-labeled sulfate showed that the uptake of sulfate per weight unit of the acid mucopolysaccharides isolated from the tissue decreased definitely with age and with increasing arteriosclerosis [8].

These results indicate that *the acid mucopolysaccharides in the vessel wall may play an essential part in the aging of arterial tissue*, but at the present time little or nothing is known about the underlying mechanism.

1. Faber, M. *A.M.A. Arch. Pathol.* **48** (1949) 342.
2. Kirk, J. E. and Dyrbye, M., *J. Gerontol.* **11** (1956) 273.
3. Liang, I. and Kirk, J. E. *J. Gerontol.* **12** (1957) 388.

4. Meyer, K., Hoffman, Ph. and Linker, A. *CIOMS symposium on connective tissue*, Oxford, 1957, Blackwell Scientific Publications.
5. Dyrbye, M. and Kirk, J. E. *J. Gerontol.* **12** (1957) 20.
6. Dyrbye, M., Kirk, J. E. and Wang, J. *J. Gerontol.* **13** (1958) 149.
7. Kirk, J. E., Wang, J. and Dyrbye, M. *J. Gerontol.* **13** (1958) 362.
8. Dyrbye, M. O. *J. Gerontol.* **14** (1959) 32.

Fractionation of Gelatin by Ion-Exchange Resin

T. Tuominen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The figure shows the fractionation of rat bone gelatin (obtained with 50-fold volume of water at + 100°C for 12 h). The resin (Amberlite CG-50, 100—200 mesh) was equilibrated with pH 5.5 citrate buffer (McIlwaine) and used as 15 × 1 cm column, which was eluted first at room temperature with the same buffer and later at + 37°C with 0.1 N NaOH as described by Russell[1]. Fractions of 2 ml were collected (numbers are indicated) and protein estimated with a modified biuret reaction (Lowry). The first peak differred in amino acid composition from gelatin, the second and third resembled each other and gelatin. The fourth peak is not constant and its amino acid composition is not known.

1. Russell, G. *Nature* **181** (1958) 102; Ward, A.G. *Nature* **183** (1959) 440.

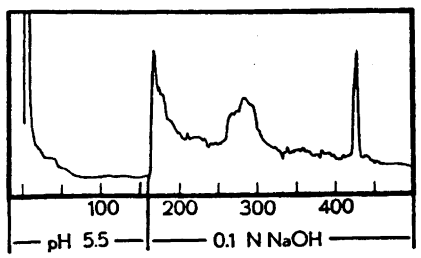


Fig. 1.

Effect of Hydrocortisone on the Nucleotides in Carrageenin Granuloma and Chick Embryo

U. K. Virtanen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The acid-soluble nucleotides were fractionated with Dowex-1 resin using elution with formiate according to Hurlbert *et al.*[1] and estimated by their absorption at 260 mμ. The peaks were further fractionated by paper chromatography and the composition of nucleotides determined by phosphate analysis and by paper chromatography of the purine bases and pyrimidine mononucleotides after acid hydrolysis.

Subcutaneous carrageenin granulomata were produced to cortisone-treated guinea pigs according to Robertson *et al.*[2] The tissue was prepared, homogenized and suspended into 0.6 N cold perchloric acid. The supernatant was neutralized and analyzed. Some differences were found between the cortisone-pretreated and control animals. The individual differences were, however, so large that no exact conclusions could be drawn.

The experiment was therefore repeated with chick embryos, which on the eighth day of incubation received 2.5 mg of hydrocortisone into the yolk sac. The embryos were prepared and analyzed as above after 1, 2, 3 or 4 days. The growth was almost totally inhibited.

Generally the energy-rich di- and triphosphates were decreased. In the region of monophosphates there appeared in all the samples from hydrocortisone-treated embryos a small but distinct peak which was totally absent from the controls. This peak is not yet positively identified. It has an absorption maximum at 262 mμ and in the paper chromatograms (using *iso*propanol-HCl) it migrates almost with the same rate as UMP. However, it cannot be the UMP, which emerges later.

On paper chromatographic evidence it seems that UMP and IMP are decreased after hydrocortisonetreatment apparently in equal degree.

1. Hurlbert, R. B., Schmitz, H., Brumm, A. F. and Potter, V. R. *J. Biol. Chem.* **209** (1954) 23.
2. Robertson, W. v. B. and Schwartz, B. *J. Biol. Chem.* **201** (1953) 681.

4. Meyer, K., Hoffman, Ph. and Linker, A. *CIOMS symposium on connective tissue*, Oxford, 1957, Blackwell Scientific Publications.
5. Dyrbye, M. and Kirk, J. E. *J. Gerontol.* **12** (1957) 20.
6. Dyrbye, M., Kirk, J. E. and Wang, J. *J. Gerontol.* **13** (1958) 149.
7. Kirk, J. E., Wang, J. and Dyrbye, M. *J. Gerontol.* **13** (1958) 362.
8. Dyrbye, M. O. *J. Gerontol.* **14** (1959) 32.

Fractionation of Gelatin by Ion-Exchange Resin

T. Tuominen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The figure shows the fractionation of rat bone gelatin (obtained with 50-fold volume of water at + 100°C for 12 h). The resin (Amberlite CG-50, 100—200 mesh) was equilibrated with pH 5.5 citrate buffer (McIlwaine) and used as 15 × 1 cm column, which was eluted first at room temperature with the same buffer and later at + 37°C with 0.1 N NaOH as described by Russell[1]. Fractions of 2 ml were collected (numbers are indicated) and protein estimated with a modified biuret reaction (Lowry). The first peak differred in amino acid composition from gelatin, the second and third resembled each other and gelatin. The fourth peak is not constant and its amino acid composition is not known.

1. Russell, G. *Nature* **181** (1958) 102; Ward, A.G. *Nature* **183** (1959) 440.

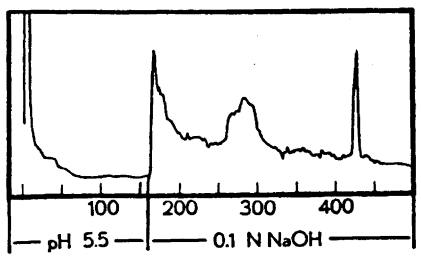


Fig. 1.

Effect of Hydrocortisone on the Nucleotides in Carrageenin Granuloma and Chick Embryo

U. K. Virtanen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The acid-soluble nucleotides were fractionated with Dowex-1 resin using elution with formiate according to Hurlbert *et al.*[1] and estimated by their absorption at 260 mμ. The peaks were further fractionated by paper chromatography and the composition of nucleotides determined by phosphate analysis and by paper chromatography of the purine bases and pyrimidine mononucleotides after acid hydrolysis.

Subcutaneous carrageenin granulomata were produced to cortisone-treated guinea pigs according to Robertson *et al.*[2] The tissue was prepared, homogenized and suspended into 0.6 N cold perchloric acid. The supernatant was neutralized and analyzed. Some differences were found between the cortisone-pretreated and control animals. The individual differences were, however, so large that no exact conclusions could be drawn.

The experiment was therefore repeated with chick embryos, which on the eighth day of incubation received 2.5 mg of hydrocortisone into the yolk sac. The embryos were prepared and analyzed as above after 1, 2, 3 or 4 days. The growth was almost totally inhibited.

Generally the energy-rich di- and triphosphates were decreased. In the region of monophosphates there appeared in all the samples from hydrocortisone-treated embryos a small but distinct peak which was totally absent from the controls. This peak is not yet positively identified. It has an absorption maximum at 262 mμ and in the paper chromatograms (using *iso*propanol-HCl) it migrates almost with the same rate as UMP. However, it cannot be the UMP, which emerges later.

On paper chromatographic evidence it seems that UMP and IMP are decreased after hydrocortisonetreatment apparently in equal degree.

1. Hurlbert, R. B., Schmitz, H., Brumm, A. F. and Potter, V. R. *J. Biol. Chem.* **209** (1954) 23.
2. Robertson, W. v. B. and Schwartz, B. *J. Biol. Chem.* **201** (1953) 681.

The Acid Mucopolysaccharides of Furth's Mastocytoma in the Mouse

Lennart Rodén and Albert Dorfman

Wenner-Grens Institut, Stockholm, Sweden, and La Rabida-University of Chicago Institute, Chicago, U.S.A.

Mast cells, though widely distributed in mammalian connective tissues, are difficult to isolate in amounts large enough to permit a detailed study of the biosynthesis of one of their most important components, heparin. The recent development of two different transplantable mastocytomas in the mouse [1,2] therefore seems to offer considerable promise in this respect.

In naturally occurring mast cells isolated from peritoneal washings Schiller and Dorfman [3] found no other mucopolysaccharide than heparin. However, before using the malignant mast cells in biosynthetic studies it was considered advisable to carry out a more detailed characterization of their mucopolysaccharide fraction.

Tumours of the Furth type * were used in the present study. The mucopolysaccharides were prepared from a boiled water homogenate by the following procedure: 1) extraction with sodium hydroxide, 2) digestion of the dialyzed extract with trypsin, 3) precipitation of undigested material with trichloroacetic acid and dialysis of supernatant and 4) fractionation of the cetyl pyridinium complexes on a cellulose column according to Borelius, Gardell and Scott [4].

In this way two fractions were obtained in approximately equal amounts, which were eluted from the column with 1.2 N NaCl and 2.0 N NaCl, respectively. The 2.0 N fraction contained glucosamine, uronic acid and sulphate in molar ratios of 1:1:2, was resistant to digestion with testicular hyaluronidase and had an anticoagulant activity of 92 units per mg of the air-dried powder. The 1.2 N fraction contained hexosamine, uronic acid and sulphate in equimolar amounts, was digested to two thirds by hyaluronidase and had an anticoagulant activity of 15 units per mg. Both glucosamine and galactosamine were present in this fraction, as shown with the method of Stoffyn and Jeanloz [5].

On the basis of these observations it is suggested that the mast cell tumours contain, in addition to heparin, a fraction with low sulphur content similar to heparin monosulphuric acid, and chondroitin sulphuric acid A and/or C.

* Kindly provided by Dr. Jacob Furth.

1. Furth, J., Hagen, P. and Hirsch, E. I. *Proc. Soc. Exptl. Biol. Med.* **95** (1957) 824.
2. Dunn, T. M. and Potter, M. *J. Natl. Cancer Inst.* **18** (1957) 587.
3. Schiller, S. and Dorfman, A. *Biochim. et Biophys. Acta.* **31** (1959) 278.
4. Borelius, E., Gardell, S. and Scott, J. *To be published.*
5. Stoffyn, P. J. and Jeanloz, R. W. *Arch. Biochem. Biophys.* **52** (1954) 373.

Turbidimetric Analysis of Inorganic Sulfate in Serum, Plasma and Urine

Fredrik Berglund and Bo Sörbo

Department of Pharmacology, Karolinska Institutet, Stockholm, and Research Institute of National Defence, Dept. I, Sundbyberg, Sweden

The method is based on a precipitation of sulfate in the acidified sample by a barium chloride-gelatine reagent. The latter contains preformed barium sulfate (as commercial gelatine is contaminated with sulfate) and this accounts for the accuracy of the method [1]. The resulting turbidity is determined absorptiometrically in the ultraviolet region. Serum is analyzed after deproteinization with an equal volume of 8 % trichloroacetic acid and to a 4 ml aliquot is added 1 ml of the barium chloride-gelatine reagent. The latter is prepared by dissolving 2 g $BaCl_2.2H_2O$ in 400 ml of a 0.5 % solution of commercial gelatine "for microbiological work" (or of equivalent purity). 10–60 min later the absorbancy at 360 mμ is determined against a reagent blank. The standard curve is linear for amounts of sulfate up to 2 μmoles in the test. The method can also be applied to plasma, if citrate is used as anticoagulant, but heparin cannot be used as it strongly interferes in the test. Urine is analyzed by diluting a suitable aliquot with 4 % TCA and proceeding as above. The standard deviation of the method (in per cent of the mean) was obtained as 5 % in case of serum and 1 % in case of urine.

Gassner, K. and Friedel, H. *Z. anal. Chem.* **152** (1956) 420.

Respiration of Tissue Slices in the Presence of β-Aminopropionitrile

K. Juva, T. Nikkari and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

Monoamino-oxidase inhibitor ("Marsilid") aggravated the symptoms of experimental lathyrism [1]. This observation prompted us to study the respiration of tissue slices in the presence of β-aminopropionitrile (β-APN).

The experiments were conducted with conventional Warburg-technique. The liver slices (dry weight 8—10 mg) of fasting Wistar rats were incubated at + 37°C in 2 ml of bicarbonate-Ringer solution containing 0.2 % glucose. Sixteen control experiments were made with no additions in the incubation medium, the mean oxygen consumption being 9.29 ± 2.94 µl/mg/h. Decreasing concentrations of β-APN were added to the medium. The resulting oxygen uptakes were

No. of expts	β-APN	% of control
2	1 M	9
2	0.1 M	81
18	0.01 M	152
2	0.001 M	112

Similar stimulating effect of 0.01 M APN was also obtained with epiphyseal cartilage.

In eleven experiments using 0.01 M concentration of APN the disappearance of APN from the medium after 1 h incubation was studied by measuring the green colour, developed of APN by ninhydrin, at 340 mµ. An average of 1.2 µmoles of APN (6 % of the APN present) had disappeared in each experiment. If detoxified to cyanoacetic acid, it would have elevated the oxygen uptake only by about half of the actual increase.

Marsilid, in 0.001 M concentration, did not have any influence on the disappearance of APN. Neither the oxygen uptake of the controls nor the stimulation of oxygen consumption by 0.01 M APN were influenced by the presence of 0.001 M Marsilid in the incubation medium, as stated in 10 experiments.

Determinations of inorganic phosphorus of the incubation fluid indicated a possible impairment of phosphorus uptake by slices incubated with 0.01 M APN.

1. Juva, K., Mikkonen, L., Tuominen, T. and Kulonen, E. *Experientia* **15** (1959) 351.

Histochemical Demonstration of Phosphorylase Activity in Tissue Sections

Arto Palkama

Department of Anatomy, University of Helsinki, Helsinki, Finland

The formation of glycogen from glucose-1-phosphate is due to phosphorylase acitivity. Our preliminary experiments showed that the techniques published as yet tend to be capricious and the results difficult to reproduce. All the published methods include glucose-1-phosphate as a substrate, from which phosphorylase and transglucosidase build glycogen and at the same time liberate phosphoric acid. The newly formed glucogen indicates the phosphorylase activity and can be demonstrated either by iodine solution or by the periodic acid Schiff method.

Fresh frozen sections were cut with a freezing microtome at 10—50 microns. 20 to 30 microns gave best results. The sections were transferred immediately in the incubation solution or dried on coverslips. In the incubation solution the glucose-1-phosphate concentration was varied from 0.1 % up to 5.0 %. Below 0.5 % the results were poor. The pH was varied from 4.5 to 6.0. After preliminary experiments pH 5.9 was found to be most suitable. As an inhibitor of acid phosphatase fluoride was used. Best results were obtained when the sodium fluoride concentration was about 1.7 %. Activators such as muscle adenylic acid, insulin and glycogen had little or no influence on the results. The composition of the final incubation mixture was: 250.0 mg glucose-1-phosphate, 2.0 ml saturated sodium fluoride, 1.0 ml 0.5-M acetate buffer, aq.dest. ad 5.0 ml. The sections were incubated for 2 h at 37°C.

There was a rather strong colour in those control sections which were stained with the periodic acid-Schiff technique. The best way to decrease the nonspecific colour was by ether-chloroform extraction after incubation in the substrate mixture.

Most clear-cut results were obtained with sceletal muscle. Similar or less intense reaction was also seen in heart and smooth muscle. A definitely positive reaction was also seen in heart and smooth muscle. A definitely positive reaction was also seen in the liver. As an observation not previously reported we found a uniform strongly positive reaction in the adrenal medulla.

The technique developed is by no means perfect. The coloured precipitate produced can only be a rough guide to the sites of the enzyme activity. Therefore efforts are continued to refine the technique.

1. Ellis, R. A. and Montagna, W. *J. Histochem. and Cytochem.* **6** (1958) 201.
2. Friede, R. L. *J. Histochem. and Cytochem.* **7** (1959) 34.
3. Goldberg, B., Wade, O. R. and Jones Jr., H. W. *J. Natl. Cancer Inst.* **13** (2), (1952) 543.
4. Shimizu, N. and Okada, M. *J. Histochem. and Cytochem.* **5** (1957) 459.
5. Takeuchi, T. *J. Histochem. and Cytochem.* **6** (1958) 208.
6. Takeuchi, T. and Kuriaki, H. *J. Histochem. and Cytochem.* **3** (1955) 153.
7. Yasuda, K., Furusawa, H. and Ogata, N. *Okajimas Folia Anat. Japon.* **31** (1958) 161.

The Phosphotransferase Activity of Alkaline Phosphatases. Studies with Hydroxyamino Acids and Amines as Acceptors

Gunnar Ågren

Department of Medical Chemistry, University of Uppsala, Sweden

In an extensive study Morton [1] recently reported that alkaline phosphatase from calf intestinal mucosa catalysed the synthesis of phosphate esters by direct transfer of the phosphate group from phosphomonoesters to suitable hydroxy compounds. He also used serine, threonine, and ethanolamine as acceptors with some presumptive evidence of a positive result. However, he had no authentic marker compounds for comparison with the suspected phosphorylated components. Clear evidence for the formation of such compounds has now been obtained.

Radioactive glycerophosphate was used as donor. It was prepared according to Bublitz and Kennedy [2] using $AT^{32}P$ and glycerokinase. A simplified method for assay of enzyme activity was used based on the observation of Ernster *et al.*[3] Some radioactive inorganic phosphate formed in the reaction was separated on a Dowex-1 column.

In a series of experiments 10 mg of glycerophosphate, containing 0.46 mC ^{32}P, and 10 mg of a preparation of alkaline bone phosphatase [4] containing 420 Portmann [5] units/mg N were incubated at pH 9.8 in a bicarbonate buffer for 10 min with 1 mmole of serine, threonine, glucosamine, or ethanolamine, respectively, in a total volume of 2 ml. After heating glycerophosphate was separated from the formed phosphorylated product by gradient elution on a Dowex-1 formate column [6]. The total radioactivity of the fractions measured in a L.K.B. Robot Scaler gave the following values in counts per minute:

	With enzyme	Without enzyme
Phosphorylserine	375 000	26 000
Phosphorylthreonine	26 000	460
Phosphorylglucosamine	67 000	41 000
Phosphorylethanolamine	912 000	150 000

Some interesting features are observed, the large proportion of phosphorylserine to phosphorylthreonine also found in hydrolysates of casein and animal phosphoproteins [6]. Of similar interest is the dominance of phosphorylethanolamine over phosphorylglucosamine also found in hydrolysates of the protein fraction from *E. coli* [7]. The significance of these observations with regard to possible ways for the formation of phosphoproteins is, however, unclear. The yield obtained in the enzymatic transphosphorylation was rather small, usually less than 1 μmole. The specific activity calculated as counts per minute per μg of phosphorus had about the same value for all compounds. In controls without enzyme smaller amounts of phosphorylated amino acids and amines were formed. Of interest for the present problem is the recent isolation of a free phosphorylated amino acid from extracts of *L. casei* [8] together with some phosphopeptides.

1. Morton, R. K. *Biochem. J.* **70** (1958) 139.
2. Bublitz, C. and Kennedy, E. P. *J. Biol. Chem.* **211** (1954) 951.
3. Ernster, L., Zetterström, R. and Lundberg, O. *Acta Chem. Scand.* **4** (1950) 942.
4. Ågren, G., Zetterquist, Ö. and Ojamäe, M. *Acta Chem. Scand.* **13** (1959) 1047.
5. Portmann, P. *Z. Physiol. Chem. Hoppe-Seyler.* **309** (1957) 87.
6. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1097.
7. de Verdier, C.-H. and Ågren, G. *Acta Chem. Scand.* **12** (1958) 361.
8. Ågren, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 379.

The technique developed is by no means perfect. The coloured precipitate produced can only be a rough guide to the sites of the enzyme activity. Therefore efforts are continued to refine the technique.

1. Ellis, R. A. and Montagna, W. *J. Histochem. and Cytochem.* **6** (1958) 201.
2. Friede, R. L. *J. Histochem. and Cytochem.* **7** (1959) 34.
3. Goldberg, B., Wade, O. R. and Jones Jr., H. W. *J. Natl. Cancer Inst.* **13** (2), (1952) 543.
4. Shimizu, N. and Okada, M. *J. Histochem. and Cytochem.* **5** (1957) 459.
5. Takeuchi, T. *J. Histochem. and Cytochem.* **6** (1958) 208.
6. Takeuchi, T. and Kuriaki, H. *J. Histochem. and Cytochem.* **3** (1955) 153.
7. Yasuda, K., Furusawa, H. and Ogata, N. *Okajimas Folia Anat. Japon.* **31** (1958) 161.

The Phosphotransferase Activity of Alkaline Phosphatases. Studies with Hydroxyamino Acids and Amines as Acceptors

Gunnar Ågren

Department of Medical Chemistry, University of Uppsala, Sweden

In an extensive study Morton [1] recently reported that alkaline phosphatase from calf intestinal mucosa catalysed the synthesis of phosphate esters by direct transfer of the phosphate group from phosphomonoesters to suitable hydroxy compounds. He also used serine, threonine, and ethanolamine as acceptors with some presumptive evidence of a positive result. However, he had no authentic marker compounds for comparison with the suspected phosphorylated components. Clear evidence for the formation of such compounds has now been obtained.

Radioactive glycerophosphate was used as donor. It was prepared according to Bublitz and Kennedy [2] using $AT^{32}P$ and glycerokinase. A simplified method for assay of enzyme activity was used based on the observation of Ernster *et al.*[3] Some radioactive inorganic phosphate formed in the reaction was separated on a Dowex-1 column.

In a series of experiments 10 mg of glycerophosphate, containing 0.46 mC ^{32}P, and 10 mg of a preparation of alkaline bone phosphatase [4] containing 420 Portmann [5] units/mg N were incubated at pH 9.8 in a bicarbonate buffer for 10 min with 1 mmole of serine, threonine, glucosamine, or ethanolamine, respectively, in a total volume of 2 ml. After heating glycerophosphate was separated from the formed phosphorylated product by gradient elution on a Dowex-1 formate column [6]. The total radioactivity of the fractions measured in a L.K.B. Robot Scaler gave the following values in counts per minute:

	With enzyme	Without enzyme
Phosphorylserine	375 000	26 000
Phosphorylthreonine	26 000	460
Phosphorylglucosamine	67 000	41 000
Phosphorylethanolamine	912 000	150 000

Some interesting features are observed, the large proportion of phosphorylserine to phosphorylthreonine also found in hydrolysates of casein and animal phosphoproteins [6]. Of similar interest is the dominance of phosphorylethanolamine over phosphorylglucosamine also found in hydrolysates of the protein fraction from *E. coli* [7]. The significance of these observations with regard to possible ways for the formation of phosphoproteins is, however, unclear. The yield obtained in the enzymatic transphosphorylation was rather small, usually less than 1 μmole. The specific activity calculated as counts per minute per μg of phosphorus had about the same value for all compounds. In controls without enzyme smaller amounts of phosphorylated amino acids and amines were formed. Of interest for the present problem is the recent isolation of a free phosphorylated amino acid from extracts of *L. casei* [8] together with some phosphopeptides.

1. Morton, R. K. *Biochem. J.* **70** (1958) 139.
2. Bublitz, C. and Kennedy, E. P. *J. Biol. Chem.* **211** (1954) 951.
3. Ernster, L., Zetterström, R. and Lundberg, O. *Acta Chem. Scand.* **4** (1950) 942.
4. Ågren, G., Zetterquist, Ö. and Ojamäe, M. *Acta Chem. Scand.* **13** (1959) 1047.
5. Portmann, P. *Z. Physiol. Chem. Hoppe-Seyler.* **309** (1957) 87.
6. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1097.
7. de Verdier, C.-H. and Ågren, G. *Acta Chem. Scand.* **12** (1958) 361.
8. Ågren, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 379.

The Enzymatic Reduction of Uracil to Dihydrouracil in Rat Liver

Per Fritzson*

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

The reduction of uracil to dihydrouracil is the rate limiting step in the breakdown of uracil to β-alanine in rat liver [1-3]. This fact indicates the central role of this reaction in the uracil-catabolizing system. The present work describes a procedure for the assay of dihydrouracil dehydrogenase in rat liver. The quantitative relation between enzymatic activity and factors which influence the reduction, has been investigated.

Spectrophotometric studies were carried out with an enzyme purified by ammonium sulphate fractionation of the particle-free supernatant. Optimal rate of reduction was in the pH-range 7.0 to 7.6. The reaction is TPNH-dependent [2,4], and the optimal concentration was found to be 7×10^{-5} M. The Michaelis constant was 1.7×10^{-5} M. The optimal uracil concentration was very small and less than 1×10^{-5} M. The reversible reduction of uracil was demonstrated, but the very slow rate suggested that this reaction is without physiological significance.

The quantitative determination of dihydrouracil dehydrogenase was carried out by anaerobic incubation of homogenate or the soluble fraction with uracil-6-^{14}C in the presence of TPNH, with G-6-P and G-6-P dehydrogenase as a TPN reducing system. ATP was found to have a stimulating effect on the reaction under these conditions [5,3]. The effect decreased, however, when the TPNH concentration was increased, indicating that ATP had a sparing action on the cofactor rather than a stimulating influence on the reduction as such. When appropriate amounts of ATP, nicotinamide [6] and fluoride were included in the incubation mixture, a linear relation between the rate of reduction and the amount of homogenate added, was obtained. The enzymatic activities of the homogenate and the soluble fraction were identical, indicating that dihydrouracil dehydrogenase is situated entirely in the soluble fraction. The enzymatic activity per g of liver, wet weight, corresponded to the reduction of about 0.7 μmole of uracil in 10 min.

The TPNH level in normal liver [7,8] is probably greater than that required for optimal enzymatic activity. Hence, the capacity to degrade uracil may be limited only by the amount of enzyme. The rate of uracil breakdown in the intact rat [5] support this view.

1. Fink, R. M., McGaughey, C., Cline, R. E. and Fink, K. *J. Biol. Chem.* **218** (1956) 1.
2. Canellakis, E. S. *J. Biol. Chem.* **221** (1956) 315.
3. Fritzson, P. *J. Biol. Chem.* **226** (1957) 223.
4. Grisolia, S. and Cardoso, S. S. *Biochim. et Biophys. Acta* **25** (1957) 430.
5. Rutman, R. J., Cantarow, A. and Paschkis, K. E. *J. Biol. Chem.* **210** (1954) 321.
6. Mann, P. J. G. and Quastel, J. H. *Biochem. J.* **35** (1941) 502.
7. Glock, G. E. and McLean, P. *Biochem. J.* **65** (1957) 413.
8. Jacobson, K. B. and Kaplan, N. O. *J. Biol. Chem.* **226** (1957) 603.

Adsorption of Water Vapour by Tissue Polysaccharide

J. Pikkarainen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

Since "water-binding" is considered as a special rôle of the connective tissue polysaccharides, the affinity of water vapour to their surface was determined using the Brunauer-Emmett-Teller-plot [1]. The preparation was isolated from human umbilical cords (using $CaCl_2$-extraction, precipitation with 66 % ethanol and repeated Sewag-procedure) and contained nitrogen 0.07 mg/ml and glucosamine 0.32 mg/ml (corresponding to about 76 % polysaccharide and 24 % protein). The solution was lyophilized. Part of this dry residue was depolymerized with hyaluronidase, and the digest was dialyzed and lyophilized. The combustion residues were about 15—17 % and the results are correlated accordingly.

The practical procedure was adopted from Bull [2], except that + 40°C temperature was used. Ten points up to $P/P_o = 0.6$ were determined. The B.E.T. plots were quite straight, and the following constants were obtained from estimating equations:

original preparation Vm 11.3 ml/100 g, C 39;
depolymerized " 12.6 " /100 g, " 99.

* Fellow of The Norwegian Cancer Society, Oslo, Norway.

Hyaluronate adsorbs water more than collagen, but the difference is not large. The effect of depolymerization is seen in the change of the constant C.

1. Brunauer, S., Emmett, P. H. and Teller, E. *J. Am. Chem. Soc.* **60** (1938) 309.
2. Bull, H. B., *J. Am. Chem. Soc.* **66** (1944) 1499; *Physical Biochemistry*, 2nd Ed. John Wiley and Sons, New York 1951.

Purification and Properties of a Bacterial Glucose Dehydrogenase

J. G. Hauge

The State Institute for Public Health, Oslo, Norway

A bacterium posessing a glucose dehydrogenase was isolated from a secondary culture infection. It has provisionally been assigned to the genus *Alcaligenes*. 100 g quantities (dry wt.) of the organism could be grown in a simple 20 liter continuous culture apparatus in a normal work day. Resting cells oxidize glucose rapidly to the level of 1 atom oxygen consumed per molecule glucose, and from then on at a reduced rate. In this first period CO_2 evolution is slight. Cell-free extracts, prepared by grinding with Ballotini glass beads, also carry out oxidation of glucose with oxygen as acceptor. The primary dehydrogenation could be studied with 2,6-dichlorophenol indophenol as hydrogen acceptor. Using this assay method, the enzyme was purified 250 fold with protamine sulphate, ammonium sulphate, DEAE cellulose, and calcium phosphate gel. At this stage the enzyme catalyzes the reduction of 40 μmoles dye per min per mg protein.

Cytochrome *c* or DPN are not reduced at a significant rate, and reoxidation with oxygen is not measurable, unless phenazine methosulphate is also added. In the presence of phenazine methosulphate and oxygen, the immediate product of the reaction was shown to be gluconolactone. The absorption spectrum of the enzyme preparation shows the presence of a b-type hemoprotein, partially reducible by glucose. The spectrum also suggests the presence of functional flavin, and preliminary inhibition and fluoresence data support this evidence. This soluble dehydrogenase appears to represent a fragment of the particulate glucose oxidation system found in the cells and the extract.

Collagen Fractions in Experimental Lathyrism

L. Mikkonen, T. Tuominen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The collagen fractions of pooled skins of lathyritic and control rats were obtained by a modified procedure by Harkness *et al.*[1]. In the disodium phosphate-soluble fractions the collagen was estimated from the content of hydroxyproline [2], the acid-soluble and insoluble fractions from the nitrogen content. Four extraction series were performed in both groups.

The insoluble collagen was nearly same in lathyritic animals as in controls. The acid-soluble collagen was higher in lathyritic samples (content 0.48 % and 0.18 % from the air-dried skin samples of lathyritic and normal rats, respectively; $P>0.10$).

The most marked difference was in the disodium phosphate-soluble collagen. Calculated from the hydroxyproline contents of the extracts, the averages were 3.15 % and 0.62 % in the lathyritic and normal samples, respectively ($P<0.001$) and after precipitation the alkali-soluble collagen amounted to 1.07 % and 0.30 % ($P<0.01$). It is concluded that in experimental lathyrism the alkali-soluble collagen is increased but there is a defect in the fibre formation.

1. Harkness, R. D., Marko, A. M., Muir, H. and Neuberger, A. *Biochem. J.* **56** (1954) 558.
2. Neuman, R. E. and Logan, M. A. *J. Biol. Chem.* **184** (1950) 299.

A New Sensitive Detector for Gas Chromatography

E. Haahti and T. Nikkari

Department of Medical Chemistry, University of Turku, Finland

We constructed Lovelock's type of gas chromatography detector [1] of Pyrex-glass using the ^{90}Sr-covered silver plate as anode. Some incidental observations prompted us to replace the anode by a non-radioactive aluminium plate.

The Enzymatic Reduction of Uracil to Dihydrouracil in Rat Liver

Per Fritzson*

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

The reduction of uracil to dihydrouracil is the rate limiting step in the breakdown of uracil to β-alanine in rat liver [1-3]. This fact indicates the central role of this reaction in the uracil-catabolizing system. The present work describes a procedure for the assay of dihydrouracil dehydrogenase in rat liver. The quantitative relation between enzymatic activity and factors which influence the reduction, has been investigated.

Spectrophotometric studies were carried out with an enzyme purified by ammonium sulphate fractionation of the particle-free supernatant. Optimal rate of reduction was in the pH-range 7.0 to 7.6. The reaction is TPNH-dependent [2,4], and the optimal concentration was found to be 7×10^{-5} M. The Michaelis constant was 1.7×10^{-5} M. The optimal uracil concentration was very small and less than 1×10^{-5} M. The reversible reduction of uracil was demonstrated, but the very slow rate suggested that this reaction is without physiological significance.

The quantitative determination of dihydrouracil dehydrogenase was carried out by anaerobic incubation of homogenate or the soluble fraction with uracil-6-^{14}C in the presence of TPNH, with G-6-P and G-6-P dehydrogenase as a TPN reducing system. ATP was found to have a stimulating effect on the reaction under these conditions [5,3]. The effect decreased, however, when the TPNH concentration was increased, indicating that ATP had a sparing action on the cofactor rather than a stimulating influence on the reduction as such. When appropriate amounts of ATP, nicotinamide [6] and fluoride were included in the incubation mixture, a linear relation between the rate of reduction and the amount of homogenate added, was obtained. The enzymatic activities of the homogenate and the soluble fraction were identical, indicating that dihydrouracil dehydrogenase is situated entirely in the soluble fraction. The enzymatic activity per g of liver, wet weight, corresponded to the reduction of about 0.7 μmole of uracil in 10 min.

The TPNH level in normal liver [7,8] is probably greater than that required for optimal enzymatic activity. Hence, the capacity to degrade uracil may be limited only by the amount of enzyme. The rate of uracil breakdown in the intact rat [5] support this view.

1. Fink, R. M., McGaughey, C., Cline, R. E. and Fink, K. *J. Biol. Chem.* **218** (1956) 1.
2. Canellakis, E. S. *J. Biol. Chem.* **221** (1956) 315.
3. Fritzson, P. *J. Biol. Chem.* **226** (1957) 223.
4. Grisolia, S. and Cardoso, S. S. *Biochim. et Biophys. Acta* **25** (1957) 430.
5. Rutman, R. J., Cantarow, A. and Paschkis, K. E. *J. Biol. Chem.* **210** (1954) 321.
6. Mann, P. J. G. and Quastel, J. H. *Biochem. J.* **35** (1941) 502.
7. Glock, G. E. and McLean, P. *Biochem. J.* **65** (1957) 413.
8. Jacobson, K. B. and Kaplan, N. O. *J. Biol. Chem.* **226** (1957) 603.

Adsorption of Water Vapour by Tissue Polysaccharide

J. Pikkarainen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

Since "water-binding" is considered as a special rôle of the connective tissue polysaccharides, the affinity of water vapour to their surface was determined using the Brunauer-Emmett-Teller-plot [1]. The preparation was isolated from human umbilical cords (using $CaCl_2$-extraction, precipitation with 66 % ethanol and repeated Sewag-procedure) and contained nitrogen 0.07 mg/ml and glucosamine 0.32 mg/ml (corresponding to about 76 % polysaccharide and 24 % protein). The solution was lyophilized. Part of this dry residue was depolymerized with hyaluronidase, and the digest was dialyzed and lyophilized. The combustion residues were about 15—17 % and the results are correlated accordingly.

The practical procedure was adopted from Bull [2], except that + 40°C temperature was used. Ten points up to $P/P_o = 0.6$ were determined. The B.E.T. plots were quite straight, and the following constants were obtained from estimating equations:

original preparation Vm 11.3 ml/100 g, C 39;
depolymerized " 12.6 " /100 g, " 99.

* Fellow of The Norwegian Cancer Society, Oslo, Norway.

Hyaluronate adsorbs water more than collagen, but the difference is not large. The effect of depolymerization is seen in the change of the constant C.

1. Brunauer, S., Emmett, P. H. and Teller, E. *J. Am. Chem. Soc.* **60** (1938) 309.
2. Bull, H. B., *J. Am. Chem. Soc.* **66** (1944) 1499; *Physical Biochemistry*, 2nd Ed. John Wiley and Sons, New York 1951.

Purification and Properties of a Bacterial Glucose Dehydrogenase

J. G. Hauge

The State Institute for Public Health, Oslo, Norway

A bacterium posessing a glucose dehydrogenase was isolated from a secondary culture infection. It has provisionally been assigned to the genus *Alcaligenes*. 100 g quantities (dry wt.) of the organism could be grown in a simple 20 liter continuous culture apparatus in a normal work day. Resting cells oxidize glucose rapidly to the level of 1 atom oxygen consumed per molecule glucose, and from then on at a reduced rate. In this first period CO_2 evolution is slight. Cell-free extracts, prepared by grinding with Ballotini glass beads, also carry out oxidation of glucose with oxygen as acceptor. The primary dehydrogenation could be studied with 2,6-dichlorophenol indophenol as hydrogen acceptor. Using this assay method, the enzyme was purified 250 fold with protamine sulphate, ammonium sulphate, DEAE cellulose, and calcium phosphate gel. At this stage the enzyme catalyzes the reduction of 40 μmoles dye per min per mg protein.

Cytochrome *c* or DPN are not reduced at a significant rate, and reoxidation with oxygen is not measurable, unless phenazine methosulphate is also added. In the presence of phenazine methosulphate and oxygen, the immediate product of the reaction was shown to be gluconolactone. The absorption spectrum of the enzyme preparation shows the presence of a b-type hemoprotein, partially reducible by glucose. The spectrum also suggests the presence of functional flavin, and preliminary inhibition and fluoresence data support this evidence. This soluble dehydrogenase appears to represent a fragment of the particulate glucose oxidation system found in the cells and the extract.

Collagen Fractions in Experimental Lathyrism

L. Mikkonen, T. Tuominen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The collagen fractions of pooled skins of lathyritic and control rats were obtained by a modified procedure by Harkness *et al.*[1]. In the disodium phosphate-soluble fractions the collagen was estimated from the content of hydroxyproline [2], the acid-soluble and insoluble fractions from the nitrogen content. Four extraction series were performed in both groups.

The insoluble collagen was nearly same in lathyritic animals as in controls. The acid-soluble collagen was higher in lathyritic samples (content 0.48 % and 0.18 % from the air-dried skin samples of lathyritic and normal rats, respectively; $P>0.10$).

The most marked difference was in the disodium phosphate-soluble collagen. Calculated from the hydroxyproline contents of the extracts, the averages were 3.15 % and 0.62 % in the lathyritic and normal samples, respectively ($P<0.001$) and after precipitation the alkali-soluble collagen amounted to 1.07 % and 0.30 % ($P<0.01$). It is concluded that in experimental lathyrism the alkali-soluble collagen is increased but there is a defect in the fibre formation.

1. Harkness, R. D., Marko, A. M., Muir, H. and Neuberger, A. *Biochem. J.* **56** (1954) 558.
2. Neuman, R. E. and Logan, M. A. *J. Biol. Chem.* **184** (1950) 299.

A New Sensitive Detector for Gas Chromatography

E. Haahti and T. Nikkari

Department of Medical Chemistry, University of Turku, Finland

We constructed Lovelock's type of gas chromatography detector [1] of Pyrex-glass using the ^{90}Sr-covered silver plate as anode. Some incidental observations prompted us to replace the anode by a non-radioactive aluminium plate.

This modification of the detector was equally useful for analysis of methyl esters of fatty acids. It seemed to possess the same characteristics of response as the ^{90}Sr-detector, with some advantages in the operation.

The response of this detector was studied in a gas-chromatography apparatus made in our laboratory according to James [2], using a 4 feet glass column of 4 mm diameter filled with Celite 545 of 100—120 mesh containing 20 % (w/w) Apiezon L grease as stationary phase. Commercial argon (99.8 %) at an inlet pressure of 0.5 atm was used as carrier gas. The column and the detector were maintained at a temperature of 180°C. The ionization current was amplified by a feedback electrometer amplifier with 5 000 MΩ input resistance and recorded by a Honeywell recorder. Different anode-voltages ranging from 450 V to 2 000 V could be applied. Using a potential of 2 000 V and applying 0.1 μl of a 1:200 000 dilution (w/v) of methyl laurate in chloroform, the detector still gave a well detectable response, which corresponds to 5×10^{-4} μg of methyl laurate.

The sensitivity of this detector is at least of the same order as that of Lovelock's detector. The absence of the strontium plate causes a considerable fall of the basic ionization current and increases stability of the detector at higher anode potentials and gives a low noise level.

1. Lovelock, J. E. *J. Chromatography* **1** (1958) 35.
2. James, A. T. *Personal Communication* 1958.

The Mechanism of Inactivation of SH-Enzymes by X-Rays

Rolf Lange* and Alexander Pihl

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

Previous work in this laboratory has established [1,2] that the SH-enzymes muscle glyceraldehyde dehydrogenase (GAPDH) and yeast alcohol dehydrogenase (ADH) are inactivated by X-rays with ionic yields which fall in the same range as those of non-SH-enzymes. This finding, which stands in contrast to previous claims [3,4], raises the questions as to the radiosensitivity of protein SH-groups and the role of such groups in the radiation induced inactivation of SH-enzymes. In order to throw light on these questions the X-ray inactivation of GAPDH and ADH in dilute solution has been correlated with the concomitant disappearance of titratable SH-groups.

In the case of GAPDH, the disappearance of the protein SH-groups as well as the enzyme inactivation was found to be a linear function of the radiation dose. Complete inactivation of the enzyme occurred when 3 SH-groups per enzyme molecule had been destroyed. Since GAPDH is completely inactivated by the addition of 3 moles of *p*-chloromercuriphenyl sulphonate [5] the present data strongly indicate that the radiation induced inactivation of the enzyme is entirely due to radiochemical destruction of SH-groups.

The ADH was inactivated by X-ray to a somewhat greater extent than could be expected on the basis of the disappearance of the titratable SH-groups. Thus, from a comparison of the experimentally determined number of SH-groups with the theoretical number calculated on the basis of the remaining enzymatic activity [6], it was estimated that approximately 60—80 % of the enzyme inactivation can be accounted for by the radiochemical destruction of SH-groups.

In both enzymes the disappearance of SH-groups proceeds with a low ionic yield compared with that observed on irradiation of low molecular thiols [7]. This finding indicates that the enzyme SH-groups are protected by the rest of the protein molecule and that the major part of the radiation energy is dissipated in reactions not leading to the loss of thiol-groups. The consequent alterations of the enzyme molecule are apparently of little significance for the enzymatic activity.

1. Pihl, A., Lange, R. and Eldjarn, L. *Nature* **182** (1958) 1732.
2. Lange, R., Pihl, A. and Eldjarn, L. *Intern. J. Rad. Biol.* **1** (1959) 73.
3. Barron, E. S. G., Dickman, S., Muntz, J. A. and Singer, T. P. *J. Gen. Physiol.* **32** (1949) 537.
4. Barron, E. S. G. and Johnson, P. *Arch. Biochem. Biophys.* **48** (1954) 149.
5. Velick, S. F. *J. Biol. Chem.* **203** (1953) 563.
6. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 17.
7. Barron, E. S. G. and Flood, V. *J. Gen. Physiol.* **33** (1950) 229.

* Fellow of The Norwegian Cancer Society, Oslo, Norway.

Hyaluronate adsorbs water more than collagen, but the difference is not large. The effect of depolymerization is seen in the change of the constant C.

1. Brunauer, S., Emmett, P. H. and Teller, E. *J. Am. Chem. Soc.* **60** (1938) 309.
2. Bull, H. B., *J. Am. Chem. Soc.* **66** (1944) 1499; *Physical Biochemistry*, 2nd Ed. John Wiley and Sons, New York 1951.

Purification and Properties of a Bacterial Glucose Dehydrogenase

J. G. Hauge

The State Institute for Public Health, Oslo, Norway

A bacterium posessing a glucose dehydrogenase was isolated from a secondary culture infection. It has provisionally been assigned to the genus *Alcaligenes*. 100 g quantities (dry wt.) of the organism could be grown in a simple 20 liter continuous culture apparatus in a normal work day. Resting cells oxidize glucose rapidly to the level of 1 atom oxygen consumed per molecule glucose, and from then on at a reduced rate. In this first period CO_2 evolution is slight. Cell-free extracts, prepared by grinding with Ballotini glass beads, also carry out oxidation of glucose with oxygen as acceptor. The primary dehydrogenation could be studied with 2,6-dichlorophenol indophenol as hydrogen acceptor. Using this assay method, the enzyme was purified 250 fold with protamine sulphate, ammonium sulphate, DEAE cellulose, and calcium phosphate gel. At this stage the enzyme catalyzes the reduction of 40 μmoles dye per min per mg protein.

Cytochrome *c* or DPN are not reduced at a significant rate, and reoxidation with oxygen is not measurable, unless phenazine methosulphate is also added. In the presence of phenazine methosulphate and oxygen, the immediate product of the reaction was shown to be gluconolactone. The absorption spectrum of the enzyme preparation shows the presence of a b-type hemoprotein, partially reducible by glucose. The spectrum also suggests the presence of functional flavin, and preliminary inhibition and fluoresence data support this evidence. This soluble dehydrogenase appears to represent a fragment of the particulate glucose oxidation system found in the cells and the extract.

Collagen Fractions in Experimental Lathyrism

L. Mikkonen, T. Tuominen and E. Kulonen

Department of Medical Chemistry, University of Turku, Finland

The collagen fractions of pooled skins of lathyritic and control rats were obtained by a modified procedure by Harkness *et al.*[1]. In the disodium phosphate-soluble fractions the collagen was estimated from the content of hydroxyproline [2], the acid-soluble and insoluble fractions from the nitrogen content. Four extraction series were performed in both groups.

The insoluble collagen was nearly same in lathyritic animals as in controls. The acid-soluble collagen was higher in lathyritic samples (content 0.48 % and 0.18 % from the air-dried skin samples of lathyritic and normal rats, respectively; $P>0.10$).

The most marked difference was in the disodium phosphate-soluble collagen. Calculated from the hydroxyproline contents of the extracts, the averages were 3.15 % and 0.62 % in the lathyritic and normal samples, respectively ($P<0.001$) and after precipitation the alkali-soluble collagen amounted to 1.07 % and 0.30 % ($P<0.01$). It is concluded that in experimental lathyrism the alkali-soluble collagen is increased but there is a defect in the fibre formation.

1. Harkness, R. D., Marko, A. M., Muir, H. and Neuberger, A. *Biochem. J.* **56** (1954) 558.
2. Neuman, R. E. and Logan, M. A. *J. Biol. Chem.* **184** (1950) 299.

A New Sensitive Detector for Gas Chromatography

E. Haahti and T. Nikkari

Department of Medical Chemistry, University of Turku, Finland

We constructed Lovelock's type of gas chromatography detector [1] of Pyrex-glass using the ^{90}Sr-covered silver plate as anode. Some incidental observations prompted us to replace the anode by a non-radioactive aluminium plate.

This modification of the detector was equally useful for analysis of methyl esters of fatty acids. It seemed to possess the same characteristics of response as the ^{90}Sr-detector, with some advantages in the operation.

The response of this detector was studied in a gas-chromatography apparatus made in our laboratory according to James [2], using a 4 feet glass column of 4 mm diameter filled with Celite 545 of 100—120 mesh containing 20 % (w/w) Apiezon L grease as stationary phase. Commercial argon (99.8 %) at an inlet pressure of 0.5 atm was used as carrier gas. The column and the detector were maintained at a temperature of 180°C. The ionization current was amplified by a feedback electrometer amplifier with 5 000 MΩ input resistance and recorded by a Honeywell recorder. Different anode-voltages ranging from 450 V to 2 000 V could be applied. Using a potential of 2 000 V and applying 0.1 μl of a 1:200 000 dilution (w/v) of methyl laurate in chloroform, the detector still gave a well detectable response, which corresponds to 5×10^{-4} μg of methyl laurate.

The sensitivity of this detector is at least of the same order as that of Lovelock's detector. The absence of the strontium plate causes a considerable fall of the basic ionization current and increases stability of the detector at higher anode potentials and gives a low noise level.

1. Lovelock, J. E. *J. Chromatography* **1** (1958) 35.
2. James, A. T. *Personal Communication* 1958.

The Mechanism of Inactivation of SH-Enzymes by X-Rays

Rolf Lange* and Alexander Pihl

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

Previous work in this laboratory has established [1,2] that the SH-enzymes muscle glyceraldehyde dehydrogenase (GAPDH) and yeast alcohol dehydrogenase (ADH) are inactivated by X-rays with ionic yields which fall in the same range as those of non-SH-enzymes. This finding, which stands in contrast to previous claims [3,4], raises the questions as to the radiosensitivity of protein SH-groups and the role of such groups in the radiation induced inactivation of SH-enzymes. In order to throw light on these questions the X-ray inactivation of GAPDH and ADH in dilute solution has been correlated with the concomitant disappearance of titratable SH-groups.

In the case of GAPDH, the disappearance of the protein SH-groups as well as the enzyme inactivation was found to be a linear function of the radiation dose. Complete inactivation of the enzyme occurred when 3 SH-groups per enzyme molecule had been destroyed. Since GAPDH is completely inactivated by the addition of 3 moles of *p*-chloromercuriphenyl sulphonate [5] the present data strongly indicate that the radiation induced inactivation of the enzyme is entirely due to radiochemical destruction of SH-groups.

The ADH was inactivated by X-ray to a somewhat greater extent than could be expected on the basis of the disappearance of the titratable SH-groups. Thus, from a comparison of the experimentally determined number of SH-groups with the theoretical number calculated on the basis of the remaining enzymatic activity [6], it was estimated that approximately 60—80 % of the enzyme inactivation can be accounted for by the radiochemical destruction of SH-groups.

In both enzymes the disappearance of SH-groups proceeds with a low ionic yield compared with that observed on irradiation of low molecular thiols [7]. This finding indicates that the enzyme SH-groups are protected by the rest of the protein molecule and that the major part of the radiation energy is dissipated in reactions not leading to the loss of thiol-groups. The consequent alterations of the enzyme molecule are apparently of little significance for the enzymatic activity.

1. Pihl, A., Lange, R. and Eldjarn, L. *Nature* **182** (1958) 1732.
2. Lange, R., Pihl, A. and Eldjarn, L. *Intern. J. Rad. Biol.* **1** (1959) 73.
3. Barron, E. S. G., Dickman, S., Muntz, J. A. and Singer, T. P. *J. Gen. Physiol.* **32** (1949) 537.
4. Barron, E. S. G. and Johnson, P. *Arch. Biochem. Biophys.* **48** (1954) 149.
5. Velick, S. F. *J. Biol. Chem.* **203** (1953) 563.
6. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 17.
7. Barron, E. S. G. and Flood, V. *J. Gen. Physiol.* **33** (1950) 229.

* Fellow of The Norwegian Cancer Society, Oslo, Norway.

Purification of Pepsin by Gel Filtration

Bertil Gelotte and Anna-Britta Krantz

Research Laboratories, Pharmacia, Uppsala, Sweden

A new separation method called gel filtration has recently been developed [1]. It is an extension of the observations made by Lathe and Ruthven [2] that molecules of different size can be separated, when filtered through a column of starch. In these experiments the cross-linked polysaccharide Sephadex G-50 (manufactured by Pharmacia, Uppsala, Sweden) was used as bed material.

Sephadex consists of small grains, which swell in aqueous solutions. In the swollen state the gel grains are packed to a column in a glass tube. When a solution is filtered through the bed, solutes of large molecular size are prevented from entering the gel phase and will thus move in the aqueous phase surrounding the grains. Low molecular weight solutes, however, penetrate the gel phase and will pass the column in the water inside as well as outside the grains. They will therefore be eluted from the column later than the larger ones.

The method is very useful for desalting colloid solutions, for fractionation of polymer homologues and for group separation of biological extracts [1,3-5]. It can also be used as a step in a purification procedure, which is exemplified by a purification of pepsin.

A crude pepsin (Pepsin 1:10 000 from A/S Orthana, Copenhagen) was used as starting material. The proteolytic activity was tested spectrophotometrically at 280 mμ with hemoglobin as substrate and expressed as activity per mg of nitrogen. The activity of our preparations was compared with that of a twice crystallized pepsin (Worthington Biochemical). The gel filtration experiments were made on a column 7.5 × 49 cm equilibrated with 0.2 M sodium acetate pH 4.9.

100 ml of the acetate buffer containing 37 g of crude pepsin was filtered through the column with the same buffer as eluent, and with an elution rate of 30 ml/min. After partial concentration in vacuum the eluted pepsin was desalted by a second gel filtration on the same column using distilled water as eluent. The saltfree pepsin was then lyophilized.

A good purification and a quantitative recovery was obtained in the filtration experiments. Unfortunately, there was some loss of activity in the concentration steps. Therefore, in another series of experiments, the concentration was made with Sephadex. The pepsin solution was mixed with the dry powder, which takes up water but leaves the protein molecules outside the grains. By filtration with careful suction a concentrated enzyme solution was obtained without any loss of activity.

The experimental results are summarized in Table 1.

Table 1.

Treatment	Yield, %		Pepsin activity	
	Nitrogen	Activity	Conc. in vacuum	Conc. with Sephadex
Orig. solution	100	100	23	23
1st gel filtration	35	99	65	59
Concentration	35	84	55	64
2nd gel filtration	23	84	84	87
Concentration	23	72	72	95
2 × cryst. pepsin			117	

1. Porath, J. and Flodin, P. *Nature* **183** (1959) 1657.
2. Lathe, G. H. and Ruthven, C. R. J. *Biochem J.* **62** (1956) 665.
3. Flodin, P. *In preparation.*
4. Porath, J. *Biochim. et Biophys, Acta. In press.*
5. Gelotte, B. *J. Chromatography. In press.*

New Pigments Derived from Vitamin B_{12} by the Use of a Gram Negative Organism

K. Helgeland, J. Jonsen and S. Laland

Department of Biochemistry, University of Oslo, Blindern, Oslo, Norway

We wish to report the bacterial conversion of cyanocobalamin into several different coloured substances by a gram negative rod isolated from soil. The gram negative rod has been classified as *Aerobacter aerogenes*.

The microorganism was grown in a synthetic fluid medium containing 0.8 % glucose, salts and 0.4 mg cyanocobalamin per ml. After incubation at 37°C for 12 days the culture was centrifuged and the supernatant extracted with phenol. The phenol layer was separated by centrifugation, washed with water to remove salts and diluted with ether. Unchanged vitamin B_{12} and the pigments were then re-extracted into water.

By electrophoresis in N acetic acid the extracted material separated into two coloured substances with mobilities different from that of unchanged cyanocobalamin. They both carried a positive charge and exhibited a mobility of 0.75 (I) and 0.25 (II), respectively, relative to aquo-cobalamin.

Substance (I) had a brownish-yellow colour, while substance (II) was yellow. The yield of these two substances was very low and only substance (I) has been further investigated. It was purified by descending chromatography in the usual *sec.* butanol water-1 % acetic acid-0.01 % KCN solvent. In this solvent it had an R_F of 0.35 whereas the R_F of cyanocobalamin was 0.25.

When dissolved in distilled water it exhibited the following absorption maxima, 262, 342 and 474 mμ, and when examined in 0.1 M KCN the brownish-yellow substance turned pink with a concomitant shift in the maxima to 280, 344 and 494–502 mμ. This shift in colour and maxima, as with cyanocobalamin, was shown to be reversible. In water it showed the absorption ratios $O.D._{342}/O.D._{474} = 1.0$ and $O.D._{342}/O.D._{262} = 0.4$, and in 0.1 M KCN $O.D._{344}/O.D._{494} = 1.0$ and $O.D._{344}/O.D._{280} = 0.6$. The absorption spectrum resembles that of cyanocobalamin, the main difference being the lack of the large maximum in the region 300–400 mμ.

The presence of Co as indicated by the absorption spectrum in KCN was verified by the use of ^{58}Co labeled cyanocobalamin.

Substance (I) was purified twice by electrophoresis in N acetic acid and twice by paper chromatography in the previously mentioned solvent and showed then small but definite activity in the *Lactobacillus leichmannii* and *Euglena gracilis* test. Since the presence of contaminating vitamin B_{12} cannot be completely excluded, the microbiological activity of the substance must be interpreted with some caution.

No conversion was found with *E.coli* or several gram positive bacteria tested (*B. subtilis, B. cereus, Str. faecalis* and *Staph. aureus*).

We wish to thank Glaxo Lab. Ltd. for the gift of vitamin B_{12} and *Nansenfondet* for financial support.

A Cytochemical Study of Enzymes in the Alveolar Phagocytes

Mikko Niemi

Department of Anatomy, University of Helsinki, Helsinki, Finland

Alveolar phagocytes of several vertebrates have been studied using histochemical techniques. These cells have been shown to exhibit a strong activity of acid phosphatase, non-specific esterase, cathepsin and leucine aminopeptidase. They were also found to contain a high activity of DPNH diaphorase as well as succinic dehydrogenase. Furthermore, the enzymatic activity of eight out of the nine histochemically demonstrable pyridine nucleotide-linked dehydrogenases was found to be intense, β-hydroxybutyrate dehydrogenase activity, however, being absent in the lungs of all the species studied. This obvious blockage of fatty acid breakdown in alveolar phagocytes may be correlated with some pathological changes in the lungs.

The DPNH diaphorase activity was also demonstrated in the fetal mouse lungs. Here, the staining reaction was, however, localized in the endodermal cells only; thus, towards the end of the foetal life, the forming alveoli were found to be filled with cells of moderate enzyme activity, whereas the mesodermal connective tissue cells exhibited no enzyme activity at all. This finding strongly suggests that the alveolar cells are derived from the alveolar epithelium, *i.e.* of endodermal origin.

The Oxidation of TPNH by Rat Liver Microsomes and its Stimulation by Inducing Agents

Alexandra von der Decken and Tore Hultin

The Wenner-Gren Institute, Stockholm, Sweden

A number of enzymatic reactions related to metabolic detoxication have been shown to be associated with the microsomal structures of the liver. The reactions are aerobic but take place only in the presence of reduced pyridine nucleotides, primarily TPNH. The activity of these enzymes temporarily increases after the intraperitoneal injection of small amounts of methylcholanthrene (MC)[1]. It has been shown that TPNH can be oxidized by rat liver microsomes in the presence of *i.a.* cytochrome c, dichlorophenolindophenol or tetrazolium salts. Cytochrome c, however, is not present in the microsomes, which instead contain another related compound, cytochrome b_5. Preparations of cytochrome b_5 become reduced in the presence of liver microsomes and TPNH.[2]

In our experiments the oxidative demethylation of *p*-methylaminoazobenzene by liver microsomes of young rats was stimulated about 3 times as a result of MC treatment. The reduction of cytochrome b_5 in the presence of TPNH by the same microsomal preparations was measured by following the development of the difference spectrum in the region of 557 mμ. Normal microsomes usually showed an activity of the order 3×10^{-3} μmoles of reduced cytochrome b_5 per mg protein per minute. The cytochrome b_5 reduction was significantly increased in the microsomes from MC-treated animals. Also the TPNH-diaphorase (measured by dichlorophenolindophenol or neotetrazolium) was stimulated, while on the other hand the TPNH cytochrome c reductase showed a significant decrease. The content of cytochrome b_5 in the microsomes did not change appreciably under these conditions.

After partial hepatectomy the activity per mg protein of oxidative demethylase and the content of cytochrome b_5 in rat liver microsomes decrease by about 50 %[3]. Hepatectomized rats were treated with MC 24 hours after the operation. In comparison with non-treated, hepatectomized rats the demethylase activity increased about 4 times. At the same time the reduction of cytochrome b_5 by TPNH was stimulated. The TPNH diaphorase activity increased by 15—25 %, while the TPNH cytochrome c reductase again showed a decrease. In the regenerating livers the cytochrome b_5 content showed a positive response to MC.

The experimental results may imply that an oxidation of TPNH by cytochrome b_5 is somehow involved in the microsomal reactions stimulated by the MC-treatment.

1. Conney, A. H., Miller, E. C. and Miller, J. A. *Cancer Research* **16** (1956) 450.
2. Strittmatter, P. and Velick, S. F. *J. Biol. Chem.* **221** (1956) 277.
3. v. d. Decken, A. and Hultin, T. *Exptl. Cell Research. (In press).*

Ethanol and Liver Metabolism

Olof Forsander and Niels Räihä

Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland

During the oxidation of ethanol to acetic acid *ca.* 1/3 of its energy content is liberated in the liver. If it is assumed that the need of energy is constant in the liver, and that the energy derived from ethanol can be utilized as well as that of other substrates, it would seem reasonable to postulate that the breakdown of other substrates would decrease correspondingly during the oxidation of ethanol.

During the artificial perfusion of an isolated normal rat liver the addition of ethanol to the perfused blood decreased the amount of CO_2 liberated, whereas the fasted liver with a low initial CO_2 production was unaffected. The oxidation of ethanol changed the type of metabolism in a normal liver from a complete oxidation of substrate to CO_2 to a partial oxidation to intermediates, whereas the metabolism of the fasted liver, where the partial oxidation already was considerable, was unaffected. In the normal liver the partial oxidation was increased by ethanol and an accumulation of organic acids proportional to the decrease of the complete oxidation was observed. During the perfusion of normal liver 2.27 mg of CO_2 per min was produced. When ethanol was added to the blood the production of CO_2 decreased to 1.48 mg per min and additional energy was produced from the partial oxidation to acetic acid of 1.26 mg of ethanol per min. If it is assumed that the CO_2 production in the normal liver is derived solely from complete oxidation of carbohydrates the energy production would be 5.7 cal per min. In the case where ethanol was added to the blood the calculated energy production would be 6.6 cal per min.

Apparent Phosphate Ion Effect on Activity of Phosphoprotein Phosphatase

Bo Norberg

Kemiska Centrallaboratoriet, Sabbatsbergs Sjukhus, Stockholm, Sverige

Phosphoprotein phosphatase activity was lost upon dialysis of the extract (rat liver and erythrocytes). The studies (to be reported in detail elsewhere) show an activating effect of a small amount of phosphate ion (to a final concentration of 0.1 to 0.3 mM PO_4 depending on substrate concentration) whereas higher concentrations are inhibitory, as found earlier [1].

1. Norberg, B. *Acta Chem. Scand.* 4 (1950) 1206.

Effects of Carcinogenic Amines on the Protein and Ribonucleic Acid Metabolism of the Liver

E. Arrhenius and T. Hultin

The Wenner-Gren Institute, University of Stockholm, Sweden

The incorporation in vitro of [^{14}C]-DL- valine into the proteins of rat liver slices was studied after different periods of preincubation with 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), 7-fluoro-2-aminofluorene (FAF), 7-fluoro-2-acetylamino-fluorene (FAAF), 2-aminonaphtalene (AN) and aniline at varied concentrations. While a marked reduction of the incorporation was caused by the polynuclear amines, no significant inhibition was observed in the case of aniline even at relatively high concentrations (10^{-3} M). Similar experiments were performed with AF and AN, with [^{14}C] adenine as isotopic component. Adenine and guanine were isolated from RNA, and their specific activities were determined. Also in this case a marked inhibition was observed, less strong however than in the [^{14}C] valine experiments. Within the group of polynuclear amines the capacity of inhibition was not in detail correlated to the carcinogenic potency. As a rule AN was at least as inhibitory as AF, although AN is known to exert its carcinogenic effect on other target organs than the liver. FAF had a slightly lower inhibitory effect than AF. An inhibition by AF and FAF was observed not only in rat liver slices but also in liver slices of guinea pig, although this animal is refractory to the carcinogenic action of AF derivatives. Rats were treated *in vivo* with AN (100 mg/kg in 1.0 ml of propylene glycol, *per os*). The incorporation of [^{14}C] L-leucine into proteins by a mitochondriafree liver preparation was studied 4 h after the AN- treatment. The activity of the preparation for leucine incorporation *in vitro* was reduced when compared with similar preparations from untreated rats. The microsomes of the system showed a decreased activity in comparison with normal microsomes.

Inhibitor Effects on Light-Induced Phosphorylation. A Comparison between Plant and Bacterial Systems

Herrick Baltscheffsky

Wenner-Gren Institute, University of Stockholm, Sweden

Electron transport in mitochondrial oxidative phosphorylation is strongly inhibited by amytal (I), atebrin (II),antimycin A (III) and 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (IV). The effects of these inhibitors were tested on light-induced phosphorylation (LIP) in washed spinach chloroplasts, and compared with earlier results obtained with washed bacterial (*Rhodospirillum rubrum*) chromatophores [1-4]. The following results were obtained in an ascorbate containing medium, whether or not flavin mononucleotide had been added (aerobic conditions, saturating light intensity, 20°C). A 2×10^{-3} M concentration of I gave only little inhibition, as in bacterial LIP [4]. 5×10^{-5} M concentrations of II, III or IV gave inhibitions between 50 and 100 %. The inhibition with II occurs in the same inhibitor concentration region as with chromatophores and indicates participation of chloroplast flavin in the electron transport in chloroplast LIP. For III and IV, however, the concentrations needed are about 1 000-fold higher than with chromatophores (or animal mitochondria).

Earlier results with these inhibitors have indicated that electron transport in bacterial (*R. rubrum*) LIP proceeds over a chain with at least two electron carriers [3,4]. The present results will be discussed in relation to current schemes of electron transport in plant LIP, and the electron transport chains in plant and bacterial LIP will be compared.

1. Smith, L. and Baltscheffsky, M. *Federation Proc.* **15** (1956) 357.
2. Geller, D.M. *VIIth International Congress for Microbiology, Abstracts*, Stockholm 1958, p. 73.
3. Baltscheffsky, H. *Biochim. et Biophys. Acta.* **40** (1960) 1.
4. Baltscheffsky, H. and Baltscheffsky, M. *To be published.*

Diphosphothiamine Disulfide in Baker's Yeast

Heikki Suomalainen, Sakari Rihtniemi and Erkki Oura

Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland

About 15 years ago Myrbäck and coworkers [1] indicated that baker's yeast, produced in vigorously aerated conditions, contains nearly all its thiamine as diphosphothiamine disulfide. His findings were met with criticism and his viewpoint was not generally accepted, also owing to the fact that the physiological function of cocarboxylase disulfide has not been conclusively verified.

We have now, in contrary to our earlier attempts [2] been able to confirm the appearance of diphosphothiamine disulfide in aerobically cultivated baker's yeast. We first repeated the experiments of Myrbäck and found a thiamine derivative in baker's yeast, which after cystein reduction acted like cocarboxylase in the thiochrome reaction. With ionexchange chromatography, using Dowex 1-X resin in formiate form, a thiamine derivative was separated from the perchloric acid extract of the commercial stage of baker's yeast, which was eluted by gradient eluation with formic acid or with ammonium formiate considerably later than thiamine, thiamine disulfide or thiamine monophosphate and cocarboxylase, the speed corresponding to that of synthetic diphosphothiamine disulfide (Merck). When oxidized after cystein reduction it gives a thiochrome reaction corresponding to that of cocarboxylase.

1. Myrbäck, K., Wallin, I. and Magnell, I. *Svensk Kem. Tidskr.* **57** (1945) 124.
2. Forsander, O. and Suomalainen, H. *Acta Chem. Scand.* **11** (1957) 1094.

Changes in the Microbial Amino Acid Metabolism Induced by 4-Deoxypyridoxine

J. Jännes and T. Lahikainen

Aurora Hospital, Helsinki, Finland

Normal strains of *E.coli* were grown in media containing 4-deoxypyridoxine in amounts of 10, 20, 30 and 40 mg per 10 ml of the culture media. Asparagine was the only source of nitrogen, and the incubation time was 7 to 8 days at 37 C°. At the end of the incubation time the media were centrifuged. The clear supernatants were treated with Amberlite IR 120 resins in acid form and eluated with 1 N ammonium hydroxide. The analyses of the amino acids were performed with paper chromatography. The most characteristic finding was the disappearance of the spots of both glutamic and γ-amino-butyric acids when 4-deoxypyridoxine was present in the culture media. The effect of 4-deoxypyridoxine on the growth was also noticed.

Two Unusual U.V. Absorbing Substances Associated with Amino Acids in Bacterial Extracts

Jon Jonsen, S. Laland and I. Smith-Kielland

Department of Biochemistry, University of Oslo, Blindern, Oslo, Norway

During an investigation of the nucleotides in various extracts from different bacteria we have detected unusual ultraviolet absorbing acidic substances associated with amino acids.

Crude acid-soluble extracts were made by cold perchloric acid treatment of bacteria disrupted in a bacterial press.

Crude water-soluble extracts were obtained by heating living cells in boiling water for 10 min.

The bacteria were harvested during rapid growth. In some cases the extracted material was adsorbed on Norite and again eluted with 50 % ethanol containing 2 % of conc. ammonia. The extracts were fractionated on a Dowex

Apparent Phosphate Ion Effect on Activity of Phosphoprotein Phosphatase

Bo Norberg

Kemiska Centrallaboratoriet, Sabbatsbergs Sjukhus, Stockholm, Sverige

Phosphoprotein phosphatase activity was lost upon dialysis of the extract (rat liver and erythrocytes). The studies (to be reported in detail elsewhere) show an activating effect of a small amount of phosphate ion (to a final concentration of 0.1 to 0.3 mM PO_4 depending on substrate concentration) whereas higher concentrations are inhibitory, as found earlier [1].

1. Norberg, B. *Acta Chem. Scand.* 4 (1950) 1206.

Effects of Carcinogenic Amines on the Protein and Ribonucleic Acid Metabolism of the Liver

E. Arrhenius and T. Hultin

The Wenner-Gren Institute, University of Stockholm, Sweden

The incorporation in vitro of [^{14}C]-DL- valine into the proteins of rat liver slices was studied after different periods of preincubation with 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), 7-fluoro-2-aminofluorene (FAF), 7-fluoro-2-acetylamino-fluorene (FAAF), 2-aminonaphtalene (AN) and aniline at varied concentrations. While a marked reduction of the incorporation was caused by the polynuclear amines, no significant inhibition was observed in the case of aniline even at relatively high concentrations (10^{-3} M). Similar experiments were performed with AF and AN, with [^{14}C] adenine as isotopic component. Adenine and guanine were isolated from RNA, and their specific activities were determined. Also in this case a marked inhibition was observed, less strong however than in the [^{14}C] valine experiments. Within the group of polynuclear amines the capacity of inhibition was not in detail correlated to the carcinogenic potency. As a rule AN was at least as inhibitory as AF, although AN is known to exert its carcinogenic effect on other target organs than the liver. FAF had a slightly lower inhibitory effect than AF. An inhibition by AF and FAF was observed not only in rat liver slices but also in liver slices of guinea pig, although this animal is refractory to the carcinogenic action of AF derivatives. Rats were treated *in vivo* with AN (100 mg/kg in 1.0 ml of propylene glycol, *per os*). The incorporation of [^{14}C] L-leucine into proteins by a mitochondriafree liver preparation was studied 4 h after the AN- treatment. The activity of the preparation for leucine incorporation *in vitro* was reduced when compared with similar preparations from untreated rats. The microsomes of the system showed a decreased activity in comparison with normal microsomes.

Inhibitor Effects on Light-Induced Phosphorylation. A Comparison between Plant and Bacterial Systems

Herrick Baltscheffsky

Wenner-Gren Institute, University of Stockholm, Sweden

Electron transport in mitochondrial oxidative phosphorylation is strongly inhibited by amytal (I), atebrin (II),antimycin A (III) and 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (IV). The effects of these inhibitors were tested on light-induced phosphorylation (LIP) in washed spinach chloroplasts, and compared with earlier results obtained with washed bacterial (*Rhodospirillum rubrum*) chromatophores [1-4]. The following results were obtained in an ascorbate containing medium, whether or not flavin mononucleotide had been added (aerobic conditions, saturating light intensity, 20°C). A 2×10^{-3} M concentration of I gave only little inhibition, as in bacterial LIP [4]. 5×10^{-5} M concentrations of II, III or IV gave inhibitions between 50 and 100 %. The inhibition with II occurs in the same inhibitor concentration region as with chromatophores and indicates participation of chloroplast flavin in the electron transport in chloroplast LIP. For III and IV, however, the concentrations needed are about 1 000-fold higher than with chromatophores (or animal mitochondria).

Earlier results with these inhibitors have indicated that electron transport in bacterial (*R. rubrum*) LIP proceeds over a chain with at least two electron carriers [3,4]. The present results will be discussed in relation to current schemes of electron transport in plant LIP, and the electron transport chains in plant and bacterial LIP will be compared.

1. Smith, L. and Baltscheffsky, M. *Federation Proc.* **15** (1956) 357.
2. Geller, D.M. *VIIth International Congress for Microbiology, Abstracts*, Stockholm 1958, p. 73.
3. Baltscheffsky, H. *Biochim. et Biophys. Acta.* **40** (1960) 1.
4. Baltscheffsky, H. and Baltscheffsky, M. *To be published.*

Diphosphothiamine Disulfide in Baker's Yeast

Heikki Suomalainen, Sakari Rihtniemi and Erkki Oura

Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland

About 15 years ago Myrbäck and coworkers [1] indicated that baker's yeast, produced in vigorously aerated conditions, contains nearly all its thiamine as diphosphothiamine disulfide. His findings were met with criticism and his viewpoint was not generally accepted, also owing to the fact that the physiological function of cocarboxylase disulfide has not been conclusively verified.

We have now, in contrary to our earlier attempts [2] been able to confirm the appearance of diphosphothiamine disulfide in aerobically cultivated baker's yeast. We first repeated the experiments of Myrbäck and found a thiamine derivative in baker's yeast, which after cystein reduction acted like cocarboxylase in the thiochrome reaction. With ionexchange chromatography, using Dowex 1-X resin in formiate form, a thiamine derivative was separated from the perchloric acid extract of the commercial stage of baker's yeast, which was eluted by gradient eluation with formic acid or with ammonium formiate considerably later than thiamine, thiamine disulfide or thiamine monophosphate and cocarboxylase, the speed corresponding to that of synthetic diphosphothiamine disulfide (Merck). When oxidized after cystein reduction it gives a thiochrome reaction corresponding to that of cocarboxylase.

1. Myrbäck, K., Wallin, I. and Magnell, I. *Svensk Kem. Tidskr.* **57** (1945) 124.
2. Forsander, O. and Suomalainen, H. *Acta Chem. Scand.* **11** (1957) 1094.

Changes in the Microbial Amino Acid Metabolism Induced by 4-Deoxypyridoxine

J. Jännes and T. Lahikainen

Aurora Hospital, Helsinki, Finland

Normal strains of *E.coli* were grown in media containing 4-deoxypyridoxine in amounts of 10, 20, 30 and 40 mg per 10 ml of the culture media. Asparagine was the only source of nitrogen, and the incubation time was 7 to 8 days at 37 C°. At the end of the incubation time the media were centrifuged. The clear supernatants were treated with Amberlite IR 120 resins in acid form and eluated with 1 N ammonium hydroxide. The analyses of the amino acids were performed with paper chromatography. The most characteristic finding was the disappearance of the spots of both glutamic and γ-amino-butyric acids when 4-deoxypyridoxine was present in the culture media. The effect of 4-deoxypyridoxine on the growth was also noticed.

Two Unusual U.V. Absorbing Substances Associated with Amino Acids in Bacterial Extracts

Jon Jonsen, S. Laland and I. Smith-Kielland

Department of Biochemistry, University of Oslo, Blindern, Oslo, Norway

During an investigation of the nucleotides in various extracts from different bacteria we have detected unusual ultraviolet absorbing acidic substances associated with amino acids.

Crude acid-soluble extracts were made by cold perchloric acid treatment of bacteria disrupted in a bacterial press.

Crude water-soluble extracts were obtained by heating living cells in boiling water for 10 min.

The bacteria were harvested during rapid growth. In some cases the extracted material was adsorbed on Norite and again eluted with 50 % ethanol containing 2 % of conc. ammonia. The extracts were fractionated on a Dowex

Table 1.

	Spectra in acid max	min	250/260	280/260	Spectra in alkali max	min	250/260	280/260
Substance I	266	237	0.76	0.82	275	244	0.79	1.16
Substance II	261	237	0.63	0.13	261		0.76	0.17

1 × 8 column in the formate form by gradient elution with the formic acid-ammonium formate system of Hurlbert *et al.* A slight modification was introduced as the elution prodecure was started with very dilute formic acid (0.001 M) in the reservoir.

In addition to amino acid complexes containing adenine nucleotides, two other complexes with amino acids were isolated which had U.V. spectra different from any of the usual purines and pyrimidines. Both were eluted early in the fractionation procedure. One (substance I) appeared earlier than any of the known nucleotides, the other (substance II) together with CMP-5' or in a fraction following immediately after CMP-5'.

In paper chromatography both substances moved as single spots in five different solvents. In most solvents the R_F values exceeded those of the known nucleotides. No change in the mobility of the U.V. absorbing substances was observed after hydrolysis for 18 h in N HCl at 120°C. The two substances were homogeneous and negatively charged at pH 7.0 as indicated by electrophoresis in phosphate buffer.

Whereas the unhydrolyzed material was ninhydrine negative, hydrolysis in 6 N HCl gave for both substances ninhydrine positive spots corresponding to the position of alanine, serine, aspartic acid, glutamic acid, glycine and lysine in two dimensional paper chromatography. Small amounts of these amino acids also split off when the unhydrolyzed material was subjected to paper chromatography. None of the substances contained phosphate. One of them (substance I) could be reduced with sodium amalgam and the reduced material gave positive test for ribose.

The two substances exhibited a slight difference in the electrophoretic mobility at acid pH values and pronounced differences in the spectra values as shown in Table 1.

We wish to thank *Norges Almenvitenskapelige Forskningsråd* for financial support.

1. Jonsen, J., Laland, S., Smith-Kielland, I. and Sömme, R. *Acta Chem. Scand.* **13** (1959) 838.

Biologically Active Fractions from *Haemophilus Pertussis*

H. Billaudelle, L. Edebo, E. Hammarsten, C.-G. Hedén, B. Malmgren and H. Palmstierna

Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden

Cells from *H. pertussis*, cultivated in the 500 litres scale, were disintegrated in the frozen state in the Hughes press. The disintegrate was extracted by a series of aqueous salt solutions. The supernatant was highly neurotoxic and dermatonecrotic. The neurotoxin was purified by column electrophoresis on a stabilized medium (320 cm columns). Due to the presence of acid polyelectrolytes the conditions for a purification of the neurotoxin on cellulose ion exchangers are unfavourable. They were consequently removed by electrophoresis before chromatography. The neurotoxin occurs in the same pK region (around 7.4) as a blue coloured protein. There exist dermatonecrotic proteins in other pK regions. The neurotoxin is reasonably stable only in its isoelectric region.

The neurotoxin has been purified to a MLD per 10 g mouse of 10 μg. Further purification is tried.

The residue after extractions forms very stable suspensions after the salts have been removed. It can be spun down at 100 000 *g*. It is salted out by monovalents salts. It can be solubilised again by either removing the salts or by making it 2 M with regard to urea. The residue contains two lipid fractions, protein and (amino-)polysaccharide. It is nontoxic up to at least 2 mg per 10 g mouse, and protects mice against 1 000 MLD of *H. pertussis* down to at least 50 μg per 10 g mouse. Electron microscopy shows that it consists primarily of bacterial cell walls. A *Haemophilus pertussis* immunogen has thus been found that shows no toxic activity on mice even in high dosages.

The work has been performed under a grant from the *U.S. Army European Research Office.*

Table 1.

	Spectra in acid max	min	250/260	280/260	Spectra in alkali max	min	250/260	280/260
Substance I	266	237	0.76	0.82	275	244	0.79	1.16
Substance II	261	237	0.63	0.13	261		0.76	0.17

1 × 8 column in the formate form by gradient elution with the formic acid-ammonium formate system of Hurlbert *et al.* A slight modification was introduced as the elution prodecure was started with very dilute formic acid (0.001 M) in the reservoir.

In addition to amino acid complexes containing adenine nucleotides, two other complexes with amino acids were isolated which had U.V. spectra different from any of the usual purines and pyrimidines. Both were eluted early in the fractionation procedure. One (substance I) appeared earlier than any of the known nucleotides, the other (substance II) together with CMP-5' or in a fraction following immediately after CMP-5'.

In paper chromatography both substances moved as single spots in five different solvents. In most solvents the R_F values exceeded those of the known nucleotides. No change in the mobility of the U.V. absorbing substances was observed after hydrolysis for 18 h in N HCl at 120°C. The two substances were homogeneous and negatively charged at pH 7.0 as indicated by electrophoresis in phosphate buffer.

Whereas the unhydrolyzed material was ninhydrine negative, hydrolysis in 6 N HCl gave for both substances ninhydrine positive spots corresponding to the position of alanine, serine, aspartic acid, glutamic acid, glycine and lysine in two dimensional paper chromatography. Small amounts of these amino acids also split off when the unhydrolyzed material was subjected to paper chromatography. None of the substances contained phosphate. One of them (substance I) could be reduced with sodium amalgam and the reduced material gave positive test for ribose.

The two substances exhibited a slight difference in the electrophoretic mobility at acid pH values and pronounced differences in the spectra values as shown in Table 1.

We wish to thank *Norges Almenvitenskapelige Forskningsråd* for financial support.

1. Jonsen, J., Laland, S., Smith-Kielland, I. and Sömme, R. *Acta Chem. Scand.* **13** (1959) 838.

Biologically Active Fractions from *Haemophilus Pertussis*

H. Billaudelle, L. Edebo, E. Hammarsten, C.-G. Hedén, B. Malmgren and H. Palmstierna

Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden

Cells from *H. pertussis*, cultivated in the 500 litres scale, were disintegrated in the frozen state in the Hughes press. The disintegrate was extracted by a series of aqueous salt solutions. The supernatant was highly neurotoxic and dermatonecrotic. The neurotoxin was purified by column electrophoresis on a stabilized medium (320 cm columns). Due to the presence of acid polyelectrolytes the conditions for a purification of the neurotoxin on cellulose ion exchangers are unfavourable. They were consequently removed by electrophoresis before chromatography. The neurotoxin occurs in the same pK region (around 7.4) as a blue coloured protein. There exist dermatonecrotic proteins in other pK regions. The neurotoxin is reasonably stable only in its isoelectric region.

The neurotoxin has been purified to a MLD per 10 g mouse of 10 μg. Further purification is tried.

The residue after extractions forms very stable suspensions after the salts have been removed. It can be spun down at 100 000 *g*. It is salted out by monovalents salts. It can be solubilised again by either removing the salts or by making it 2 M with regard to urea. The residue contains two lipid fractions, protein and (amino-)polysaccharide. It is nontoxic up to at least 2 mg per 10 g mouse, and protects mice against 1 000 MLD of *H. pertussis* down to at least 50 μg per 10 g mouse. Electron microscopy shows that it consists primarily of bacterial cell walls. A *Haemophilus pertussis* immunogen has thus been found that shows no toxic activity on mice even in high dosages.

The work has been performed under a grant from the *U.S. Army European Research Office.*

On the Enzymatic Formation of Ester Sulphates of Aliphatic Polyols

Anders Vestermark and Harry Boström

Department of Metabolic Research, Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

Sulphate conjugation of phenols [1], steroids [2,3], some amines [4], certain carbohydrate acceptors [5] and primary aliphatic alcohols [6] is known to take place in cell-free media containing sulphate-activating and sulphate-transferring enzymes [7].

Our studies on alcohols have now been extended to aliphatic polyols, and indications have been obtained of an enzymatic sulphurylation of several compounds of this type. As in earlier studies [8,6], ^{35}S-labelled sulphate was used as tracer substance; formation of sulphate esters in the incubating medium [3] (phosphate buffer, ATP, Mg ions, particle-free rat liver supernatant, ^{35}S-labelled sulphate and sample of substance to be tested) was demonstrated by means of two-dimensional paper chromatography and electrophoresis, both methods combined with autoradiography.

The results of this investigation are briefly summarized in Table 1. In most experiments, a new spot appeared in addition to the basic pattern, indicating the formation of a sulphate ester. Higher concentrations of the substance added gave stronger spots, until in some cases inhibition occurred.

As seen in the table, the different glycols studied (Group I) had about the same tendency to form sulphate esters in the system used. Increased number of HCOH groups (II) decreased the tendency to sulphate conjugation. Of the diols tested (Group III), those with a larger distance between the OH groups were more easily combined with sulphate.

1. De Meio, R. H. and Tkacz, L. *Arch. Biochem.* **27** (1950) 242.
2. De Meio, R. H. and Lewycka, C. *Endocrinology* **56** (1955) 489.
3. Roy, A. B. *Biochem. J.* **63** (1956) 294.
4. Roy, A. B. *Biochem. et Biophys. Acta* **30** (1958) 193.
5. Suzuki, S. *Federation Proc.* **18** (1959) 335.
6. Vestermark, A. and Boström, H. *Exptl. Cell Research* **18** (1959) 174.
7. Lipman, F. *Science* **128** (1958) 575.
8. Vestermark, A. and Boström, H. *Acta Chem. Scand.* **13** (1959) 827.

Group	Compound	Concentration in the medium (%) *				
		0.01	0.1	0.5	5	10
I	Glycol	−	−	+	+++	+++
	Diethylene glycol	−	+	+	+++	+++
	Triethylene glycol	−	+	++	+++	+++
	Polyethylene glycol (Approx. 200 repeating units)	−	−	+	+++	+++
II	Methanol	−	+	+	+++	+++
	Glycol	−	−	+	+++	+++
	Glycerol	−	−	−	+	+
	Erythritol	−	−	− (0.3 %)		
	Pentitols	−	−	− (0.3 %)		
	Hexitols	−	−	− (0.3 %)		
III	Glycol	−	−	+	+++	+++
	1,4-Butanediol	−	+	+++	+++	+++
	1,5-Pentanediol	+	++	+++	++	+
	1,6-Hexanediol	+	+++	+++		

* The sign − denotes absence of new spots, and +, ++, +++ appearence of a weak, medium or strong new spot.

Changes in the Phosphatase Activity and Location of Phosphatases in Baker's Yeast Cells

Erkki Oura, Heikki Suomalainen and Matti Linko

Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland

Using phenolphtalein diphosphate as a substrate it was possible to demonstrate three peaks in the pH-curve of the phosphatase activity of baker's yeast, one at the pH optimum of the acid phosphatase, 4.3, and two on the alkaline side at pH 7.9 and 8.9. The acid phosphatase of baker's yeast is mainly located on the cell surface, about one third, however, within the cell. Both alkaline phosphatase components are wholly located inside the cell membrane.

During the industrial cultivation of baker's yeast the nitrogen content decreases stage by stage, as does the phosphorus content during the last two stages. The acid phosphatase activity first increases, but falls sharply to one tenth during the growth period of the precommercial A_4 stage. The activity increases again during the cultivation of the commercial yeast stage when the phosphorus content of the yeast still more decreases. During the phosphorus starvation of the commercial yeast stage the activity of the acid phosphatase increases sharply being 3 to 8 fold in 8 h as compared with the seed yeast.

The activity of the alkaline phosphatases up to the commercial yeast stage does apparently follow the decrease in the phosphorus content of the yeast cells. A sharp increase in the activity 4 to 6 times is, however, observed during the experimental phosphorus starvation of the cells.

A more detailed report will be published elsehwere [1].

1. Suomalainen, H., Linko, M. and Oura, E. *Biochim. et Biophys. Acta. In press.*

Stereochemical Course of the Conversion of Cholesterol to Bile Acid

S. Bergström, B. Samuelsson and H. Thommen

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

With the aid of cholesterol stereospecifically tritium labelled at positions 3α, 4α, 4β, 6, 7α, 7β, or 7α 7β, 8β, the course of the transformation of cholesterol into chenodeoxycholic and cholic acid has been investigated in the rat and pig.

ACTA CHEMICA SCANDINAVICA 13 (1959) 2135–2162

Short Communications

New Secular Equations in the Problem of Molecular Vibrations

S. J. CYVIN

Institutt for teoretisk kjemi, Norges tekniske høgskole, Trondheim, Norway

The well-known secular equation in the problem of harmonic vibrations of polyatomic molecules may be put in the form [1,2]

$$| GF - E\lambda | = 0 \qquad (1)$$

Here G and F are the energy matrices introduced by Wilson,[1] and E is the identity matrix. The G matrix is composed of the atomic masses and occasionally the structural parameters of the molecule, the F matrix containing the force constants included the interaction terms. Eqn. (1) yields the familiar relations connecting the force constants with the normal frequencies of the molecule. The values of λ are proportional to the squares of the frequencies (ν) according to the equation

$$\lambda_k = 4\pi^2\nu_k^2 \qquad (2)$$

It should be emphasized that the number of normal frequencies of a polyatomic molecule in most cases is not sufficient for the complete determination of the harmonic force field. Therefore usually a simplified force field, *e.g.* a valence force field is assumed, or additional information, *e.g.* normal frequencies from isotopically substituted molecules, or force constant values from related molecules, are utilized.

Let now S be a column matrix consisting of a complete set of internal displacement coordinates and connected with the vibrational normal coordinates (Q) through the linear transformation

$$S = LQ \qquad (3)$$

The following relations involving the transformation matrix L exist.

$$\tilde{L}FL = \Lambda \qquad (4)$$

$$G = L\tilde{L} \qquad (5)$$

Here $\sim$ denotes the transpose of a matrix, and Λ is a diagonal matrix with the elements λ_k given by eqn. (2). The symbol $\langle\rangle$ will be used to indicate that the mean value of each element of the matrix in question has been taken, and the matrix

$$\Sigma = \langle S\tilde{S} \rangle \qquad (6)$$

will be considered. The elements on its main diagonal, *viz.*

$$\Sigma_{ii} = \langle S_i^2 \rangle \qquad (7)$$

have been called the "mean-square amplitudes of vibration" and are of considerable interest in molecular structure studies (see, *e.g.* Refs.[3-5]).

The matrix Σ of eqn. (6) may be computed by means of the L matrix according to

$$\Sigma = L \triangle \tilde{L} \qquad (8)$$

where $\triangle = \langle Q\tilde{Q} \rangle$ is a diagonal matrix with the elements

$$\triangle_k = (h/8\pi^2\nu_k)\coth(h\nu_k/2kT) \qquad (9)$$

If the F matrix has been established, and with the knowledge of the normal frequencies, the L matrix may be obtained according to eqn. (1) by means of the standard method of characteristic vectors applied to the GF matrix. This is the method for computing mean-square amplitudes of vibration which has been evaluated by Morino *et al.*[4]

The progress in gas electron diffraction techniques has made it possible to produce experimental values of root-mean-square

amplitudes of vibration,[5] and it has been proposed to utilize such measurements in force constant determinations. It seems to be doubtful whether the present electron diffraction data of root-mean-square amplitudes are of sufficient accuracy to be of practical value for this purpose. Nevertheless, we may hope that the accuracy still will increase in the future. This discussion indicates the desire of evaluating a method for obtaining some information about the force constants from given values of root-mean-square amplitudes of vibration. The above mentioned method of Morino *et al.*[4] is in practice not convenient for such computations.

From eqns. (5) and (8) a new secular equation may be derived and written in the form

$$| \Sigma G^{-1} - E\Delta | = 0 \qquad (10)$$

This equation makes it possible to treat the mean-square amplitudes of vibration similarly to the treatment of force constants. It may be applied to establish relations connecting the normal frequencies and the mean-square amplitudes including the interaction terms, *i.e.* the off-diagonal Σ matrix elements. Here again the number of normal frequencies is usually not sufficient for the complete determination of the Σ matrix, but approximations may be performed, and additional information about the mean-square amplitudes may be utilized. This method represents a new approach to the calculation of mean-square amplitudes of vibration from spectroscopic data, being different from the method of Morino *et al.*[4] Furthermore, if the complete Σ matrix could be established, it would be possible to determine the L matrix by the method of characteristic vectors applied to the ΣG^{-1} matrix. In consequence, the potential energy matrix could be obtained from the equation *

$$F = \widetilde{L}^{-1} \wedge L^{-1} \qquad (11)$$

being a modification of eqn. (4).

Still another secular equation will be reported, *viz.*

$$| \Sigma F - E\lambda\Delta | = 0 \qquad (12)$$

which may be deduced from eqns. (4) and (8). According to eqns. (2) and (9), the characteristic values of the ΣF matrix are

$$\lambda_k \Delta_k = \tfrac{1}{2} h\nu_k \coth (h\nu_k/2kT) \qquad (13)$$

Eqn. (12) yields relations containing the elements from both of the matrices Σ and F. These relations will be useful in combination with the relations obtained from (1) and (12) if it is desirable to apply some information about the Σ matrix elements for determination of the elements of the F matrix, and *vice versa*. Accordingly, such computations may be performed without determination of the L matrix.

It should be pointed out that the new secular eqns. (10) and (12) will factorize in precisely the same manner as the ordinary secular eqn. (1) if a set of *symmetry* coordinates is chosen.

Finally an isotope rule for mean-square amplitudes of vibration will be reported. The ordinary product rule for isotopically substituted molecules as derived from eqn. (1) may be written in the form [1]

$$\prod_i (\nu_i / \nu_i^*) = | G |^{\frac{1}{2}} / | G^* |^{\frac{1}{2}} \qquad (14)$$

where the quantities of the isotopically substituted molecule are identified by an asterisk. Using the analogous notation, one obtains from eqn. (12)

$$| \Sigma | / | \Sigma^* | = \prod_i \frac{\nu_i \coth(h\nu_i/2kT)}{\nu_i^* \coth(h\nu_i^*/2kT)} \qquad (15)$$

If the mean-square amplitudes at the absolute zero are considered, and the corresponding matrices are identified by the subscript $_0$, one obtains

$$|\Sigma_0| / |\Sigma_0^*| = \prod_i (\nu_i/\nu_i^*) = |G|^{\frac{1}{2}} / |G^*|^{\frac{1}{2}} \qquad (16)$$

Some applications of the here presented secular equations to specific problems, are under preparation.

* The L^{-1} matrix may be computed directly by means of a modified form of equation (10), *viz.* $| G^{-1}\Sigma - E\Delta | = 0$.

1. Wilson, E. B. Jr. *J. Chem. Phys.* **7** (1939) 1047; **9** (1941) 76.
2. Wilson, E. B. Jr., Decius, J. C. and Cross, P. C. *Molecular Vibrations*, McGraw-Hill Book Co. New York 1955.
3. Morino, Y. *J. Chem. Phys.* **18** (1950) 395.
4. Morino, Y., Kuchitsu, K. and Shimanouchi, T. *J. Chem. Phys.* **20** (1952) 726.
5. Bastiansen, O. and Cyvin, S. J. *Nature* **180** (1957) 980.

Received November 5, 1959.

Optical Rotatory Dispersion

I. A Recording Spectropolarimeter Based on the Cary 11MS—50 Spectrophotometer

FLEMMING WOLDBYE

Chemistry Department A, Technical University of Denmark, Copenhagen, Denmark

The increasing interest in measurements of optical rotatory dispersion recently developed within several branches of chemistry [1-6] has accentuated the instrumentation problems of spectropolarimetry. The solution of the structural and theoretical problems formulated in the works referenced will require a detailed knowledge of the rotatory dispersion within the widest possible wavelength range, especially within the ranges of absorption. Much effort is being spent, therefore, in developing recording spectropolarimeters, none of which, however, seem to be commercially available as yet.

In the course of studies of the optical rotatory dispersion of some transition metal complex ions [6] we have developed an accessory for the Cary 11MS—50 spectrophotometer which permits the automatic recording of optical rotatory dispersion in the wavelength range $800 \geq \lambda \geq 228$ mμ with a relative accuracy of *ca.* 1 %.

Although unsatisfactory for high precision quantitative analysis optical rotatory dispersion curves of this accuracy may certainly meet the requirements of the structural and theoretical investigations.

The principle by which the rotation, α, of a sample is converted into an apparent optical density which may be recorded by the spectrophotometer is illustrated in Fig. 1 *. If the samples, S, are optically inactive, the two systems, P—S—A, have identical optical densities

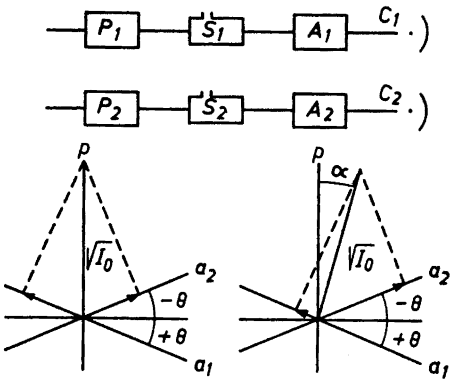


Fig. 1. $P_{1,2}$ polarizers, $S_{1,2}$ identical samples, $A_{1,2}$ analyzers at angles $90° + \Theta$ and $90° - \Theta$, respectively, with the planes of polarization of P_1 and P_2, and C_1 and C_2 sample and standard photomultipliers of the spectrophotometer. p, a_1 and a_2 planes of the electric vector of light polarized by $P_{1 \text{ or } 2}$, A_1 and A_2 viewed from $C_{1 \text{ or } 2}$. The intensities transmitted by the systems are obtained by squaring the lengths of the projections of the electric vector, $\sqrt{I_0}$, upon a_1 and a_2.

$$D_1 = D_2 = \log \frac{I_0}{I} = \log \frac{I_0}{I_0 \cos^2(\pm(90° - \Theta))} + D_u = -2 \log \sin\Theta + D_u$$

where the first term represents the density caused by the relative setting of polarizer and analyzer while D_u represents the density caused by the initial polarization, the absorption in the sample, reflections *etc.* Thus the density recorded by the spectrophotometer, $D_{app} = D_1 - D_2$, will be equal to zero.

If identical, optically active samples, S, causing a rotation $\alpha < \Theta$, are inserted consideration of Fig. 1 shows that $D_1 = -2 \log \sin(\Theta - \alpha) + D_u$ and $D_2 = -2 \log \sin(\Theta + \alpha) + D_u$ so that

$$D_{app} = D_1 - D_2 = 2 \log \frac{\sin(\Theta + \alpha)}{\sin(\Theta - \alpha)} \quad (1)$$

Provided $\Theta + \alpha < 10°$, ($\alpha < \Theta$), the approximate solution [6,9] of (1)

* This principle has been utilized in polarimetry before, *e.g.* by Keston [7]. Also the accessory [8] marketed by the Perkin-Elmer Corporation, which has been developed simultaneously with, but independently of our accessory, is based on this principle.

$$\alpha \approx \Theta \tanh\left(\frac{1}{4 \log e} D_{app}\right) = \Theta \tanh (0.576\, D_{app}) \qquad (2)$$

is correct to within 0.5 %.

For $D_{app} < 1$ this relation between α and D_{app} is nearly linear. The curves recorded are, therefore, very similar to plots of α against wavelength. The exact measuring is most conveniently done by means of a rule graduated so as to permit the direct reading of tanh (0.576 D_{app}).

The necessary attachments to the spectrophotometer consist of two identical units made from brass rod of a square cross section fitting the cell holders of the spectrophotometer equipped with a cylindrical bore accomodating polarizer, polarimeter tube and analyzer. For reasons discussed below the standard light sources of the spectrophotometer have been replaced by a special projector tungsten filament lamp and a 2 kW hydrogen arc for use in the visible and the ultraviolet regions, respectively.

The angles, Θ, are set by means of a special setting apparatus in which adjustments may be made with an accuracy of $\pm$ 0.002°. However, the value of Θ is not measured by means of this instrument but is calculated from measurements of D_{app} for solutions of sucrose of known concentrations by means of (2). The relative accuracy and reproducibility of these determinations of Θ are the same as those obtained in the recording of absorption spectra, *i.e.* $\pm$ 1 % or better.

The details of the construction, operation and performance of the spectropolarimeter together with theoretical considerations regarding the sources of error in the system (ellipticity of the light transmitted by absorbing samples, inequality of the two angles $\pm \Theta$, optical anisotropy of the windows in the sample cells, stray light *etc.*) will be dealt with in a later paper[10], but a few remarks concerning the sensitivity and the accuracy so far obtained should be made.

The main difficulty in measuring optical rotatory dispersion within wavelength ranges of absorption, namely that of getting sufficient light through the sample to be able to measure the rotation, may be expressed quantitatively as

$$A = \frac{D}{|\alpha|} = \frac{100\ \varepsilon}{|[M]|}$$

i.e. the optical density of a sample which will produce a rotation of 1° (ε and $[M]$ being the molar extinction coefficient and rotation, respectively). Being a function of the wavelength this quantity equals zero outside the ranges of absorption but in the vicinity of the peaks (and troughs) of the rotatory dispersion curves which are usually situated within the optically active absorption bands it may often assume values of 7 or 8. Since these parts of the curves are of a particular theoretical interest, a method of measuring rather small rotations with a relatively high accuracy is needed.

Consideration of (2) shows that the sensitivity of the present instrument

$$\frac{D_{app}}{\alpha} \approx \frac{1}{0.576\,\Theta}$$

(tanh $x \approx x$ for small values of x) may, in principle, be increased to any desired value by decreasing Θ. This is, of course, subject to several limitations, the most important of which is the fact that the optical density of the polarimeter units (polarizer-analyzer) increases very rapidly with decreasing Θ at small values of Θ. We are thus facing the problem of finding the optimum value of Θ.

This problem may be attacked as follows [6]. For a given spectrophotometer there will exist a maximum permissible optical density, D_{max}, which cannot be exceeded by the systems inserted into the light paths without seriously affecting the signal-to-noise ratio or the resolution. D_{max} will be a function of the wavelength *and* of the resolution demanded. This maximum condition may be written

$$D_{max} \geq D_1 = -2 \log \sin(\Theta - \alpha) + D_u$$

Splitting D_u into two terms, the optical density due to the absorption in the sample, $D_s = A \times \alpha$, and a constant term, D_p, due to the initial polarization, reflection losses *etc.* we obtain

$$D_m \equiv D_{max} - D_p \geq -2 \log \sin(\Theta - \alpha) + A \times \alpha \qquad (3)$$

Approximating (2) by

$$\alpha = \Theta \times 0.576 \times D_{app} \qquad (2')$$

and $\sin(\Theta - \alpha)$ by $(\Theta - \alpha)$ and eliminating Θ from (2') and (3) we get

$$\frac{dD_{app}}{d\alpha} = 0 \text{ for } \alpha = \frac{2}{A \ln 10} = \frac{0.87}{A}$$

Inserting this value of α into (3) we may calculate the corresponding minimum value (*i.e.* the desired optimum value) of Θ.

If a rough estimate of A has been obtained from preliminary measurements we may adjust the setting of the prisms according to this result and we may use a combination of sample concentration and tube length such that $\alpha = 0.87/A$. Doing this we ensure the highest pen deflection (D_{app}) which may be produced by any sample of the substance in question subject to our requirements of resolution.

The exchange of the ordinary light sources of the spectrophotometer for more powerful ones serves the purpose of increasing D_{max}. In our present setup this has enabled us to work with values of Θ as small as 1.5°, *i.e.* a rotation of 0.001° corresponds to an apparent optical density of *ca.* 0.001, and we believe that it will prove difficult to increase this sensitivity very much. (The Perkin-Elmer attachment [8] is operated at $\Theta \geq 10°$).

The accuracy of the values of α obtained is comparable, or a little inferior, to that with which absorption spectra are recorded by the spectrophotometer, *i.e.* α is correct to within *ca.* $\pm$ 1 %, provided $A < 5$. Substances with appreciably higher values of A must be measured in very dilute solutions, and the correspondingly lower values of D_{app} cannot be evaluated with this relative accuracy.

This work has been sponsored in part by the *European Research Office, U. S. Department of the Army*, Frankfurt a.M.

1. Moffitt, W. *J. Chem. Phys.* **25** (1956) 467, 1189.
2. Djerassi, C. and Klyne, W. *Proc. Chem. Soc.* **1957** 55.
3. Schellman, J. A. *Compt. rend. Lab. Carlsberg Ser. chim.* **30** (1958) 363.
4. Djerassi, C. *Optical Rotatory Dispersion*, McGraw Hill, New York 1960.
5. Hidaka, J., Yamada, S. and Tsuchida, R. *Bull. Chem. Soc. Japan* **31** (1958) 921.
6. Woldbye, F. *Optical Rotatory Dispersion of Transition Metal Complexes*, European Research Office, U S Army, Frankfurt a. M. 1959.
7. Keston, A. and Lospalluto, J. *Federation Proc.* **12** (1953) 229.
8. Savitzky, A., Slavin, W. and Salinger, R. E. *VIth Meeting on Molecular Spectroscopy*, Pergamon Press, London 1960.
9. Gallop, P. M. *Rev.Sci. Instr.* **28** (1957) 209.
10. Woldbye, F. To be published in *Acta Chem. Scand.*

Received November 3, 1959.

Isolation of a New Neutral Amino Acid from *Lactarius helvus*

J. CASIMIR* and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

During chromatographic investigations on the soluble nitrogen compounds in the mushroom *Lactarius helvus* we found relatively large amounts of an amino acid which could not be identified with any knovn amino acid already found to occur in plant tissues. This new substance corresponded to spot A in Fig. 1. When treated with ninhydrin on paper, it was easily recognized by a strong yellow colour at 80°C. The colour soon turned violet brown. On two-dimensional chromatograms the substance moved just above glutamine when phenol-water-ammonia and butanolacetic acid-water were used as solvents. The R_F-values

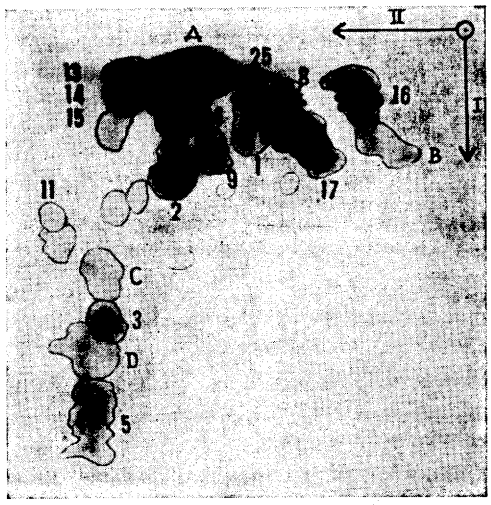


Fig. 1. Two-dimensional paper chromatogram of a 70 % ethanol extract of *Lactarius helvu.*. Spots A, C, and D apparently represent unknown neutral amino acids and spot B an acidic one. 1 gly, 2 ala, 3 val, 5 leu, 8 ser, 9 threo, 11 pro, 13—15 basic, 16 asp, 17 glu, 24 glu-NH_2, 25 asp-NH_2. I butanol-acetic acid-water, II phenol-water-NH_3.

* Present address: Institut Agronomique de l'Etat, Gembloux, Belgique.

If a rough estimate of A has been obtained from preliminary measurements we may adjust the setting of the prisms according to this result and we may use a combination of sample concentration and tube length such that $\alpha = 0.87/A$. Doing this we ensure the highest pen deflection (D_{app}) which may be produced by any sample of the substance in question subject to our requirements of resolution.

The exchange of the ordinary light sources of the spectrophotometer for more powerful ones serves the purpose of increasing D_{max}. In our present setup this has enabled us to work with values of Θ as small as 1.5°, *i.e.* a rotation of 0.001° corresponds to an apparent optical density of *ca.* 0.001, and we believe that it will prove difficult to increase this sensitivity very much. (The Perkin-Elmer attachment [8] is operated at $\Theta \geq 10°$).

The accuracy of the values of α obtained is comparable, or a little inferior, to that with which absorption spectra are recorded by the spectrophotometer, *i.e.* α is correct to within *ca.* $\pm$ 1 %, provided $A < 5$. Substances with appreciably higher values of A must be measured in very dilute solutions, and the correspondingly lower values of D_{app} cannot be evaluated with this relative accuracy.

This work has been sponsored in part by the *European Research Office, U. S. Department of the Army*, Frankfurt a.M.

1. Moffitt, W. *J. Chem. Phys.* **25** (1956) 467, 1189.
2. Djerassi, C. and Klyne, W. *Proc. Chem. Soc.* **1957** 55.
3. Schellman, J. A. *Compt. rend. Lab. Carlsberg Ser. chim.* **30** (1958) 363.
4. Djerassi, C. *Optical Rotatory Dispersion*, McGraw Hill, New York 1960.
5. Hidaka, J., Yamada, S. and Tsuchida, R. *Bull. Chem. Soc. Japan* **31** (1958) 921.
6. Woldbye, F. *Optical Rotatory Dispersion of Transition Metal Complexes*, European Research Office, U S Army, Frankfurt a. M. 1959.
7. Keston, A. and Lospalluto, J. *Federation Proc.* **12** (1953) 229.
8. Savitzky, A., Slavin, W. and Salinger, R. E. *VIth Meeting on Molecular Spectroscopy*, Pergamon Press, London 1960.
9. Gallop, P. M. *Rev.Sci. Instr.* **28** (1957) 209.
10. Woldbye, F. To be published in *Acta Chem. Scand.*

Received November 3, 1959.

Isolation of a New Neutral Amino Acid from *Lactarius helvus*

J. CASIMIR* and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

During chromatographic investigations on the soluble nitrogen compounds in the mushroom *Lactarius helvus* we found relatively large amounts of an amino acid which could not be identified with any knovn amino acid already found to occur in plant tissues. This new substance corresponded to spot A in Fig. 1. When treated with ninhydrin on paper, it was easily recognized by a strong yellow colour at 80°C. The colour soon turned violet brown. On two-dimensional chromatograms the substance moved just above glutamine when phenol-water-ammonia and butanolacetic acid-water were used as solvents. The R_F-values

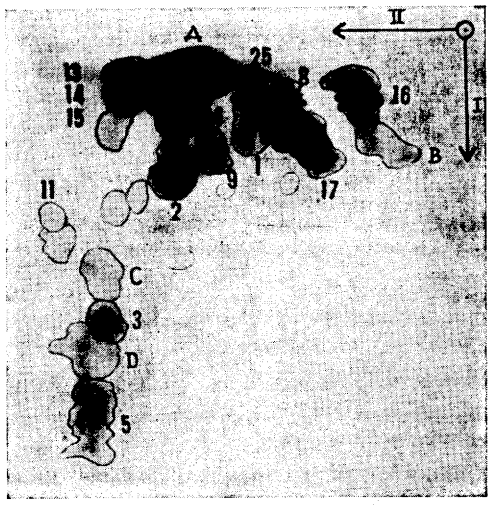


Fig. 1. Two-dimensional paper chromatogram of a 70 % ethanol extract of *Lactarius helvu.*. Spots A, C, and D apparently represent unknown neutral amino acids and spot B an acidic one. 1 gly, 2 ala, 3 val, 5 leu, 8 ser, 9 threo, 11 pro, 13—15 basic, 16 asp, 17 glu, 24 glu-NH_2, 25 asp-NH_2. I butanol-acetic acid-water, II phenol-water-NH_3.

* Present address: Institut Agronomique de l'Etat, Gembloux, Belgique.

(Whatman 4) in the two solvents are 0.54 and 0.06, respectively. Additional unknown spots coloured with ninhydrin are also present on the chromatogram. A substance which gives a yellow spot B near aspartic acid, moves with the acidic fraction on paper electrophoresis at pH 5.6. Brown spots C and D were present in smaller amounts than A, moving with phenol-water-ammonia as valine and with butanol-acetic acid-water just above and below valine, respectively. A, C, and D appeared to be neutral.

Isolation of the amino acid A. About 10 kg (fresh weight) of *Lactarius helvus* were extracted with 40 l of ethanol. After partial decolorization with active charcoal (Darco G 60), which absorbs tyrosine and phenylalanine but not substances A, B, C, or D, the extract was concentrated and passed through an Amberlite IR-120 column. Amino acids were eluted from the column with 1 N ammonium hydroxide. The eluate was evaporated to dryness *in vacuo* giving a yellow powder (26 g). From a 10 g portion of this powder dissolved in 70 ml of 0.5 N acetic acid the acidic amino acids were separated from the neutral and basic ones in a Dowex 1 column (4.5 × 75 cm). 300 fractions, 25 ml each, were first taken using 0.5 N acetic acid and then 100 similar fractions using 1 N HCl as solvent. Fractions 19—28 contained the neutral and basic amino acids, 82—101 glutamic acid, 220—239 aspartic acid, and 358—369 the acidic substance partly decomposed during fractionation and giving two additional yellow spots with phenol-ammonia on a paper chromatogram.

The neutral eluate was evaporated to dryness and the residue dissolved in 65 ml of 1.5 N HCl. The solution was applied to a column of Dowex 50 (4 × 100 cm) which had been treated with 1.5 N HCl. The amino acids were eluted firstly with 1.5 N HCl (150 fractions each of 30 ml), then with 2.5 N HCl (150 fractions of 30 ml), and finally with 4 N HCl (100 fractions of 30 ml). Fractions 175—195 contained spots C and D, and fractions 334—384 only spot A. In another separation it appeared possible to elute the new substance from Dowex 50 with 2.5 N HCl, substance A coming between leucine and lysine.

Fractions 334—384 were combined and evaporated to dryness. Attempts to obtain crystals from methanol or ethanol were unsuccessful. Yellow impure crystals were obtained from *iso*propanol, from a small part of the residue. The unknown A was crystallized as hydrochloride from 6 N HCl and 1.75 g of pure, colourless crystals were obtained. This material corresponded to the unknown A and was found free from contaminants on a two-dimensional chromatogram with the usual solvents. The crystals were soluble in water, methanol, and ethanol. The free amino acid could be prepared by removing the HCl with a small bed of Dowex 1. The colourless crystals were moderately soluble in water but almost insoluble in methanol and ethanol.

Melting point determination on A. The new substance did not melt and on heating it began to turn yellow at about 200°C with a little sublimation and continued to darken until 300°C, when the heating was stopped.

On *total hydrolysis* with 6 N HCl for 24 h at 108°C the amino acid was stable, as it was on *alkaline hydrolysis* with saturated $Ba(OH)_2$ for 1 h at 100°C.

On *catalytic reduction with* PtO_2 *and* H_2 about two moles hydrogen were taken up per 1 mole. Spot A disappeared and a new spot A_1 was formed just above alanine on the two-dimensional chromatogram. The spot was immediately coloured violet by ninhydrin.

The free amino acid showed no absorption maxima in the UV region of the spectrum.

A micro test for the aldehyde or keto group (with 2,4-dinitrophenylhydrazine) was negative. Isatin spraying (proline and its homologue) gave no colour.

Thanks are due to Dr. Heinemann for providing us with the samples of mushrooms.

Received November 14, 1959.

On the Structure of the New Neutral Amino Acid A Isolated from *Lactarius helvus*

ATSUSHI KOMAMINE and
ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Some characteristics of the amino acid A isolated from *Lactarius helvus* in this laboratory were presented in a previous paper by Casimir and Virtanen [1]. We have continued the investigations on the chemical composition and the structure of this amino acid. Some results of these studies are presented in this paper.

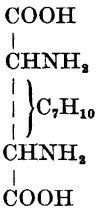
The hydrochloride of the new amino acid (Dr. Casimir's preparation) was dissolved in 0.5 M acetic acid and then passed through a column containing Dowex 1 in the acetate form. The resulting solution was evaporated to dryness *in vacuo* and the residue was dissolved in water. White crystals (needles) separated in an ice box. They were recrystallized from water three times. In accordance with the behaviour of the amino acid on a Dowex 1 column the result of paper electrophoresis showed that it is neutral.

Elementary analysis: Found: C 54.28; H 7.52; N 11.53; O 26.70 (determined). Calc. for $C_{11}H_{18}N_2O_4$: C 54.55; H 7.50; N 11.57; O 26.45. This preparation was used in the present work. An estimation of the molecular weight by isothermal distillation according to Childs [2] gave the value 251 (average of three determinations). The formula of the amino acid is accordingly $C_{11}H_{18}N_2O_4$.

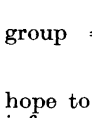
Estimation of α-amino and carboxyl groups. The formation of CO_2 and NH_3 by ninhydrin was determined according to Linko [3]. From 2.562 mg of substance 0.895 mg or 34.9 % of CO_2 was formed, which is 96.9 % of the theoretical value assuming two carboxyl groups in one amino acid molecule. 0.4199 mg of NH_3 or 96.4 % of the total N was found. These results suggest that the amino acid contains two α-amino groups and two carboxyl groups.

The estimation of amino nitrogen by van Slyke's method gave 99.5 % of the total N to be amino N. This result confirms the presence of two α-amino groups in the new amino acid.

By titration of the carboxyl groups in 90 % ethanol, 2.331 mg of substance consumed 1.387 ml of 0.01195 N KOH. This corresponds to 86.2 % of the carboxyl groups, assuming two carboxyl groups in one molecule. Under the same conditions 83.8 % of the carboxyl groups in α,ε-diaminopimelic acid were titratable. Accordingly the titration confirms the presence of two carboxyl groups in the new diamino acid. All the findings presented above suggest that the new amino acid is an aliphatic diamino-dicarboxylic acid



The positions of seven C atoms and ten H atoms in the molecule have thus still to be elucidated. Our knowledge about this part of the molecule is at the moment as follows:

As described in the paper of Casimir and Virtanen, the amino acid takes up 2 moles of H_2 per mole of amino acid on catalytic hydrogenation. The bromine uptake was estimated according to Rossmann [4] and it was found that 1.1075 mg of the amino acid absorbed 0.2055 mg, or 1.9 mole of bromine per molecule of amino acid. Both the uptake of hydrogen and bromine suggest that the amino acid contains two double bonds. The rate of decolorization of $KMnO_4$ in alkaline solution by the amino acid confirms the presence of double bonds in the latter molecule. The amino acid is also attacked by ozone. Acetone, oxalic acid, and acetic acid could be found among the ozonization products. On the basis of the formation of acetone the unknown part of the molecule, C_7H_{10}, contains the group  . In the next paper we hope to be able to present more detailed information about this part of the molecule.

1. Casimir, J. and Virtanen, A. I. *Acta Chem. Scand.* **13** (1959) 2139.
2. Childs, C. E. *Anal. Chem.* **26** (1954) 1963.
3. Linko, P. *Suomen Kemistilehti B* **28** (1955) 96.
4. Rossmann, E. *Ber.* **65** (1932) 1847.

Received November 14, 1959.

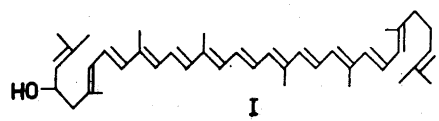
Bacterial Carotenoids

IV *. A Comparative Study of Lycoxanthin and Rhodopin

SYNNÖVE LIAAEN JENSEN

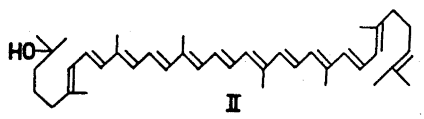
Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

It has been claimed by Goodwin and Land [1] that lycoxanthin (I), first isolated by Zechmeister and Cholnoky [2] from *Solanum dulcamara*, and rhodopin, first isolated by Karrer and Solmssen [3] from purple bacteria, were identical.



I

A critical examination of the literature [1-6] reveals, however, certain data contradictory to this statement. This has recently been pointed out by the present author [6], who proposed the structure (II) for rhodopin in accordance with the properties reported for this carotenoid [1,3-6].



II

It was therefore found desirable to repeat a direct, comparative study of the two carotenoids.

In the present investigation lycoxanthin has been isolated from *Solanum dulcamara*, and rhodopin from *Rhodospirillum rubrum* in the exponential growth stage and also from dried cells of two strains of *Rhodopseudomonas palustris*, as previously described [6].

Lycoxanthin was isolated from the ripe berries of *Solanum dulcamara* by acetone extraction followed by chromatography of the unsaponifiable matter on deactivated alumina. Lycoxanthin crystallized in dark, red needles mp. 167.5—168.5 °C with λ_{max} in petroleum ether at (422), 445, 472.5, 503.5 mμ. The spectrum had a pronounced fine-structure and was indistinguishable from that of rhodopin as well as that of lycopene.

When lycoxanthin was co-chromatographed with a chromatographically homogeneous sample of all-*trans* rhodopin on a column of deactivated alumina, a clear separation into two zones was obtained. This is not in agreement with the statement of Goodwin and Land [1] that rhodopin and lycoxanthin could not be separated on an alumina column. Rhodopin was eluted with petroleum ether containing 14 % of acetone, while lycoxanthin required petroleum ether containing 25 % of acetone for elution.

Paper chromatographical studies according to the method described by Jensen *et al.*[7] gave R_F-values as presented in Table 1.

Table 1. R_F-values on Schleicher and Schüll No. 287 paper.

Carotenoid	R_F-value 5 % acetone *	10 % acetone *
Rhodopin	0.39	0.75
Lycoxanthin	0.35	0.69

* In petroleum ether bp. 60—70°C.

Again rhodopin and lycoxanthin were clearly separated into two zones. As expected, rhodopin with a shielded, tertiary hydroxyl group moved somewhat quicker than lycoxanthin with a secondary hydroxyl group, on alumina as well as on paper.

Further the IR-spectrum (KBr) of lycoxanthin was different from that of rhodopin in the 1 200—1 000 cm^{-1} region. Lycoxanthin showed a fairly strong band at 1 008 cm^{-1}. This band was absent from rhodopin, which exhibits a band of somewhat weaker intensity at 1 140 cm^{-1} characteristic of tertiary hydroxyl groups [6]. It has previously been pointed out [6] that bicyclic carotenoids with secondary hydroxyl groups all exhibit an absorption band around 1 030 cm^{-1}. The shift to 1 008 cm^{-1} for lycoxanthin is possibly due to the aliphatic nature of this carotenoid.

When treated with acetic anhydride in pyridine, lycoxanthin was quantitatively acetylated in 6 h at room temperature. Rhodopin gave no acetate at all under similar conditions. The result of the acetylation again demonstrates the different character of the hydroxyl groups of lycoxanthin and rhodopin.

* Number I, II, III of this series in *Acta Chem. Scand.* **12** (1958) 1698, **13** (1959) 381, **13** (1959) 842.

An additional support of the non-identity of the two carotenoids was obtained by a paper chromatographical study [7] of the stereoisomers obtained by iodine catalysis of the all-*trans* compounds. The composition of the iodine catalyzed equilibrium mixtures was different for the two compounds both qualitatively and quantitatively.

As a consequence of the above study it may be considered as proved that lycoxanthin and rhodopin are not identical. This further strenghtens the suggested structure (II) for rhodopin.

This work will be published in more detail elsewhere.

The author wishes to express her gratitude to Prof. N. A. Sörensen for collecting the berries of *Solanum dulcamara* and for his inspiring interest in this work. Dried cells of two strains of *Rhodopseudomonas palustris* most kindly were supplied by Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, California, USA. A maintenance grant from *Norges Tekniske Högskole* is gratefully acknowledged.

1. Goodwin, T. W. and Land, D. G. *Arch. Mikrobiol.* **24** (1956) 305.
2. Zechmeister, L. and Cholnoky, L. v. *Ber.* **69** (1936) 422.
3. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **18** (1935) 1306.
4. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 1019.
5. Karrer, P., Solmssen, U. and Koenig, H. *Helv. Chim. Acta* **21** (1938) 454.
6. Liaaen Jensen, S. *Acta Chem. Scand* **13** (1959) 842.
7. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 1863.

Received November 18, 1959.

Bacterial Carotenoids

V. A Note on the Constitution of Rhodovibrin (OH-P481)

SYNNÖVE LIAAEN JENSEN

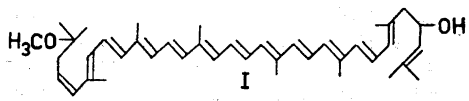
Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

During their investigations on the carotenoids of the purple bacteria, Karrer *et al.*[1-3] described a polyene alcohol with absorption maxima in carbon disulphide at 517 and 556 mμ. In the chromatographic purification procedure employed by these workers, this carotenoid could be separated only with great difficulty from the upper part of the rhodopin zone. A crystalline sample, by Karrer *et al.* considered not to be completely pure, melted at 168°C. Combustion analysis gave 83.97 % C and 10.09 % H, which suggested that two oxygen atoms were present in the carotenoid molecule. According to partition tests, not more than one hydroxyl group could be present. Methoxyl determination was negative. This carotenoid which was rather well characterized in the early work of Karrer *et al.*, was named rhodovibrin.

From a number of purple bacteria, Goodwin *et al.*[4,5] later isolated a similar mono-hydroxy-carotenoid, designated OH-P481. Goodwin and Land [5] suggested that OH-P481 might be identical with rhodovibrin.

OH-P481 was shown by Stanier and collaborators [6] to be an intermediate between lycopene and spirilloxanthin in the biosynthesis of carotenoids in *Rhodospirillum rubrum*. It was pointed out that the absorption spectrum of OH-P481 in visible light corresponded to a chromophore of twelve conjugated carbon-carbon double bonds in an aliphatic system. The presence of one methoxyl group in OH-P481 has been established later [7].

In a speculative transformation scheme for the biochemical conversion of lycopene to spirilloxanthin, Weedon and collaborators [8] on basis of the properties previously reported for OH-P481 [5-7], suggested the structure (I) for this carotenoid.



I

This structure, which contains a secondary hydroxyl group and one *iso*propropylidene end-group is, however, not in agreement with the chemical evidence presented here.

In the present investigation OH-P481 has been isolated from cells of *Rhodospirillum rubrum* in the exponential growth stage and from dried cells of two strains of *Rhodopseudomonas palustris*. The carotenoid was extracted with acetone and isolated from the unsaponifiable matter by repeated chromatography on deactivated alumina. It crystallized as dark,

An additional support of the non-identity of the two carotenoids was obtained by a paper chromatographical study [7] of the stereoisomers obtained by iodine catalysis of the all-*trans* compounds. The composition of the iodine catalyzed equilibrium mixtures was different for the two compounds both qualitatively and quantitatively.

As a consequence of the above study it may be considered as proved that lycoxanthin and rhodopin are not identical. This further strenghtens the suggested structure (II) for rhodopin.

This work will be published in more detail elsewhere.

The author wishes to express her gratitude to Prof. N. A. Sörensen for collecting the berries of *Solanum dulcamara* and for his inspiring interest in this work. Dried cells of two strains of *Rhodopseudomonas palustris* most kindly were supplied by Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, California, USA. A maintenance grant from *Norges Tekniske Högskole* is gratefully acknowledged.

1. Goodwin, T. W. and Land, D. G. *Arch. Mikrobiol.* **24** (1956) 305.
2. Zechmeister, L. and Cholnoky, L. v. *Ber.* **69** (1936) 422.
3. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **18** (1935) 1306.
4. Karrer, P. and Solmssen, U. *Helv. Chim. Acta* **19** (1936) 1019.
5. Karrer, P., Solmssen, U. and Koenig, H. *Helv. Chim. Acta* **21** (1938) 454.
6. Liaaen Jensen, S. *Acta Chem. Scand* **13** (1959) 842.
7. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 1863.

Received November 18, 1959.

Bacterial Carotenoids

V. A Note on the Constitution of Rhodovibrin (OH-P481)

SYNNÖVE LIAAEN JENSEN

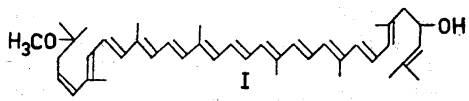
Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

During their investigations on the carotenoids of the purple bacteria, Karrer *et al.*[1-3] described a polyene alcohol with absorption maxima in carbon disulphide at 517 and 556 mμ. In the chromatographic purification procedure employed by these workers, this carotenoid could be separated only with great difficulty from the upper part of the rhodopin zone. A crystalline sample, by Karrer *et al.* considered not to be completely pure, melted at 168°C. Combustion analysis gave 83.97 % C and 10.09 % H, which suggested that two oxygen atoms were present in the carotenoid molecule. According to partition tests, not more than one hydroxyl group could be present. Methoxyl determination was negative. This carotenoid which was rather well characterized in the early work of Karrer *et al.*, was named rhodovibrin.

From a number of purple bacteria, Goodwin *et al.*[4,5] later isolated a similar mono-hydroxy-carotenoid, designated OH-P481. Goodwin and Land [5] suggested that OH-P481 might be identical with rhodovibrin.

OH-P481 was shown by Stanier and collaborators [6] to be an intermediate between lycopene and spirilloxanthin in the biosynthesis of carotenoids in *Rhodospirillum rubrum*. It was pointed out that the absorption spectrum of OH-P481 in visible light corresponded to a chromophore of twelve conjugated carbon-carbon double bonds in an aliphatic system. The presence of one methoxyl group in OH-P481 has been established later [7].

In a speculative transformation scheme for the biochemical conversion of lycopene to spirilloxanthin, Weedon and collaborators [8] on basis of the properties previously reported for OH-P481 [5-7], suggested the structure (I) for this carotenoid.



I

This structure, which contains a secondary hydroxyl group and one *iso*propropylidene end-group is, however, not in agreement with the chemical evidence presented here.

In the present investigation OH-P481 has been isolated from cells of *Rhodospirillum rubrum* in the exponential growth stage and from dried cells of two strains of *Rhodopseudomonas palustris*. The carotenoid was extracted with acetone and isolated from the unsaponifiable matter by repeated chromatography on deactivated alumina. It crystallized as dark,

red needles from petroleum ether-acetone solution. The chromatographic fractions of OH-P481 were accompanied by oily substances in the *Rhodospirillum rubrum* extracts and a white substance mp. 282°C (subl.) in the *Rhodopseudomonas palustris* extracts, which reduced the yield of the pure, crystalline pigment. After several recrystallizations the carotenoid melted at 190.5°C.

Crystalline OH-P481 was readily soluble in acetone and carbon disulphide, fairly soluble in chloroform, benzene and methanol, less soluble in ether and nearly insoluble in cold petroleum ether.

Absorption maxima for OH-P481 in various solvents as recorded quickly after dissolution is given in Table 1. The spectra were determined in a Zeiss PMQ2 spectrophotometer. Reading of correct maxima was checked by a didymium standard filter.

Table 1. The absorption maxima of all-*trans* OH-P481 in various solvents.

Solvent	Absorption maxima in mμ	
Petroleum ether bp. 60–70°C	358 374	455 483 516
Acetone	363 378	460 488 522
Chloroform	370 385	469 498 532
Benzene	372 388	473 503 535
Carbon disulphide	(408)	491 522 559

The two maxima in the first column indicate the position of the *cis*-peak, which was very weak in the spectrum of the all-*trans* compound. The purest sample measured had $E^{1\%}_{1\ cm} = 2\,940$ at 483 mμ in petroleum ether. This sample contained 5.06 % ash as determined by combustion. When corrected for 5.06 % ash the value $E^{1\%}_{1\ cm} = 3\,100$ is obtained. By extrapolation between the extinction coefficients for spirilloxanthin $E^{1\%}_{1\ cm} = 2\,620$ [9] and lycopene $E^{1\%}_{1\ cm} = 3\,460$ [10] at λ_{max} in petroleum ether, and correcting for the presence of an additional oxygen atom the value $E^{1\%}_{1\ cm} = 2\,970$ is obtained. It is therefore likely that the value $E^{1\ cm}_{1\%} = 3\,100$ for OH-P481 at λ_{max} in petroleum ether is close to the correct value. Iodine catalysis caused a shift to 358, 374, (455), 479 and 511 mμ in petroleum ether with 25 % decrease in extinction coefficient at the middle maximum. OH-P481 crystallizes normally in the all-*trans* form, but was readily *cis*-isomerized in solution. The stereochemical lability was studied by means of the paper-chromatographical method reported by Jensen *et al.*[11] The main *cis*-isomers designated Neo A and Neo B showed absorption maxima in acetone at 376, 459, 485, 517 mμ and 376, 453, 481, 513 mμ, respectivly. In the iodine catalyzed equilibrium mixture a content of 58 % all-*trans*, 18 % Neo A and 24 % Neo B was determined colourimetrically. Otherwise the stereochemical lability was comparable with that of spirilloxanthin [9].

In the quantitative partition test recommended by Zechmeister and Petracek [12], the following partition ratio was found:

Petroleum ether/95 % methanol	66:34
Petroleum ether/85 % methanol	95:5

The carotenoid thus distributed as a monohydroxy-carotenoid. The presence of a hydroxyl group further was established by an IR absorption band at 3 420 cm^{-1} in KBr. There was, however, no absorption band in the 1 030 cm^{-1} region, which is characteristic for secondary hydroxyl groups, but a band of weaker intensity at 1 148 cm^{-1}, which previously has been discussed and found typical of carotenoids containing tertiary OH-groups [13]. The tertiary character of the hydroxyl group further was confirmed by the behaviour of the pigment upon acetylation. A series of attempts to acetylate OH-P481 with acetic anhydride or acetyl chloride in pyridine, never gave a compound with properties corresponding to an acetate in yields exceeding 5 % of the unchanged starting material. This resistance to acetylation is similar to that reported for rhodopin [3,13,14] with a tertiary hydroxyl group. Parallel experiments with lutein resulted in quantitative formation of lutein diacetate.

Treatment with $CHCl_3$-HCl according to Entschel and Karrer [15] gave no product with extended conjugated chain. Neither the hydroxyl group, nor the methoxyl group of OH-P481 are therefore likely to be allylic.

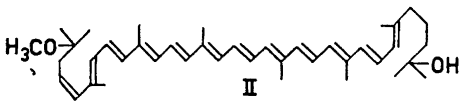
Quantitative determination of *iso*propylidene groups according to the method of Kuhn and Roth [16] gave 0.62 moles of acetone per mole of carotenoid compared with the value 1.70 simultaneously obtained for lycopene which contains two *iso*propylidene end-groups. The value obtained for OH-P481 is lower than expected for one *iso*propylidene group, and is interpreted as being due to the presence of one end-group with a tertiary methoxyl group as in spirilloxanthin [6,17] and another containing a tertiary hydroxyl group as in rhodopin [13].

The possibility for these types of end-groups to yield a small amount of acetone upon ozonolysis has already been pointed out.[17,18]

Catalytic micro-hydrogenation of a specimen with $E_{1\ cm}^{1\%} = 2\,850$ at 483 mμ in petroleum ether gave an uptake of 11.32 and 11.40 moles of hydrogen per mole of carotenoid calculated on the basis of a molecular formula $C_{40}H_{56}(OH)(OCH_3)$. The absorption spectrum in visible light indicates a chromophoric system containing twelve conjugated double bonds, hence the hydrogenation value is obviously somewhat low. Accepting $E_{1\ cm}^{1\%} = 3\,100$ for the pure pigment, the sample used in the hydrogenation has been 92 % pure. Chromatographic purity tests had revealed that the impurities present in the sample were not of carotenoid nature. The hydrogen uptake could then be corrected to a maximum of 12.3 moles per mole of carotenoid, assuming that the impurities do not consume any hydrogen.

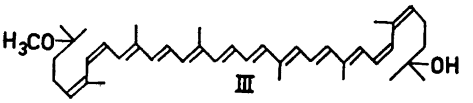
Exact analytical data could not be obtained because of the difficulty of isolating an ash-free substance. The figures given below are corrected for the presence of 5.06 % ash of unknown composition. (Found: C 85.88, H 10.16, O indirect 3.96, direct 3.61. Calc. for $C_{40}H_{56}(OH)(OCH_3)$: C 84.20, H 10.34, O 5.47.) The values obtained correspond to a molecular formula $C_{41}H_{60}O_{1.4}$. The presence of two oxygen atoms in the molecule is, however, clearly demonstrated from (1) the quantitative determination of methoxyl [7], and (2) partition behaviour, chromatographic behaviour, IR-spectrum and acetylation which reveal the presence of a hydroxyl group.

As a consequence of the data presented above, the structure (II) is possible for OH-P481:



II

This formulation is consistent with a chromophoric system of twelve conjugated double bonds in an aliphatic system, one non-allylic methoxyl group at a similar position as in spirilloxanthin, one non-allylic, tertiary hydroxyl group and no *iso*propylidene end-group. The available chemical data, however, do not exclude a symmetrical position of the chromophore as in (III).



III

The structures (II) or (III) for OH-P481 together with the structures previously suggested for rhodopin [13], P481 [8,17] and spirilloxanthin [8,17] make possible a more definite interpretation in chemical terms of the reactions involved in the later steps of carotenoid biosynthesis in the genus *Rhodospirillum* [16], for which the kinetics already has been established [6].

In view of the similar source of isolation, the accompanying carotenoids, the visible absorption spectrum, partition tests and chromatographic behaviour, there is good reason to believe that OH-P481 is identical with rhodovibrin, first described by Karrer and Solmssen [1]. As long as rhodovibrin was not isolated from a definite organism and not in a completely pure state, this cannot be definitely proved. In accordance with the generally accepted nomenclature it is suggested that OH-P481 from now on should be referred to as rhodovibrin. A further reason for changing the name of OH-P481 is the fact that the main absorption maximum of the all-*trans* compound is located at 483 mμ and not at 481 mμ in petroleum ether.

This work will be published in more detail elsewhere.

The author is gratefully indebted to Prof. Richard Kuhn for the catalytic micro-hydrogenations carried out in his laboratory at Max-Planck Institut für Biologie, Heidelberg, to Dr. C. B. van Niel for a generous supply of dried cells of two strains of *Rhodopseudomonas palustris*, to Prof. N. A. Sörensen for his inspiring interest and advice in this work and to *Norges Tekniske Högskole* for a maintenance grant.

1. Karrer, P. and Solmssen, U. *Helv.Chim. Acta.* **19** (1936) 3.
2. Karrer, P. and Solmssen, U. *Helv.Chim. Acta.* **19** (1936) 1019.
3. Karrer, P., Solmssen, U. and Koenig, H. *Helv.Chim. Acta* **21** (1938) 454.
4. Goodwin, T. W. *Arch. Mikrobiol.* **24** (1956) 313.
5. Goodwin, T. W. and Land, D. G. *Arch. Mikrobiol.* **24** (1956) 305.

6. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochem. et Biophys. Acta* **29** (1958) 477.
7. Liaaen Jensen, S. *Acta Chem. Scand.* **12** (1958) 1698.
8. Barber, M. S., Jackman, L. M. and Weedon, B. C. L. *Proc. Chem. Soc.* **1959** 96.
9. Polgár, A., van Niel, C. B. and Zechmeister, L. *Arch. Biochem.* **5** (1944) 243.
10. Zechmeister, L., Le Rosen, A. L., Schroeder, W. A., Polgár, A. and Pauling, L. *J.Am. Chem. Soc.* **65** (1943) 1940.
11. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 1863.
12. Zechmeister, L. and Petracek, F. J. *Anal. Chem.* **28** (1956) 1484.
13. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 842.
14. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 2142.
15. Entschel, R. and Karrer, P. *Helv. Chim. Acta* **41** (1958) 402.
16. Kuhn, R. and Roth, H. *Ber.* **65** (1932) 1285.
17. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 381.
18. Liaaen Jensen, S. *To be published.*

Received November 18, 1959.

Note on the Crystal Structure of Vanadium Dichloride

JØRGEN VILLADSEN

The Haldor Topsøe Research Laboratories, Bøunegaardsvej 73, Hellerup, Denmark

In a paper on transition metal halides [1] Klemm and Grimm were unable to determine the structure of VCl_2 and $TiCl_2$. They stated, however, that VCl_2 was not isomorphous with TiI_2 which was found to have the CdI_2-type structure. Later Baenziger and Rundle have found a preparation of $TiCl_2$ to be isomorphous with CdI_2[2].

We have carried out an X-ray investigation of VCl_2 prepared by K. Riishede of this laboratory by heating vanadium powder in a stream of dry HCl at 950°C. The product was obtained in the form of apple-green, muscovite-like leaflets of a more or less hexagonal appearance. The following data were obtained:

Unit cell dimensions: $a_0 = 3.60 \pm 0.01$ Å, $c_0 = 5.83 \pm 0.01$ Å. Observed density[1]: 3.09 g/cm³, calculated density: 3.09 g/cm³. Diffraction symbol: $\bar{3}mP...$; test for pyroelectricity: negative. Probable space group: $P\bar{3}m$ (No. 164). Arrangement of atoms:

1 V at 0,0,0
2 Cl at $\pm$ (1/3, 2/3, u) with $u \sim 1/4$.

Shortest V-Cl distance: 2.55 ± 0.05 Å. Structure type: CdI_2 ($C6$).

Experimental: Laue photographs were taken along the c-axis. The lattice constants were calculated from Debye-Scherrer diagrams taken in a 19 cm camera using Cr$K\alpha$ radiation ($\lambda = 2.2909$ Å). Intensities were recorded on an X-ray diffractometer with Cu$K\alpha$ radiation. The value of the parameter u was found to deviate only insignificantly from the ideal value 1/4 from a Fourier projection along the c-axis using the $00l$ reflections. The agreement between observed and calculated intensities of the $00l$ and $10l$ reflections is satisfactory but a different scaling factor had to be used because preferred orientation could not be totally depressed.

From the above it may be concluded either that $TiCl_2$ and VCl_2 both exist in two modifications or that the materials investigated by Klemm and Grimm have been partly decomposed before or during their X-ray exposure.

Added in proof: Very recently[3] Ehrlich and Seifert have recorded the structure of VCl_2 in complete agreement with the results given above.

The author is indebted to the head of the Chemistry Department of the Royal Veterinary and Agricultural College in Copenhagen, professor A. Tovborg Jensen, for permission to use the X-ray diffractometer there and to Mr. Haldor Topsøe for permission to publish this note.

1. Klemm, W. and Grimm, L. *Z. anorg. allgem. Chem.* **249** (1942) 198.
2. Baenziger, N. C. and Rundle, R. E. *Acta Cryst.* **1** (1948) 274.
3. Ehrlich, P. and Seifert, H.-J. *Z. anorg. allgem. Chem.* **301** (1959) 282.

Received November 27, 1959.

6. Liaaen Jensen, S., Cohen-Bazire, G., Nakayama, T. O. M. and Stanier, R. Y. *Biochem. et Biophys. Acta* **29** (1958) 477.
7. Liaaen Jensen, S. *Acta Chem. Scand.* **12** (1958) 1698.
8. Barber, M. S., Jackman, L. M. and Weedon, B. C. L. *Proc. Chem. Soc.* **1959** 96.
9. Polgár, A., van Niel, C. B. and Zechmeister, L. *Arch. Biochem.* **5** (1944) 243.
10. Zechmeister, L., Le Rosen, A. L., Schroeder, W. A., Polgár, A. and Pauling, L. *J.Am. Chem. Soc.* **65** (1943) 1940.
11. Jensen, A. and Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 1863.
12. Zechmeister, L. and Petracek, F. J. *Anal. Chem.* **28** (1956) 1484.
13. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 842.
14. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 2142.
15. Entschel, R. and Karrer, P. *Helv. Chim. Acta* **41** (1958) 402.
16. Kuhn, R. and Roth, H. *Ber.* **65** (1932) 1285.
17. Liaaen Jensen, S. *Acta Chem. Scand.* **13** (1959) 381.
18. Liaaen Jensen, S. *To be published.*

Received November 18, 1959.

Note on the Crystal Structure of Vanadium Dichloride

JØRGEN VILLADSEN

The Haldor Topsøe Research Laboratories, Bøunegaardsvej 73, Hellerup, Denmark

In a paper on transition metal halides [1] Klemm and Grimm were unable to determine the structure of VCl_2 and $TiCl_2$. They stated, however, that VCl_2 was not isomorphous with TiI_2 which was found to have the CdI_2-type structure. Later Baenziger and Rundle have found a preparation of $TiCl_2$ to be isomorphous with CdI_2[2].

We have carried out an X-ray investigation of VCl_2 prepared by K. Riishede of this laboratory by heating vanadium powder in a stream of dry HCl at 950°C. The product was obtained in the form of apple-green, muscovite-like leaflets of a more or less hexagonal appearance. The following data were obtained:

Unit cell dimensions: $a_0 = 3.60 \pm 0.01$ Å, $c_0 = 5.83 \pm 0.01$ Å. Observed density[1]: 3.09 g/cm³, calculated density: 3.09 g/cm³. Diffraction symbol: $\bar{3}mP...$; test for pyroelectricity: negative. Probable space group: $P\bar{3}m$ (No. 164). Arrangement of atoms:

1 V at 0,0,0
2 Cl at $\pm$ (1/3, 2/3, u) with $u \sim 1/4$.

Shortest V-Cl distance: 2.55 ± 0.05 Å. Structure type: CdI_2 ($C6$).

Experimental: Laue photographs were taken along the c-axis. The lattice constants were calculated from Debye-Scherrer diagrams taken in a 19 cm camera using Cr$K\alpha$ radiation ($\lambda = 2.2909$ Å). Intensities were recorded on an X-ray diffractometer with Cu$K\alpha$ radiation. The value of the parameter u was found to deviate only insignificantly from the ideal value 1/4 from a Fourier projection along the c-axis using the $00l$ reflections. The agreement between observed and calculated intensities of the $00l$ and $10l$ reflections is satisfactory but a different scaling factor had to be used because preferred orientation could not be totally depressed.

From the above it may be concluded either that $TiCl_2$ and VCl_2 both exist in two modifications or that the materials investigated by Klemm and Grimm have been partly decomposed before or during their X-ray exposure.

Added in proof: Very recently[3] Ehrlich and Seifert have recorded the structure of VCl_2 in complete agreement with the results given above.

The author is indebted to the head of the Chemistry Department of the Royal Veterinary and Agricultural College in Copenhagen, professor A. Tovborg Jensen, for permission to use the X-ray diffractometer there and to Mr. Haldor Topsøe for permission to publish this note.

1. Klemm, W. and Grimm, L. *Z. anorg. allgem. Chem.* **249** (1942) 198.
2. Baenziger, N. C. and Rundle, R. E. *Acta Cryst.* **1** (1948) 274.
3. Ehrlich, P. and Seifert, H.-J. *Z. anorg. allgem. Chem.* **301** (1959) 282.

Received November 27, 1959.

Separation and Identification of Alkoxyglycerols

BO HALLGREN and SAM OLOF LARSSON

Department of Medical Biochemistry, University of Gothenburg, Sweden

In the liver oils of several Elasmobranch fishes a relatively large proportion of the unsaponifiable matter consists of alkoxyglycerols [1]. Hitherto three such compounds have been isolated, *viz.* chimyl, batyl and selachyl alcohols. Through the use of the technique described below we were able to discover several new alkoxyglycerols.

We separated the unsaponifiable matter of marine oils or of other biological materials by adsorption on a column of activated aluminium oxide. After elution of the hydrocarbons by light petroleum and the cholesterol by methylene dichloride, the alkoxyglycerols were eluted by 10 % methanol in methylene dichloride. The quantity of alkoxyglycerols was then determined gravimetrically. In order to be able to study the composition of the mixtures of the alkoxyglycerols by gas chromatography we converted the free hydroxy groups into methyl ether groups by the method described by Müller and Rundel [2], treating the hydroxy compound with diazomethane and boron trifluoride. Next the dimethyl ethers of the alkoxyglycerols were separated from monomethyl ethers and unchanged alkoxyglycerols by chromatography on aluminium oxide. The dimethyl ethers were eluted by 25 % diethyl ether in light petroleum and afterwards submitted to gas chromatography.

For the gas chromatographic analyses we used a Perkin Elmer apparatus (model 116) with an outlet system modified to permit collecting of fractions of high-boiling substances. Silicone grease and a polyester Reoplex 400 (Geigy) [3] were used as stationary phases and helium as the carrier gas. The samples were analyzed before and after hydrogenation. The components were identified by mass spectrometry and in the case of batyl alcohol also by comparing the retention time with that of synthetic batyl alcohol. The mass spectra of the dimethyl ethers of the alkoxyglycerols will be published later.

Besides glyceryl ethers with hexadecanyl, octadecanyl or octadecenyl as the long-chain component, we found compounds with tetradecanyl, with monounsaturated C_{16}, C_{20} and C_{22} chains, and with a C_{18} chain with two double bonds. There were also small amounts of glyceryl ethers with a long chain containing an odd number of carbon atoms. In Table 1 the composition of the alkoxyglycerols from the liver oils of three marine species of Elasmobranch fish are presented. The chimyl, batyl and selachyl alcohols to-

Table 1. The percentage composition (wt) of the alkoxyglycerols from liver oils. The alkoxyglycerols are represented by the long-chain part of the molecule.

Alkoxyglycerol	Grey dogfish *Squalus acanthias*	Greenland shark *Somniosus microcephalus*	Ratfish *Chimaera monstrosa*
C_{12}	trace	trace	trace
C_{14}, *n*-tetradecanyl	5.7	2.0	1.7
C_{15}	1.9	0.7	1.1
C_{16}, *n*-hexadecanyl (chimyl)	13.2	9.1	10.4
C_{16}, *n*-hexadecenyl	10.6	10.8	9.1
C_{17}	3.0	3.6	4.7
C_{18}, *n*-octadecanyl (batyl)	3.4	2.8	6.7
C_{18}, *n*-octadecenyl (selachyl)	47.8	59.4	53.6
C_{18}, octadecadienyl	2.4	1.6	2.5
C_{18}, octadecatrienyl	trace	?	?
C_{19}, *n*-nonadecenyl	1.2	1.5	2.4
C_{20}, *n*-eicosenyl	8.0	6.2	6.4
C_{21}	trace	?	?
C_{22}, *n*-docosenyl	2.7	2.2	1.0

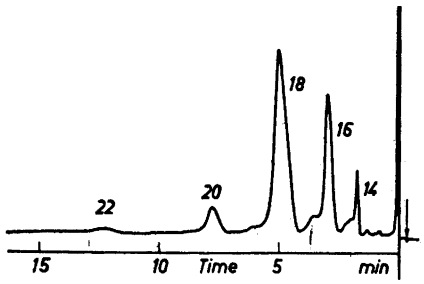


Fig. 1. Gas chromatogram of the dimethyl ethers of the alkoxyglycerols in the liver oil of *Squalus acanthias*. Stationary phase: Silicone grease. Temperature: 265°C. Column length: 2 m. The figures beside the peaks denote the number of carbon atoms in the long-chain part of the molecule.

gether constitute only 64 % of the alkoxyglycerols in the liver of the grey dogfish studied and 71 % in the Greenland shark and ratfish. The rest is composed of alkoxyglycerols not previously identified.

A typical gas chromatogram on silicone of the dimethyl ethers of the alkoxyglycerols in the liver oil from *Squalus acanthias* is shown in Fig. 1. and the corresponding

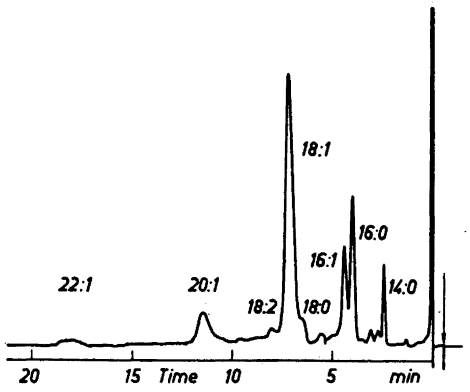


Fig. 2. Gas chromatogram of the dimethyl ethers of the alkoxyglycerols in the liver oil of *Squalus acanthias*. Stationary phase: Reoplex 400. Temperature: 247°C. Column length: 3 m. The figures beside the peaks refer to the long-chain part of the molecule. The figures to the left of the colons denote the number of carbon atoms and the figures after the colon the number of double bonds.

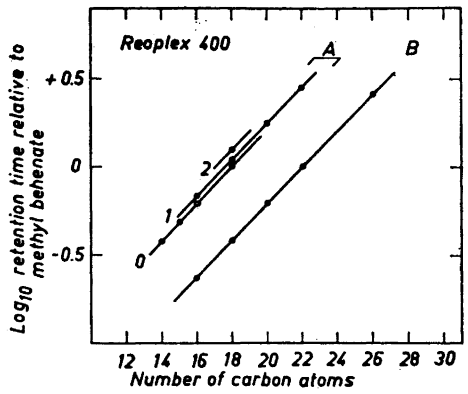


Fig. 3. Relationships between $\log_{10}$ retention times relative to methyl behenate in Reoplex 400 at 247°C and the number of carbon atoms for the alkoxyglycerols (A) and for the saturated straight chain fatty acids (B). For the alkoxyglycerols the number of carbon atoms refer to the long chain part of the molecule. The number of double bonds in the long chains are denoted by 0, 1 or 2.

chromatogram on Reoplex 400 in Fig. 2. The retention time for the component with the highest molecular weight (1,2-dimethoxy-3-docosenoxy-propan) was about 12 min on the silicone and 18 min on the Reoplex column. The logarithms of the retention times on Reoplex 400 relative to methyl behenate were calculated and plotted against the number of carbon atoms (Fig. 3). The saturated ethers fell on a straight line and the mono-unsaturated ones on a line parallell to it.

Oxidation of 1,2-dimethoxy-3-hexadecenoxy-propan by chromium trioxide gave heptanoic acid. This could be established by gas chromatography. Thus the double bond in the hexadecenyl chain is situated between carbon atoms 9 and 10.

This study is a part of investigations supported by grants from the *Swedish Medical Research Council.*

1. Hilditch, T. P. *The Chemical Constitution of Natural Fats.* Chapman & Hall Ltd, London 1956.
2. Müller, E. and Rundel, W. *Angew. Chem.* **70** (1958) 105.
3. Orr, C. H. and Callen, J. E. *J. Am. Chem. Soc.* **80** (1958) 249.

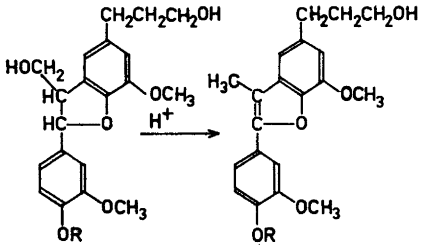
Received November 24, 1959.

Phenylcoumaran Elements in Spruce Lignin

ERICH ADLER, SUNE DELIN and KNUT LUNDQUIST

Institutionen för organisk kemi, Chalmers Tekniska Högskola, Göteborg, Sweden

Dihydro-dehydrodiconiferyl alcohol (I) [1] and its monomethyl ether (II) [1], on heating with 0.2 N HCl in methanol or in dioxane-water (9:1), are converted into the phenylcoumarone derivatives III and IV, respectively [2]. This conversion of a hydroxymethyl-substituted phenylcoumaran into a methyl-substituted phenylcoumarone can readily be accounted for by a sequence of ring opening, allylic rearrangement and recyclisation [2].

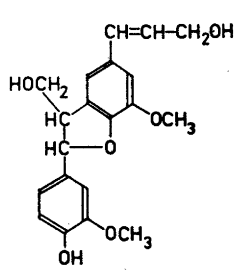


I : R = H
II : R = CH_3
III : R = H
IV : R = CH_3

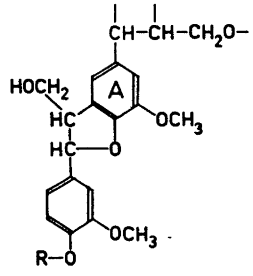
The absorption spectrum of the phenolic coumarone derivative (III), m.p. 172—173° (diacetate, m.p. 114°), shows a maximum at 310 mμ (ε, 27 000) in ethanolic solution and a maximum at 326 mμ (ε, 31 400) in alkaline ethanolic solution. These spectra and the "ionisation $\Delta\varepsilon$" curve (λ_{max} 338 mμ, ε, 27 000) obtained from them are very similar to those previously reported [3] for a related coumarone[4] obtained by dehydrogenation of dihydro-dehydrodi*iso*-eugenol with sulphur. The absorption spectrum of the non-phenolic coumarone IV in ethanol is similar to that of III in the same solvent. Spectrophotometric examination of the reactions I → III and II → IV revealed that a maximum conversion of about 70 % was obtained after 20 h in a boiling 0.2 N HCl dioxane-water mixture.

Dehydrodiconiferyl alcohol (V) is formed, together with other products, by enzymatic dehydrogenation of coniferyl alcohol [1], and the phenylcoumaran system (VIa and VIb, with various modifications of the side-chain of the phenylpropane moiety A) is assumed [5] to be present in lignin.

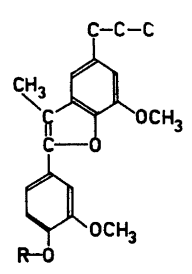
In the present investigation an attempt has been made to demonstrate, by spectrophotometric analysis, the formation of phenylcoumarone systems (type VII) from phenylcoumaran elements (type VI) on treatment of spruce Björkman lignin with hot acid and to estimate the frequency of phenylcoumaran elements in the lignin. A solution of Björkman lignin in dioxane-water (9:1) containing 0.2 N HCl was refluxed for 21 h. As shown in previous work [6], such treatment of lignin ("acidolysis") converts arylglycerol-β-aryl ether elements into arylpropanone structures of various types ("Hibbert ketone" structures), including arylpropanone-1 and arylpropanedione-1,2 structures. These being



V



VIa : R = H
VIb : R = C of propane side-chain of adjacent monomer



VIIa : R = H
VIIb : R = R in VIb

aryl α-ketones have strong light-absorption in the region of 310 mμ and thus would interfere with the intended spectrophotometric determination of the arylcoumarone systems (VII). The acid-treated lignin solution was therefore made alkaline and treated with sodium borohydride to eliminate any carbonyl groups present [7]. From the absorption curve of the reacidified solution a $\Delta\varepsilon$ curve was obtained by "subtraction" of the absorption curve of a similar lignin solution which had been reduced with borohydride and then reacidified but which had not been subjected to "acidolysis". This $\Delta\varepsilon$ curve showed a distinct band with a maximum at 310 mμ and a shape very similar to that of the main absorption band of the arylcoumarones III and IV. This indicates that arylcoumarone systems (VIIa + VIIb) were actually formed during the hot acid treatment of the lignin and, consequently, that arylcoumaran elements (VI) were present in the original lignin. Comparison of the $\Delta\varepsilon$ value at 310 mμ with the corresponding value for the model conversion I→ III indicated that in the Björkman lignin sample used, about 18 % of the phenylpropane units were present in dimeric elements of the phenylcoumaran type (VIa + VIb).

To investigate the proportion of phenolic coumarone elements (VIIa) formed, "ionisation $\Delta\varepsilon$" curves were obtained for the carbonyl-free acidolysis lignin, and for carbonyl-free lignin which had not been subjected to acidolysis. From these two curves a difference curve was prepared which was expected to be the "ionisation $\Delta\varepsilon$" curve for the phenolic guaiacylcoumarone systems (VIIa) formed in the acidolysis. The position of the main band of this difference curve (max 338 mμ) was in fact identical with that of the corresponding band obtained in a model experiment (I → III). Comparison of the $\Delta\varepsilon$ values at 338 mμ for both experiments indicated that about 16 % of the phenylpropane units in the Björkman lignin used had given a phenolic coumarone system (VIIa). The fact that the major part of the coumarone was of the phenolic type (VIIa) rather than the ether type (VIIb) is explained by the previous finding [6] that acidolysis of lignin liberated a considerable amount of phenolic hydroxyl groups.

Preceding work [6] has shown that about 25 % of the phenylpropane units in spruce Björkman lignin are of the phenylglycerol type linked by a β-aryl ether bridge to the adjacent monomer, or in other words, that up to 50 % of the lignin may consist of dimeric elements of the guaiacylglycerol-β-dihydroconiferyl ether type. The present study indicates that nearly 20 % of the lignin is made up of phenylcoumaran dimers (VIa + VIb). Although the various dimeric elements may "overlap" to some extent, one or both of the two phenylpropane moieties of a dimeric element also forming part of a following one, the results obtained account for the structural elements of an appreciable part of the total average lignin molecule.

1. Freudenberg, K. and Hübner, H. H. *Chem. Ber.* **85** (1952) 1181.
2. Adler, E. *Ind. Eng. Chem.* **49** (1957) 1377.
3. Aulin-Erdtman, G. *Svensk Papperstidn.* **56** (1953) 91, 287.
4. Spets, Å. *Acta Chem. Scand.* **8** (1954) 360.
5. Freudenberg, K. and Sakakibara, A. *Ann.* **623** (1959) 129; Freudenberg, K. *Chem. Ber.* **92** (1959) LXXXIX.
6. Adler, E., Pepper, J. M. and Eriksoo, E. *Ind. Eng. Chem.* **49** (1957) 1391.
7. Adler, E. and Marton, J. *Acta Chem. Scand.* **13** (1959) 75.

Received November 25, 1959.

Determination of ε-Aminocaproic Acid in Urine by Means of High-Voltage Paper Electrophoresis

ALBERT SJOERDSMA* and ARNE HANSON

Medical Clinic and Department of Clinical Chemistry, General Hospital, Malmö, Sweden

It has been reported that ε-amino-*n*-caproic acid (ε-ACA) is a potent inhibitor of plasminogen activation *in vitro* [1,2]. Preliminary studies in this hospital by Nilsson *et al.*[3] suggest that the compound may also be effective *in vivo* in the treatment of fibrinolysis associated with various diseases. It was therefore desirable to obtain information about the fate of ε-ACA in the body and to determine whether it is absorbed efficiently from the intestinal tract. To accomplish this a method for the estimation of ε-ACA in urine was needed.

* Visiting scientist, National Heart Institute, Bethesda 14, Maryland, U.S.A.

aryl α-ketones have strong light-absorption in the region of 310 mμ and thus would interfere with the intended spectrophotometric determination of the arylcoumarone systems (VII). The acid-treated lignin solution was therefore made alkaline and treated with sodium borohydride to eliminate any carbonyl groups present [7]. From the absorption curve of the reacidified solution a $\Delta\varepsilon$ curve was obtained by "subtraction" of the absorption curve of a similar lignin solution which had been reduced with borohydride and then reacidified but which had not been subjected to "acidolysis". This $\Delta\varepsilon$ curve showed a distinct band with a maximum at 310 mμ and a shape very similar to that of the main absorption band of the arylcoumarones III and IV. This indicates that arylcoumarone systems (VIIa + VIIb) were actually formed during the hot acid treatment of the lignin and, consequently, that arylcoumaran elements (VI) were present in the original lignin. Comparison of the $\Delta\varepsilon$ value at 310 mμ with the corresponding value for the model conversion I→ III indicated that in the Björkman lignin sample used, about 18 % of the phenylpropane units were present in dimeric elements of the phenylcoumaran type (VIa + VIb).

To investigate the proportion of phenolic coumarone elements (VIIa) formed, "ionisation $\Delta\varepsilon$" curves were obtained for the carbonyl-free acidolysis lignin, and for carbonyl-free lignin which had not been subjected to acidolysis. From these two curves a difference curve was prepared which was expected to be the "ionisation $\Delta\varepsilon$" curve for the phenolic guaiacylcoumarone systems (VIIa) formed in the acidolysis. The position of the main band of this difference curve (max 338 mμ) was in fact identical with that of the corresponding band obtained in a model experiment (I → III). Comparison of the $\Delta\varepsilon$ values at 338 mμ for both experiments indicated that about 16 % of the phenylpropane units in the Björkman lignin used had given a phenolic coumarone system (VIIa). The fact that the major part of the coumarone was of the phenolic type (VIIa) rather than the ether type (VIIb) is explained by the previous finding [6] that acidolysis of lignin liberated a considerable amount of phenolic hydroxyl groups.

Preceding work [6] has shown that about 25 % of the phenylpropane units in spruce Björkman lignin are of the phenylglycerol type linked by a β-aryl ether bridge to the adjacent monomer, or in other words, that up to 50 % of the lignin may consist of dimeric elements of the guaiacylglycerol-β-dihydroconiferyl ether type. The present study indicates that nearly 20 % of the lignin is made up of phenylcoumaran dimers (VIa + VIb). Although the various dimeric elements may "overlap" to some extent, one or both of the two phenylpropane moieties of a dimeric element also forming part of a following one, the results obtained account for the structural elements of an appreciable part of the total average lignin molecule.

1. Freudenberg, K. and Hübner, H. H. *Chem. Ber.* **85** (1952) 1181.
2. Adler, E. *Ind. Eng. Chem.* **49** (1957) 1377.
3. Aulin-Erdtman, G. *Svensk Papperstidn.* **56** (1953) 91, 287.
4. Spets, Å. *Acta Chem. Scand.* **8** (1954) 360.
5. Freudenberg, K. and Sakakibara, A. *Ann.* **623** (1959) 129; Freudenberg, K. *Chem. Ber.* **92** (1959) LXXXIX.
6. Adler, E., Pepper, J. M. and Eriksoo, E. *Ind. Eng. Chem.* **49** (1957) 1391.
7. Adler, E. and Marton, J. *Acta Chem. Scand.* **13** (1959) 75.

Received November 25, 1959.

Determination of ε-Aminocaproic Acid in Urine by Means of High-Voltage Paper Electrophoresis

ALBERT SJOERDSMA* and ARNE HANSON

Medical Clinic and Department of Clinical Chemistry, General Hospital, Malmö, Sweden

It has been reported that ε-amino-*n*-caproic acid (ε-ACA) is a potent inhibitor of plasminogen activation *in vitro* [1,2]. Preliminary studies in this hospital by Nilsson *et al.*[3] suggest that the compound may also be effective *in vivo* in the treatment of fibrinolysis associated with various diseases. It was therefore desirable to obtain information about the fate of ε-ACA in the body and to determine whether it is absorbed efficiently from the intestinal tract. To accomplish this a method for the estimation of ε-ACA in urine was needed.

* Visiting scientist, National Heart Institute, Bethesda 14, Maryland, U.S.A.

However, there seemed to be no specific reaction available allowing a direct assay of this amino acid and we regarded the conventional two-dimensional paper chromatography too difficult and time-consuming for our purpose. We therefore developed a quicker and simpler method for the determination of ε-ACA, principally similar to methods already developed in this laboratory for the determination of some other amino acids, *e.g.* γ-aminobutyric and glutamic acids [4]. The method consists of isolation of ε-ACA by high-voltage paper electrophoresis, development of color with a ninhydrin and copper spray, elution of the spots from paper and measurement of the color in a spectrophotometer.

Method. The following reagents were used: 1) Buffer for electrophoresis, a mixture of formic acid, acetic acid, acetone and water (15:10:15:65 v/v) with a final pH of 1.2. 2) Spray reagents, a) Ninhydrin, 0.5 % in *n*-butanol previously saturated with a mixture of pyridine, acetic acid and water (10:1:95) with a pH of 6, and b) Copper reagent [5], prepared by mixing a saturated solution of cupric nitrate, 10 % nitric acid, water and methanol (1:0.2:4:95). 3) Methanol, as eluent, 4) ε-ACA, reference standard solution containing 1 mg per ml of water. The ε-ACA used here was obtained from L. Light & Co. Ltd. Colnbrook, England. The method for high-voltage paper electrophoresis was that of Wieland and Pfleiderer [6] and Wieland and Dose [7] using Whatman No. 3 filter paper.

Preliminary experiments indicated excellent separation of ε-ACA from normally-occurring amino acids in urine except alanine. Separation from the latter could be achieved with paper chromatography in the second dimension in a quick-running solvent mixture of methanol, water and pyridine (80:20:4). However, during the first 24 h after administration of ε-ACA in the usual clinical dosage (6.0 g), the excretion of the compound was of such a magnitude that accurate assays could be performed on diluted urine in which interference from alanine was insignificant. Thus in the procedure to be described, a volume of urine equivalent to 1 minute's excretion was diluted to 10 ml with water prior to analysis. 0.005 to 0.04 ml samples of diluted urine and reference standards (5 to 40 μg) were applied to the paper with micropipettes at a distance of 8 cm from the anode end. With an operating voltage of 2 000 V and a current of 60 mA — resulting in a potential gradient of 50 V/cm — the ε-ACA migrated about 10 cm towards the cathode in an experimental period of 1 h. On removal from the apparatus the paper was dried in an oven at 80°C for at least 1 h. It was then sprayed with the ninhydrin reagent and the color developed by heating in an oven at 80°C for 30 min. The purple color obtained was then converted to a more stable light-red color by spraying with the copper-complexing reagent and the paper was allowed to dry in a cool current of air. Each colored spot was then cut out, placed in a small glass-stoppered test-tube and eluted for 30 min with 4 ml of methanol. A clear portion of the paper of corresponding size was eluted also and used as a blank. The measurements of the color intensity were made at 508 mμ in a Beckman spectrophotometer model B. The extinction values of reference spots were proportionate to concentration over a range 0 to 30 μg, with 1 μg giving an average value of 0.02 to 0.025. From the extinction values obtained with the reference standards a graph was constructed and from this the concentration of the urine sample was read. Recovery of ε-ACA added to diluted urine was satisfactory (95 ± 10 %). In three patients who received on different days single 6.0 g doses of ε-ACA orally and intravenously the compound was found to be excreted rapidly in the urine, 40 to 65 % of the dose being recovered in the first 12 h. Since the percentage excreted after oral and intravenous administration was similar, the compound is thought to be completely absorbed from the intestinal tract.

This work was supported by a grant from the *Swedish Medical Research Council.*

1. Ablondi, F. B., Hagan, J. J., Philips, M. and De Renzo, E. C. *Arch. Biochem. Biophys.* **82** (1959) 153.
2. Alkjaersig, N., Fletcher, A. P. and Sherry, S. *J. Biol. Chem.* **234** (1959) 832.
3. Nilsson, I. M., Sjoerdsma, A. and Waldenström, J. *To be published.*
4. Hanson, A. and von Studnitz, W. *Acta Chem. Scand.* **12** (1958) 1332.
5. Kawerau, E. and Wieland, T. *Nature* **168** (1951) 77.
6. Wieland, T. and Pfleiderer, G. *Angew. Chem.* **67** (1955) 257.
7. Wieland, T. and Dose, K. in Berl. W.G. (Ed.) *Physical Methods in Chemical Analysis*, Academic Press Inc., New York (1956), vol. 3.

Received November 27, 1959.

Photoinduced Oxidation of *Iso*propanol by Acid Chromate Studied by Flash Photolysis

ULRIK KLÄNING

Laboratoriet for Fotokemi, Fotografi og Reproduktionsteknik, Technical University, Copenhagen, Denmark

In aqueous solutions at low acidity the oxidation of *iso*propanol by acid chromate is a very slow process. The reaction is accelerated when the solution is irradiated with ultraviolet light [1]. A kinetic study of the photochemically induced oxidation led to the conclusion that the primary process is the decomposition of mono*iso*propyl chromate to give Cr^{IV} and acetone [1,2]. Further it was found that a reaction sequence which included the step

1) $Cr^{IV} + (CH_3)_2CHOH \rightarrow Cr^{II} + (CH_3)_2CO$

satisfactorily explained the kinetic data [2]. However it has now been proved that Cr^{IV} does not react directly with the alcohol [3,4]. An alternative mechanism which fits the results so far obtained [3] is

2) $Cr^{IV} + Cr^{VI} \rightarrow 2Cr^{V}$
3) $Cr^{V} + (CH_3)_2CHOH + H^+ \rightarrow Cr^{III} + (CH_3)_2CO$
4) $Cr^{V} + Cr^{IV} \rightarrow Cr^{VI} + Cr^{III}$

In search for the correct mechanism it is feasible to attempt the direct observation of the chromium intermediates by the flash photolysis technique [5]. Some preliminary experiments making use of this technique are reported here. Deaerated solutions, containing 2.5–10 M *iso*propanol. 5×10^{-4}–3×10^{-3} M perchloric acid, and 5×10^{-4}–2×10^{-3} acid chromate in a 50 ml pyrex cell, 20 cm long, were illuminated in a flash photolysis apparatus similar to those described by Porter and Windsor [6], and Christie, Norrish and Porter [7]. Photographs of the spectrum of the reaction mixtures taken 30 μsec and 200 μsec after an 1 150 Joule photolysis flash revealed a transient absorption extending from 650 mμ (the limit of the photographic plate) towards green and a transient decrease of the Cr^{VI} absorption band in the near ultraviolet. The change of the transient absorption in time was followed photoelectrically [7] at 590 mμ after irradiation with an 810 Joule flash. Three steps in the reaction could be detected, a fast increase of the optical density completed in about 200 μsec, a fast decrease of the optical density with a halftime of about 600 μsec at $C_{HCrO_4^-} = 10^{-3}$ M, $C_{HClO_4} = 10^{-3}$ M, and $C_{alcohol} = 10$ M, and finally a third slow step consisting of a further decrease in optical density, the rate of which seems to be of second order independent of conditions. In the presence of 10^{-3} M manganous sulfate in the solution the third slow step disappeared and a brownish colour developed after the flash indicating a formation of MnO_2. Figs. 1 and 2 show photographs of oscillographic recordings of the transient events. The lines A and B designate 0 % and 100 % light absorption, respectively. C is the decay curve for the intermediates and the straight line D is the absorption to infinite time giving the amount of Cr^{III} formed. In experiments as shown in Fig. 2 the sweep rate on the oscilloscope screen decreased exponentially over the sweep range with a halftime of 6.3×10 sec. The second fast step appears in these measurements as a hook on the decay curve (Fig. 2). Only two measurements were

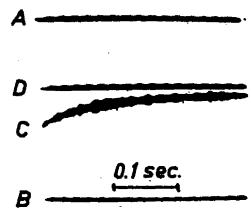


Fig. 1. Experimental decay curve for the third step. ($C_{HClO_4} = 3 \times 10^{-3}$ M, $C_{HCrO_4^-} = 10^{-3}$ M, $C_{alc.} = 10$ M).

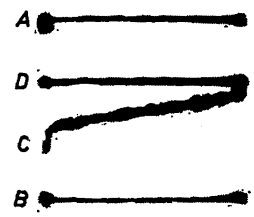


Fig. 2. Experimental decay curve. The sweep rate decreases exponentially over the sweep range, halftime for the sweep is 6.3×10^{-2} sec. ($C_{HClO_4} = 3 \times 10^{-3}$ M, $C_{HCrO_4^-} = 10^{-3}$ M, $C_{alc.} = 10$ M)

Table 1. Results (Temp. 22°C ± 2°C).

$C_{alc.}$ mole litre	C_{HClO_4} mole/litre	$C_{HCrO_4^-}$ mole/litre	D_0	D_∞	$\Delta D/D_0 - D_\infty$	$10^4 \times C_{Cr^{III}}$ mole/litre	$k/\Delta\varepsilon$ sec^{-1}
2.5	10^{-3}	10^{-3}	0.1	0.03	0.7	0.3	—
5	10^{-3}	10^{-3}	0.34	0.10	0.62	1.0	1 800
10	5×10^{-4}	5×10^{-4}	0.54	0.17	0.32	1.8	2 000
10	10^{-3}	5×10^{-4}	—	—	—	—	1 800
10	3×10^{-3}	5×10^{-4}	0.39	0.12	0.37	1.3	2 300
10	10^{-3}	10^{-3}	0.75	0.25	0.37	2.7	2 000
10	3×10^{-3}	10^{-3}	0.58	0.19	0.46	2.0	1 700
10	10^{-3}	2×10^{-3}	0.81	0.28	0.28	3.0	2 000
10	3×10^{-3}	2×10^{-3}	0.62	0.22	0.45	2.3	2 000
10*	10^{-3}	10^{-3}	0.74	—	—	—	—

* $C_{MnSO_4} = 10^{-3}$ M.

made with a halftime of 5×10^{-4} sec for the sweep, revealing the two first fast steps. The findings are compiled in Table 1. D_0 is the maximum transient optical density taken from the experiments where the sweep rate decreased exponentically. D_∞ is the optical density after infinite time. In the expression for the relative extent of step two $\Delta D/D - D_\infty$, ΔD is somewhat arbitrarily taken as the change of the optical density in 2×10^{-3} sec (Fig. 2). The concentration of Cr^{III} formed on photolysis was calculated using 47 litre/mole cm for the molar extinction coefficient of Cr^{III}, a value found by reducing thermally 5×10^{-4} M acid chromate in an aqueous solution, 10 M in *iso*propanol and 3×10^{-3} M in perchloric acid. The last column shows second order rate constants k for the third step divided by the difference in the extinction coefficients $\Delta\varepsilon$ between the intermediate in question and Cr^{III}; $k/\Delta\varepsilon$ is calculated from experiments with constant sweep rates (Fig. 1). Fig. 3 shows a second order plot for the third slow process. From Table 1 one may note that D_0/D_∞ does not vary appreciably with conditions, whereas $\Delta D/D_0 - D_\infty$ varies somewhat with conditions This indicates that reactions by which chromium intermediates disappear by disproportionation are faster than is the oxidation of the alcohol by the intermediates. Further it seems that the relative extent of the second step $\Delta D/D_0 - D_\infty$ increases with increasing acidity.

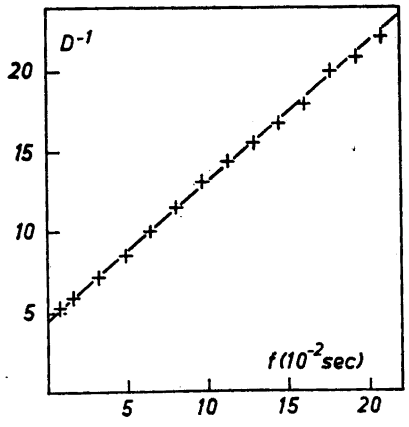


Fig. 3. The experimental decay curve in Fig. 1 as a second order decay.

Although the data are not sufficient to establish the mechanism we may tentatively ascribe the first step to be the breakdown of excited Cr^{VI} esters to give Cr^{IV} and acetone. In presence of Mn^{II} the next step is probably [8]

6) $Mn^{II} + Cr^{IV} \rightarrow Mn^{III} + Cr^{III}$
$(2\ Mn^{III} \rightarrow MnO_2 + Mn^{II})$

The variation of the extent of the second fast step with conditions suggests reactions like

7) $Cr^{IV} + Cr^{VI} + H^+ \rightarrow 2Cr^V$
8) $Cr^{IV} + Cr^{IV} \rightarrow Cr^V + Cr^{III}$
9) $Cr^{IV} + Cr^V + H^+ \rightarrow Cr^{VI} + Cr^{III}$

the third slow step could then be the reverse reaction of 7)

$-7)\ 2\ Cr^V \rightarrow Cr^{VI} + Cr^{IV} + H^+$

The experimental work was made at the Department of Physical Chemistry, University

of Sheffield. The author's sincere thanks are due to professor G. Porter for information and help in the work and for loan of apparatus.

1. Bowen, E. J. and Chatwin, J. E. *J. Chem. Soc.* **1932** 2081.
2. Kläning, U. *Acta Chem. Scand.* **12** (1958) 807.
3. Kläning, U. and Symons, M. C. R. *Proc. Chem. Soc.* **1959** 95.
4. Kläning, U. and Symons, M. C. R. *J. Chem. Soc. In press.*
5. Porter, G. *Proc. Roy. Soc.* **A 200** (1950) 284.
6. Porter, G. and Windsor, M. W. *Discussions Faraday Soc.* **17** (1954) 178.
7. Christie, M. I., Norrish, R. G. W. and Porter, G. *Proc. Roy. Soc.* **A 216** (1953) 152.
8. Watanabe, W. and Westheimer, F. H. *J. Chem. Phys.* **17** (1949) 61.

Received November 27, 1959.

Note on the Infrared Spectra of Pro-γ-carotene and Neo-γ-carotene P

K. LUNDE

Universitetets Kjemiske Institutt, Blindern, Norway

These two *cis* isomers of γ-carotene are natural products and can be isolated easily from ripe berries of *Pyracantha angustifolia* [1,2]. In the visible spectral region, hexane solutions of pro-γ-carotene show two almost equal maxima (434, 457 mμ) while the neo-γ-carotene curve contains only a single main maximum (459 mμ) [2,3]. In spite of this difference a comparison of the infrared spectra showed near-identity[4].

Although, as was pointed out [4], several pairs of *cis* forms are possible in this sterically unsymmetrical set whose IR spectra are expected to be (almost) identical, an experimental error such as mislabeling of samples could not be excluded considering the great number of stereoisomeric carotenoids studied.

At the recommendation of Prof. L. Zechmeister, the author has now reinvestigated the IR spectra using solutions of authentic samples of pro-γ-carotene (m. p. 133—134°) and neo-γ-carotene P (m. p. 89—90°). The identity of the two curves was confirmed in the 2—12 μ region; an important difference was observed, however, in the 12—14 μ region. As Fig. 1 shows, only the neo P curve possesses a band at 13.02—13.19 μ (doublet). This band lies in the region that has been assigned to unmethylated *cis* double bonds [4]. Consequently, our earlier interpretations must be revised. It is proposed that the neo-γ-carotene P molecule contains both methylated and unmethylated *cis* double bond(s) while the latter type is absent from pro-γ-carotene. This difference has a profound influence on the spectra observed in the visible region.

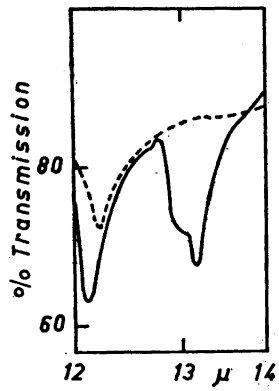


Fig. 1. Infrared spectra of: ———, neo-γ-carotene P and - - -, pro-γ-carotene (1 % solutions in carbon disulfide, 1.0 mm cells).

1. Zechmeister, L. and Schroeder, W. A. *J. Biol. Chem.* **144** (1942) 315.
2. Zechmeister, L. and Pinckard, *J. Am. Chem. Soc.* **69** (1947) 1930; Pinckard, H. J. *Thesis.* Pasadena, Calif. 1949.
3. Zechmeister, L., LeRosen, A. L., Schroeder, W. A., Polgar, A. and Pauling, L. *J. Am. Chem. Soc.* **65** (1943) 1940.
4. Lunde, K. and Zechmeister, L. *J. Am. Chem. Soc.* **77** (1955) 1647.

Received December 1, 1959.

of Sheffield. The author's sincere thanks are due to professor G. Porter for information and help in the work and for loan of apparatus.

1. Bowen, E. J. and Chatwin, J. E. *J. Chem. Soc.* **1932** 2081.
2. Kläning, U. *Acta Chem. Scand.* **12** (1958) 807.
3. Kläning, U. and Symons, M. C. R. *Proc. Chem. Soc.* **1959** 95.
4. Kläning, U. and Symons, M. C. R. *J. Chem. Soc. In press.*
5. Porter, G. *Proc. Roy. Soc.* **A 200** (1950) 284.
6. Porter, G. and Windsor, M. W. *Discussions Faraday Soc.* **17** (1954) 178.
7. Christie, M. I., Norrish, R. G. W. and Porter, G. *Proc. Roy. Soc.* **A 216** (1953) 152.
8. Watanabe, W. and Westheimer, F. H. *J. Chem. Phys.* **17** (1949) 61.

Received November 27, 1959.

Note on the Infrared Spectra of Pro-γ-carotene and Neo-γ-carotene P

K. LUNDE

Universitetets Kjemiske Institutt, Blindern, Norway

These two *cis* isomers of γ-carotene are natural products and can be isolated easily from ripe berries of *Pyracantha angustifolia* [1,2]. In the visible spectral region, hexane solutions of pro-γ-carotene show two almost equal maxima (434, 457 mμ) while the neo-γ-carotene curve contains only a single main maximum (459 mμ) [2,3]. In spite of this difference a comparison of the infrared spectra showed near-identity[4].

Although, as was pointed out [4], several pairs of *cis* forms are possible in this sterically unsymmetrical set whose IR spectra are expected to be (almost) identical, an experimental error such as mislabeling of samples could not be excluded considering the great number of stereoisomeric carotenoids studied.

At the recommendation of Prof. L. Zechmeister, the author has now reinvestigated the IR spectra using solutions of authentic samples of pro-γ-carotene (m. p. 133—134°) and neo-γ-carotene P (m. p. 89—90°). The identity of the two curves was confirmed in the 2—12 μ region; an important difference was observed, however, in the 12—14 μ region. As Fig. 1 shows, only the neo P curve possesses a band at 13.02—13.19 μ (doublet). This band lies in the region that has been assigned to unmethylated *cis* double bonds [4]. Consequently, our earlier interpretations must be revised. It is proposed that the neo-γ-carotene P molecule contains both methylated and unmethylated *cis* double bond(s) while the latter type is absent from pro-γ-carotene. This difference has a profound influence on the spectra observed in the visible region.

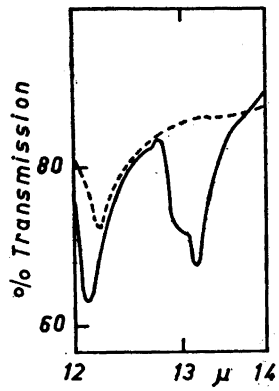


Fig. 1. Infrared spectra of: ———, neo-γ-carotene P and - - -, pro-γ-carotene (1 % solutions in carbon disulfide, 1.0 mm cells).

1. Zechmeister, L. and Schroeder, W. A. *J. Biol. Chem.* **144** (1942) 315.
2. Zechmeister, L. and Pinckard, *J. Am. Chem. Soc.* **69** (1947) 1930; Pinckard, H. J. *Thesis.* Pasadena, Calif. 1949.
3. Zechmeister, L., LeRosen, A. L., Schroeder, W. A., Polgar, A. and Pauling, L. *J. Am. Chem. Soc.* **65** (1943) 1940.
4. Lunde, K. and Zechmeister, L. *J. Am. Chem. Soc.* **77** (1955) 1647.

Received December 1, 1959.

Reactions of Phenyltellurium Trichloride with Thiourea

OLAV FOSS and SVERRE HAUGE

Chemical Institute, University of Bergen, Bergen, Norway

Tellurium dioxide dissolved in hydrochloric or hydrobromic acid reacts with four moles of thiourea to give covalent halides $Te(tu)_2X_2$ (tu = thiourea) and with six moles of thiourea to give salts of the cation $Te(tu)_4^{++}$. These are planar, four-co-ordinated complexes of divalent tellurium [1].

It has been found that phenyltellurium trichloride reacts similarly with thiourea, to give thiourea derivatives of divalent tellurium. The reactions are, with three moles of thiourea:

$$C_6H_5TeCl_3 + 3\ SC(NH_2)_2 = C_6H_5Te[SC(NH_2)_2]Cl + [SC(NH_2)_2]_2^{++} + 2\ Cl^-$$

and with four moles:

$$C_6H_5TeCl_3 + 4\ SC(NH_2)_2 = C_6H_5Te[SC(NH_2)_2]_3^+ + [SC(NH_2)_2]_2^{++} + 3\ Cl^-$$

The following compounds have been prepared and characterized through single-crystal X-ray photographs (Cu$K\alpha$ radiation, $\lambda = 1.542$ Å). Melting points are corrected.

Thiourea-benzenetellurenyl chloride, $C_6H_5Te(tu)Cl$. To 3.11 g (10 mmoles) of phenyltellurium trichloride dissolved in 20 ml of methanol was added 2.28 g (30 mmoles) of thiourea dissolved in 20 ml of warm water. A clear, orange red solution resulted, from which the divalent tellurium compound crystallized on standing. Yield, about 2.5 g (80 %). The product was recrystallized from methanol (2 g dissolved in about 25 ml at boiling temperature). M. p. 166°. (Found: S 9.96; Te 40.11. Calc. for $C_7H_9ClN_2STe$: S 10.14; Te 40.35.)

The crystals are orange red and appear as well developed, monoclinic prisms {011} with $a = 6.32$ Å, $b = 10.62$ Å, $c = 15.16$ Å, $\beta = 90\frac{1}{2}°$. There are four molecules per unit cell; density, calc. 2.06, found 2.07 g/cm³. The space group, from systematic absences, is $C_{2h}^5 - P2_1/n$.

Thiourea-benzenetellurenyl bromide, $C_6H_5Te(tu)Br$. The crystals are isomorphous with those of the chloride, and show the same colour and morphology. The unit cell dimensions are, $a = 6.46$ Å, $b = 10.73$ Å, $c = 15.36$ Å, $\beta = 91\frac{1}{2}°$, and the density, calc. 2.25, found 2.24 g/cm³.

The compound was prepared as described above for the chloride, by dissolving about 3.6 g (30 mmoles) of potassium bromide in the aqueous thiourea solution before mixing with the methanolic phenyltellurium trichloride solution. Yield, about 3.6 g (99 %). M. p. 178° (recrystallized from methanol, 2 g dissolved in about 45 ml at boiling temperature). (Found: Te 35.82. Calc. for $C_7H_9BrN_2STe$: Te 35.37.)

The compounds $C_6H_5Te(tu)X$ dissolve in warm aqueous thiourea to give yellow to orange yellow solutions from which salts of the cation $C_6H_5Te(tu)_3^+$ crystallize on cooling. The chloride was also prepared directly from phenyltellurium trichloride.

Dithiourea-benzenetellurenyl chloride, $C_6H_5Te(tu)_2Cl$. To 3.11 g (10 mmoles) of phenyltellurium trichloride dissolved in 15 ml of methanol was added one drop of conc. hydrochloric acid and, with stirring, 3.8 g (50 mmoles, 25 % excess) of thiourea dissolved in 25 ml of warm water, and then 25 ml of water. On standing, the chloride slowly crystallized, as clusters of yellow needles which ultimately filled the whole liquid. Yield, about 3.4 g (85 %); m. p. 168°. (Found: S 16.30; Te 32.33. Calc. for $C_9H_{13}ClN_4S_2Te$: S 16.34; Te 32.52.) It may be recrystallized from aqueous thiourea, preferrably in presence of a small amount of hydrochloric acid.

The salt forms long orthorhombic prisms elongated in the direction of the c axis. The axial lengths are, $a = 11.98$ Å, $b = 20.80$ Å, $c = 5.79$ Å, and the space group, from systematic absences, is $D_2^4 - P2_12_12_1$. There are four formula units per unit cell; density, calc. 1.81, found 1.81 g/cm³.

The nitrate and perchlorate crystallize on addition of nitric or perchloric acid to aqueous thiourea solutions of the chloride (1 g chloride in 30 ml of warm 10 % thiourea, 3 ml of *ca.* 35 % nitric acid or 2 ml of *ca.* 60 % perchloric acid. Yields on standing to room temperature, about 1 g of each). The salts form long, yellow prisms elongated along the b axis.

Dithiourea-benzenetellurenyl nitrate, $C_6H_5Te(tu)_2NO_3$. M. p. 135°. (Found: S 15.49; Te 29.88. Calc. for $C_8H_{13}N_5O_3S_2Te$: S 15.31; Te 30.46.) Monoclinic, $a = 24.30$ Å, $b = 5.88$ Å, $c = 21.30$ Å, $\beta = 90\frac{1}{2}°$. There are eight formula units per unit cell; density, calc. 1.83, found 1.83 g/cm³. On the basis of the systematic absences, hkl when $h + k$ is odd, $h0l$ when h is odd or l is odd, the space group is either C_{2h}^6—$C2/c$ or C_s^4—Cc.

Dithiourea-benzenetellurenyl perchlorate, $C_6H_5Te(tu)_2ClO_4$. M. p. 136°. (Found: Te 27.97. Calc. for $C_8H_{13}ClN_4O_4S_2Te$: Te 27.96.) Monoclinic prismatic, $a = 12.24$ Å, $b = 5.86$ Å, $c = 22.76$ Å, $\beta = 96°$. There are four formula units per unit cell; density, calc. 1.87, found 1.87 g/cm³. The space group, from systematic absences, is C_{2h}^5—$P2_1/c$.

Dithiourea-benzenetellurenyl thiocyanate, $C_6H_5Te(tu)_2SCN$, was prepared *via* a compound, presumably $C_6H_5Te(tu)SCN$, obtained from phenyltellurium trichloride in the same way as $C_6H_5Te(tu)Br$, by use of 30 mmoles of potassium thiocyanate instead of potassium bromide. The micro-crystalline solid (3 g) which separated on standing at ice temperature, was filtered off and dissolved in 60 ml of warm 10 % aqueous thiourea. On filtering, a clear, orange yellow solution resulted which on standing deposited yellow crystals (2.4 g) of the thiocyanate. M. p. 109°. (Found: S 22.98; Te 30.47. Calc. for $C_9H_{13}N_5S_3Te$: S 23.18; Te 30.75.)

The crystals occur as long, monoclinic prisms extended along the b axis. The unit cell dimensions are, $a = 15.37$ Å, $b = 5.83$ Å, $c = 17.47$ Å, $\beta = 96°$, and the space group, from systematic absences, C_{2h}^5—$P2_1/n$. There are four formula units per unit cell; density, calc. 1.77, found 1.78 g/cm³.

The crystal structures of representatives of the compounds are being studied with a view towards a possible bearing on the transition state in nucleophilic displacements on divalent tellurium, selenium and sulphur.

1. Foss, O. and Hauge, S. *Acta Chem. Scand.* **13** (1959) 1252.

Received December 5, 1959.

Specificity of a Purified Hog Intestinal Maltase Fraction

Competitive Inhibition of Maltase Activity by Other Substrates

ARNE DAHLQVIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

In previous papers the separation of hog intestinal maltase activity into three fractions (maltase I—III) with different enzymatic properties has been described [1-3]. In the present paper the specificity of one of these fractions, *maltase III*, will be further discussed.

Maltase III preparations hydrolyze *phenyl-α-D-glucopyranoside* [2], *isomaltose* (6-(α-D-glucopyranosyl)-D-glucose) [2], *turanose* (3-(α-D-glucopyranosyl)-D-fructose) [2], and *melezitose* (α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) [2]. (Melezitose is hydrolyzed to glucose and sucrose). Heat inactivation experiments support the theory that these activities and the maltase activity of the purified maltase III preparation are caused by the same enzyme [2,3].

When two substrates are hydrolyzed by the same enzyme, they act as competitive inhibitors for the hydrolysis of each other. The study of this inhibition usually requires a method for the separate determination of the hydrolysis products of each substrate, when the substrates are present in a mixture [3]. With maltase III, however, the hydrolysis of the other substrates proceeds slowly compared with the hydrolysis of maltose (measured at 0.1388 M substrate concentration the rate for their hydrolysis is 1/20 or less of the rate for the hydrolysis of maltose). When maltose is present in a mixture with one of the other substrates, therefore, it may be assumed that all the glucose produced is derived from maltose, and thus the rate for the hydrolysis of maltose may be measured simply by measuring the increase in reducing power, as if no other substrate were present *.

* This approximation will cause the K_i value measured to be somewhat too high. The error to be expected was calculated from the relative rate of hydrolysis of the different substrates at 0.1388 M substrate concentration and their K_s values. With phenyl-α-D-glycopyranoside as inhibitor the K_i value measured should be about 15 % too high, with *iso*-maltose and turanose about 30 % too high, and with melezitose less than 10 % too high.

Dithiourea-benzenetellurenyl nitrate, $C_6H_5Te(tu)_2NO_3$. M. p. 135°. (Found: S 15.49; Te 29.88. Calc. for $C_8H_{13}N_5O_3S_2Te$: S 15.31; Te 30.46.) Monoclinic, $a = 24.30$ Å, $b = 5.88$ Å, $c = 21.30$ Å, $\beta = 90\frac{1}{2}°$. There are eight formula units per unit cell; density, calc. 1.83, found 1.83 g/cm³. On the basis of the systematic absences, hkl when $h + k$ is odd, $h0l$ when h is odd or l is odd, the space group is either C_{2h}^6—$C2/c$ or C_s^4—Cc.

Dithiourea-benzenetellurenyl perchlorate, $C_6H_5Te(tu)_2ClO_4$. M. p. 136°. (Found: Te 27.97. Calc. for $C_8H_{13}ClN_4O_4S_2Te$: Te 27.96.) Monoclinic prismatic, $a = 12.24$ Å, $b = 5.86$ Å, $c = 22.76$ Å, $\beta = 96°$. There are four formula units per unit cell; density, calc. 1.87, found 1.87 g/cm³. The space group, from systematic absences, is C_{2h}^5—$P2_1/c$.

Dithiourea-benzenetellurenyl thiocyanate, $C_6H_5Te(tu)_2SCN$, was prepared *via* a compound, presumably $C_6H_5Te(tu)SCN$, obtained from phenyltellurium trichloride in the same way as $C_6H_5Te(tu)Br$, by use of 30 mmoles of potassium thiocyanate instead of potassium bromide. The micro-crystalline solid (3 g) which separated on standing at ice temperature, was filtered off and dissolved in 60 ml of warm 10 % aqueous thiourea. On filtering, a clear, orange yellow solution resulted which on standing deposited yellow crystals (2.4 g) of the thiocyanate. M. p. 109°. (Found: S 22.98; Te 30.47. Calc. for $C_9H_{13}N_5S_3Te$: S 23.18; Te 30.75.)

The crystals occur as long, monoclinic prisms extended along the b axis. The unit cell dimensions are, $a = 15.37$ Å, $b = 5.83$ Å, $c = 17.47$ Å, $\beta = 96°$, and the space group, from systematic absences, C_{2h}^5—$P2_1/n$. There are four formula units per unit cell; density, calc. 1.77, found 1.78 g/cm³.

The crystal structures of representatives of the compounds are being studied with a view towards a possible bearing on the transition state in nucleophilic displacements on divalent tellurium, selenium and sulphur.

1. Foss, O. and Hauge, S. *Acta Chem. Scand.* **13** (1959) 1252.

Received December 5, 1959.

Specificity of a Purified Hog Intestinal Maltase Fraction

Competitive Inhibition of Maltase Activity by Other Substrates

ARNE DAHLQVIST

Department of Physiological Chemistry, University of Lund, Lund, Sweden

In previous papers the separation of hog intestinal maltase activity into three fractions (maltase I—III) with different enzymatic properties has been described [1-3]. In the present paper the specificity of one of these fractions, *maltase III*, will be further discussed.

Maltase III preparations hydrolyze *phenyl-α-D-glucopyranoside* [2], *isomaltose* (6-(α-D-glucopyranosyl)-D-glucose) [2], *turanose* (3-(α-D-glucopyranosyl)-D-fructose) [2], and *melezitose* (α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) [2]. (Melezitose is hydrolyzed to glucose and sucrose). Heat inactivation experiments support the theory that these activities and the maltase activity of the purified maltase III preparation are caused by the same enzyme [2,3].

When two substrates are hydrolyzed by the same enzyme, they act as competitive inhibitors for the hydrolysis of each other. The study of this inhibition usually requires a method for the separate determination of the hydrolysis products of each substrate, when the substrates are present in a mixture [3]. With maltase III, however, the hydrolysis of the other substrates proceeds slowly compared with the hydrolysis of maltose (measured at 0.1388 M substrate concentration the rate for their hydrolysis is 1/20 or less of the rate for the hydrolysis of maltose). When maltose is present in a mixture with one of the other substrates, therefore, it may be assumed that all the glucose produced is derived from maltose, and thus the rate for the hydrolysis of maltose may be measured simply by measuring the increase in reducing power, as if no other substrate were present *.

* This approximation will cause the K_i value measured to be somewhat too high. The error to be expected was calculated from the relative rate of hydrolysis of the different substrates at 0.1388 M substrate concentration and their K_s values. With phenyl-α-D-glycopyranoside as inhibitor the K_i value measured should be about 15 % too high, with *iso*-maltose and turanose about 30 % too high, and with melezitose less than 10 % too high.

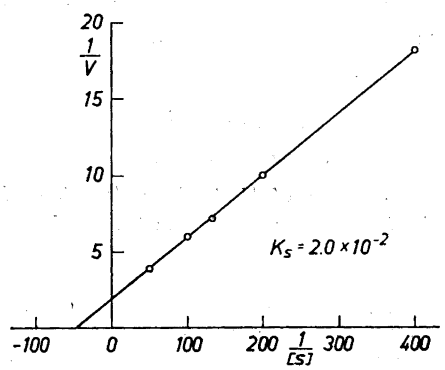


Fig. 1. Determination of K_s for phenyl-α-D-glucopyranosidase activity of purified hog intestinal maltase III. Reaction performed in 0.05 M maleate buffer pH 6.5 at 37°C.

Maltase III was obtained by mutual displacement chromatography [4]. By measuring the rate of hydrolysis (at 37°C in 0.05 M maleate buffer pH 6.5) in a series of maltose solutions containing 0.0020—0.0050 M maltose (chromatographically pure [4]) the K_s for maltase III was determined at 4.2×10^{-3} in agreement with the value obtained earlier [4]. The degree of hydrolysis was measured with acid copper reagent (Caputto's modification) [4,5]. It was not allowed to exceed 20 %. Then the inhibition of maltase III activity was studied, using the same series of maltose solutions in the presence of a suitable amount of the other substrates. The values of K_s and K_i were calculated by Dixon's [8,9] modification of the method of Lineweaver and Burk [10] as described earlier [3]. The calculations for phenyl-α-D-glucopyranoside are seen in Figs. 1 and 2.

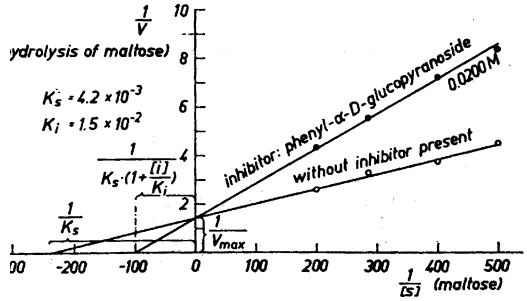


Fig. 2. Inhibition of hog intestinal maltase III activity by phenyl-α-D-glucopyranoside. Reaction performed in 0.05 M maleate buffer pH 6.5 at 37°C. The inhibition is *competitive* (the V_{max} is not altered by the presence of inhibitor). K_s (for maltase III activity) and K_i (for the inhibition by phenyl-α-D-glucopyranoside) are calculated from the intercept with the abscissa as seen in the figure.

The inhibition of maltase III activity was *purely competitive* in all cases (which is seen by the fact that V_{max} was unaltered by the presence of inhibitor). The value for K_i in the different cases are given below, with the concentration of inhibitor used given within parentheses:

Phenyl-α-D-glucopyranoside (0.0200 M) $K_i = 1.5 \times 10^{-2}$ (Fig. 2). The K_s for phenyl-α-D-glucopyranosidase activity was determined to be 2.0×10^{-2} (substrate concentration 0.0025—0.0200 M, and the degree of hydrolysis measured with Somogyi-Nelson's reagents [3], Fig. 1).

Isomaltose (0.0050 M or 0.0100 M) $K_i = 2 \times 10^{-2}$. The K_s for *iso*maltase activity was measured to be 2×10^{-2} (substrate concentration 0.0100—0.0250 M, and the degree of hydrolysis measured by quantitative paper chromatography [6] with 25 μl of the reaction mixture being applied).

Turanose (0.0020 M) $K_i = 3.3 \times 10^{-3}$. The K_s for turanase activity was determined to be 2.2×10^{-3} (substrate concentration 0.0030—0.0200 M, and the degree of hydrolysis determined with glucose oxidase [7]).

Melezitose (0.100 M or 0.200 M*) $K_i = 5.0 \times 10^{-1}$. The K_s for melezitase activity was measured to be 4.5×10^{-1} (substrate concentration 0.0500—0.1400 M, and the degree of hydrolysis determined with 3,5-dinitrosalicylic acid reagent [4]).

Thus in all cases the K_i for the inhibition of the maltase III activity by the other substrates tested was of the same order of magnitude as the K_s for the same substance as substrate. This strongly supports the theory that the phenyl-α-D-glucopyranosidase, *iso*maltase, turanase, and melezitase activities of the purified maltase III preparation are caused by the maltase *per se*, and not by contaminating enzymes.

* In the experiments where 0.200 M melezitose was used as inhibitor, the inhibitor concentration was 100 times the lowest substrate (maltose) concentration. Even during these circumstances, however, it was calculated (from the relative rate of hydrolysis at 0.1388 M substrate concentration and the K_s values) that the amount of glucose produced from melezitose was less than 1 % of the amount produced from maltose.

Acknowledgment. The author's thanks are due to Professor Bengt Borgström for his interest in my work and for valuable discussions. This work has been supported by grants from the *Medical Faculty of the University of Lund*. The skillful technical assistance of Mrs. K. Hagebris is gratefully acknowledged.

1. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 945.
2. Dahlqvist, A. *Acta Chem. Scand.* **14** (1960) 9.
3. Dahlqvist, A. *Acta Chem. Scand.* **14** (1960). *In the press.*
4. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 1817.
5. Caputto, R., Leloir, F. and Trucco, R. E. *Enzymologia* **12** (1947) 350.
6. Dahlqvist, A. and Borgström, B. *Acta Chem. Scand.* **13** (1959) 1659.
7. Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 2012.
8. Dixon, M. *Biochem. J.* **55** (1953) 170.
9. Dixon, M. and Webb, E. C. *Enzymes*, London 1958.
10. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.

Received December 9, 1959.

Sewage as a Substitute for Casein Hydrolyzates in Fermentations with *Propionibacterium shermannii*

HALINA Y. NEUJAHR

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

The bacteria belonging to the genus *Propionibacterium* are characterized by an outstanding ability to produce vitamin B_{12} as reported by several authors [1,2]. Janicki, Pawelkiewicz *et al.*[3-5] used *Propionibacterium shermannii* in their comprehensive studies on vitamin B_{12} production by propionic acid bacteria and isolated several vitamin B_{12} factors from cultures of this organism. These authors found that the addition of small amounts of Aureomycin or sulfathiazole stimulated the synthesis of cyanocobalamin at the expense of other vitamin B_{12} factors [6,7] as is also the case with the addition of 5,6-dimethyl benzimidazole (DMB)[6]. The semi-synthetic medium used in fermentations with *Propionibacterium shermannii* is composed of casein hydrolyzates, glucose, phosphates, certain mineral salts and the vitamins, calcium pantothenate and biotin. Since the most expensive constituents of the medium are the casein hydrolyzates, it was considered

Table 1. Semi-continuous stationary fermentations with *Propionibacterium shermannii* in 2 l conical flasks.

Expt. No.	1	2	3
Medium	semi-synth.	semi-synth. + DMB 1 μg/ml	semi-synth. + corn steep liquor, **
E. coli activity after 5 days μg/ml *	1.8	0.7	0.9
E. coli activity after 12 days μg/ml *	3.6	0.5	2.6

* calculated as cyanocobalamin in cup plate assay.
** 4 % calculated on the basis of dry product containing 4 % N.

Acknowledgment. The author's thanks are due to Professor Bengt Borgström for his interest in my work and for valuable discussions. This work has been supported by grants from the *Medical Faculty of the University of Lund*. The skillful technical assistance of Mrs. K. Hagebris is gratefully acknowledged.

1. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 945.
2. Dahlqvist, A. *Acta Chem. Scand.* **14** (1960) 9.
3. Dahlqvist, A. *Acta Chem. Scand.* **14** (1960). *In the press.*
4. Dahlqvist, A. *Acta Chem. Scand.* **13** (1959) 1817.
5. Caputto, R., Leloir, F. and Trucco, R. E. *Enzymologia* **12** (1947) 350.
6. Dahlqvist, A. and Borgström, B. *Acta Chem. Scand.* **13** (1959) 1659.
7. Dahlqvist, A. *Acta Chem. Scand.* **12** (1958) 2012.
8. Dixon, M. *Biochem. J.* **55** (1953) 170.
9. Dixon, M. and Webb, E. C. *Enzymes*, London 1958.
10. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.

Received December 9, 1959.

Sewage as a Substitute for Casein Hydrolyzates in Fermentations with *Propionibacterium shermannii*

HALINA Y. NEUJAHR

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

The bacteria belonging to the genus *Propionibacterium* are characterized by an outstanding ability to produce vitamin B_{12} as reported by several authors [1,2]. Janicki, Pawelkiewicz *et al.*[3-5] used *Propionibacterium shermannii* in their comprehensive studies on vitamin B_{12} production by propionic acid bacteria and isolated several vitamin B_{12} factors from cultures of this organism. These authors found that the addition of small amounts of Aureomycin or sulfathiazole stimulated the synthesis of cyanocobalamin at the expense of other vitamin B_{12} factors [6,7] as is also the case with the addition of 5,6-dimethyl benzimidazole (DMB)[6]. The semi-synthetic medium used in fermentations with *Propionibacterium shermannii* is composed of casein hydrolyzates, glucose, phosphates, certain mineral salts and the vitamins, calcium pantothenate and biotin. Since the most expensive constituents of the medium are the casein hydrolyzates, it was considered

Table 1. Semi-continuous stationary fermentations with *Propionibacterium shermannii* in 2 l conical flasks.

Expt. No.	1	2	3
Medium	semi-synth.	semi-synth. + DMB 1 μg/ml	semi-synth. + corn steep liquor, **
E. coli activity after 5 days μg/ml *	1.8	0.7	0.9
E. coli activity after 12 days μg/ml *	3.6	0.5	2.6

* calculated as cyanocobalamin in cup plate assay.
** 4 % calculated on the basis of dry product containing 4 % N.

Table 2. Fermentation and vitamin B_{12} production by *Propionibacterium shermannii* grown for 5 days in different media. Suppl.-supplemented with the salts, vitamins and glucose of the semi-synthetic medium.

No.	Medium	Suppl.	pH	Increase in *E. coli* activity μg/ml *
1	Sewage fraction 1	−	4.9	0.1
2	» » 1	+	4.7	0.3
3	» » 2	−	4.7	0.1
4	» » 2	+	4.7	0.3
5	» » 3	−	4.8	0.1
6	» » 3	+	4.6	0.2
7	» » 4	−	6.5	0.0
8	» » 4	+	4.5	2.4
9	» » 5	−	5.1	0.1
10	» » 5	+	5.1	0.1
11	» » 6	−	5.7	0.0
12	» » 6	+	4.8	1.0
13	» » 7	−	5.4	0.0
14	» » 7	+	4.7	0.3
15	» » 8	−	4.8	0.1
16	» » 8	+	4.6	0.2
17	Milk whey	−	4.7	1.0
18	» »	+	4.8	0.5
19	*Anabaena supernat.*	−	4.9	0.1
20	» »	+	4.9	0.1
21	Yeast autolyzate	−	5.2	0.0
22	» »	+	4.3	0.2
23	Semi-synthetic	−	4.3	0.5
24	» »		4.5	0.5

* calculated as cyanocobalamin in plate assay.

to be of interest to find a suitable cheap substitute for this substrate.

A preliminary series of experiments was devoted to finding ways for stimulating the production of vitamin B_{12} in the semi-synthetic medium used by other authors. This series consisted of three fermentations in 2 l conical flasks using the semi-synthetic medium * supplemented as indicated in Table 1. The fermentations lasted over a period of 12 days and were operated on a semi-continuous basis — 100 ml of the fermented medium was replaced every day by fresh medium. The glucose content was adjusted every day to 1 % and the pH to 6.6. The vitamin B_{12} activity was determined every day using *E. coli* 113—3 in

* Composition of the semi-synthetic medium: Casaminoacids Technical (Difco), 14 g; Bactogen (Vitrum), 12.5 g; $NaH_2PO_4 \cdot 2H_2O$, 2.2 g; K_2PO_4, 1.8 g; $MgCl_2 \cdot 6\ H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CoCl_2 \cdot 6H_2O$, 10 p. p.m.; dist. water to 1 000 ml.

cup plate assay and bioautography as described elsewhere [8]. The results are given in Table 1.

The *E. coli* activity obtained separated upon bioautography into four spots. One of them could be identified as cyanocobalamin and another one as factor B whereas the remaining two were slow moving spots similar to the spots corresponding to factors Z[8] or GDP-factor B[9,10]. Approximately 20–30 % of the activity was due to cyanocobalamin. The addition of DMB increased the cyanocobalamin content to about 50 % of the total *E. coli* activity but had a markedly inhibiting effect upon the total activity (*cf.* Table 1). The addition of corn steep liquor seemed to decrease somewhat the *E. coli* activity formed but had no effect on the distribution of this activity between the different factors.

The fermentation liquors contained, in addition, a factor with a growth inhibiting activity for *L. leichmannii* 313. This factor had a mobility similar to that of factor B upon electrophoresis at pH 2.5 (2 M HAc). The presence of similar inhibitors in cultures of vitamin B_{12}-producing organisms has been reported earlier from this laboratory and by other authors [11,12].

A second series of experiments was performed in 24 test tubes, each containing 20 ml medium. Two of the tubes contained the complete semi-synthetic medium while the remaining 22 tubes contained instead either different fractions of fresh and treated sewage or milk whey, yeast autolyzate or supernatant from cultures of a blue-green alga (*Anabaena*). Each of these substrates was used both without any further supplementation and also supplemented with the constituents of the synthetic medium apart from the casein hydrolyzates. The pH of all media was adjusted to 6.6 and their *E. coli* activity determined. No re-adjustments of the glucose content or pH were made and the fermentations were interrupted after 5 days. The results can be seen in Table 2.

It seems from Table 2 that the sewage fraction 4 supplemented as described above may provide a good substitute for casein hydrolyzates in the semi-synthetic medium. While, under the conditions described, a five days fermentation in the semi-synthetic medium gave only 0.5 μg *E. coli* activity per ml, a corresponding fermentation using the sewage fraction 4 gave 2.4 μg/ml. Bioautographic determinations did not reveal any significant differences in the distribution of the *E. coli* activity between different factors. It seems also that the semi-continuous method and repeated adjustments of pH and glucose content are of great importance for the yield of *E. coli* activity (*cf.* Tables 1 and 2).

Further experiments devoted to these problems are in progress and the results will be reported within the near future.

1. Leviton, A. and Hargrove, R. E. *Ind. Eng. Chem.* **44** (1952) 2651.
2. Janicki, J. and Pawelkiewicz, J. *Acta Biochim. Polon.* **1** (1954) 307.
3. Pawelkiewicz, J. *Acta Biochim. Polon.* **1** (1954) 313.
4. Janicki, J. and Pawelkiewicz, J. *Acta Biochim. Polon.* **2** (1955) 329.
5. Pawelkiewicz, J. and Nowakowska, K. *Acta Biochim. Polon.* **2** (1955) 259.
6. Pawelkiewicz, J. *Acta Biochim. Polon.* **2** (1955) 321.
7. Janicki, J. and Pedziwilk, Fr. *Acta Biochim. Polon.* **5** (1958) 299.
8. Neujahr, H. Y. *Acta Chem. Scand.* **10** (1956) 917.
9. DiMarco, A., Boretti, G., Migliacci, P. J. and Minghetti, A. *Boll. soc. ital. biol. sper.* **33** (1957) 1513.
10. Pawelkiewicz, J., Walerych, W. and Bartosinski, B. *Abstr. III Internat. Symp. Vit.* Poznan, Poland, p. 224.
11. Neujahr, H. Y. *Acta Chem. Scand.* **13** (1959) 1960.
12. Juillard, M. *Zentr. Bakteriol. Parasitenk.* Abt. II **110** (1957) 701.

Received December 7, 1959.

Inhibition of *Pediococcus cerevisiae* ATCC 8081 by Deoxyuridine

ARNE E. BOLINDER and WOLFGANG G. KURZ

Division of Food Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Plate assays for folinic acid factors and thymidine as well as the bioautography of these growth factors have been carried out in this laboratory using *Pediococcus cerevisiae (Leuconostoc citrovorum)* ATCC 8081 as the test organism [1-3]. This paper deals with the growth inhibitory effect of deoxyuridine on *P. cerevisiae* in cup plate and tube assays generally used for folinic acid * and thymidine determinations.

* Leucovorin (synthetic N^5-formyl tetrahydropteroylglutamic acid) was used as a source of folinic acid throughout these studies.

cup plate assay and bioautography as described elsewhere [8]. The results are given in Table 1.

The *E. coli* activity obtained separated upon bioautography into four spots. One of them could be identified as cyanocobalamin and another one as factor B whereas the remaining two were slow moving spots similar to the spots corresponding to factors Z[8] or GDP-factor B[9,10]. Approximately 20–30 % of the activity was due to cyanocobalamin. The addition of DMB increased the cyanocobalamin content to about 50 % of the total *E. coli* activity but had a markedly inhibiting effect upon the total activity (*cf.* Table 1). The addition of corn steep liquor seemed to decrease somewhat the *E. coli* activity formed but had no effect on the distribution of this activity between the different factors.

The fermentation liquors contained, in addition, a factor with a growth inhibiting activity for *L. leichmannii* 313. This factor had a mobility similar to that of factor B upon electrophoresis at pH 2.5 (2 M HAc). The presence of similar inhibitors in cultures of vitamin B_{12}-producing organisms has been reported earlier from this laboratory and by other authors [11,12].

A second series of experiments was performed in 24 test tubes, each containing 20 ml medium. Two of the tubes contained the complete semi-synthetic medium while the remaining 22 tubes contained instead either different fractions of fresh and treated sewage or milk whey, yeast autolyzate or supernatant from cultures of a blue-green alga (*Anabaena*). Each of these substrates was used both without any further supplementation and also supplemented with the constituents of the synthetic medium apart from the casein hydrolyzates. The pH of all media was adjusted to 6.6 and their *E. coli* activity determined. No re-adjustments of the glucose content or pH were made and the fermentations were interrupted after 5 days. The results can be seen in Table 2.

It seems from Table 2 that the sewage fraction 4 supplemented as described above may provide a good substitute for casein hydrolyzates in the semi-synthetic medium. While, under the conditions described, a five days fermentation in the semi-synthetic medium gave only 0.5 μg *E. coli* activity per ml, a corresponding fermentation using the sewage fraction 4 gave 2.4 μg/ml. Bioautographic determinations did not reveal any significant differences in the distribution of the *E. coli* activity between different factors. It seems also that the semi-continuous method and repeated adjustments of pH and glucose content are of great importance for the yield of *E. coli* activity (*cf.* Tables 1 and 2).

Further experiments devoted to these problems are in progress and the results will be reported within the near future.

1. Leviton, A. and Hargrove, R. E. *Ind. Eng. Chem.* **44** (1952) 2651.
2. Janicki, J. and Pawelkiewicz, J. *Acta Biochim. Polon.* **1** (1954) 307.
3. Pawelkiewicz, J. *Acta Biochim. Polon.* **1** (1954) 313.
4. Janicki, J. and Pawelkiewicz, J. *Acta Biochim. Polon.* **2** (1955) 329.
5. Pawelkiewicz, J. and Nowakowska, K. *Acta Biochim. Polon.* **2** (1955) 259.
6. Pawelkiewicz, J. *Acta Biochim. Polon.* **2** (1955) 321.
7. Janicki, J. and Pedziwilk, Fr. *Acta Biochim. Polon.* **5** (1958) 299.
8. Neujahr, H. Y. *Acta Chem. Scand.* **10** (1956) 917.
9. DiMarco, A., Boretti, G., Migliacci, P. J. and Minghetti, A. *Boll. soc. ital. biol. sper.* **33** (1957) 1513.
10. Pawelkiewicz, J., Walerych, W. and Bartosinski, B. *Abstr. III Internat. Symp. Vit.* Poznan, Poland, p. 224.
11. Neujahr, H. Y. *Acta Chem. Scand.* **13** (1959) 1960.
12. Juillard, M. *Zentr. Bakteriol. Parasitenk.* Abt. II **110** (1957) 701.

Received December 7, 1959.

Inhibition of *Pediococcus cerevisiae* ATCC 8081 by Deoxyuridine

ARNE E. BOLINDER and WOLFGANG G. KURZ

Division of Food Chemistry, Royal Institute of Technology, Stockholm 70, Sweden

Plate assays for folinic acid factors and thymidine as well as the bioautography of these growth factors have been carried out in this laboratory using *Pediococcus cerevisiae (Leuconostoc citrovorum)* ATCC 8081 as the test organism [1-3]. This paper deals with the growth inhibitory effect of deoxyuridine on *P. cerevisiae* in cup plate and tube assays generally used for folinic acid * and thymidine determinations.

* Leucovorin (synthetic N^5-formyl tetrahydropteroylglutamic acid) was used as a source of folinic acid throughout these studies.

For the cup plate tests and the bioautography large plates were used, each containing 150 ml of agar medium inoculated with *P. cerevisiae* [1]. Three types of media were used; unsupplemented media and media supplemented with either 15 mμg of leucovorin or 30 μg of thymidine per plate. The plates with supplemented media were used in order to obtain a good background growth and thereby give a more pronounced appearance of growth inhibition. For the cup plate tests, 0.03 ml aliquots of the test solutions were pipetted into cups, 6 mm in diameter, and the plates were incubated over night at 37°C. Tube tests were made using a final volume of 10 ml per tube. After suitable periods of incubation at 37°C, cell growth was measured turbidimetrically. For making the plates and the tube assays, washed and optically standardized inocula were used. A detailed report of these assays will be published later in this journal.

The growth inhibitory effect of deoxyuridine was discovered while investigating the contamination of a deoxyuridine preparation by thymidine. When checking the results obtained bioautographically with *Lactobacillus leichmannii* 313 ATCC 7830 as the test organism [2] (which responds to vitamin B_{12}-factors, deoxyribonucleosides, and deoxyribonucleotides) against bioautography with *P. cerevisiae*, we found that deoxyuridine produced a spot of nearly complete growth inhibition in the background growth of the *P. cerevisiae* plates made with unsupplemented media or with media containing leucovorin *. Bioautography using four different solvent systems showed that the UV-absorbing spot of deoxyuridine always corresponded to the growth inhibition spot in the *P. cerevisiae* plates and to a dense growth spot in the *Lb. leichmannii* 313 plates. In the *P. cerevisiae* plates, 3—4 μg of deoxyuridine could be detected bioautographically, which was the amount required for detection by in-spection in UV-light.

In the cup plate tests, deoxyuridine formed inhibition zones only when tested at concentrations of 100 μg/ml and above. In typical cup tests made in *P. cerevisiae* plates using unsupplemented media or media supplemented with leucovorin, the almost completely clear inhibition zones with fairly well defined zone edges produced by deoxyuridine (100 and 1 000 μg/ml) measured 17 mm and 27 mm, respectively. The growth inhibition produced by deoxyuridine was reversed by leucovorin or by thymidine, if either of the latter compounds were present in sufficient concentration.

In the *P. cerevisiae* plates with unsupplemented media leucovorin produced zones of heavy growth, whereas thymidine produced somewhat fainter growth zones.

In a series of cup plate experiments several nucleic acid compounds were tested for growth stimulation or growth inhibition using the three types of *P. cerevisiae* media. However, of all the compounds tested, deoxyuridine was the only one that showed strong growth inhibition. Very faint and diffuse inhibition zones were formed by uridine, cytidine, adenosine, and guanosine when these compounds were tested at concentrations of 1 mg/ml. These inhibitions were also reversed by leucovorin or thymidine.

Small zones of growth stimulation were formed by 5-methyldeoxycytidine (500 μg/ml) which was confirmed bioautographically. This finding is under further investigation.

No inhibition zones could be detected in the plates when the following compounds were tested separately at concentrations of 1 mg/ml (if not otherwise stated in brackets): (a) uracil (500 μg/ml), cytosine, 5-methylcytosine, thymine (500 μg/ml), orotic acid (500 μg/ml), dihydrouracil, and dihydrothymine, (b) adenine (500 μg/ml), guanine (200 μg/ml), hypoxanthine (500 μg/ml), and xanthine (200 μg/ml), (c) deoxycytidine, deoxyadenosine, deoxyguanosine, and deoxyinosine, (d) inosine and xanthosine (500 μg/ml), (e) the 5′-monophosphates of thymidine, deoxyuridine, deoxycytidine, 5-methyldeoxycytidine (500 μg/ml), deoxyadenosine, and deoxyguanosine, (f) the 5′-monophosphates of uridine, cytidine, adenosine, guanosine, and inosine, (g) uridylic, cytidylic, adenylic, and guanylic acids (mixtures of the 2′- and 3′-monophosphates), (h) the cyclic 2′,3′-monophosphates of uridine, cytidine, and adenosine, and (i) the 5′-di- and -tri-phosphates of adenosine and inosine. Each of the compounds uracil, adenine, guanine, and xanthine were present in the basal medium at a concentration of 10 μg/ml.

The growth inhibitory activity of deoxyuridine and some other nucleic acid compounds was also studied by turbidimetric tube tests using *P. cerevisiae*. Read-

* In *P. cerevisiae* plates made with media containing thymidine, only faint zones of growth inhibition were formed by deoxyuridine in cup tests or bioautographic tests.

ings were made after 24, 48, and 68 h of incubation. For these tube tests a batch of deoxyuridine free of contaminating thymidine was prepared by chromatography on Whatman No. 3MM filter paper followed by elution with distilled water. We found that the growth promoting effects of suboptimal amounts of leucovorin or thymidine were strongly inhibited by deoxyuridine and to a much lesser extent by uridine and adenosine. Under the same testing conditions cytidine, guanosine, and inosine were only very slightly growth inhibitory, whereas no growth inhibition was caused by deoxycytidine, deoxyadenosine, deoxyguanosine or xanthosine. These findings are in accordance with the plate tests mentioned above.

The growth promoting effect of suboptimal amounts of leucovorin was inhibited noncompetitively by deoxyuridine, the inhibition index * increased as the amount of leucovorin was increased in the tubes. However, the growth promoting effect of suboptimal amounts of thymidine was competitively inhibited by deoxyuridine. An inhibition index of about 30 was obtained after 48 h of incubation when thymidine was present in the medium in amounts of 0.1 to 3 μg per tube. Larger amounts of thymidine increased the inhibition index. No inhibitions occurred in the tube tests when leucovorin or thymidine were present in amounts sufficient to promote optimal growth of *P. cerevisiae*.

Earlier work on the nutritional requirements of *P. cerevisiae* using the tube assay method, have shown that thymidine was the only deoxyribonucleoside which supported growth when substituted for leucovorin [4,5]. However, in these investigations only low concentrations of deoxyribonucleosides were tested. Some synthetic nucleosides of thymine (*e. g.* 1-D-glycosylthymine) have been reported to have a slight inhibitory effect upon the growth of *P. cerevisiae* when cultured in the presence of limiting amounts of thymidine [6].

Further work is needed before any conclusions can be drawn concerning the inhibition mechanism of *P. cerevisiae* by deoxyuridine. Our findings for *P. cerevisiae* are very similar to those obtained by Lansford *et al.*[7] using 2,4-diamino-6,7-diphenylpteridine-inhibited *Lactobacillus arabinosus*. This latter is a microbiological testing system in which the biosynthesis of thymidine is inhibited. Thus, these workers found that deoxyuridine was the only deoxyribonucleoside that showed any growth inhibition. Adenosine, inosine, guanosine, cytidine, and uridine were also found to be growth inhibitory, whereas deoxyadenosine, deoxyguanosine, deoxycytidine, and xanthosine showed neither inhibitory nor stimulatory effects upon growth. However, we found that no inhibition of growth was exerted by kinetin (100, 300, and 1 000 μg/ml) in plate tests with *P. cerevisiae*. This is in contrast to the *Lb. arabinosus* testing system of Lansford *et al.*[7] in which kinetin and related compounds showed growth inhibitory effects.

* The inhibition index was calculated as the molar ratio of deoxyuridine to growth factor, present in the medium at 50 % inhibition of growth.

1. Bolinder, A., Widoff, E. and Ericson, L.-E. *Arkiv Kemi* **6** (1953) 487.
2. Ericson, L.-E. *Arkiv kemi* **6** (1953) 503.
3. Bolinder, A., Kurz, W. and Lundin, H. *J. Inst. Brewing* **62** (1956) 497.
4. Kitay, E., MacNutt, W. S. and Snell, E. E. *J. Biol. Chem.* **177** (1949) 993.
5. MacNutt, W. S. *Biochem. J.* **50** (1951) 384.
6. Sauberlich, H. E. *Arch. Biochem.* **24** (1949) 224.
7. Lansford, E. M., Skinner, C. G. and Shive. W. *Arch. Biochem. Biophys.* **73** (1958) 191.

Received December 10, 1959.