ACTA CHEMICA SCANDINAVICA 2 (1948) 1—14

On Hydrogen Peroxide Decomposition

Ferric Salt Catalysis

V. STEN ANDERSEN

The Tecnical University of Denmark, Chemistry Department A, and The Royal Danish School of Pharmacy, Department of Inorganic Chemistry, Copenhagen, Denmark

Bertalan [1] has investigated the reaction

$$2\ H_2O_2 = 2\ H_2O + O_2$$

in water acidified with sulphuric acid, and with ferrous as well as ferric sulphate as catalyst. He is of the opinion of having shown that:

1. The course of the process is independent of the oxidation state of iron, because immediately after addition hydrogen peroxide will oxidize ferrous iron to ferric iron.

2. The reaction is of the first order.

3. The rate of decomposition is directly proportional to the concentration of ferric salt, but inversely proportional to the concentration of hydrogen ions.

Bohnson and Robertson [2] have put forward the hypothesis, that ferric acid (H_2FeO_4) is formed by interaction between hydrogen peroxide and ferric salt, the ferric acid being subsequently reduced with the evolution of oxygen. They have tested this hypothesis by comparing the absorption spectra of solutions of 1) barium ferrate, 2) potassium ferrate, 3) mixtures of hydrogen peroxide and ferric chloride, and 4) ferric chloride alone. Furthermore they have investigated the effect of neutral salts on the rate of the reaction and found, that addition of potassium chloride and potassium nitrate has an only slight effect, while addition of potassium sulphate and especially of potassium phosphate causes a considerable decrease in the velocity constant, possibly due to the removal of iron as a complex ion. Bohnson and Robertson have calculated the rate constants on the basis of the amount of oxygen evolved in the interval between 25 % to 50 % destruction of the hydrogen peroxide initially present.

Simon and co-workers [3] have investigated the effect of diverse ferric salts on the hydrogen peroxide decomposition. They have shown, that the catalytic effect of ferric salts decreases with increasing degree of complexity.

Haber and Weiss [4] discuss on the basis of measurements, done partly by themselves and partly by other investigators, the mechanism of reaction of the hydrogen peroxide decomposition in the presence of ferrous and ferric salts and propose, that the ferric salt catalysed reaction takes place in the following steps:

$$Fe^{+3} + HO_2^- = Fe^{+2} + HO_2$$
$$HO_2 + H_2O_2 = O_2 + H_2O + OH$$
$$OH + H_2O_2 = H_2O + HO_2$$

EXPERIMENTAL

In this paper the hydrogen peroxide decomposition in presence of ferric ions, the solution acidified with nitric acid, is subjected to a critical investigation. As source of ferric ions is used ferric nitrate, as this can be obtained pure and the nitrate ion usually forms complex compounds only to a small extent.

Reagents

Hydrogen peroxide 30 %, *pro analysi*, Merck.

0.1 *M* nitric acid; the titer of the acid was determined by titration of sodium tetraborate (tested purity, Danish Pharmacopoeia, 1933).

0.1 *M* ferric nitrate solution, which at the same time is 0.1 *M* as to nitric acid. This solution was made by dissolving 40.40 *g* ferric nitrate [$Fe(NO_3)_3 + 9\ H_2O$, *pro analysi*, Merck] in 100.0 ml 1 *M* nitric acid and then diluting with redestilled water to 1000 ml in a volumetric flask. The ferric ion content was controlled by a gravimetric iron determination.

0.1 *N* (0.02 *M*) potassium permanganate solution; the titer was determined by titration of sodium oxalate (Sørensen).

All water used was redistilled water, made in a glass distilling apparatus.

Experimental study of the rate of reaction

The reacting mixture was made by mixing equal volumes of 1) a solution (hereafter called catalyst mixture) containing ferric nitrate and nitric acid, and 2) a solution of hydrogen peroxide and nitric acid, the concentration of nitric acid being the same as in the catalyst mixture.

1. The catalyst mixture was prepared from ferric nitrate solution acidified with nitric acid, 0.1 *M* nitric acid and redistilled water in a 250 ml volumetric flask. After mixing carefully the solution was poured into a one liter bottle (pyrex), which then was mounted in a water thermostat at the required temperature.

2. For each experiment 500 ml hydrogen peroxide solution was made by mixing 30 %'s hydrogen peroxide, redistilled water and as much of 0.1 *M* nitric acid, that the concentration of nitric acid was exactly the same as in the catalyst mixture. 250 ml was then placed in a 250 ml volumetric flask, which was mounted in the water thermostat next to the catalyst mixture. The rest of the hydrogen peroxide solution was used for determination of the concentration of hydrogen peroxide by titration with 0.1 *N* potassium permanganate.

The experiments themselves were started by emptying — as fast as possible — the volumetric flask of hydrogen peroxide into the bottle with the catalyst mixture, which was kept steadily moving by rotating the bottle. The mixing lasts about 8 seconds. From time to time a sample of the reacting mixture is taken out with a 30 ml pipette, the delivery orifice of which being fairly large. The sample is at once allowed to run into a previously tared Erlenmeyer flask containing 20 ml 2 *M* sulfuric acid; hereby the reaction will practically stop. The flask is now weighed within an accuracy of 0.01 g. Due to the content of oxygen bubbles in the reacting mixture it was impossible to take out an accurate volume of the solution.

The hydrogen peroxide content was determined by titration with 0.1 *N* potassium permanganate; in cases, where the amount of permanganate required was less than 10 ml, a 10 ml burette, the smallest subdivision being 0.02 ml, was used.

On the basis of the titration results the concentration of non-decomposed hydrogen peroxide at the various times was calculated. The concentration of hydrogen peroxide to time zero was calculated from the direct titration of the hydrogen peroxide solution used.

CALCULATIONS

The decomposition of hydrogen peroxide can not — as Bertalan asserted — be satisfactorily calculated from the kinetic equation for a reaction of the first order:

$$0.4343 \cdot k \cdot t = \log \frac{a}{x} \qquad (1)$$

where a is the initial molar concentration of hydrogen peroxide and x the concentration at the time t. This equation requires, that a plot of $\log \frac{a}{x}$ against t should be linear if the reaction is of first order.

In Table 1 is given the experimental results from ferric ion catalysed hydrogen peroxide decomposition at 25° C, in a solution which is 0.004 *M* as to ferric nitrate and 0.01 *M* as to nitric acid. In column 4 is given the values found of $\log \frac{a}{x}$. These values are in Fig. 1 plotted against the corresponding t values, and the points hereby produced are fitted with a curve (marked I). This is not a straight line plot, but drops towards the t-axis with increasing t values. The reason, why Bertalan has not noticed this phenomenon, is probably, that he has only followed the reaction to 80 % decomposition of the hydrogen peroxide.

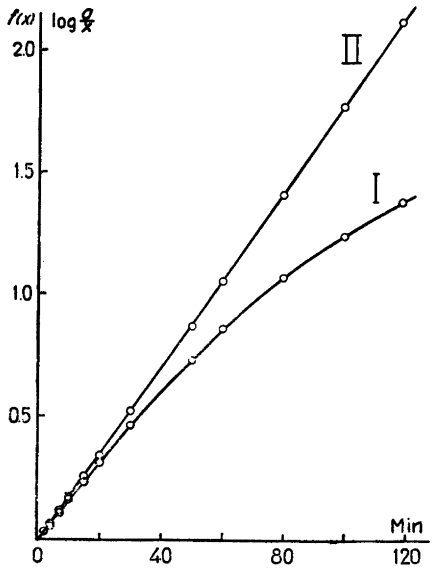


Fig. 1. The decomposition of hydrogen peroxide at 25° C in solution 0.004 M as to ferric nitrate and 0.01 M as to nitric acid. Curve I is a plot of $\log \frac{a}{x}$ *against time t. Curve II represents the function* $(fx) = \log \frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right)$ *plotted against time t;* $B \cdot t + \tau = f(x)$; $A = 1.5 \cdot 10^{-3}$; $B = 1.78 \cdot 10^{-2}$ *and* $\tau = -0.01$.

A function of $c_{H_2O_2}$, which to its whole extent except in the very beginning, is a linear function of time, is the following expression:

$$f(x) = B \cdot t + \tau = \log \frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right) \qquad (2)$$

where t, a and x have the same meanings as in (1), and A and B are constants.

The constants A and B are calculated by substituting A in (2) with different values, until a plot of f (x) against t is a straight line; B will then be the slope of this line. The significance of the deviations in the very beginning of an experiment will be dealt with later. While B in this way can be calculated with a reasonable accuracy, that will not apply to A, which roughly estimated may vary up to 15 % without much effect on the magnitude of B. The calculated values of A and B are given in the text to Table 1. In column 8 are given the values of $f(x) = \log \frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right)$ calculated from the found value of A. For sake of control the values of $t = \frac{1}{B} \cdot [f(x) - \tau]$ are calculated by substituting B with the found value (column 9).

A comparison of columns 1 and 9 together with the graph shows the applicability of the calculation method.

Table 1. The decomposition of hydrogen peroxide at 25° C in solution 0.004 M as to ferric nitrate and 0.01 M as to nitric acid. The experiments are calculated after (2). In column 8 is given $f(x) = \log\frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right)$; *the constant A is calculated graphically, so that f(x) becomes a linear function of t, i. e.* $B \cdot t + \tau = f(x)$. *(cf. Fig. 1).* $A = 1.5 \cdot 10^{-3}$, $B = 1.78 \cdot 10^{-2}$, $\tau = -0.01$.

1	2	3	4	5	6	7	8	9
t min	$x = c_{H_2O_2}$	log x	$\log\frac{a}{x}$	$\frac{1}{x}$	$\frac{1}{x} - \frac{1}{a}$	(6) · A	$f(x) =$ (4)+(7)	t
0.0	0.04710	0.6730-2		21.23				
2.0	0.04426	0.6460	0.0270	22.59	1.36	0.0020	0.0290	2.2
4.0	0.04139	0.6169	0.0561	24.16	2.93	0.0044	0.0605	4.0
7.0	0.03707	0.5690	0.1040	26.98	5.75	0.0086	0.1126	6.9
10.0	0.03264	0.5137	0.1593	30.64	9.41	0.0141	0.1734	10.3
15.0	0.02754	0.4400	0.2330	36.31	15.08	0.0226	0.2556	14.9
20.0	0.02305	0.3627	0.3103	43.38	22.15	0.0333	0.3436	19.9
30.0	0.01622	0.2101	0.4629	61.65	40.42	0.0606	0.5235	30.0
40.0	0.01168	0.0675	0.6055	85.62	64.39	0.0966	0.7021	40.0
50.0	0.008672	0.9431-3	0.7299	115.31	94.08	0.1411	0.8710	49.5
60.0	0.006554	0.8165	0.8565	152.57	131.34	0.1970	1.0535	59.7
80.0	0.004021	0.6044	1.0686	248.69	227.46	0.3413	1.4099	79.7
100.0	0.002686	0.4291	1.2439	372.30	351.07	0.5266	1.7705	100.0
119.0	0.001959	0.2920	1.3810	510.72	489.49	0.7343	2.1153	119.3

EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON RATE OF REACTION

In order to establish the effect of the hydrogen peroxide concentration two experiments were carried out, one 0.05 *M* and the other 0.009 *M* as to hydrogen peroxide. The molar concentration of ferric nitrate was 0.004 and that of nitric acid 0.01 in both experiments. The calculations were carried out according to (2) and the results are given in Table 2.

Table 2. The effect of hydrogen peroxide concentration on the constants A and B. 25° C.

$c_{H_2O_2}$	$c_{Fe(NO_3)_3}$	c_{HNO_3}	A	B
0.009	0.004	0.01	$1.5 \cdot 10^{-3}$	$1.78 \cdot 10^{-2}$
0.05	0.004	0.01	$1.5 \cdot 10^{-3}$	$1.78 \cdot 10^{-2}$

As appears from the table, the constants A and B are independent of the initial concentration of hydrogen peroxide.

EFFECT OF FERRIC NITRATE CONCENTRATION ON RATE OF REACTION

A series of experiments were run, in which all initial concentrations — except the ferric nitrate concentration — were the same; the calculations were carried out according to (2), and the results are given in Table 3. The table shows, that A and B are directly proportional to the concentration of ferric nitrate, *i. e.*

$$A = \alpha \cdot c_{\text{Ferric}} \qquad (3) \qquad \text{and } B = \beta \cdot c_{\text{Ferric}} \qquad (4)$$

Table 3. Decomposition at 25° C of hydrogen peroxide in solution 0.01 M as to nitric acid, but with varying amounts of ferric nitrate added. The constants A and B are calculated according to (2).

$c_{H_2O_2}$	c_{Ferric}	c_{HNO_3}	A	B	$\frac{A}{c_{\text{Ferric}}}$	$\frac{B}{c_{\text{Ferric}}}$
0.05	0.001	0.01	0.00040	0.0045	0.40	4.50
0.05	0.002	0.01	0.00075	0.0089	0.38	4.45
0.05	0.003	0.01	0.00108	0.0132	0.36	4.40
0.05	0.004	0.01	0.00150	0.0176	0.38	4.40

EFFECT OF HYDROGEN ION CONCENTRATION ON RATE OF REACTION

In order to establish the effect of the hydrogen ion concentration on the rate of reaction four experiments were carried out. The initial concentrations of hydrogen peroxide and ferric nitrate were the same, but varying amounts of nitric acid added. On the basis of the experimental results the constants A and B were calculated according to (2). The results are given in Table 4, columns 4 and 5. The figures in columns 6, 7, 8 and 9 will be dealt with later.

Table 4. Effect of hydrogen ion concentration at 25° C on the rate of reaction. The constants A and B are calculated according to (2).

1	2	3	4	5	6	7	8	9
$c_{H_2O_2}$	$c_{Fe(NO_3)_3}$	c_{HNO_3}	A	B	$\frac{1}{B}$	A Corr.	B Corr.	$\frac{1}{B}$ Corr.
0.05	0.004	0.01	$1.50 \cdot 10^{-3}$	$1.76 \cdot 10^{-2}$	56.8	$1.50 \cdot 10^{-3}$	$1.76 \cdot 10^{-2}$	56.8
0.05	0.004	0.02	$1.50 \cdot 10^{-3}$	$1.00 \cdot 10^{-2}$	100	$1.64 \cdot 10^{-3}$	$1.01 \cdot 10^{-2}$	99
0.05	0.004	0.03	$1.50 \cdot 10^{-3}$	$0.67 \cdot 10^{-2}$	149	$1.70 \cdot 10^{-3}$	$0.68 \cdot 10^{-2}$	147
0.06	0.004	0.04	$1.50 \cdot 10^{-3}$	$0.52 \cdot 10^{-2}$	192	$1.72 \cdot 10^{-3}$	$0.53 \cdot 10^{-2}$	189

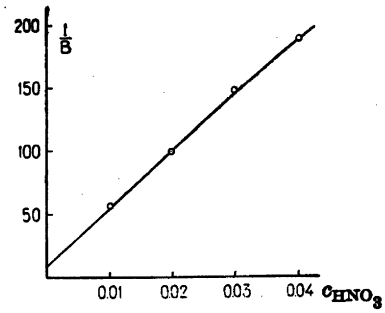


Fig. 2. Relation between the constant B and the concentration of nitric acid, the concentration of ferric nitrate being 0.004 M.

The table shows, that the concentration of nitric acid, *i. e.* the concentration of hydrogen ions, has a marked influence on the constant B, but that A, within the accuracy of the method, is independent of the hydrogen ion concentration. From Fig. 2, in which $\frac{1}{B}$ is plotted against c_{HNO_3}, it is evident, that

$$\frac{1}{B} = \gamma \cdot c_{HNO_3} + K \tag{5}$$

γ is the slope of the straight line and K the intercept on $\frac{1}{B}$-axis.

In the solutions in question it is allowable to put c_{HNO_3} equal to c_{H^+}. (5) can then be written:

$$\frac{1}{B} = \gamma \cdot c_{H^+} + K \tag{5a}$$

or

$$B = \frac{1}{\gamma} \cdot \frac{1}{c_{H^+} + K'} \tag{5b}$$

On Fig. 2 can be measured: $K = 0.9$ and $\gamma = 4600$, which gives $K' = 1.96 \cdot 10^{-3}$.

EXPERIMENTAL RESULTS SUMMARIZED

1. The ferric ion catalysed decomposition of hydrogen peroxide, the reacting solution being acidified with nitric acid, can be calculated from

$$f(x) = B \cdot t + \tau = \log \frac{a}{x} + A \left(\frac{1}{x} - \frac{1}{a} \right) \tag{2}$$

The straight line $f(x)$ does not pass through the origin of the coordinates, but τ is the intercept on the ordinate-axis. That it is not possible to calculate A and B so, that $f(x)$ passes through the origin, may be due to the start of the process having a certain — but usually rather small — time lag.

2. The constants A and B are independent of the initial concentration of hydrogen peroxide.

3. At constant concentration of hydrogen ions the constants A and B are proportional to the concentration of ferric nitrate.

4. The constant A is — within the accuracy of the calculation method — independent of the hydrogen ion concentration, while B is approximately inversely proportional to the latter.

THE REACTION MECHANISM

Upon differentiation of (2) with respect to x is found:

$$-B \cdot \frac{dt}{dx} = 0.4343 \frac{1}{x} + A \cdot \frac{1}{x^2} \tag{6}$$

or

$$\frac{1}{s} = -\frac{dt}{dx} = \frac{0.4343}{B \cdot x} + \frac{0.4343 \cdot A}{B \cdot 0.4343 \cdot x^2} \tag{6a}$$

where $\frac{1}{s}$ is the reciprocal velocity.

The simplest way, in which to explain the expression (6a), is to assume the following mechanism:

$$Fe^{+3} + HO_2^- \underset{-1}{\overset{1}{\rightleftharpoons}} FeOH^{+2} + O \qquad \text{I}$$

$$O + HO_2^- \xrightarrow{2} OH^- + O_2 \qquad \text{II}$$

while we at the same time have the equilibria:

$$Fe^{+3} + H_2O \rightleftharpoons FeOH^{+2} + H^+ \qquad \text{III}$$

$$H_2O_2 \rightleftharpoons HO_2^- + H^+ \qquad \text{IV}$$

When assuming, as originally proposed by Bodenstein [5], that soon after starting the reaction, a stationary state is attained, in which the velocity of consumption of the instable intermediate (here the oxygen atoms) is just the same as the velocity of the production, the expression for the reciprocal velocity can be written (Christiansen [6]):

$$\frac{1}{s'} = \frac{dt}{d[O_2]} = -2\frac{dt}{d[H_2O_2]} = \frac{1}{k_1 \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} + \frac{k_{-1} \cdot c_{FeOH^{+2}}}{k_1 \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} \cdot \frac{1}{k_2 \cdot c_{HO_2^-}} \quad (7)$$

or
$$-\frac{dt}{d[H_2O_2]} = \frac{1}{2 \cdot k_1 \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} + \frac{k_{-1} \cdot c_{FeOH^{+2}}}{2 \cdot k_1 \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} \cdot \frac{1}{k_2 \cdot c_{HO_2^-}} \quad (7a)$$

The expression for the equilibrium constant of equation III, which correctly ought to be written:

$$Fe(H_2O)_6^{+3} \rightleftharpoons FeOH(H_2O)_5^{+2} + H^+$$

is abbreviated to:

$$\frac{c_{FeOH^{+2}} \cdot c_{H^+}}{c_{Fe^{+3}}} = K_{Fe} \quad (8)$$

and for IV we have:

$$\frac{c_{H^+} \cdot c_{HO_2^-}}{c_{H_2O_2}} = K_{H_2O_2} \quad (9)$$

From (8) is derived — when $c_{Ferric} = c_{Fe^{+3}} + c_{FeOH^{+2}}$

$$c_{FeOH^{+2}} = \frac{K_{Fe}}{K_{Fe} + c_{H^+}} \cdot c_{Ferric} \quad (10)$$

$$c_{Fe^{+3}} = \frac{c_{H^+}}{K_{Fe} + c_{H^+}} \cdot c_{Ferric} \quad (11)$$

and from (9) is derived:

$$c_{HO_2^-} = K_{H_2O_2} \cdot \frac{c_{H_2O_2}}{c_{H^+}} \quad (12)$$

(7a) modified by the introduction of (10), (11) and (12) gives

$$-\frac{dt}{d[H_2O_2]} = \frac{K_{Fe} + c_{H^+}}{2k_1 \cdot K_{H_2O_2} \cdot c_{Ferric}} \cdot \frac{1}{c_{H_2O_2}} + \frac{K_{Fe} + c_{H^+}}{2k_1 \cdot K_{H_2O_2} \cdot c_{Ferric}} \cdot \frac{k_{-1} \cdot K_{Fe} \cdot c_{H^+} \cdot c_{Ferric}}{k_2 \cdot K_{H_2O_2} \cdot (K_{Fe} + c_{H^+})} \cdot \frac{1}{c_{H_2O_2}^2} \quad (13)$$

If the reaction mechanism, as proposed in I and II, is true, (13) and (6a) ought to be identical, *i. e.*

$$B = 0.4343 \cdot \frac{2k_1 \cdot K_{H_2O_2}}{K_{Fe} + c_{H^+}} \cdot c_{Ferric} \quad (14)$$

$$A = 0.4343 \cdot \frac{k_{-1} \cdot K_{Fe}}{k_2 \cdot K_{H_2O_2}} \cdot \frac{c_{H^+}}{K_{Fe} + c_{H^+}} \cdot c_{Ferric} \quad (15)$$

Consequently B has to be directly proportional to the ferric salt concentration and inversely proportional to $K_{Fe} + c_{H^+}$, which is in accordance with the experimental results as expressed in (4) and (5b), when assuming that the constant $K' = 1.96 \cdot 10^{-3} = 10^{-2.7}$ in (5b) is the first acid dissociation constant of the hexaaquo ferric ion — *i. e.* K_{Fe} in (8). This constant has previously been determined as $10^{-2.5}$ at 25° C by Lamb and Jaques [7] by means of a glass electrode in solutions containing ferric nitrate and nitric acid.

A has to be directly proportional to the concentration of ferric salt, which is in agreement with the experiments (*cf.* (3)); furthermore A has to be proportional to the term $\frac{c_{H^+}}{K_{Fe} + c_{H^+}}$, *i. e.* in strong acid solution practically independent of the hydrogen ion concentration. In the experiments dealt with in Table 4 the factor of proportionality ought to vary with the hydrogen ion concentration as shown in Table 5. Therefore the variations in A are not

Table 5 The variation of $\frac{c_{H^+}}{K_{Fe^+} + c_{H^+}}$ *(15) with different* c_{H^+}; $K_{Fe} = 2 \cdot 10^{-3}$.

c_{H^+}	0.01	0.02	0.03	0.04
$\frac{c_{H^+}}{K_{Fe} + c_{H^+}}$	0.83	0.91	0.94	0.95

bigger than can be concealed by the errors of determination. If the A value, which is calculated on the basis of the investigation of hydrogen peroxide decomposition in 0.01 M nitric acid (*cf.* Table 1 and Fig. 1), is regarded to be just about true, and that seems reasonable, because the time lag in this experiment is rather small, then the other A values and the corresponding B values in Table 4 are to be corrected in accordance with Table 5. These corrected values are shown in Table 4, columns 7 and 8. If — graphically (*cf.* Fig. 2) — the relation between the corrected B values and the hydrogen ion concentration is found, the relationship still agrees with (5a); the slope of the straight line will be slightly different, but K' in (5b) does not alter noticeably.

From the above conciderations it will be clear, that nothing in the experimental results goes against the proposed reaction mechanism I and II.

It has to be emphasized, that the above does not invalidate the theory of Bohnson and Robertson about the formation of ferric acid as an intermediate. If the mechanism of the reaction is supposed to be:

$$Fe^{+3} + HO_2^- \underset{-1a}{\overset{1a}{\rightleftharpoons}} FeOOH^{+2} \qquad \text{Ia}$$

$$FeOOH^{+2} \underset{-1b}{\overset{1b}{\rightleftharpoons}} FeOH^{+2} + O \qquad \text{Ib}$$

$$O + HO_2^- \overset{2}{\rightarrow} OH^- + O_2 \qquad \text{II}$$

then the expression for the reciprocal velocity is:

$$\frac{1}{s} = \frac{1}{k_{1a} \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} \cdot \left(1 + \frac{k_{-1a}}{k_{1b}}\right) + \frac{1}{k_{1a} \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} \cdot \frac{k_{-1a} \cdot k_{-1b} \cdot c_{FeOH^{+2}}}{k_{1b} \cdot k_2 \cdot c_{HO_2^-}} \qquad (16)$$

That is an expression analogous to (7).

But the postulate of Bohnson and Robertson, that the ferric acid immediately will be reduced by hydrogen peroxide, does not seem to agree with the experiments. If we assume the reaction mechanism to be:

$$Fe^{+3} + HO_2^- \underset{-1a}{\overset{1a}{\rightleftharpoons}} FeOOH^{+2} \qquad \text{Ia}$$

$$FeOOH^{+2} + H_2O_2 \overset{2a}{\rightarrow} FeOH^{+2} + O_2 + H_2O \qquad \text{IIa}$$

then the expression for the reciprocal velocity is:

$$\frac{1}{s} = \frac{1}{k_{1a} \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} + \frac{1}{k_{1a} \cdot c_{Fe^{+3}} \cdot c_{HO_2^-}} \cdot \frac{k_{-1a}}{k_{2a} \cdot c_{H_2O_2}} \qquad (17)$$

This would imply, that A in (6) had to be independent of the ferric salt concentration, which does not agree with (3).

CALCULATION OF THE VELOCITY CONSTANTS

According to (14) and (15) we have:

$$B = 0.4343 \cdot 2 \cdot k_1 \cdot K_{H_2O_2} \cdot \frac{c_{Ferric}}{K_{Fe} + c_{H^+}} \qquad (14)$$

and

$$A = 0.4343 \cdot \frac{k_{-1} \cdot K_{Fe}}{k_2 \cdot K_{H_2O_2}} \cdot \frac{c_{H^+}}{K_{Fe} + c_{H^+}} \cdot c_{Ferric} \qquad (15)$$

or when $K_{Fe} = 2 \cdot 10^{-3}$ and $K_{H_2O_2} = 2.4 \cdot 10^{-12}$ at 25° C [8]):

$$0.4343 \cdot k_1 = \frac{B}{2 \cdot 2.4 \cdot 10^{-12}} \cdot \frac{2 \cdot 10^{-3} + c_{H+}}{c_{Ferric}}$$

$$0.4343 \cdot \frac{k_{-1}}{k_2} = A \cdot \frac{2.4 \cdot 10^{-12}}{2 \cdot 10^{-3}} \cdot \frac{2 \cdot 10^{-3} + c_{H+}}{c_{H+} \cdot c_{Ferric}}$$

In Table 6 are given the values of $0.4343 \cdot k_1$ and $0.4343 \cdot \frac{k_{-1}}{k_2}$ calculated on the basis of the results in Table 3.

Table 6. $0.4343 \cdot k_1$ and $0.4343 \cdot \frac{k_{-1}}{k_2}$ calculated on the basis of Table 3.

1	2	3	4	5	6
c_{H+}	c_{Ferric}	A	B	$0.4343 \cdot k_1$	$0.4343 \cdot \frac{k_{-1}}{k_2}$
0.01	0.001	0.00040	0.0045	$1.13 \cdot 10^{10}$	$5.8 \cdot 10^{-10}$
0.01	0.002	0.00075	0.0089	$1.11 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$
0.01	0.003	0.00108	0.0132	$1.10 \cdot 10^{10}$	$5.2 \cdot 10^{-10}$
0.01	0.004	0.00150	0.0176	$1.10 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$

In table 7 are given the values of $0.4343 \cdot k_1$ and $0.4343 \cdot \frac{k_{-1}}{k_2}$ calculated on the basis of the results in Table 4.

Table 7. $0.4343 \cdot k_1$ and $0.4343 \cdot \frac{k_{-1}}{k_2}$ calculated on the basis of the results in Table 4.

c_{H+}	c_{Ferric}	A Corr.	B Corr.	$0.4343 \cdot k_1$	$0.4343 \cdot \frac{k_{-1}}{k_2}$
0.01	0.004	$1.50 \cdot 10^{-3}$	0.0176	$1.10 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$
0.02	0.004	$1.64 \cdot 10^{-3}$	0.0101	$1.14 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$
0.03	0.004	$1.70 \cdot 10^{-3}$	0.0068	$1.13 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$
0.04	0.004	$1.72 \cdot 10^{-3}$	0.0053	$1.16 \cdot 10^{10}$	$5.4 \cdot 10^{-10}$

SUMMARY

The decomposition of hydrogen peroxide in acid solution in the presence of ferric nitrate is investigated at 25° C.

It is shown that:

1. The process is not of the first order, but the experimental results can be calculated from the empiric expression:

$$B \cdot t + \tau = \log \frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right) \qquad (2)$$

in which a is the initial concentration of hydrogen peroxide, and x is the concentration after the lapse of time t. A and B are constants, which can be calculated graphically.

2. The constants A and B in (2) are proportional to the concentration of ferric nitrate. B is approximately inversely proportional to the hydrogen ion concentration, while A — within the accuracy of the method — is independent of the hydrogen ion concentration.

3. The experimental results agree with the reaction mechanism:

$$Fe^{+3} + HO_2^- \underset{-1}{\overset{1}{\rightleftharpoons}} FeOH^{+2} + O \qquad \text{I}$$

$$O + HO_2^- \xrightarrow{2} OH^- + O_2 \qquad \text{II}$$

This investigation was undertaken at The Technical University of Denmark, Chemistry Department A.

The autor wishes to thank the head of department, Prof. J. A. Christiansen, for his interest and advice especially as to the theoretical treatment of the experimental results.

I also wish to thank Prof. Carl Faurholt, The Royal Danish School of Pharmacy for his interest in my work.

During the investigation I have recieved financial aid from *Prof. Emil Koefoeds Mindelegat* and *Etatsraad, cand. pharm. C. A. Olesens Studielegat.*

REFERENCES

1. van Bertalan, J. *Z. physik. Chem.* **95** (1920) 328.
2. van Bohnson, L., and Robertson, A. C. *J. Am. Chem. Soc.* **45** (1923) 2493.
3. Simon, A., Haufe, W., Reetz, Th., and Preissler, R. *Z. anorg. u. allgem. Chem.* **230** (1936) 129.
4. Haber, F., and Weiss, J. *Proc. Roy. Soc. London* **A 147** (1934) 332.
5. Bodenstein, M. *Z. physik. Chem.* **85** (1913) 329.
6. Christiansen, J. A. *Handbuch der Katalyse* (1941) 261.
7. Lamb, A. B., and Jacques, A. *J. Am. Chem. Soc.* **60** (1938) 1215.
8. Joyner, R. A. *Z. anorg. Chem.* **77** (1912) 103.

Received November 29, 1947.

A C T A C H E M I C A S C A N D I N A V I C A 2 (1 9 4 8) 1 4 — 3 3

On Accumulation and Active Transport in Biological Systems

I. Thermodynamic Considerations *

THOMAS ROSENBERG

Universitetets Fysisk-Kemiske Institut, København, Denmark

The extensive experimental studies on the phenomena associated in biological literature with the concepts of accumulation and active transport give evidence of the significance attached to this problem. As regards the literature on the subject reference shall be made to Höber's thorough treatise [1] and moreover to several monographs and congress reports on the problem of membrane diffusion [2–6]. The processes in question include for example absorption processes in intestine and kidney, various kinds of secretion processes, *e. g.* those involved in the formation of gastric juice, absorption of inorganic salts by plant cells, and the unequal distribution of alkali ions between extracellular and intracellular fluids.

Active transport may generally be characterised as a transfer of matter which for energetic reasons cannot take place spontaneously, its performance requiring the coupling to compensating processes. In accordance with this we shall define active transport as a transfer of chemical matter from a lower to a higher chemical (in case of charged components: electrochemical) potential.

The terminology generally employed is rather heterogeneous on this point, a fact which has possibly contributed to make the theoretical treatment of the subject somewhat casual. Thus two of the terms most frequently used: free energy and osmotic work cannot be used as a matter of course in the definition of active transport. With regard to the former term it is not as a rule feasible to define the system in such a way that the change in free energy can be taken as a measure for the extent of the active transport; furthermore it may be mentioned that the concept: supply of free energy to a system is

* The viewpoints of this paper were presented in a lecture at Wenner-Gren's Institute for Experimental Biology, Stockholm, in February 1947.

not included in the definition of this term. The term: osmotic work is often employed with different meanings. As a rule, however, it indicates the work associated with the movement of a semipermeable membrane in a system in osmotic equilibrium, a process characterised by potential equality of the substances transported through the membrane. Finally the definition of active transport as a transfer of matter depending on metabolic activity is inconsistent as the concept of metabolic activity is not well defined and can hardly be defined in such a way that it does not include all kinds of biological transports.

Active transport is generally assumed to take place by a coupling to intracellular chemical processes. It has not, however, been possible in any case to clear up the mechanism of such couplings. One of the main difficulties of the problem is presumably the fact that an identification and isolation of the compensating processes concerned cannot be achieved on account of our incomplete knowledge of the processes which take place interdependently or independently in these complicated systems. The question of the so-called »driving forces» is of a more general nature. What sort of a coupling with a chemical reaction is able to force upon a chemical substance a direction of movement opposite to its natural diffusion tendency? For the elucidation of this question it will generally be necessary to study model systems, and a number of such investigations which aim at and in certain cases do achieve accumulation effects similar to those of living organisms have been made.

It is the object of the present paper to contribute to the elucidation of the energetic mechanism in processes of this kind by means of certain basic model conceptions. The means used for this purpose consist in generally recognised fundamental principles and facts, and consequently it does not lead to the recognition of new principles. If this paper thus contains some commonplaces, several points may be alleged in excuse hereof: First that there exists no analysis of the present problems on the basis of basic thermodynamic principles; secondly that on the whole we have no explanations of the capacity of cells to accomplish such energy transformations. Or to quote one of the recent discussions on the subject [7], »One of the most important unsolved problems of physiology is how the cell does work — work of any kind. The problem of heat production has been extensively investigated and is understood; but how the body transforms chemical energy into mechanical or into osmotic work is a complete blank.» Finally the present treatise on the subject, which deviates somewhat from the ordinary thermodynamic treatments, being based on Brønsted's [8] energetic formulations of the principles of thermodynamics, seems to favour a better understanding of the very mechanism of energy transformations in general.

FORMULATION OF THE PROBLEM

We shall consider transfer of chemical matter in defined model systems. In our investigation of the conditions which determine the direction of transference we shall prefer to consider the transport of infinitesimal quantities of matter. In this way the interdependence between the process in question and the other properties of the system cannot be obscured by the secondary changes caused by the transport proper.

A chemical substance A is assumed to be localised in two phases (i) and (o) of given compositions which do not vary in time. As a rule we shall assume homogeneity with respect to temperature and pressure. Through a connecting link, M, the properties of which shall be further defined for each individual system, the possibility of communication of A between the two phases is maintained. The object is to examine the conditions associated with the transport of an infinitesimal quantity dn_A through M.

We shall distinguish between three essentially different possibilities by which the stability in such a system may be secured. The three states we propose to consider are termed total equilibrium, partial equilibrium, and stationary states respectively. Particularly the latter two are of interest in the present context.

BASIC ENERGETIC PROCESSES

Transport in total equilibrium

Here a brief summing-up of the terminology used shall be given: temperature (T), pressure with negative sign ($-p$) and the chemical potentials (μ_A, μ_B, ... μ_K) are designated as energetic potentials. Corresponding to each of them we have an energetic quantity: entropy (S), volume (v), and the chemical components (A, B, ... K) respectively. Analogous extensions are made if there is a possibility of electric or gravitational processes in the system considered, each of these processes involving a potential and a quantity. A basic energetic process consists in transport of an energetic quantity between two potentials the values of which express the tendency of the transport concerned, transport from higher to lower potential indicating a positive tendency. Two different transport tendencies may balance each other, and a reversible total process consists of at least two balanced basic processes if the potentials involved have different values. A neutral basic process is a transport of quantity between identical potential values, and such a transfer may take place isolatedly without other energetic changes.

In a system in total equilibrium all energetic potentials are constant in the whole system. It appears from this that here transports of chemical components can never be active. If the two phases have different properties, the state corresponds to an ordinary phase equilibrium, *e. g.* between two solutions. Here the stability requires no connecting link with special properties. That these systems are mentioned at all in the present connection, is because it is often difficult in actual practice to ascertain whether a given system is in total equilibrium. The first indication of an active transport is as a rule that a substance accumulates in certain places. If different solvents are involved, such differences in concentration do not indicate corresponding differences in the chemical potentials. In living organisms this applies particularly to solvents of a lipoid character, and to phase boundaries the solvent properties of which usually differ greatly from the properties of the adjacent macrophases and where accumulation takes place according to Gibbs' adsorption equation. If on the other hand the two phases only differ slightly in composition, an unequal distribution of a substance may be regarded as indicative of an active transport. In this connection it is worth mentioning that in special cases the equilibrium distribution of a substance between two phases may be one-sided even if the properties of the phases only differ slightly in other respects. Such conditions are associated with the distribution of large molecules, and extreme distribution ratios between two phases of very nearly the same composition have been observed in some colloids [9].

Transport in partial equilibrium

If individual potentials have different values in the two phases considered, this is generally an expression of a reaction tendency in the total system which without special arrangements will result in the occurrence of spontaneous processes. It is well known that in many cases it is possible by special constructions of the connecting link M to prevent the occurrence of such processes, and thus establish a state of partial equilibrium. Osmotic equilibria, including Donnan equilibria, certain reversible galvanic cells etc. belong to such systems, which may in general be regarded as membrane equilibria. As there may be a possibility of transport of energetic quantities contrary to their natural diffusion tendency in these systems, a thermodynamic description of the energetic mechanism will also throw light upon the present problem.

If the system is not in total equilibrium, an elementary thermodynamic consideration will show that at least two kinds of energetic potentials must have different values in the two phases. This appears for instance from the basic equation for the changes of potentials:

$$SdT - vdp + n_A d\mu_A + n_B d\mu_B + \ldots\ldots\ldots + n_K d\mu_K = 0 \qquad (1)$$

which applies to arbitrary infinitesimal changes in a homogeneous phase. The equation shows that two infinitesimally different phases which differ with regard to one potential will necessarily differ with regard to another or several other potentials. So the same must be valid if the difference between the two phases is finite.

We shall assume that A's chemical potential is different in (o) and (i), *e. g.* $\mu_{A(i)} > \mu_{A(o)}$, and that the maintenance of this difference is not due to the fact that M is impermeable to A in any form. The latter condition can as a rule be proved experimentally by means of radioactive or other isotopes. If on these assumptions a transport of A shall be practicable in equilibrium, it follows as a condition of equilibrium that the transport must be coupled to another process with an opposite tendency so that the two tendencies exactly balance each other. In the particular system of two homogeneous phases this is only possible if the transfer of A, *e. g.* from (o) to (i), is coupled the transport of another energetic quantity K', which thereby goes from a higher to a lower potential. What is transported between the two phases is consequently a complex of A and K', possibly together with other quantities, which in case of potential equality will not yield any energetic contribution to the transport. Having regard to the rôle of the connecting link in such systems, we can formulate the result in the following way: The maintenance of equilibrium requires that M permits the passage of a complex of A and K' and does not permit the passage of A or K' in any other form. Purely energetic considerations thus involve the condition that the connecting link between the two phases shall be impermeable to certain quantities and permeable to others.

We shall as a, somewhat unbiological, example consider two different dilute solutions of Pb in Hg, *i. e.* two amalgams, which are separated from each other by an aqueous $Pb(NO_3)_2$-solution. In this system, which is known as a particularly simple reversible galvanic cell, we shall notice the following properties:

1. By the use of radioactive lead it will be proved that there is a possibility of communication for Pb between the two amalgams.

2. No spontaneous equalisation will take place between the two solutions.

3. The maintenance of the difference in concentration does not require the occurrence of energy-supplying »metabolic» reactions.

4. A transport of lead from the weaker to the stronger solution may be established if the weaker solution is combined with an electron acceptor and the stronger with an electron donator. This can for instance be effected by connecting the two solutions with the two poles of a condenser.

5. By such a transport chemical work is gained, and an equal amount of electric work is lost. The process can be described as a transformation of electric to chemical energy.

The energetic mechanism which is responsible for the stability of the system and for the coupling can be described in the following way: The communication between the two amalgams is maintained exclusively by the ion Pb^{++} which from a macroenergetic point of view can be regarded as a complex of Pb and positive charge. The diffusion tendency of this complex is determined by the chemical potential difference of lead between the two amalgams and the electric potential difference (or more precisely: the electrochemical potential difference of the electron) between them. At equilibrium the actions of these potential differences on the complex compensate each other. In this system the $Pb(NO_3)_2$-solution thus acts as an ideal semipermeable membrane which is impermeable to Pb and »free» electrons and permeable to Pb^{++}. In such a system it is usual to operate with the potential concept of the actual complex which is transferable. Thus a state of equilibrium is due to potential equality of all energetic quantities and complexes for which transport possibilities exist. Accordingly, equilibrium in the system of two amalgams is based on the fact that the electrochemical potential of Pb^{++} is constant in the entire system. In the two amalgam phases this potential is defined by Pb's chemical potential and the electrochemical potential of the electron.

Analogous systems in which the complex transferred consists of a compound of different chemical substances have not been made the subject of investigation and description.* This may be mainly due to the fact that the conditions required with regard to the permeability of the connecting link are far more difficult to realise experimentally than in the case of the corresponding electrochemical coupling. However, as such systems may be used as a basis for considering the coupling between transport of a chemical substance from a lower to a higher potential and other chemical processes, we shall deal with them in some detail.

The two phases (i) and (o) are assumed to contain the two substances A and B where $\mu_{A(i)} > \mu_{A(o)}$ and $\mu_{B(o)} > \mu_{B(i)}$. The connecting link M is assumed to be impermeable to A and B and permeable to a compound (AB) of the two substances. At equilibrium μ_{AB} is constant in the whole system, and if the compound is assumed to consist of 1 mole A and 1 mole B, the relation:

$$\mu_{A(i)} - \mu_{A(o)} = \mu_{B(o)} - \mu_{B(i)} \qquad (2)$$

* Compare, however, the »purely chemical cells» introduced by E. A. Guggenheim (*Modern Thermodynamics* London (1933) 143).

is valid. At the neutral transport of the compound between (o) and (i) thus equal molar amounts of A and B are transferred through numerically identical potential differences with opposite signs. Already at an early date it was recognised that such a process, which can be taken as a mutual transformation of chemical energies, is associated with stoichiometrical relations. Thus Ostwald [10] writes, »Eine unmittelbare gegenseitige Umwandlung chemischer Energien ist also nur insofern möglich, als chemische Energien miteinander in Beziehung gesetzt werden können, d. h. *innerhalb solcher Vorgänge, die durch eine stöchiometrische Gleichung dargestellt werden können.*» It shall be shown later that such a limitation of the possibilities is not generally valid. On the other hand the principle holds for the systems considered here, the equilibrium depending on a constant stoichiometrical ratio between the two coupled transported quantities A and B. Supposing that besides AB a complex AB_2 could be transported through M, we find that the equilibrium condition for this process would be:

$$\mu_{AB_2(i)} = \mu_{AB_2(o)} \tag{3}$$

or:

$$\mu_{A(i)} - \mu_{A(o)} = 2\mu_{B(o)} - 2\mu_{B(i)} \tag{4}$$

(4) and (2) can only be valid simultaneously when $\mu_{A(i)} = \mu_{A(o)}$ and $\mu_{B(i)} = \mu_{B(o)}$. At possibility for simultaneous transport of the two complexes of different composition the transported quantities of A and B are not stoichiometrically related, and consequently it will result in a potential equalisation between the two phases. Corresponding conditions apply to the analogous electrochemical coupling.

If M is selectively permeable to AB, an infinitesimal transport of the complex in one direction will cause an infinitesimal tendency towards transport in the opposite direction at equilibrium. A onesided transport of major quantities therefore cannot take place without co-operation with the surroundings. Also this is quite analogous to the electrochemical coupling. If the phase (o) is connected to a B-donator and the phase (i) to a B-acceptor, finite quantities of the complex can be transported from (o) to (i), this transport resulting in an accumulation of A in (i) opposite to its chemical potential gradient. This may for example take place if the two phases are in communication with two B-reservoirs in the surroundings. In biological systems the most frequent case is that a certain accumulation is supposed to be associated with a chemical reaction in the phase (i). It is obvious that such a coupling can be established in the present case if this reaction works as a B-acceptor, *i. e.* if B is consumed during the reaction. Suppose that in phase (i) B reacts with a non-diffusible substance C, which results in the formation of the compound

BC, and that this reaction takes place in equilibrium. In this way the coupling with a chemical reaction sought for is established. Besides equation (2) the relation:

$$\mu_{B(i)} + \mu_{C(i)} = \mu_{BC(i)} \tag{5}$$

holds in this system. By combining (5) with (2) we obtain:

$$\mu_{A(i)} - \mu_{A(o)} = \mu_{B(o)} + \mu_{C(i)} - \mu_{BC(i)} \tag{6}$$

From (6) it appears that the chemical energy necessary for the active transport of *A* originates from the chemical reaction:

$$B_{(o)} + C_{(i)} \rightarrow BC_{(i)} \tag{7}$$

in which neither the complex *AB* nor the substance *A* in any other form are included. Hereby an obscuration of the transport mechanism may arise, but the mechanism is also in this case based on the selective permeability of the connecting link to *AB*. Moreover it is seen from (6) that the energy transformation is complete, the energetic efficiency being 100 %. This condition holds in general for systems in partial equilibrium.

Transport in stationary systems

As shown above the maintenance of a state of partial equilibrium is associated with very stringent requirements on the permeability of the connecting link. In such systems the latter may be regarded as a phase in the sense of the phase rule. Impermeability to certain substances is based on their absolute insolubility in the connecting phase. The system represents an ideal limiting case which is practicable in certain cases with a very great approximation. Thus it holds for the lead amalgam cell considered that the insignificant solubility of lead metal in the $Pb(NO_3)_2$-solution and its consequent irreversible diffusion will not result in recognisable changes for very long periods. We shall now pass to systems in which corresponding processes occur to such an extent that they can no more be ignored when compared to the coupled processes.

In such systems the reaction tendency of the total system will manifest itself in the occurrence of spontaneous processes, and a system isolated from the surroundings will finally attain a state of equilibrium. On the other hand it is possible to sustain states of potential inequality in the phases (o) and (i) by co-operation with the surroundings. A potential gradient for a quantity

can be established by supplying the latter from the surroundings to one phase at a higher potential and removing the same quantity from the other phase at a lower potential. Hereby a flow of this quantity through the system is produced which will necessarily bring about and maintain other potential differences. We shall assume that the phases (i) and (o) are kept homogeneous, and that the potential gradients are limited to the connecting link. The term: stationary state shall be used for such a system if its properties are not changed in time.

The question to what a degree such systems are accessible to a thermodynamic analysis is discussed by Brønsted [11] in a recently published paper. Here it is shown that in stationary systems reversible and irreversible elements may be separated, and that also here the conception of energetic transport complexes can be used to describe the transformation of energy. Quantitative deductions are made for certain systems with thermoelectric, electrochemical, and thermochemical coupling.

As to the present problem we can assume that temperature gradients and pressure gradients will rarely play a major part in active transports. As a rule the mechanism has to be sought in the coupling of different chemical processes, and the problem is to explain an unequal distribution by maintenance of chemical potential gradients in the connecting link and flow of matter through it. Consequently we shall also in the following considerations assume homogeneity of temperature and pressure in the entire system.

The tendency of a mixture of chemical substances to be transferred between two phases, is expressed by the work gained in the surroundings by the reversible transfer of a conventionally fixed standard quantity of the mixture. If a given quantity of a mixture I containing n_A moles of the substance A and n_B moles of the substance B is transferred to a mixture II of the same substances, this work is given by:

$$W = n_A\,(\mu_{A(\mathrm{I})} - \mu_{A(\mathrm{II})}) + n_B\,(\mu_{B(\mathrm{I})} - \mu_{B\,\mathrm{II}}) \tag{8}$$

provided that the potentials in II only change infinitesimally during the process. If the two mixtures only differ infinitesimally, we obtain from (8), using (1):

$$dW = n_A d\mu_A + n_B d\mu_B = 0 \tag{9}$$

Consequently the mixture as such has no transference tendency here, the opposite tendencies of A and B neutralising each other. We shall regard a stationary system involving various potential gradients as thermodynamically equivalent to a series of phases of infinitesimally varying composition. In

each of these phases the components of the mixture: A and B are not in equilibrium with the neighbouring phases, while on the other hand the mixture as such is in equilibrium with them. An infinitesimal transport of the mixture, which consequently may be regarded as a reversible process, represents a coupling of a positive basic process for one component with a negative basic process or active transport for the other component. There are some points of resemblance between the reversible transport of such a mixture and the corresponding transport of a chemical compound in a system in equilibrium. The fundamental difference is that the composition of the compound is constant in the connecting link, whereas the mixtures which in different places are in equilibrium with the neighbouring phases differ in composition. The complex which can be transported reversibly in such systems through finite potential differences, therefore, changes its composition continuously. A continuous flow of matter through the system is required to maintain the stationary state, *i. e.* an irreversible process which is the contribution of the surroundings towards stabilisation of the system. According to our basic presuppositions this process and its extent are not, however, included in our considerations of the reversible movements of the complex. Equation (9), or in its extended form equation (1), may thus be considered the thermodynamic basis of the coupling mechanism in active transport both at equilibrium and in stationary systems.

Under the transport of a chemical compound in partial equilibrium its components are held together by chemical forces which do not permit a dissociation of the complex in the connecting link. Different chemical components may, however, also be held together in other and looser ways than in a chemical compound. In a solution of electrolytes electrostatic forces will counteract an isolation of individual ions. Even if there exists no mutual chemical interaction of the components as for instance in mixtures of ideal gases, they will oppose an isolation, and the movement of one component will produce a diffusion tendency in and unequal distribution of the other components. Thus it is plausible that a stationary diffusion of one substance will result in an unequal distribution of other nondiffusing substances. This effect was investigated by Thovert [12] for salt solutions and later described by the same author [13] for solutions of non-electrolytes.

That this »diffusion rétrograde» can serve as a coupling mechanism in active transport is seen most immediately when the components considered are ideal gases. We shall consider a system with the phases (i) and (o) containing the two gases A and B, and with a equal pressure all over, which is secured by the use of a wide tube as connecting link. By communication with the surroundings through semipermeable walls or the like B's partial pressure

is kept higher in (o) than in (i) so that a constant diffusion of B will take place through the system from (o) to (i). In this stationary state the non-diffusing component A can be transported actively from (o) to (i), *e. g.* by an infinitesimal increase of A's or B's partial pressure in (o).

Phenomena of the same kind may occur in liquid mixtures. In a mixture of acetone and water which is kept in a stationary state by a constant diffusion of acetone the chemical potential of water is increasing from places with a higher to places with a lower concentration of acetone. If a third non-diffusing substance is dissolved, no general predictions can be made with regard to sign and magnitude of its potential gradient in the stationary state, the substance being influenced by the potential gradients of both solvent components. It has been claimed [14] that such a substance will be distributed according to its solubility so that its chemical potential assumes the same value in the whole system. According to the present considerations such a distribution is not, however, more probable than other distributions. Apart from the fact that an equipotential distribution is unlikely for symmetrical reasons, cases can easily be pointed out in which no such distribution occurs. If for instance deuterium oxide is dissolved in the mixture considered, its distribution will be nearly the same as that of the ordinary water, and consequently it will also assume the same potential gradient.

The distribution which takes place in a dilute electrolyte solution when a stationary flow of a salt passes through it is one of the few cases of stationary systems of which a quantitative treatment has been given [15]. An electric potential gradient which arises on account of different mobilities of the diffusing anions and cations will affect the non-diffusing ions in a way solely determined by their charge (Teorell's diffusion effect). The distribution arising hereby is analogous to the corresponding equilibrium system where one of the ions cannot diffuse at all (Donnan equilibrium). The calculations are based upon the assumption that a non-diffusing ion has the same electrochemical potential in the whole system. In concentrated solutions with great potential gradients and also in solvents with a small dielectric constant great individual deviations from such a distribution may be expected.

Active transport caused by the different diffusion effects discussed will as a rule be characterised by similar distributions of substances with similar chemical or electrochemical properties. It is very probable that such effects occur to a large extent in biological systems. This will, however, render it difficult to explain the specific active transports which for various reasons are the most interesting. Cells which transport potassium from and sodium to a solvent poor in K^+ and rich in Na^+, other membranes which prefer hexoses to pentoses require mechanisms working more specifically. The substances

which form the components of the transported complex must be more specifically attached to each other than it is the case in the »diffusion complexes» mentioned above. If a substance A is transported actively through a connecting link through which a chemically similar substance, A_1, cannot be transported actively, this is tantamount to the two substances being affected by different forces or potential gradients in the connecting link. For the purpose of explaining such active transports it will be difficult to get round the assumption that chemical compounds in which the actively transported substances are included as components participate to a great extent in the transport between the two phases. Under the treatment of transport in partial equilibrium the reversible limiting case for such couplings, which is characterised by equilibrium with regard to a compound AB, was discussed. In a stationary system the movement of such a compound through M may be influenced with regard to rate and direction by other simultaneously diffusing substances. Special conditions exist when there is also a possibility of diffusion for the substances A and B besides for AB. Simultaneous diffusion of other substances which have no chemical relation to the compound AB may be assumed not to affect the latter specifically and consequently shall be ignored in the following discussion.

We shall assume that the phases (o) and (i) are two homogeneous solutions of A and B in the same solvent, and that potential gradients are maintained in the connecting link by the supply from outside of B to (o) and removal of B from (i). On the other hand such possibilities of communication are not assumed to exist for A. If $\mu_{B(o)}$ in this system is greater than $\mu_{B(i)}$, and if M is permeable to a compound AB, a potential difference $\mu_{A(i)} - \mu_{A(o)} > 0$ will be maintained by B's flow from (o) to (i). The stationary state involves that equal quantities of A as such or in the form of AB pass through each section of the system in opposite directions. By an infinitesimal increase of A's transference tendency in the direction (o) to (i) this substance is transported actively. But while the efficiency was 100 % in the equilibrium system, it is always less in the stationary system.

To examine what factors are decisive for the efficiency, we shall consider two simplified limiting cases in which the concentrations of the components concerned are assumed to be so low that their diffusion takes place independently of each other: 1) Only AB and B but not A, can diffuse. As A is transported through M exclusively in the form of AB in this system, AB is in equilibrium in the whole system, and accordingly equation (2) holds here just as for the system in partial equilibrium. In contradistinction to the latter this state must be stabilised by B's irreversible diffusion through the system. Hereby a constant quantity of B passes through each section of the system in

unit time. For the maintenance of given potential differences this quantity will be the smaller, the smaller the concentration gradient of B is in the connecting link. This means that the efficiency increases with decreasing solubility of the substance B in M. If an active transport of A is accomplished by increased supply of B from outside to (o), B will flow through M partly in the form of B as such and partly in the form of AB. Hence it appears that the efficiency increases with increasing solubility and rate of diffusion of AB in M. 2) Only AB and A but not B, can diffuse. In this system B is transported through M exclusively in the form of AB so that $\mu_{AB(o)} > \mu_{AB(i)}$, and consequently $\mu_{A(i)} - \mu_{A(o)} < \mu_{B(o)} - \mu_{B(i)}$. In the stationary state a constant amount of AB passes through each section of M in the direction (o) to (i), and a corresponding amount of A passes in the opposite direction in unit time. Moreover this amount is equal to the amount of B supplied to (o) and removed from (i). Analogous to 1) a low solubility and mobility of A and a high solubility and mobility of AB in the connecting link will tend to increase the efficiency.

It has not been attempted to make quantitative calculations by the use of hypothetical distribution ratios and insertion of hypothetical values for concentration gradients and diffusion coefficients in Fick's law of diffusion, which — as pointed out by Danielli [3] — has a very limited applicability in the systems considered. Qualitatively it appears from the two cases discussed that the efficiency of the coupling is determined by the ratio of the solubilities of the compound and its components in M and by the difference between their mobilities. As mobility is mainly determined by molecular volume, which for ordinary molecules only varies within comparatively narrow limits, the solubility conditions seem to be decisive for the accumulation effect*. Thus the difference in μ_A will only assume a major value when the amounts of A and B in the connecting link are small compared to the amount of AB, in other words when the deviations from the ideal system in partial equilibrium are not too great. Such conditions can only be expected to exist if the chemical character of the connecting link as a solvent differs essentially from those of two phases.

B's function in the systems mentioned may be taken as a carrier function, the active transport of A being induced by B's positive transport tendency in the total transport. In our foregoing considerations the difference in μ_B is assumed to be maintained by transference processes of B between the phases

---

* After the manuscript of the present paper was finished, a paper by J. Franck and J. E. Mayer appeared (*Arch. Biochem.* 14 (1947) 297), in which the authors propose some models mainly based upon differences in rate of diffusion. From quantitative calculations it is derived that for the energetic efficiency such high values as 30 % can be reached.

and the surroundings. This potential difference may, however, also be maintained if the said transference processes are replaced by different chemical reactions which consume and produce B respectively.

For the further elucidation of this case we shall choose a system where the phase (i) — just as a single cell — is completely surrounded by the phase (o) and separated from the latter by the connecting link or membrane M. A potential difference $\mu_{A(i)} - \mu_{A(o)} > 0$ is assumed to be maintained by supply of the »metabolite» G from the surroundings to (o) and removal of the metabolic end product C from the same phase. As, in contradistinction to the above-mentioned systems, (i) has no possibility of direct communication with external surroundings, the removal of the carrier substance from this phase can be effected only by a chemical reaction therein. This condition is generally valid — also for the non-specific diffusion coupling — for such systems in which one phase is completely surrounded by the other: The stationary maintenance of potential gradients — including pressure and temperature gradients — for quantities which are transportable through the membrane is only possible by simultaneous chemical reactions in the inner phase.

The carrier function must be ascribed to either the substance G or an intermediary reaction product X. Particularly the latter case is of interest in connection with the mechanism in biological systems. As it is a necessary presupposition for the performance of the active transport of A that the substance X has a positive diffusion tendency from (o) to (i), it follows that the latter must be produced in the phase (o) or on the boundary between the latter and the membrane. Consequently, in this model chemical reactions in which the carrier substance participates take place on both sides of the membrane. As before, the actual coupling mechanism is due to the transfer of a chemical compound including both the carrier and the actively transported substance. But the occurrence of the carrier substance may be limited to the membrane proper, a fact which, considering the dimensions of biological membranes, will render a direct observation of its presence and transport function extremely difficult. The different localisation of chemical reactions in biological systems may be ascribed to the presence of different metabolic enzyme systems on the two sides of the membrane.

DISCUSSION

The basis of the present considerations is the conditions of stability in systems in which potential differences can exist persistently between two phases although possibilities of exchange are assumed to exist for the components not in equilibrium. In the ideal limiting case, the system in partial

equilibrium, the maintenance of this state requires no coupling to spontaneous chemical reactions. The conditions here required with regard to the selectivity of the membrane permeability cannot, however, be assumed to be complied with in biological systems. On the other hand it is not impossible that systems with a low metabolism and high chemical potential differences are not far from the equilibrium system. Such conditions may exist at the distribution of alkali ions between erythrocytes and plasma where it was shown by means of radioactive indicators [16, 17] that a formerly presumed absolute impermeability to cations does not seem to exist. That there is an interdependence between this distribution and cell metabolism appears *i. a.* from Wilbrandt's [18] results.

In a stationary system for the maintenance of which spontaneous processes are required a substance A which is not in equilibrium must be influenced by other forces besides its chemical potential gradient in the connecting link between the two phases. Such forces can generally be identified with a comparatively small number of energetic potential gradients, including the gradients for temperature and pressure. Here the limitation is the same as the limitation of the different forms of energy possible which is used in Gibbs' differential equation for the total energy of a system. The condition that A is influenced by other energetic potential gradients must be due to the fact that the system considered evidently does not permit an isolated transport of A between the two phases, each infinitesimal transfer of this component being coupled to the transfer of one or possibly several other components which are not in equilibrium either. This qualitative description of the energetic mechanism is unambiguous and exhaustive for systems in which the connecting link is of macroscopic dimensions. The question may, however, be raised whether the use of concepts with a statistical basis such as temperature, pressure, chemical potential etc. is permissible for biological membranes the thicknesses of which frequently correspond to a few molecules only. As regards this question it can be stated that even if the fluctuations about the probable values will naturally increase with decreasing dimensions, the conception of a continuity of these quantities will justify their application to the present problem. As mentioned subsequently, diffusion through cell membranes seems to be mainly determined by a kind of membrane solubility, a relation which may be taken as an experimental confirmation of the applicability of chemical potentials to express diffusion tendencies.

The same mechanism which is responsible for the maintenance of the stationary state of the system can in all cases be employed for the performance of active transport. An infinitesimal relative increase of the forces which act on A in the opposite direction of its chemical potential gradient in the station-

ary state will thus result in the transfer of A from a lower to a higher chemical potential. Here we have distinguished between unspecific and specific active transports. It is characteristic for the former that chemically similar substances are affected similarly. In such a diffusion complex the binding between actively and passively transported components of the complex is analogous. Thus the effect of electric potential gradients on Na^+ and K^+ is the same — *e. g.* in Teorell's diffusion effect — because the ratio between charge and chemical matter is identical for the two components. A diffusion coupling may be assumed in many cases to contribute towards the maintenance of non-equilibria in living organisms. A special case of this kind is active transfer of water which is of a more special character on account of the rôle played by water as a solvent. A model which can reproduce such transfers by the maintenance of other potential gradients is described by Hartley [14].

In specific active transports the composition of the transport complexes is quite different for components with similar chemical properties. This can be due to differences in chemical affinities or — particularly in biological systems — to the participation of specific enzymes in the formation of the complex, in which chemical compounds consequently must be included. The coupling efficiency is mainly determined by the difference in the distribution ratios of the compound and its components between phases and membrane. In order to attain major effects the membrane must have solvent properties quite different from those of the phases. In living systems the latter consist of aqueous solutions, and therefore substances of a nonpolar or a lipoid character will from a purely chemical point of view be considered best suited as structural constituents of such membranes. This point of view is in accordance with the obtaining views concerning the character of the plasma membrane which were originally set forth by Overton [19], and which have been verified subsequently by numerous results. The theory of the lipoid character of plasma membranes is mainly based on the close parallelism which is found between the distribution ratios of chemical substances between water and solvents as ether or olive oil and their rate of diffusion through cell membranes. Numerous attempts have been made at a close reproduction of the solvent properties of the cell membrane by model studies, and for this purpose both pure substances of a lipoid character and mixtures of such have been used, without particularly satisfactory results. From many studies on permeability, especially by Collander [20] and his school, it appears that the solvent properties of the cell membrane may vary within very wide limits for different species, and that besides solubility also a pore mechanism may possibly be of importance to the rate of diffusion. But moreover it must be assumed that the particular orientation which is supposed to exist in the cell membrane, and

which may be paralleled with liquid crystals, will result in solvent properties which cannot be reproduced completely in a macrophase.

With these modifications active transports can thus be ascribed to the lipoid solubility or »membrane solubility» of the transport complex which must be much higher than that of the components. In many papers Osterhout [21] has emphasised similar views with regard to active salt transport, which is assumed to take place in undissociated form. In numerous models the existence of analogies between cell membranes and guajacol solutions is shown, particularly with respect to the diffusion of Na^+ and K^+. These views are consistent with those stated in this paper; it must, however, be observed that the lipoid solubility of an undissociated salt is not sufficient by itself for the explanation of the mechanism of active transport, but for energetic reasons it is required that the diffusion tendency of one ion shall be overcompensated by the opposite tendency of the other ion in the total transport. Some conditions involved in the transport of alkali ions, and in this connection the models of Osterhout, will be further discussed in a following paper.

The component the diffusion tendency of which determines the transport direction of the complex may be described as an energetic carrier. Various results in literature seem to affirm the existence of substances or systems with such a function. Especially some findings may be mentioned by which it is demonstrated that a given form of transport can have a maximum capacity so that an increase in concentration exceeding a certain limiting value does not increase the amount of matter transported hereby. Such results are particularly apparent in the case of glucose absorption in the intestine and glucose reabsorption in the kidney tubules where different transport mechanisms can also be distinguished by means of metabolic poisons [22—24].

In a discussion on the character of the binding to the carrier system Höber [25] proposes three different possibilities: 1) The carrier operates as a solvent, 2) as an adsorbent, and 3) it combines chemically with the actively transported substances. According to Höber's own results [26] with active transport of certain dyestuffs in kidney and liver a nonpolar-polar molecular structure favours the possibility of active transport simultaneously with favouring the adsorption on the membrane boundary according to well known laws. Thus it is assumed that adsorption can possibly be conceived as the first stage in active transport although adsorption as such does not require an energetic coupling. On the basis of the conception proposed here, the said results, *e. g.* with azo dyestuffs, might be explained by the assumption that the system is able to block enzymatically the hydrophilic sulphonate groups which are situated on the same side of the molecule and thereby make this intermediarily lipoid-soluble, whereas a corresponding blocking in molecules

with many and fairly equally distributed sulphonate groups should not be possible. The effective molecular orientation in such a blocking process may be assumed to be contrary to the normal, the hydrophilic groups being directed towards the membrane. The transport direction of the whole complex would be determined by the blocking part, which according to the above suppositions must include components with a positive diffusion tendency in the transport direction. Such a mechanism would be most consistent with the third of the above alternatives. It is, however, rather doubtful whether there exists a possibility of distinguishing sharply between these alternatives in the systems considered.

A consequense of the present views on the nature of the transport mechanism is that compounds, which are per se strongly lipoid soluble, cannot be accumulated by active transport. This also is in full accordance with Höber's observations on the transport of dyestuffs.

On the other hand it is a necessary condition, irrespective of the character of binding, that the carrier passes the membrane just like the actively transported substance. If this movement has the direction from the outer phase into a single cell, the carrier must be transformed in the latter by a chemical reaction in order that the diffusion tendency should continue to be maintained. In case of transport in the opposite direction the analogous condition will involve that the carrier must be formed by a chemical reaction within the cell. By these conditions a dependence is established between active transport and metabolism, two functions closely connected in living systems. The said continuous reactions in the cell must be regarded as a part of metabolism so that the carrier can be considered an intermediary product thereof. The connection between active transport and metabolism appears most clearly from systems in which the normal concentration of an actively transported substance A is varied. When the carrier does transport A through the membrane at all, it is due to an existing restraint on the isolated transport of the carrier, *e. g.* its slight membrane solubility. If A is removed from the system, this restraint will manifest itself more clearly and in accordance with the above retard the metabolic reactions, a phenomenon frequently observed. Conversely, if the outer concentration of A is increased, there is a possibility of an increased transport of the carrier and thereby an acceleration of metabolism. This case seems to be realised in Steward's[27] results with potato discs where an increased concentration of potassium results in an increase of both carbohydrate and nitrogen metabolism.

While the association of intracellular enzymes with active transport is immediately plausible, this is perhaps less so with regard to the enzyme action on the outer surface of the membrane. This problem can be elucidated by the

consideration of systems to which no substances which can act as carriers are supplied from outside. Many plant cells are able to accumulate salts, especially potassium salts, from very dilute salt solutions which are supplied with oxygen [28]. As no other metabolites are supplied from outside, the particular carrier must be delivered from the cell where the increased metabolism manifests itself in *i. a.* mobilisation of the carbohydrate stores. However, a substance produced inside the cell will have an outwardly directed diffusion tendency and, accordingly, will not be able to act as a carrier in this case. Therefore, the most plausible explanation of this function is obtained on the assumption that the carrier in question is formed from another intermediary product on the outer side of the membrane, a reaction which may be assumed to be controlled enzymatically. A few observations of a more direct character support the assumption of the enzyme localisation on the outer surface of the membrane. A lactase is presumably present on the outside of yeast cells [29], and an enzyme which phosphorylises glucose seems to act on the outside of muscle cells in fasting animals [30, 31]. The presence of proteins in general on the outer membrane surface seems to be a well established fact.

The picture of a living mechanism given above on a thermodynamic basis naturally can only be very rough and schematic. However, in connection with biological facts some more concrete details have been recognised. As a main result a general and simple mechanism is proposed, based upon the normal permeability and solvent properties of the cell membrane combined with the action of metabolic enzyme systems which are involved in the building up and breaking down of transport complexes on both sides of the membrane. Such membrane soluble complexes may include both intermediary metabolic products and normal membrane components as steroids or phospholipids. Some models on this basis concerning the active transport of glucose and of alkali ions will be presented in following papers. With respect to model studies in general it may be concluded that it will not be possible to realise a close reproduction of active transports in living systems without the use of metabolic enzymes. Finally it must be assumed that an analogous mechanism also acts in many cases of biological transport which are not included in the definition of active transport.

SUMMARY

The thermodynamics of active transport of chemical matter in equilibrium and in stationary systems are discussed. A general mechanism for active transport is proposed on the basis of the thermodynamic results combined with known properties of living membranes and other characteristics related to the problem in biological systems.

REFERENCES

1. Höber, R. *Physical chemistry of cells and tissues* 2nd ed. Philadelphia (1946).
2. Wilbrandt, W. *Ergebn. Physiol.* **40** (1938) 204.
3. Davson, H., and Danielli, J. F. *The permeability of natural membranes* Cambridge (1943).
4. Krogh, A. *Proc. Roy. Soc. London B* **133** (1946) 140.
5. *Trans. Farad. Soc.* **33** (1937) 911 ff.
6. *Cold Spring Harbor Symposia Quant. Biol.* **8** (1940).
7. Bull, H. B. *Physical biochemistry* 2nd ed. New York (1943) 324.
8. Brønsted, J. N. *J. Phys. Chem.* **44** (1940) 699 (with further references).
9. Brønsted, J. N., and Warming, E. *Z. physik. Chem. A* **155** (1931) 343.
10. Ostwald, W. *Z. Phys. Chem.* **34** (1900) 248.
11. Brønsted, J. N. *Københavns Universitet, Festskrift* (1946) (in Danish).
12. Thovert, I. *Ann. chim. phys.* **26** (1902) 419.
13. Thovert, I. *Ann. phys.* **2** (1914) 405.
14. Hartley, G. S. *Trans. Farad. Soc.* **33** (1937) 1021.
15. Teorell, T. *Proc. Nat. Acad. Sci. U. S.* **21** (1935) 152; see also Meyer, K. H., and Sievers, J. F. *Helv. Chim. Act.* **19** (1936) 649.
16. Hahn, L. A., Hevesy, G., and Rebbe, O. H. *Biochem. J.* **33** (1939) 1549.
17. Dean, R. B., Noonan, T. R., Haege, L., and Fenn, W. O. *J. Gen. Physiol.* **24** (1940) 353.
18. Wilbrandt, W. *Trans. Farad. Soc.* **33** (1937) 956.
19. Overton, E. *Pflügers Arch. ges. Physiol.* **92** (1902) 115.
20. Collander, R. *Trans. Farad. Soc.* **33** (1937) 985.
21. Osterhout, W. J. V. *Cold Spring Harbor Symposia Quant. Biol.* **8** (1940) 51 (with many references).
22. Wilbrandt, W., and Laszt, L. A. *Biochem. Z.* **259** (1933) 398.
23. Lundsgaard, E. *Biochem. Z.* **264** (1933) 209.
24. Verzàr, F. *Biochem. Z.* **276** (1935) 17.
25. Höber, R. *Physical chemistry of cells and tissues* 2nd ed. (1946) 618.
26. Höber, R. *Cold Spring Harbor Symposia Quant. Biol.* **8** (1940) 40.
27. Steward, F. C. *Trans. Farad. Soc.* **33** (1937) 1006.
28. *Cf. e. g.* Hoagland, D. R. *Cold Spring Harbor Symposia Quant. Biol.* **8** (1940) 181.
29. Myrbäck, K., and Vasseur, E. *Z. Physiol. Chem.* **277** (1943) 171.
30. Sacks, J. *Am. J. Physiol.* **143** (1945) 157.
31. Kalckar, H. M., Dehlinger, J., and Mehler, A. *J. Biol. Chem.* **154** (1944) 275.

Received December 3, 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 34—41

Bromierungsprodukte des Pinosylvindimethyläthers

HOLGER ERDTMAN und BENGT LEOPOLD

Organisk-kemiska Institutet, Kungl. Tekniska Högskolan, Stockholm, Schweden

Cox[1] hat vor einigen Jahren die Isolierung von Pinosylvindimethyläther aus dem unverseifbaren Anteil eines »wood resin» von einer Kiefer nicht näher angegebener Art mitgeteilt.

Durch vorsichtige Bromierung erhielt er daraus ein Monobromderivat vom Schmelzpunkt 86—86,5°. Die Analyse ergab wenig zufriedenstellende Werte für Methoxyl und Brom (Br gef. 28,2 %, ber. 25,0 %). Durch weitere Bromierung wurde daraus ein Dibromderivat vom Schmelzpunkt 85,5—86,5° erhalten, dessen Bromgehalt ebenfalls etwas zu hoch gefunden wurde (41,1 statt 40,2 %).

Der eine von uns hat mit Aulin-Erdtman[2] durch Bromierung von Pinosylvindimethyläther unter anderem einen Dibrompinosylvindimethyläther vom Schmelzpunkt 135—136° (neuerdings 137—139°) erhalten, welcher bei der Oxydation mit Kaliumpermanganat Benzoesäure und 2,6-Dibrom-3,5-dimethoxy-benzoesäure (BB; vgl. das Formelschema) lieferte. Die Substanz stellt somit das 2,6-Dibrom-3,5-dimethoxy-stilben (B) dar. Um diese Diskrepanz bezüglich der Schmelzpunktsangaben zu erklären, wurde die Bromierung des Pinosylvindimethyläthers aufs neue untersucht.

Durch Bromierung des Pinosylvindimethyläthers mit einem Mol Brom wurde ein Monobromderivat vom Schmelzpunkt 91—92° erhalten. Unter Umständen wurden aber Präparate vom Schmelzpunkt 124—125° gewonnen. Wurde das Präparat vom Schmelzpunkt 91—92° auf 115° erhitzt und mit dem Präparat vom Schmelzpunkt 124—125° geimpft, so erstarrte die Schmelze, um bei weiterem Erhitzen bei 124—125° wieder zu schmelzen. Die beiden Substansen sind somit dimorph.

Aus der Mutterlauge des Mono-bromderivates konnte durch mühevolles Fraktionieren eine kleine Menge eines anderen Bromkörpers vom Schmelzpunkt 150—151° isoliert werden, der sich ebenfalls als ein Monobromderivat erwies. Der Rückstand war sehr schwer krystallisierbar.

Bei der Weiterbromierung dieses Rückstandes, der offenbar ein kompliziertes Gemisch von Pinosylvindimethyläther und verschieden hoch bromierten Derivaten desselben darstellt, wurde ein krystallines Produkt erhalten, aus welchem eine Substanz vom Schmelzpunkt 225—227° herauspräpariert werden konnte. Sie stellt ein Tribromderivat des Pinosylvindimethyläthers dar. Ausserdem wurden die früher beschriebenen Tetrabromderivate isoliert, nämlich das »niedrigschmelzende Tetrabromderivat» vom Schmelzpunkt 172—173° und das »hochschmelzende Tetrabromderivat». Das letztere schmilzt nicht, wie früher angegeben, bei 190—192° bzw. bei 195—197°, sondern nach öfterem Umkrystallisieren konstant bei 225—227° unter Zersetzung.

Durch geeignete Bromierung lieferte das Monobromderivat vom Schmelzpunkt 150—151° ein Dibromderivat vom Schmelzpunkt 131—132° und das Tribromderivat vom Schmelzpunkt 225—227°, welche beide durch vollständige Bromierung in das »niedrigschmelzende Tetrabromderivat» (172—173°) übergeführt werden konnten. Bessere Ausbeuten an Monobromderivat vom Schmelzpunkt 150—151° wurden erhalten, wenn die oben erwähnten schwer krystallisierbaren Rückstände zuerst in alkoholischer Lösung mit Zinkstaub gekocht wurden und das resultierende Produkt fraktioniert wurde. Durch diese Behandlung werden nämlich Stilbenbromide in Stilbene übergeführt, wodurch die Zahl der Komponenten erniedrigt und die Aufarbeitung des Gemisches erheblich erleichtert wird.

Das Monobromderivat vom Schmelzpunkt 91—92° bzw. 124—125° muss die Struktur A besitzen (2-Brom-3,5-dimethoxy-stilben), weil es zu 2,6-Dibrom-3,5-dimethoxy-stilben (B) bromierbar ist. Bei der weiteren Bromierung der ganz reinen Verbindung wurde *ausschliesslich* das »hochschmelzende Tetrabromderivat» des Pinosylvindimethyläthers erhalten. Das »hochschmelzende Tetrabromderivat» ist eines der beiden diastereoisomeren 2,6-Dibrom--3,5-dimethoxy-stilbendibromide (C), denn durch Kochen in alkoholischer Lösung mit Zinkstaub wird es glatt in 2,6-Dibrompinosylvin-dimethyläther (B) zurückverwandelt. Ein Tribromderivat der Formel B' konnte aus A nicht erhalten werden. Das Bromatom in Stellung 2 erschwert offenbar aus sterischen Gründen die Bromierung der Äthylendoppelbindung.

Das Monobromderivat A dürfte mit Cox' Monobromderivat vom Schmelzpunkt 86—86,5° identisch sein. Cox' Dibromderivat vom Schmelzpunkt 85,5—86,5° stellt entweder unreinen 2,6-Dibrompinosylvindimethyläther (B) dar, oder ist, was sehr wohl möglich erscheint, mit diesem dimorph. Wir haben es jedoch nie beobachten können.

Das Monobromderivat vom Schmelzpunkt 150—151° könnte die Struktur a (4-Brom-3,5-dimethoxy-stilben) und das daraus erhältliche Dibromderivat vom Schmelzpunkt 131—132° die Struktur b (2,4-Dibrom-3,5-dimethoxy-

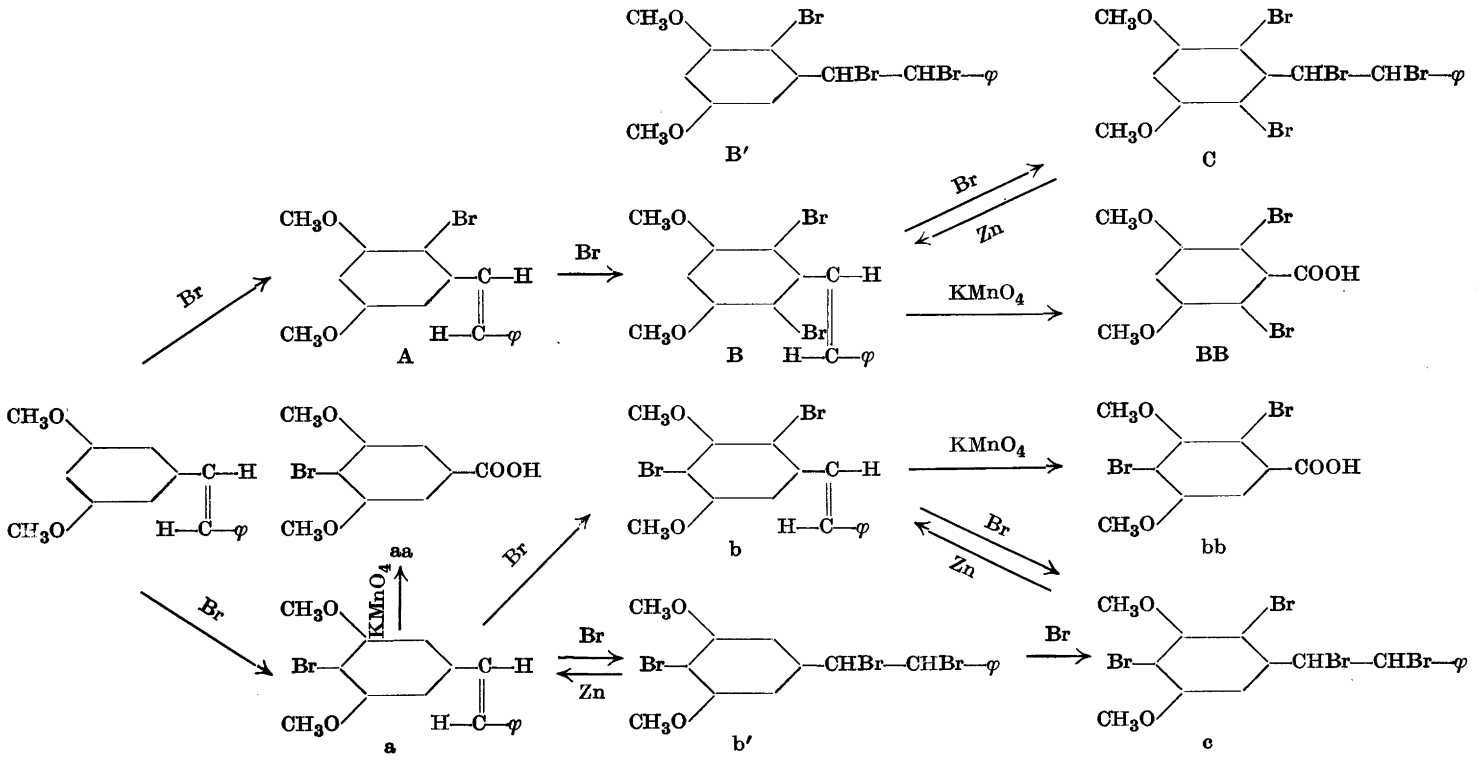
stilben) besitzen. Das Tribromderivat vom Schmelzpunkt 225—227° wäre dann b' (eines der beiden diastereoisomeren 4-Brom-3,5-dimethoxy-stilbendibromide) und das »niedrigschmelzende Tetrabromderivat» (Schmelzpunkt 172—173°) c (eines der beiden diastereoisomeren 2,4-Dibrom-3,5-dimethoxystilbendibromide).

In Übereinstimmung mit dieser Annahme wird das Tribromderivat b' bzw. das »niedrigschmelzende Tetrabromderivat» c mit Zinkstaub in 4-Brompinosylvin-dimethyläther (a) bzw. 2,4-Dibrompinosylvindimethyläther (b) übergeführt. Die Bildung des Tribromderivates ist verständlich, da das Bromatom in Stellung 4 (Formel a) keine sterische Hinderung der Bromierung der Äthylendoppelbindung, sondern nur eine Verminderung der Reaktionsfähigkeit des methoxylierten Kernes bewirkt.

Dass der Monobrom-pinosylvin-dimethyläther vom Schmelzpunkt 150—151° wirklich die Struktur a besitzt, geht aus dem Ergebnis der Oxydation mit Kaliumpermanganat hervor. Es wurden Benzoesäure und eine Monobrom-dimethoxybenzoesäure (aa) vom Schmelzpunkt 252° erhalten, welche mit Diazomethan den entsprechenden Methylester vom Schmelzpunkt 119—120° lieferte.

4-Brom-3,5-dioxybenzoesäure war von Barth und Senhofer [3] durch Bromierung von α-Resorcylsäure mit kaltem Bromwasser gewonnen worden. Ihre Struktur wurde von den gleichen Verfassern durch eine Reihe von Umwandlungen gesichert. Mit Diazomethan methyliert, lieferte die Säure den 4-Brom-3,5-dimethoxybenzoesäuremethylester, der bei 120—120,5° schmolz und mit dem Methylester der Abbausäure (aa) keine Schmelzpunktserniedrigung gab.

Durch Oxydation des Dibrom-pinosylvindimethyläthers vom Schmelzpunkt 131—132° (b) wurden Benzoesäure und eine Dibrom-dimethoxybenzoesäure (bb) vom Schmelzpunkt 183—184° erhalten, welch letztere mit Diazomethan in einen Methylester vom Schmelzpunkt 76—77° übergeführt wurde. v. Hemmelmayr [4] bromierte α-Resorcylsäure in Eisessig und erhielt eine Dibrom-α-resorcylsäure, die seiner Ansicht nach die 2,4-Dibrom-3,5-dioxybenzoesäure sein sollte. Die v. Hemmelmayr'sche Säure lieferte aber bei der Methylierung mit Diazomethan einen Dibrom-dimethoxy-benzoesäure-methylester vom Schmelzpunkt 148—148,5°. Aus 2,6-Dibrom-3,5-dimethoxybenzoesäure (BB) mittels Diazomethan gewonnener Methylester schmolz bei 152—153° und gab keine Schmelzpunktsdepression mit dem aus v. Hemmelmayr's Säure dargestellten Ester vom Schmelzpunkt 148—148,5°. v. Hemmelmayr's vermeintliche 2,4-Dibrom-3,5-dioxybenzoesäure ist somit 2,6-Dibrom-3,5-dioxybenzoesäure. v. Hemmelmayr hatte auch ein Diacetat (Schmp. 182—183°) sowie ein Nitroderivat (Schmp. 208°, Zers.) beschrieben und diese Sub-



Zusammenstellung der Bromierungs- und Spaltungsreaktionen des Pinosylvindimethyläthers.

stanzen als 2,4-Dibrom-3,5-diacetoxy-benzoesäure bzw. 4,6-Dibrom-2-nitro--3,5-dioxy-benzoesäure bezeichnet. Auf Grund unserer Ergebnisse müssen diese Körper jedoch die 2,6-Dibrom-3,5-diacetoxy-benzoesäure bzw. 2,6-Dibrom-4-nitro-3,5-dioxy-benzoesäure darstellen.

4-Brom-3,5-dimethoxybenzoesäure-methylester konnte trotz zahlreicher unter variierten Bedingungen ausgeführter Versuche nicht zum 4,6-Dibrom--3,5-dimethoxybenzoesäure-methylester bromiert werden.

EXPERIMENTELLER TEIL

Bromierung von Pinosylvindimethyläther mit einem Mol Brom

Pinosylvindimethyläther (10,00 g) wurde in Chloroform (75 ml) gelöst, und eine Lösung von Brom (6,67 g) in Chloroform (75 ml) wurde hinzugefügt. Das Brom wurde sofort verbraucht. Nach Schütteln mit Wasser und Abdestillieren des Chloroforms wurde ein Öl erhalten, welches beim Reiben mit Methanol durchkrystallisierte. Die Krystalle (10 g) wurden abgesaugt (A), Mutterlauge = B. A schmolz bei 75—85° und lieferte bei der Fraktionierung aus Methanol Krystalle (7,1 g) von *2-Brom-3,5-dimethoxy-stilben* vom Schmelzpunkt 92—93° bzw. 124—125° ($C_{16}H_{15}O_2Br$: Ber. Br = 25,0, Gef. 25,5 %). Aus der bei der Fraktionierung von A anfallenden Mutterlauge wurde durch Eindampfen und Auskochen des krystallinen Rückstandes mit wenig Äthanol eine schwer lösliche Fraktion erhalten, die aus viel Methanol oder Äthanol-Eisessig Krystalle gab, welche konstant bei 151—153° schmolzen und das *4-Brom-3,5-dimethoxy-stilben* darstellen.

$C_{16}H_{15}O_2Br$ Ber. Br 25,0 Gef. Br 25,1

Die Mutterlauge B wurde mit den Mutterlaugen von der Gewinnung des 4-Brom-3,5-dimethoxy-stilbens vereinigt und 30 Minuten unter Rückfluss mit Zinkstaub gekocht. Aus der alkoholischen Lösung fiel beim Erkalten ein feinkörniges Material (0,82 g) aus, welches schnell abgesaugt wurde. Im Filtrat erschienen gröbere Krystalle (1,8 g). Das erstgenannte Material lieferte leicht reines 4-Brom-3,5-dimethoxy-stilben (0,68 g), das letzgenannte 2-Brom-3,5-dimethoxy-stilben.

2-Brom-3,5-dimethoxy-stilben gibt, in Methanol gelöst, mit *symm.* Trinitrobenzol ein gelbes Addukt. Konzentrierte Schwefelsäure löst mit gelber Farbe. Tetranitromethan gibt bei Zimmertemperatur eine schwach gelbbraune Farbreaktion, die beim Erwärmen vertieft wird.

4-Brom-3,5-dimethoxy-stilben gibt mit *symm.* Trinitrobenzol ein ziegelrotes Addukt. Die Schwefelsäurereaktion ist negativ. Die Tetranitromethan-reaktion ähnelt der des 2-Brom-isomeren.

Dasselbe Material, welches beim obigen Versuch mit Zinkstaub behandelt wurde, wurde in einem anderen Versuch in Chloroformlösung gebracht und mit Brom in Chloroform versetzt, bis die Entfärbung nur noch sehr langsam vonstatten ging. Das krystalline Reaktionsprodukt wurde in 200 ml heissem Eisessig gelöst. Beim Erkalten schieden sich Krystalle aus, die aus Eisessig umkrystallisiert wurden. Schmelzpunkt 225—227°.

$C_{16}H_{15}O_2Br_3$ Ber. Br 50,1 Gef. Br 50,7

Die in siedendem Äthanol sehr schwer lösliche Verbindung (4-Brom-3,5-dimethoxy-stilbendibromid) gab keine Farbreaktion mit Tetranitromethan (auch nicht in der Wärme) und lieferte mit Brom unter Bromwasserstoffentwicklung den »niedrigschmelzenden Tetrabrom-pinosylvindimethyläther». Schmelzpunkt und Mischschmelzpunkt 171—173°. Durch Kochen mit Äthanol und Zink wurde 4-Brom-3,5-dimethoxy-stilben erhalten (Mischschmelzpunkt).

Bromierung von Pinosylvindimethyläther mit zwei Mol Brom

Pinosylvindimethyläther (5,00 g) wurde in einem Gemisch von Chloroform (30 ml) und Pyridin (10 ml) gelöst. Die Lösung wurde mit Wasser (50 ml) überschichtet und eine Lösung von Brom (6,8 g) in Chloroform (30 ml) tropfenweise hinzugefügt. Das Brom wurde anfangs schnell, später etwas langsamer entfärbt. Durch wiederholtes Schütteln mit Wasser wurde der gebildete Bromwasserstoff entfernt, und dessen Menge wurde durch Titration mit Natronlauge ermittelt (93,8 % d. Th.). Das Chloroform hinterliess beim Abdestillieren eine Krystallmasse, aus welcher sich durch Umkrystallisieren aus Äthanol 2,6-Dibrom-3,5-dimethoxystilben (3,63 g) gewinnen liess. Die Mutterlaugen wurden eingedampft und in Chloroform (30 ml) gelöst, worauf Pyridin (10 ml) und schliesslich Brom (4,0 g) in Chloroform (30 ml) zugesetzt wurde. Nach kurzer Zeit wurde mit Wasser ausgeschüttelt, Chloroform und Brom wurden durch Aufblasen von heisser Luft abgetrieben und die feste Masse wiederholt mit kleinen Mengen Methanol ausgekocht. Das Ungelöste wurde abfiltriert und die Lösung eingedampft. Der Rückstand wurde wiederum mehrmals mit Methanol ausgekocht. Das Ungelöste *(2,6-Dibrom-3,5--dimethoxystilben-dibromid)* wurde vereinigt und aus Eisessig mehrmals umkrystallisiert. Grobe Prismen vom Schmelzpunkt 225—227° unter Gasentwicklung.

$C_{16}H_{14}O_2Br_4$	Ber. C 34,4	H 2,53	Br 57,3
	Gef. » 34,3	» 2,53	» 57,5

Das im Methanol leichter lösliche Material wurde mehrmals aus Äthanol umkrystallisiert. Es wurden 1,25 g reines 2,4-Dibrom-3,5-dimethoxystilben-dibromid erhalten.

Reduktion des niedrig- bzw. hochschmelzenden Tetrabrom-pinosylvindimethyläthers mit Zink

Niedrigschmelzender Tetrabrompinosylvindimethyläther (2,4-Dibrom-3,5-dimethoxystilben-dibromid) (1,25 g) wurde in Äthanol (50 ml) gelöst und mit Zinkstaub (1 g) 45 Minuten unter Rückfluss gekocht. Beim Kühlen fielen watteähnliche Krystalle von 2,4-Dibrom-3,5-dimethoxystilben aus (0,72 g, 81 % d. Th.), die bei 122—127° schmolzen. Nach zweimaligem Umkrystallisieren aus Äthanol 0,55 g Substanz vom Schmelzpunkt 131—132°.

$C_{16}H_{14}O_2Br_2$ Ber. Br 40,2 Gef. Br 40,6

In ähnlicher Weise wurde aus hochschmelzendem Tetrabrompinosylvin-dimethyläther (2,6-Dibrom-3,5-dimethoxystilben-dibromid) 2,6-Dibrom-3,5-dimethoxystilben erhalten.

Oxydation von 4-Brom-3,5-dimethoxystilben mit Kaliumpermanganat

4-Brom-3,5-dimethoxystilben (0,53 g) wurde in Aceton (15 ml) gelöst und zu der Lösung wurden bei Zimmertemperatur 0,7 g Kaliumpermanganat in kleinen Portionen hinzugefügt. Nachdem alles Permanganat verbraucht war, wurde eingedampft, verdünnte Schwefelsäure hinzugegeben und Schwefeldioxyd eingeleitet. Die Fällung wurde abgesaugt und aus Methanol umkrystallisiert. So wurde *4-Brom-3,5-dimethoxybenzoesäure* (0,23 g, entspr. 54 % d. Th.) gewonnen, die bei 249—250° schmolz. Die Säure wurde direkt mit Diazomethan in den *Methylester* übergeführt, Schmelzpunkt 119—120°, Mischschmelzpunkt mit synthetischem 4-Brom-3,5-dimethoxybenzoesäuremethylester 119—120°. Aus der Mutterlauge der 4-Brom-3,5-dimethoxybenzoesäure wurde mit Äther Benzoesäure isoliert.

Oxydation von 2,4-Dibrom-3,5-dimethoxystilben mit Kaliumpermanganat

Die Oxydation wurde prinzipiell wie im vorigen Versuch ausgeführt. Es wurden aus 0,5 g Brom-dimethoxystilben 0,12 g Benzoesäure und 0,2 g rohe 2,4-Dibrom-3,5-dimethoxy-benzoesäure isoliert. Letztere wurde aus 25 %igem wässrigem Methanol umkrystallisiert. Feine Krystallkörner vom Schmelzpunkt 183—184° (Ausbeute 0,11 g).

$C_9H_8O_4Br_2$	Ber. C 31,8	H 2,35	OCH_3 18,2
	Gef. » 31,9	» 2,35	» 18,1

Mit Diazomethan wurde *2,4-Dibrom-3,5-dimethoxybenzoesäuremethylester* gewonnen. Aus 25 %igem wässrigem Methanol watteähnliche Krystalle vom Schmelzpunkt 76—77°.

$C_{10}H_{10}O_4Br_2$ Ber. OCH_3 26,3 Gef. OCH_3 25,8

Methylierung der v. Hemmelmayr'schen Dibrom-α-resorcylsäure mit Diazomethan

Die von v. Hemmelmayr als 2,4-Dibrom-3,5-dioxybenzoesäure aufgefasste Säure vom Schmelzpunkt 192° wurde nach den Angaben der Literatur dargestellt[4] und (3,1 g) in Äther (15 ml) gelöst. Nach Zusatz von Diazomethan (2,49 g) in Äther (100 ml) wurde über Nacht stehen gelassen. Nach Abdestillieren des Äthers wurden Krystalle (3,82 g, entspr. 97 % d. Th.) erhalten, die bei 142—145° schmolzen. Aus Methanol Nadeln (3,3 g) vom Schmelzpunkt 148—149°.

$C_{10}H_{10}O_4Br_2$ Ber. OCH_3 26,3 Gef. OCH_3 25,9

Der Mischschmelzpunkt mit einem Präparat, welches aus 2,6-Dibrom-3,5-dimethoxybenzoesäure und Diazomethan dargestellt worden war, zeigte keine Erniedrigung,

Der Methylester ist sehr schwer, wenn überhaupt hydrolysierbar.

2,4,6-Tribrom-3,5-dimethoxy-benzoesäure-methylester

2,4,6-Tribrom-3,5-dioxybenzoesäure wurde nach Barth und Senhofer[5] dargestellt und mit Diazomethan methyliert. Der mit einer Ausbeute von 93 % der Theorie gewonnene Ester schmolz bei 103—104°.

$C_{10}H_9O_4Br_3$ Ber. OCH_3 21,5 Gef. OCH_3 21,5

4-Brom-3,5-dimethoxybenzoesäure-methylester

Aus 4-Brom-3,5-dioxy-benzoesäure[3] mit Diazomethan in Äther. Ausbeute 90 % d. Th. Aus 50 %igem Methanol lange Nadeln vom Schmelzpunkt 120—120,5°.

$C_{10}H_{11}O_4Br$ Ber. OCH_3 33,1 Gef. OCH_3 33,2

ZUSAMMENFASSUNG

Die Bromierung des methoxylierten Kernes des Pinosylvin-dimethyläthers (*trans*-3,5-dimethoxystilbens) verläuft schneller als die der Äthylendoppelbindung. Hierbei werden nicht nur die 2,6-Stellungen sondern auch die 4-Stellung substituiert. Bei der vollständigen Bromierung entstehen deshalb strukturverschiedene, nicht wie früher behauptet worden ist, diastereoisomere Tetrabromderivate.

Die Kosten dieser Arbeit wurden teilweise durch Mittel aus *Statens Tekniska Forskningsråd* bestritten.

LITERATUR

1. Cox, R. F. B. *J. Am. Chem. Soc.* **62** (1940) 3512.
2. Aulin-Erdtman, G., und Erdtman, H. *Ber.* **74** (1941) 50.
3. Barth, L., und Senhofer, C. *Ann.* **164** (1872) 115.
4. v. Hemmelmayr, F. *M.* **33** (1912) 994.
5. Barth, L., und Senhofer, C. *Ann.* **159** (1871) 225.

Eingegangen am 6. Dezember 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 42—48

Kinetische Untersuchung über die Spaltung von Chloralhydrat mit Natriumhydroxyd in wässriger Lösung

CHARLEY GUSTAFSSON und MONICA JOHANSON

Chemisches Institut der Universität Helsinki, Finnland

Chloral liegt in wässriger Lösung als Hydrat vor. Von starken Basen wird das Chloralhydrat bekanntlich quantitativ in Chloroform und Formiat gespalten. In vorliegender Arbeit wird der Mechanismus dieser Reaktion kinetisch untersucht.

Messungen von Enklaar u. a.[1] zeigen, dass das Chloralhydrat eine schwache Säure ist, die die spaltende Base unter Salzbildung teilweise neutralisiert, wonach das Chloralation in Chloroform und Formiat zerfällt. Dieser Zerfall scheint von der unverbrauchten Base katalytisch beschleunigt zu werden.

Wir haben die Wasserstoffionenkonzentration verdünnter, wässriger Chloralhydrat — Natriumhydroxydlösungen unmittelbar nach dem Mischen mittels Thymolphtalein als Indikator und einer Reihe von Glycinat-Puffern als Standard bestimmt und die Säuredissoziationskonstante des Chloralhydrats bei Zimmertemperatur zu $1{,}5 \times 10^{-10}$ berechnet [2]. Diese Vorversuche, die hier nicht näher beschrieben werden, zeigen somit, dass das Chloralhydrat bei einem Überschuss an Natriumhydroxyd praktisch vollständig als Chloralationen vorliegt.

Man muss mit der Möglichkeit rechnen, dass der Zerfall des Chloralations nicht nur von Hydroxylionen, sondern auch von Chloralationen selbst und von Wasser (»spontan») katalysiert verlaufen kann. Die Geschwindigkeit der Reaktion zwischen Chloralhydrat und Natriumhydroxyd in verdünnter, wässriger Lösung könnte somit durch die Gleichungen 1 und 2 beschrieben werden.

$$\frac{dx}{dt} = (k_0 + k_1\,[\mathrm{A^-}] + k_2\,[\mathrm{OH^-}])\,[\mathrm{A^-}] \qquad (1)$$

$$[\mathrm{OH^-}] = \frac{K_w}{K_c} \times \frac{[\mathrm{A^-}]}{[\mathrm{HA}]} \qquad (2)$$

[HA] Die laufende Konzentration der undissozierten Form des Chloralhydrats

[A^-] Die laufende Konzentration des Chloralations

x Die laufende Konzentration des Formiats

k_0 Die Geschwindigkeitskonstante des von Wasser katalysierten (»spontanen») Zerfalls des Chloralations

k_1 Die Geschwindigkeitskonstante des von Chloralationen katalysierten Zerfalls des Chloralations

k_2 Die Geschwindigkeitskonstante des von Hydroxylionen katalysierten Zerfalls des Chloralations

K_c Die Säuredissoziationskonstante (bezüglich der Konzentration) des Chloralhydrats

K_w Das Ionenprodukt (bezüglich der Konzentration) des Wassers

t Die Zeit

VERSUCHE

Die Versuche wurden mit den von Tommila [3] beschriebenen, zweiteiligen Kolben durchgeführt. 5 ml Chloralhydratlösung und 5 ml Natriumhydroxydlösung wurden in je einen Schenkel der Versuchsgefässe abgemessen und in einem Thermostaten temperiert. Die Reaktion wurde mit Salzsäure unterbrochen und der Überschuss an Säure mit Bariumhydroxyd und Methylrot als Indikator zurücktitriert. Kontrollversuche zeigten, dass die Messungen von der besonders bei höherer Temperatur als Nebenreaktion eintretenden Bildung von Chlorionen praktisch nicht gestört wurden.

Die Reaktions- und Messflüssigkeiten wurden in üblicher Weise aus reinen Reagentien und ausgekochtem, destilliertem Wasser bereitet und gegen Kaliumbijodat standardisiert. Der Gehalt der Chloralhydratlösungen wurde durch vollständige Spaltung mit einem Überschuss an Lauge kontrolliert.

Die Ionenkonzentration der Natriumhydroxydlösungen wurde mit Natriumchlorid auf 0,2 N eingestellt. Die Ionenkonzentration der Reaktionslösungen (nach dem Vermischen) war somit immer 0,1 N. In dieser Arbeit sind alle Konzentrationen in Äquivalenten pro Liter bei 20 C° und die Zeit immer in Sekunden angegeben.

DIE ERGEBNISSE BEI EINEM ÜBERSCHUSS AN NATRIUMHYDROXYD

Bei einem genügend grossen Überschuss an Natriumhydroxyd ist $[OH^-] = (b—a)$ und $[A^-] = (a—x)$, wenn die Anfangskonzentration des Chloralhydrats mit a und die Anfangskonzentration des Natriumhydroxyds mit b bezeichnet wird. Wenn man die Gleichung 1 unter diesen Bedingungen integriert und aus den Versuchsergebnissen die Geschwindigkeitskonstanten k_0, k_1 und k_2 zu berechnen versucht, ergibt sich, dass k_0 und k_1 $(a—x)$, verglichen mit $k_2(b—a)$, unmessbar klein sind. Die Gleichung 1 geht deshalb in diesem Falle in Gleichung 3 über.

$$\frac{dx}{dt} = k_2\ (b—a)\ (a—x) \qquad (3)$$

Die Reaktion ist somit bei gegebenen a- und b-Werten von erster Ordnung bezüglich des Chloralhydrats. In Tabelle 1 sind die Ergebnisse bei einem Überschuss an Natriumhydroxyd zusammengestellt worden. Jeder $k_2\ (b—a)$-Wert, berechnet nach der bekannten Gleichung für Reaktionen erster Ordnung, ist das Mittel von wenigstens acht Messungen mit verschiedenen Reaktionszeiten.

Tabelle 1. Die Werte von $k_2\ (b—a)$ und k_2 berechnet aus den Messungen bei einem Überschuss an Natriumhydroxyd.

C°	a	$(b—a)$	$k_2\ (b—a)$	k_2
15	0,0052	0,0048	0,0051	1,06
15	0,0103	0,0097	0,0103	1,06
15	0,0082	0,0118	0,0130	1,10
15	0,0052	0,0148	0,0155	1,05
20	0,0052	0,0048	0,0096	1,99
20	0,0103	0,0088	0,0180	2,05
20	0,0082	0,0118	0,0240	2,03
20	0,0052	0,0139	0,0277	1,99
25	0,0054	0,0052	0,0185	3,56
25	0,0103	0,0088	0,0316	3,59
25	0,0082	0,0118	0,0434	3,68
25	0,0052	0,0139	0,0491	3,53
30	0,0054	0,0052	0,0333	6,40
30	0,0103	0,0088	0,0566	6,43
30	0,0082	0,0118	0,0747	6,33
30	0,0052	0,0139	0,0836	6,01

Die Geschwindigkeit einer Reaktion zwischen zwei Ionen ist von der Gesamtionenkonzentration abhängig. Um die scheinbare Aktivierungsenergie und die Aktionskonstante des von Hydroxylionen katalysierten Zerfalls der Chloralationen berechnen zu können, müssen deshalb die bei der Ionenkonzentration 0,1 N experimentell erhaltenen k_2-Werte auf unendliche Verdünnung extrapoliert werden. In Tabelle 2 sind die Mittelwerte der experimentell gefundenen k_2-Werte und die mit Hilfe der bekannten Brönsted-Debye-Hückel'schen Gleichung auf unendliche Verdünnung extrapolierten k_2-Werte zusammengestellt. Aus diesen lässt sich die scheinbare Aktivierungsenergie mittels der bekannten Arrhenius'schen Gleichung zu $E = 20{,}22$ Kcal und der Logarithmus der Aktionskonstante zu $\log A = 15{,}06$ berechnen. Die sich aus diesen errechneten k_2-Werte sind in der letzten Spalte der Tabelle 2 wiedergegeben.

Tabelle 2. Die Geschwindigkeitskonstante k_2 bei der Ionenkonzentration 0,1 N, bei unendlicher Verdünnung und aus den Arrhenius'schen Konstanten berechnet.

C°	k_2		
	0,1 *N*	Unendliche Verdünnung	Aus den Arrhenius'schen Konstanten
15	1,07	0,52	0,52
20	2,02	0,97	0,96
25	3,59	1,72	1,71
30	6,29	2,99	3,01

DIE ERGEBNISSE BEI EINEM ÜBERSCHUSS AN CHLORALHYDRAT

Wenn $[A^-]/[HA]$ nicht zu gross oder zu klein ist, ist bei einem Überschuss an Chloralhydrat $[A^-] = (b—x)$ und $[HA] = (a—b)$. Wenn man unter diesen Bedingungen die Gleichung 1 integriert, erhält man für die Halbwertszeit $t_{½}$ die Gleichungen 4 und 5.

$$t_{½} = \frac{2{,}303}{k_0} \log \left(1 + \frac{1}{1 + k'\ b}\right) \tag{4}$$

$$k' = \frac{k_1}{k_0} + \frac{k_2 K_w}{k_0 K_c} \frac{1}{(a—b)} \tag{5}$$

Aus der Halbwertszeit der Reaktion bei verschiedenen b-Werten kann man k_0 und k' graphisch berechnen und aus den k'-Werten bei verschiedenen $(a—b)$ nach Einsetzen von k_0, k_2 und K_w (nach Harned und Cook [4] berechnet) die Werte von k_1 und K_c berechnen. Die Ergebnisse sind in den Tabellen 3, 4, 5 und 6 zusammengestellt. Die Halbwertszeiten wurden graphisch aus wenigstens acht Messungen mit verschiedenen Reaktionszeiten bestimmt. Die scheinbare Aktivierungsenergie und die Aktionskonstante des von Wasser katalysierten (»spontanen») Zerfalls des Chloralations sind aus den Mittelwerten der experimentellen k_0-Werte zu $E = 29{,}58$ Kcal, $\log A = 17{,}79$ und die des von Chloralationen katalysierten Zerfalls des Chloralations aus den auf unendliche Verdünnung extrapolierten k_1-Werten zu $E = 29{,}47$ Kcal, $\log A = 19{,}07$ berechnet worden. Aus diesen wurden wieder die Geschwindigkeitskonstanten berechnet und in den Tabellen 4 und 5 zusammengestellt.

Tabelle 3. Die Werte von k_0 und k', berechnet aus den Messungen bei einem Überschuss an Chloralhydrat.

C°	b	$(a-b)$	$t_{½}$	$k_0 \times 10^5$	k'
15	0,0099	0,0122	9760	2,25	
					310
15	0,0200	0,0124	5820	2,23	
15	0,0100	0,0224	13185	2,25	
					190
15	0,0300	0,0227	6170	2,25	
20	0,0099	0,0122	4080	5,63	
					290
20	0,0200	0,0124	2550	5,38	
20	0,0100	0,0224	5670	5,39	
					180
20	0,0300	0,0227	2580	5,62	
25	0,0099	0,0122	1910	12,6	
					270
25	0,0200	0,0124	1120	13,0	
25	0,0100	0,0224	2430	13,0	
					170
25	0,0300	0,0227	1185	12,8	
30	0,0099	0,0122	870	28,7	
					255
30	0,0200	0,0124	520	29,2	
30	0,0100	0,0224	1120	29,1	
					160
30	0,0300	0,0227	550	28,9	

Tabelle 4. Die Geschwindigkeitskonstante k_0 aus den experimentellen Ergebnissen und aus den Arrhenius'schen Konstanten berechnet.

C°	$k_0 \times 10^5$	
	Experimentell	Berechnet
15	2,2	2,3
20	5,5	5,5
25	12,9	12,8
30	29,0	29,2

Tabelle 5. Die Geschwindigkeitskonstante k_1 bei der Ionenkonzentration 0,1 N, bei unendlicher Verdünnung und aus den Arrhenius'schen Konstanten berechnet.

C°	$k_1 \times 10^3$		
	0,1 *N*	Unendliche Verdünnung	Aus den Arrhenius'schen Konstanten
15	1,04	0,50	0,52
20	2,64	1,27	1,25
25	6,45	3,09	2,92
30	13,3	6,34	6,64

Tabelle 6. Die Säuredissoziationskonstante K_c bei der Ionenkonzentration 0,1 N.

C°	$K_c \times 10^{10}$
15	1,1
20	1,4
25	1,7
30	2,0

DISKUSSION DER ERGEBNISSE

Die Messungsergebnisse, die Verifizierbarkeit der Arrhenius'schen Gleichung durch die Geschwindigkeitskonstanten und die Übereinstimmung zwischen den kinetisch und kolorimetrisch bestimmten Säuredissoziationskonstanten des Chloralhydrats sprechen für die Richtigkeit der Theorie.

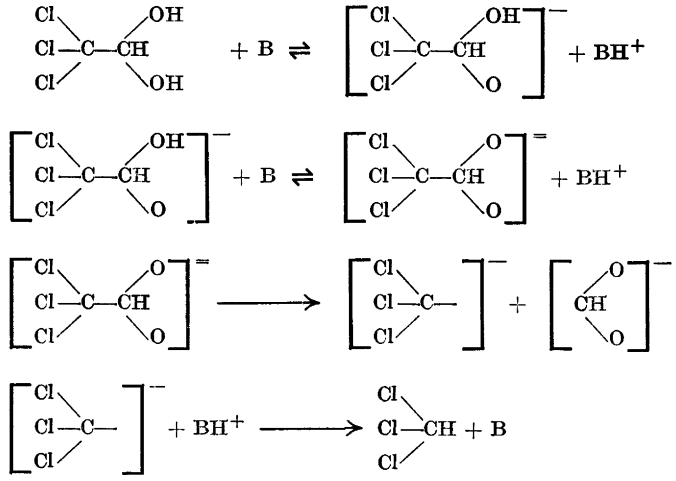
Die Säuredissoziationskonstante des Chloralhydrats ist von gleicher Grössenordnung wie die des Phenols. Sie kann durch die acidifizierende Wirkung der Chloratome und durch die Tatsache, dass in Chloralhydrat zwei Hydroxylgruppen an demselben Kohlenstoffatom gebunden sind, verstanden werden.

Der Zerfall des Chloralations wird sowohl von Hydroxylionen wie auch von Chloralationen selbst katalytisch beschleunigt, und wir haben es somit mit einer Basenkatalyse zu tun. Dies deutet darauf hin, dass das Chloralation unter Bildung von einem negativ zweiwertigen Ion noch ein Proton an die Base abgibt. Erst dieses zweiwertige Ion zerfällt unter Rückbildung der Base in Chloroform und Formiation.

Auch der von der Reaktionsgeschwindigkeitskonstanten k_0 bestimmte Zerfall des Chloralations kann am besten als eine von den Wassermolekülen bewirkte Basenkatalyse erklärt werden. Wenn dies richtig ist, müssen sowohl

k_0 wie die entsprechende Aktionskonstante mit 55,5 (der Molkonzentration des Wassers) dividiert werden.

Der Mechanismus der Reaktion zwischen Chloralhydrat und Basen könnte somit durch folgende Reaktionsgleichungen beschrieben werden.



ZUSAMMENFASSUNG

Die Geschwindigkeit der Reaktion zwischen Chloralhydrat und Natriumhydroxyd wurde in wässriger Lösung bei der Gesamtionenkonzentration 0,1 *N* bei vier verschiedenen Temperaturen gemessen.

Die Säuredissoziationskonstante des Chloralhydrats wurde aus den kinetischen Daten berechnet (Tabelle 6).

Die Reaktionsgeschwindigkeitskonstanten des von Wasser, von Chloralationen und von Hydroxylionen katalysierten Zerfalls des Chlorations (Tabellen 4, 5 und 2) sowie die scheinbare Aktivierungsenergie und die Aktionskonstante der Reaktionen wurden berechnet.

Der Reaktionsmechanismus wurde diskutiert.

LITERATUR

1. Enklaar, J. E. *Rec. trav. chim.* **23** (1904) 419, **24** (1905) 419, **25** (1906) 297, **29** (1910) 173; Böttger, W., und Kötz, A. *J. prakt. Chem.* **65** (1902) 481; Arenson, S. B., Roller, P. E., und Brown, D. J. *J. Phys. Chem.* **30** (1926) 620; Vergl. auch Müller, E. *Neuere Anschauungen der Organischen Chemie* Berlin (1940) 150.
2. Vergl. Euler, H. v. *Ber.* **39** (1906) 347.
3. Tommila, E. *Ann. Acad. Sci. Fennicae* **A 59**:3 (1942) 9.
4. Harned, H. S., und Cook, M. A. *J. Am. Chem. Soc.* **59** (1937) 2304.

Eingegangen am 15. Dezember 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 49—52

A Note on the Chemistry of Enterogastrone

HÅKAN WINBERG and KARL JOHAN ÖBRINK

The Central Laboratories, A. B. Astra, Södertälje, and the Institute of Physiology, University of Uppsala, Uppsala, Sweden

From the upper part of the small intestine a substance can be extracted that inhibits the histamine induced gastric secretion. This substance has been given the name *enterogastrone*. Papers concerning the chemical properties of enterogastrone are very few, but according to Harris, Gray and Ivy [1] it might be of a protein nature, as they found an inactivation of their preparations by proteolysis with pepsin.

Recently one of us [2] showed that the acitive principle in our preparations had *no electrophoretic mobility between pH 2.06 and 7.42*. Furthermore it was very *easy to dialyse* through a cellophane membrane [3].

As these observations do not accord with the idea that enterogastrone might be a protein it was found necessary to reinvestigate the effect of proteolysis on our preparations.

EXPERIMENTAL

The preparation process of the enterogastrone was mainly that recommended by Gray, Bradly and Ivy [4]. The extract obtained was dialysed through a cellophane membrane, thus giving the active principle in the dialysate. (The method will be described in detail in a forthcoming paper.)

The activity was tested on dogs with denervated gastric pouches according to Heidenhain. Histamine dihydrochloride was injected intravenously at a slow constant rate thus giving a constant secretion rate of gastric juice [5]. Then a 50 mg dose of the enterogastrone preparation was given intravenously causing an inhibition of the secretion, which was sometimes complete and lasted for several hours [6].

RESULTS

Effect of pepsin

In this experiment 50 mg of a very active preparation were incubated for 75 minutes at 37° C with 1 ml gastric juice from a dog and 4 ml citrate buffer,

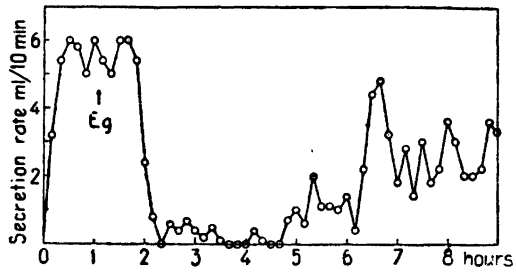


Fig. 1. The effect of a trypsin-treated enterogastrone preparation on the histamine-induced gastric secretion.

pH = 1.5. As a control experiment the peptic activity of this gastric juice was determined by the method given by Riggs and Stadie [6]. It was found that 1 ml of the juice could easily cause a »complete» proteolysis — as obtained with this method — of an egg-white solution containing 50 mg protein at the given conditions.

The pepsin-treated enterogastrone preparation was then given to a dog and found to possess the same inhibiting effect as the untreated one.

An injection of a mixture of 1 ml gastric juice and 4 ml citrate was ineffective.

Effect of trypsin

50 mg of the same enterogastrone preparation were dissolved in 4 ml borate buffer, pH 8.5, and incubated with 4 mg crystalline trypsin (Difco) for 14 hours. After digestion the same inhibiting effect characterized the preparation when injected into a dog. The result is given in Fig. 1.

A control injection of the buffer substance containing only the trypsin (4 mg) did not have any influence on the secretion from the gastric pouch.

Another 125 mg enterogastrone were dissolved in water free of CO_2 and adjusted to pH 8.0 with 0.1 *N* NaOH. The total volume was made 10 ml. When 10 mg trypsin had been added 1 ml was withdrawn, given one drop of toluol and titrated according to the wellknown Sörensen formol titration. The starting point was chosen pH 7.00 and after addition of 2 ml formalin (40 %, pH 8.25) the titration was continued with 0.04 *N* NaOH to pH 8.25.

The remaining 9 ml were divided into 1 ml samples each given one drop of toluol and incubated at 37° C. Every day the pH was controlled to be about 8.0. Only after the first day an adjustment with NaOH was necessary. Four of these samples were formol titrated at various times and the results are given in Table 1.

Table 1. The titration results after digestion by trypsin.

Substrate: *12.5 mg enterogastrone preparation in a water solution adjusted to pH 8.0.*
Trypsin: *Crystalline trypsin (Difco) 1 mg.*
Temperature: *37° C.*
Starting point: *pH 7.00.*
End point: *pH 8.25.*

Sample no.	Digestion time hours	Formol titration ml 0.04 *N* NaOH	Difference ml
1	0	0.53	0.00
2	26	1.08	0.55
3	51	1.21	0.68
4	91	1.26	0.73
5	140	1.28	0.75

It was found that the digestion by trypsin was almost finished in 4—6 days.

The preparation used could be precipitated by trichloroacetic acid before but not after digestion by trypsin. The biuret reaction remained, however, positive even after the proteolysis.

At the same time as sample 5 was titrated (*i. e.* after six days digestion) 4 ml (50 mg) of the same *digested mixture was tested on dog and found to retain its entire inhibiting properties.*

DISCUSSION

The experiments performed show that the inhibiting power on the histamine induced gastric acid secretion of an intestinal extract — enterogastrone — is unaffected by both pepsin and trypsin. In addition to the findings that the active principle of the enterogastrone preparation is easy to dialyse and has no or a very small electrophoretic mobility between pH 2.06 and 7.42 this may suggest that enterogastrone is no protein.

The tryptic action did not, however, give a complete hydrolysis of the existing peptides but left some unsplit which may be of a relatively low molecular weight.

SUMMARY

It was impossible to verify the suggestion that enterogastrone could be inactivated by pepsin. It was also shown that the inhibitory effect on the histamine induced gastric secretion was unaffected by trypsin action.

These observations suggest, that the enterogastrone inhibition active principle may be of a non-protein nature. This view may be confirmed by the demonstration that the substance can easely be dialysed and that its electrophoretic mobility is very small and unaffected by large pH-variations.

REFERENCES

1. Harris, S. C., Gray, J. S., and Ivy, A. C. *Fed. Proc.* **1** (1942) 37.
2. Öbrink, K. J. *Experientia* **3** (1947) 455.
3. Winberg, H. *Acta Chem. Scand.* **1** (1947) 251.
4. Gray, J. S., Bradly, W. B., and Ivy, A. C. *Am. J. Physiol.* **118** (1937) 463.
5. Öbrink, K. J. *Acta Physiol. Scand.* **12** (1946) 213.
6. Öbrink, K. J. *Acta Physiol Scand.* **15** (1948) suppl. 51 (added in proof).
7. Riggs, B. C., and Stadie, W. C. *J. Biol. Chem.* **150** (1943) 463.

Received December 10, 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 53—62

On the Thermodynamics of 'λ-Transitions'

JØRGEN KOEFOED

Universitetets Fysisk-Kemiske Institut, København, Denmark

The discovery by Keesom and his collaborators of what they named the λ-point of liquid helium, caused Ehrenfest[1] to put forward a thermodynamic theory of the transitions of the second order, characterized by discontinuity in the second derivatives of Gibbs' free energy, *viz.* in specific heat and coefficient of thermal expansion, but continuity in the first derivatives, S and V, the latter in contradistinction to the ordinary phase transitions, which are then called first order transitions.

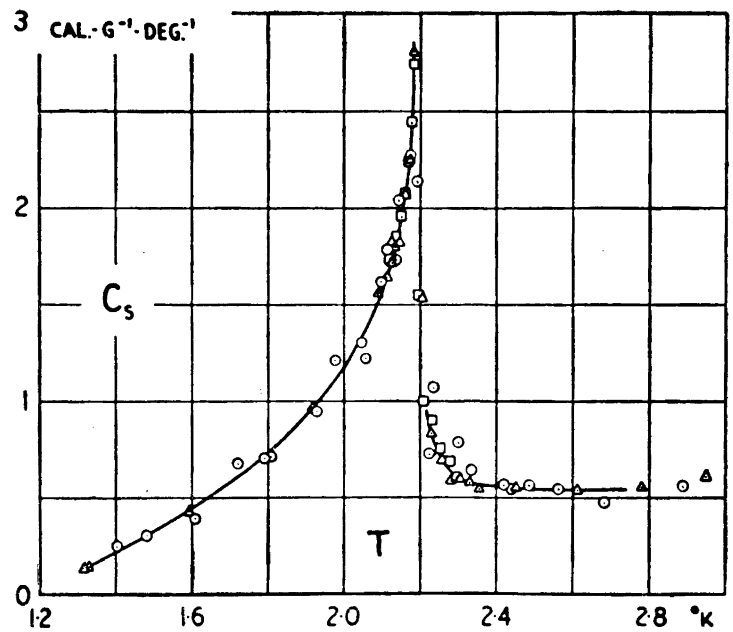


Fig. 1. The original measurements by Keesom and Keesom of the heat capacity of liquid He between 1.2° and 3° K. The heat capacity under the pressure of the saturated vapours, C_s, is given in cal/g · deg.

It is obvious that the second order transitions will show no phenomenon of two co-existing phases, and it was also pointed out at an early stage [2] that it was not permissible to regard them as occurring between two different phases each of which had fully continuous properties because an elementary calculation shows that the same phase would then be the more stable on both sides of the transition point. It is necessary, therefore, that this phase simply ceases to be physically defined, *i. e.* ceases to have even a theoretical possibility of existence when the transition point has been passed.

In his latest exposition of the theory,[3] Keesom seems to underestimate the rigour of this condition and tries to draw analogies to the possibility of experimental realization of unstable phases near ordinary transition points. The metastability of superheated or supercooled liquids, however, is easily explained by the fact that the liquid, though instable towards a macro-phase in a gaseous or solid state, respectively, is stable towards small germs of the same. Such 'germs' are therefore formed and dissolved continuously until one of them happens to grow beyond the critical size and becomes capable of forming a true germ for starting the growth of the stable macro-phase. The melting process, presumably, always finds imperfections on surfaces and interfaces where it can start without any such obstacle, and the only chance of establishing a superheated crystal is probably in working with perfect single-crystals, and no such work has hitherto been reported. But what is relevant in this connection is only that irrespective of its impracticability, the superheated crystal has perfectly well defined and continuous thermodynamic functions. The whole discussion is therefore entirely irrelevant to the problem of the second order transition.

Bridgman [4] has proposed a model of the thermodynamic discontinuity. He considers an order-disorder transition, and if the conventional conception of the same is used, his argument should read: At the transition point the disorder is complete, and the degree of order parameter equals zero. The disordering process cannot proceed further than to complete disorder, and the degree of order cannot be less than zero. Hence the prolongation of the free energy curve of the phase of progressive disorder does not correspond to any physically possible states. This pseudo-phase together with an always completely disordered phase, forms the two 'phases' between which a second order transition in Ehrenfest's sense can take place, although the free energy curve of the former may be the lower on both sides of the transition point.

Against this two arguments shall be given:

1. In many of the known order-disorder transitions there exists all through the transition interval a carrying lattice which makes it possible to distinguish unmistakably between 'right' and 'wrong' atoms. Negative degree of order then has the well defined physical meaning that more atoms are in wrong than in right places. Even in cases where a carrying lattice is not present, it is only a matter of convention to assign to a negative degree of order the same meaning as that of the numerically equal positive value.

2. The 'λ-curves' have tails. Above the transition point, the heat capacities in most of the investigated cases still have extraordinary high values, which are falling off with temperature. If we do not want to complicate our conceptions by assuming this to be some separate phenomenon, we must assume that the process, be it a disordering process or whatever else that goes on in the transition interval below the λ-point, does continue

also above the latter, though at a much smaller rate. Hence the internal parameter, be it the degree of local order or something else, cannot have reached its ultimate value at the λ-point proper.

It can be stated, therefore, that so far no satisfactory model of the pseudo-phases of Ehrenfest's theory has been given. But quite apart from that, it is important to bear in mind that the background for an introduction of pseudo-phases is in fact a dislike of discontinuities, which causes a search for an explanation of the λ-discontinuities by analogy to the theory of ordinary phase transitions in some transition between 'phases' which themselves have continuous properties. As this is impossible because, as we have seen, at least one of these pseudo-phases must possess some discontinuous properties, the whole introduction of such pseudophases, whether explicit or implicit, seems to have lost its point.

The same is true, of course, if, as is by far the most probable thing, the λ-curves of heat capacity *etc.* are not discontinuous in a strictly mathematical sense but only have an extremely steep peak. Then there is no discontinuity to explain away.

In the following treatment, therefore, we will approach the problem in a rather different way by considering the analogy to systems of two components and one phase, instead of to those of one component and two phases.

Discoveries in recent years have shown that very many pure substances are in fact equilibrium mixtures of isomeric or polymeric modifications [5]. It is possible to regard such substances as mixtures of pseudo-components and to treat them thermodynamically as such,* regardless of whether there is or is not any possibility of preventing the immediate attainment of equilibrium, and thus of experimental demonstration of the difference between the components. This in spite of the fact that in a phase-rule sense the systems are of one component only.

The partition function [6] of a mixture of the isomeric, chemically different compounds A, B, C .... in equilibrium with one another, is the sum of the partition functions of the components:

$$f = f_A + f_B + f_C + \ldots$$

* It is a matter of course that analogies to the theory of mixtures will be elucidating only if drawn to *correct* interpretations of the entities. A recent paper by Tisza (*Phys. Rev.* **72** (1947) 838) calls forth the following remark: The osmotic pressure of a solution is the pressure under which its solvent component would be in equilibrium with the pure solvent, and it has no simple kinetic interpretation. Unfortunately — from a pedagogical point of view — is it for ideal solutions numerically identical with the pressure which the solute molecules *would have exerted if* they had occupied the same volume in the ideal gas state, but they do not by any means actually exert it, neither on a wall nor on a semi-permeable membrane (added in proof).

and the partition function of a component is defined:

$$f_A = \sum_r \omega_r \cdot e^{-\varepsilon_r/kT}$$

molecules of the species A being able to exist in the degenerate quantum states r, each of the energy ε_r and with the weight factor ω_r.

Now, if for a compound A, in the pure state or in a mixture, we can divide the possible states into groups r', r'', r''' ... by some physical criteria, we can also split up the partition function accordingly:

$$f_A = f_A' + f_A'' + f_A''' + \ldots = \sum_{r'} + \sum_{r''} + \sum_{r'''} + \ldots = \sum_r$$

expressing it as the sum of the partition functions of the subspecies A', A'', A''' ... in exactly the same way as the partition function of all the interconvertible isomers is the sum of those of each of the components. As all the thermodynamic functions can be derived from the partition functions, we thus arrive at the conclusion that it is permissible to regard a chemically pure substance as a mixture of several components in equilibrium and to treat it as such according to the usual thermodynamic theory of mixtures, provided that the molecules of each of the pseudocomponents are unambiguously distinguishable from those of the other by some physical criterion.

In some way this result is quite obvious as the statistical and thermodynamic treatment cannot depend on whether the physical difference between two sorts of molecules is of such a kind that it is macroscopically recognizable, *e. g.* by different optical rotatory power, or whether it is only a difference in quantum state.

What makes it somewhat obscure at first sight, however, is the well known »Gibbs' paradox». But the pertinent offence against the laws of thermodynamics is committed only if the difference between molecules belonging to different components is not unambiguously defined because then the enumeration of states goes wrong.

On this basis it is now remarkably easy to describe the λ-transition and several related phenomena in such a way that analogies and contrasts are very clearly visualized.

Consider some compound or element which can have its atoms arranged in say two different ways. We may think of para-orto hydrogen, monocline-rhombic sulphur, monomer and trimer sulphurdioxide, solid nitrogen with rotating and non-rotating molecules, alloys with ordered unit cells and cells with some of the atoms inversed, or whatever we like, if only the difference can be unambiguously defined.

In principle we shall be able to put down a partition function for each of the two different components in pure state and to draw curves of their free energies as functions *e. g.* of temperature. The interesting part of these curves will be where the distance between them is near to or smaller than RT. If the two components are unable to mix in that interval, we observe the familiar phenomenon of a transition point where the free energy curves intersect, and

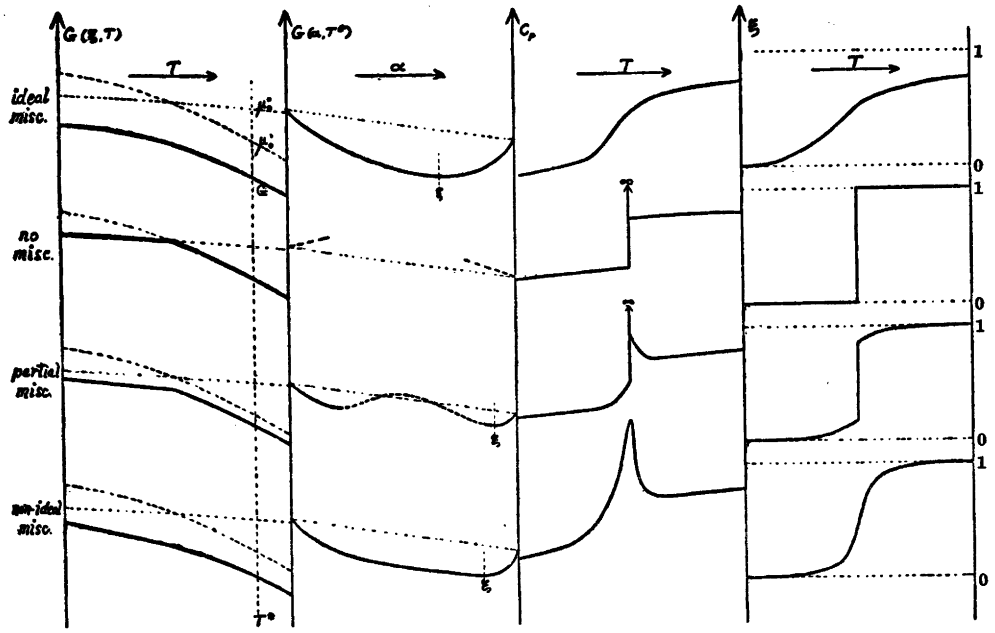


Fig. 2 shows the rough shape of the curves in four cases with different kinds of miscibility of two pseudo-components: 1) Free energy as function of temperature and compared with the free energies of the 'pure components'. 2) For a chosen temperature T: the free energy with varying mole fraction a. 3) C_p versus T. 4) The equilibrium mole fraction, ξ, versus T.*

where two phases, each consisting of a pure component, are in equilibrium. All thermodynamic functions except G change discontinuously here, while in all other points they vary quite smoothly.

If the two components are partially miscible, we shall get the same kind of transition point, but in its neighbourhood we shall observe an abnormally great heat capacity and perhaps other signs of a pre-melting like behaviour. The greater the mutual solubilities are, the more pronounced is the effect, and the less is the relative magnitude of the heat of transition proper. The miscibility may of course be one-sided, so that only one of the components is detectably soluble in the other.

If, on the other hand, the two components form ideal or nearly ideal mixtures, the equilibrium state will contain a smoothly increasing fraction of the high energy component, and the specific heat curve will show the sort of rise that is characteristic of intervals where molecules take up energy corresponding to a hitherto unexcited degree of freedom.

But if, eventually, the components are completely miscible, though with great deviations from ideality, then we can get the 'λ-transitions'. The activity coefficient of the solute component rises steeply with concentration, so that this component only appears in any appreciable fraction quite near to the temperature at which the G-curves intersect. Within a narrow range of temperature then, the two components will change their rôles as solvent and solute. The heat of transition from the one nearly pure component to the other is smeared out over the same interval and appears as the abnormal specific heat. If the great deviations from ideality do not extend to the whole range of concentrations but only to a certain interval, the above considerations will apply to the transition through this interval.

To investigate what kind of singularity we may expect to find in the thermodynamic functions, we will briefly consider a few of the relevant equations.

One mole of A consists of α moles of A' and (1—α) moles of A''. Gibbs' free energy is:

$$G = \alpha\mu' + (1—\alpha)\mu''$$

and the condition of internal equilibrium is:

$$\left(\frac{\partial G}{\partial \alpha}\right)_{p,T} = \mu' — \mu'' = 0 \qquad \text{or} \qquad \mu' = \mu'' \text{ for } \alpha = \xi$$

$$C_p = -T\left(\frac{\partial^2 G}{\partial T^2}\right)_{p,eq} = T\left\{\left(\frac{\partial S}{\partial T}\right)_{p,\xi} + \left(\frac{\partial S}{\partial \alpha}\right)_{p,T}\left(\frac{\partial \xi}{\partial T}\right)_{p,eq}\right\}$$

and the only term that may be expected to cause singularity is $\delta\xi/\delta T$. To derive an equation for this we write:

$$d\left(\frac{\partial G}{\partial \alpha}\right) = \frac{\partial}{\partial p}\left(\frac{\partial G}{\partial \alpha}\right)dp + \frac{\partial}{\partial T}\left(\frac{\partial G}{\partial \alpha}\right)dT + \frac{\partial}{\partial \alpha}\left(\frac{\partial G}{\partial \alpha}\right)d\alpha$$

and obtain:

$$\left(\frac{\partial \xi}{\partial T}\right)_{p,eq} = -\frac{\partial^2 G/\partial\alpha\partial T}{\partial^2 G/\partial\alpha^2} = \frac{(\partial S/\partial\alpha)_{p,T}}{(\partial^2 G/\partial\alpha^2)_{p,T}} = \frac{S'—S''}{(\partial^2 G/\partial\alpha^2)_{p,T}}$$

A singularity, therefore, will occur if $\partial^2 G/\partial\alpha^2$ equals zero, which condition means that the two components form a critical mixture, as is easily verified by a glance at Fig. 3. In this particular problem $\partial G/\partial\alpha$ equals zero as stated

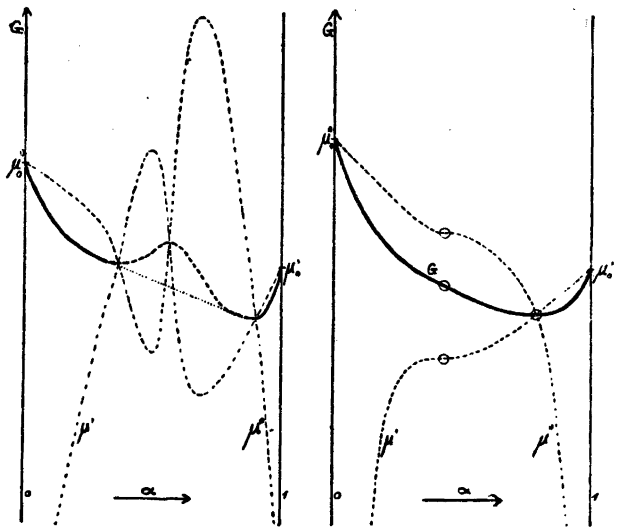


Fig. 3. The molar free energy, G, of a binary mixture and the chemical potentials of the components versus mole fraction, α, in case of 1) partial miscibility and 2) critical miscibility. In our particular case of chemical equilibrium between the 'components' the four circled points will fall in one, in which all the three curves will have horizontal tangents.

above, and for critical mixtures in general $\partial^2 G/\partial\alpha^2 = \partial^3 G/\partial\alpha^3 = 0$ if α is taken to mean the mole fraction of a component in general.

A similar singularity will then occur also in the expansion coefficient:

$$\left(\frac{\partial V}{\partial T}\right)_{p,eq} = \left(\frac{\partial V}{\partial T}\right)_{p,\xi} + \left(\frac{\partial V}{\partial \alpha}\right)_{p,T}\left(\frac{\partial \xi}{\partial T}\right)_{p,eq}$$

and in the compressibility:

$$\left(\frac{\partial V}{\partial p}\right)_{T,eq} = \left(\frac{\partial V}{\partial p}\right)_{T,\xi} + \left(\frac{\partial V}{\partial \alpha}\right)_{T,p}\left(\frac{\partial \xi}{\partial p}\right)_{T,eq}$$

where

$$\left(\frac{\partial \xi}{\partial p}\right)_{T,eq} = -\frac{\partial^2 G/\partial p\partial\alpha}{\partial^2 G/\partial\alpha^2} = -\frac{(\partial V/\partial\alpha)_{T,p}}{(\partial^2 G/\partial\alpha^2)_{T,p}}$$

If we have an example of a phase transition with a very high degree of pre-melting and pre-crystallization, as, according to the work of Ubbelohde [7], we may expect to find for the melting of pure high paraffins, and if we imagine

this degeneration of the melting point to proceed still further by increase either in pressure or in chain length, the partial miscibility of the components may reach the critical mixing point at a certain stage, and we shall see no more separation into two phases. The heat of transition will have degenerated to a singularity in the C_p-curve, which according to the above derivation will show a steep peak rising sharply to infinity from both sides. But as soon as the degeneration proceeds further, and the miscibility, though still far from ideal, is no longer critical, then the C_p-curve will become continuous, although preserving a possibly very steep peak.

The possibility of a critical point for the solid-liquid phase equilibrium was much discussed in classical thermodynamics [8], and it seems likely that under favourable conditions it may be realised in a manner like this. But so far we do not know the true characteristic difference between the molecular states in the liquid and in the solid states, and therefore we cannot say whether phenomena like those described by Ubbelohde [7] do correspond to a real 'miscibility of states' or whether the molecules can acquire only some and not all of the characteristics of the states of the other phase.

It is clear, however, that it calls for rather special assumptions for a transition of this kind to be realised in more than a point, *viz.* for instance that in a pressure interval $\partial^3 G/\partial p \partial \alpha^2 = (\partial^2 V/\partial \alpha^2)_{p,T} = 0$. The C_p-curve for the λ-transition of liquid helium as it is known to occur in the interval 0—25 ats. therefore must be supposed to be continuous however steeply it rises.

On the whole, it can be said that when we cannot observe the degeneration from two phase transition through critical transition to λ-transition, it will practically never be possible to decide definitely that a true discontinuity does occur. And anyway, in circumstances differing only infinitesimally, the discontinuity will no longer occur in a strictly mathematical sense, although the physical appearance will be practically indistinguishable. It seems the most reasonable thing, therefore, to give up the concept of second order transitions and to speak only of λ-transitions and critical λ-transition.

This whole treatment is of course of a very formal and descriptive character, as is the thermodynamic treatment of mixtures to which it relates. Only in combination with statistical mechanics under the assumption of some physical model will it attain any explanatory value, and only then will it be of interest to evaluate further the given formulae. But as pointed out by Keesom [3] himself, Keesom-Ehrenfest's equation $\frac{dp}{dT} = \frac{\triangle C_p}{TV \triangle \alpha_p}$ will preserve its validity regardless of the kind of the singularity, if only $\triangle C_p$ and $\triangle \alpha_p$ are taken as the increments in the same small temperature interval.

As regards the order-disorder transitions the very difference between Bragg-Williams' treatment and that of Bethe is in the different interaction between cells of the two different kinds. This, in connection with the present treatment, stresses the fact that what does determine the kind of a transition is the mutual interaction between molecules or cells of different kinds. The relative energy and free energy of each kind in the 'pure state' will determine only the range of temperature where it occurs. Hence it is a pronounced co-operative phenomenon.

The nature of the known λ-transitions in crystals being fairly well accounted for by the order-disorder description, the only known λ-transition in liquid phase attracts the greater interest at present. Several models of the helium transition have also been proposed, but we will reserve the discussion thereof for a separate paper.

Only as an illustration to this treatment, however, we shall briefly describe in the language used here one model which is compatible with the *thermodynamic* properties of He II, though not directly with the kinetic ones.

We assume for ordinary liquid He a structure corresponding to a coordination number 12 and with a number of nearest neighbours $z \backsimeq 11$. Ordinary liquids would at some sufficiently low temperature pass over in a crystalline state of the same coordination number, but for He at not too high pressures another structure of some lower coordination number, say 8 (or 6), must be assumed for some reason or other to be more stable, *i. e.* to be of a lower free energy. How the transition between this last mentioned state and the liquid will take place now depends not on the energies of a He-atom in the two states, but on the energy of an atom with a number of nearest neighbours in between, say 9 or 10. We may consider the two states as pseudo-components distinguished *e. g.* by the He-atoms having numbers of nearest neighbours z, a) equal to and b) greater than 8 (or 6) respectively. If now the energy of an atom varies linearly with z, the two components, if obeying Boltzman statistics, will be ideally miscible, and even in the case that quantum statistics must be applied, the deviations from ideality will not be large enough for a transition point to be detectable.

But it may be that for any such intermediate value of z the energy is much higher than that corresponding to the two states, *e. g.* if an interaction peculiar to the state of low coordination number is responsible for its stability. That will cause a clustering of the atoms of low coordination number into some kind of crystallites, and any degree of deviation from ideality may arise therefrom.

The model very much recalls Bernal and Fowler's model of liquid water and on further consideration of it, it becomes easy to understand how a model as that by Keesom and Gorter [3] of the two-phase system in an opaque vessel of constant volume may well picture the crude features of a λ-transition. When the deviations from ideal miscibility are *almost* large enough to cause a phase separation, the transfer of atoms from the crystallites to the 'serum' takes place *almost* as the transfer between two defined phases. But just in the interesting point of 'transition' that important difference appears, that the crystallites will disappear continuously though perhaps rather quickly with rising temperature, while the discontinuity is characteristic of the model system because it is *really*

of two phases and hence can be realised in a temperature interval only because the volume is kept constant. Gorter and Keesom's model, therefore, does not really bridge, as it is meant to, the gap between continuity and discontinuity.

This result does not depend upon the details of the model here assumed, but it will undoubtedly come true by any model which duly takes into account that there is always only one real phase.

SUMMARY

The degeneration of ordinary phase transition is exposed by close analogy to the theory of mixtures. Ordinary phase transition corresponds to no miscibility of molecules in the two states characteristic of each of the phases; pre-melting and analogous phenomena mean partial miscibility, and to full but non-ideal miscibility, anomalies which appear as humps on the heat capacity curve *etc.* correspond, in pronounced cases giving rise to 'λ-transitions'. Only in case of critical miscibility the transition becomes a true second order transition in Ehrenfest's sense.

It is shown by statistical arguments that such a splitting up into two pseudo-components and treatment of these as components in a mixture can be effected without inconsistencies.

REFERENCES

1. Ehrenfest, P. *Proc. Koninkl. Nederland. Akad. Wetenschap.* **36** (1933) 153.
2. Guggenheim, E. A. *Ibid.* **37** (1934) 294; Justi, E., and Laue, M. v. *S.-B. Preuss. Akad.* (1934) 237.
3. Keesom, W. H. *Helium* Amsterdam (1942).
4. Bridgman, P. W. *Phys. Rev.* **70** (1946) 425.
5. Smits, A. *Die Theorie der Komplexität und der Allotropie* Berlin (1938).
6. See *e. g.* Fowler and Guggenheim *Statistical Thermodynamics* Cambridge (1939).
7. Ubbelohde, A. R. *Trans. Faraday Soc.* **34** (1938) 292.
8. See *e. g.* the text-book of v. d. Waals and Kohnstamm or that of Schottky, Ulich and Wagner.

Received December 11, 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 63—81

The Time-Concentration Curve of Local Anesthetics

LARS EHRENBERG

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

The purpose of the present investigation is to find an adequate method to measure the activities of local anesthetics, especially some alkylaminoacyl anilides (investigated by Löfgren *et al.*[1-6]; see Table 1) such that it can be used in a theoretical study of the relation between physiological activity and physicochemical properties of anesthetics. The uncritical use of data from standard pharmacological methods for such comparisons has often led to inconclusive results and contradicting theories, because the methods, as a rule, work with too complicated systems.

It has been proposed that the activity of narcotics, also termed anesthetics — in a wide sense, substances which can reduce the ability of cells to become stimulated [8] — parallels their lipid solubilities or lipid/water partition coefficients [9—12]. In such, and similar [13] quantitative comparisons, the minimum concentration (C_m) for narcotic action, *i. e.* the concentration that just fails to produce an action at infinite time, is of the greatest interest. Measurements of depth and duration (*cf.* Girouard and Robillard [14a]) of anesthesia or the action time (T), *i. e.* the time between the moment of application and the beginning activity of an anesthetic, as a rule, involve diffusivity in an uncontrolled manner. In the commonly used wheal and cornea methods [15] the anesthetic has to diffuse from the point of application to the points of action at the same time as it is diluted and transported away by the body fluids. For example, the influence of diffusion is clearly demonstrated by the prolonged duration due to increased viscosity of the injection solution [16]. Thus, because these quantities are no simple functions of concentration, they cannot be used directly in quantitative comparisons.

Another important point which is often neglected is the great influence of pH on the composition of solutions of weak acids and bases. The alkylaminoacyl anilides and other local anesthetics are amines, B, which at physio-

logical conditions are partly ionized as BH^+, the proportion between B and BH^+ being determined by the dissociation constants, K_s, and the pH [17]. It has been claimed that B should be the active principle [18–21], and further, it is known that the proportion of uncharged molecules largely affects the diffusion through biological membranes [22, 23]. The fact that anesthesia is potentiated by a stronger basicity of the anion combined with procaine [24–27], or that it decreases with increasing concentrations in the cornea [28] or linguo-maxillary [14b] tests are probably at least partly pH effects (*cf.*, however, the phosphate effect, see page 73). The uncertainty of an activity measurement, due to a pH error of the test solution, is only partly [29] decreased (in injection methods) through the buffering action of the body fluids. It follows, that quantitative activity measurements must admit careful pH control throughout the experiment.

This control can only be affected by immersing the test object in an anesthetic solution so large that the concentrations of all components remain constant during the experiment. In addition, this is the only method to eliminate the complicated diffusion phenomena of injection experiments, although these can be somewhat simplified in cases of more localized application (*e. g.* intracutaneous and eyelid wheals, cornea test).

From the above considerations it seemed convenient for the present purpose to use a modification of the old method involving block of the frog sciatic nerve [19, 30–32] through immersing it in a sufficiently large solution the pH of which was determined. This method has been elaborated most ideally but somewhat expensively by Bennett *et al.* [33] — Naturally, the pharmacologists' determinations of depth, duration and action time are extremely valuable and necessary for the *pharmacodynamical* characterization of a compound. This fact must be distinguished from the inapplicability of these determinations for certain quantitative theoretical comparisons.

THE MINIMUM CONCENTRATION (C_m)

The C_m of an anesthetic can be determined by keeping identical nerves etc. for a long time in a series of solutions with decreasing concentrations (C) [10, 11, 34]. The C_m interval thus obtained can be made arbitrarily small but the significance of the measurement is diminished by alterations in the aging specimens (*cf.* Laubender and Saum [46]).

Between C and T (*i. e.* the action time, see above) a hyperbolic relation has been shown to exist [35–42] which, at least at intermediate concentrations can be expressed [35, 36]:

$$(C - C_m)^n \ (T - T_m) = K \qquad (1)$$

This and similar [37–43] expressions are exclusively empirical, and the constants, n and K, are without biological significance. As to membrane permeation a similar expression has been derived from Fick's diffusion law [44]. The disturbing influence of a long duration on the experiment could be avoided if a C,T-curve could be extrapolated from intermediate values to $T = \infty$, where $C = C_m$. But this has not yet been possible, because eq. (1) and similar expressions do not hold at small C values [35, 46]; *cf.*, however, Adams *et al.* [42], who have extrapolated straight lines (gold-fish method), *i. e.* n in (1) $= 1$.

For the present investigation a relation between C and T was derived, which admits such an extrapolation to $T = \infty$ and an evaluation of C_m. At the same time the diffusion constant, D, of the anesthetic in nerve tissue is calculated. The expression (eq. (2), p. 63) contains no empirical or unbiological constants. (As will be shown in Figs. 3A, 4A, 8, the intermediate part of the C, T-curve derived can be taken for a straight line; *cf.* Adams *et al.*[42]).

METHODS

The method used has many features in common with those of earlier workers [33, 31] but some details should be noted.

a. *Nervus ischiadicus*-leg preparations from *Rana arvensis* were investigated. Frogs weighing 25—40 g were used. All frogs were of the same natural race, and they were kept at identical conditions. The preparing of the nerve was performed according to Abderhalden's standard methods [45], the difference being that all the muscles beneath the knee were left. In order to decrease the metabolism and drying of the specimens an ice-cold preparation table, covered with Ringer-moistened blotting-paper, was used. After the preparing was completed the specimens were immediately immersed in Ringer solution (6.5 g of NaCl, 0.14 g of KCl, 0.12 g of $CaCl_2$, *aq. ad* 1000 ml [46]) at 15° C. They were used after 30 min.

b. In a double-walled thermostate cage, cooled with streaming tap water to 15 ± 0.5° C., and with a glass frontal wall, two preparations (as a rule, the two legs of one frog) could be mounted (Fig. 1) and tested simultaneously. The stretching of the nerve was made constant through the application of a constant pull from a 2 g weight (Fig. 1). The thermostate air was moistened thoroughly: large amounts of wet blotting-paper on the floor and walls of the cage proved to be most effective.

c. The nerves were stimulated through the application of D. C. sqare wave voltages of 0.5 s duration between two Pt wire rings 0.7 cm apart (*cf.* Laubender and Saum [46]) upon which the nerve was resting, see Fig. 1; (above 0.6 cm the nerve resistance is a linear function of the electrode distance [47]). The impulses were generated by an electronic stimulator [48]. The negative electrode was proximal to the leg. A switch permitted stimulation of one or the other preparation. The rheobase, *Rh* [49, 50a], was determined. A normal *Rh* value — about 100 mV — was taken as evidence of the normal function of an unhurt nerve. Specimens with abnormal *Rh* values, or which were known to have been hurt during the preparing, were withdrawn from further investigation.

d. The nerves were anesthetized within a proximal 2 cm region (only at regions < 0.8 cm the anesthesia is influenced by the length of the region [50b, 51]) between the point

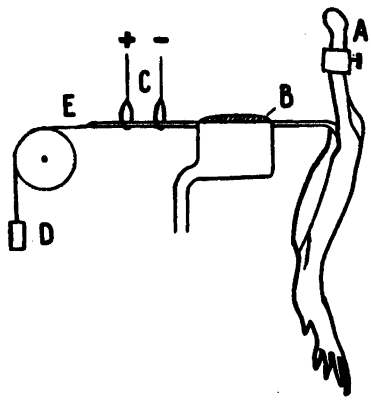


Fig. 1. Mounting of nerve-leg preparation.
A: Femur fixed in a brass holder.
B: The cup heaped up with anesthetic solution, which by reason of the paraffine does not run over.
C: Pt electrodes.
D: Weight.
E: Silk thread fixed at the end of the nerve.

of stimulation and the leg (Fig. 1), by the application of anesthetic solution in a 6 ml cup (Fig. 2; *cf.* Bennett *et. al.*[33]), which was paraffinized and provided with two diametrically situated cuts. This arrangement permitted the immersion of the nerve without hurting it through any contact with the edges of the cup. It was possible to stimulate the nerve at intervals without removing it from the anesthetic solution. Interruptions of the contact between the anesthetic solution and the test object, as a rule, are not avoidable when the test object is stimulated within the narcotized region, this giving an erroneous *C, T*-curve (*cf.* Laubender[38], Grammacioni[39]). After the application of the anesthetic solution the nerve was stimulated at 1 or 2 min intervals with one single impulse of 0.5 s duration and an amplitude somewhat greater than *Rh, i. e.* this quantity was determined continuously. In fact, *Rh* was found to be constant until block occurred. Consequently, it could also be used as evidence of normal function of the preparation during the experiment. Any drying of the nerve or the fatiguing influence of an inappropiate shape of the current impulses seem to increase *Rh* gradually. Perhaps, such effects will explain the ascending *Rh*/time curve found by other authors[46]. — The time from the application of the anesthetic until that stimulus to which there was no muscle response was noted as *T*.

e. Highly purified specimens of the anesthetics were dissolved, as chlorides or nitrates of the ions, BH^+ (*cf.* Table 1), in Ringer solution containing 0.01 mole of sodium phosphate buffer per l.* The pH at 15° C was adjusted to 7.39 by NaOH; the buffer was sufficient to keep the pH within the limits 7.39 ± 0.01 during the experiment. — The pH was measured at 25° C with a glass and a calomel electrode. Vacuum tube mV-meter (Radiometer PHM 3, Copenhagen), accuracy ± 0.002 pH. Standard: 0.05-C potassium biphthalate, pH = 4.008[52]. To the values obtained is added 0.03 to get the pH at 15° C[53].

* *I.e.* with 1.422 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.276 g of $NaH_2PO_4 \cdot H_2O$ per l.

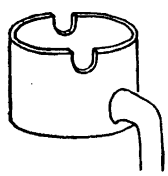


Fig. 2. Anesthetic cup with cuts and outlet for solution.

f. Every anesthetic was applied in several concentrations (Table 3), and in every concentration at least two nerves from different frogs were tested.

g. T varies as R^2 (R = nerve radius); this is found experimentally by [33] and verified by the present author (unpubl.); further, *cf.* eq. (2). This variation in T is great because R varied between 0.019 and 0.034 cm. Hence, after the completion of every experiment the mean nerve thickness within the narcotized region was determined microscopically with an ocular micrometer. The nerve was suspended horisontally and under constant pull between the objective and the stage of an ordinary microscope. Some five readings were made and averaged. By this method R could be determined within 3 %. The T values were than recalculated to a standard nerve radius, R = 0.0275 cm (Table 3), which is a mean value.

DIFFUSION THEORY

Contrary to the anesthesia of a whole nerve [54] that of a single nerve fiber follows the all-or-none law [55], and it is instantaneous after an anesthetic, at a concentration greater than C_m, has reached the nodes of Ranvier [55]. It follows, that the time before total block of the nerve, T, is due entirely to the diffusion of the anesthetic into the nerve. It is assumed that all the motoric fibers of a nerve have the same C_m, and that their distribution in the trunk includes the central part. — In order to registrate the function of all motoric elements of the nerve, whole nerve-leg preparations were made. The registration of the action current with a cathode ray oscillograph [33] would be an equivalent or preferable method. It follows that at the time (T) when the concentration, C_m, is reached at the centre of the nerve, all fibers of the trunk are blocked, *i. e.* no part of the leg responds to a stimulus.

The inner of the nerve contains a large number of small discontinuities (nerve fibers, layers of connective tissue, etc.), but, taken as a whole, it may be regarded homogeneous in the respect that an anesthetic has the same D in all parts of it. Thus, regarding the relation between C and T, the problem reduces to a special case of the heat conduction regarding the conduction of heat in an infinitely long cylinder.

Analogously to Hill's [56] calculations on the diffusion of oxygen into a cylindrical tissue, we*) arrived at the expression

$$c(t;r) = C(1 - 2 \sum_{\nu=1}^{\infty} \varphi_\nu (r) \cdot e^{-Da_\nu^2 t})$$

where

$c(t;r)$ = concentration of the anesthetic at the time t and at the distance r from the centre of the nerve,

* The derivation of eq. (2) was kindly performed by fil. lic. H. Rådström, University of Stockholm, to whom I am greatly indebted.

C = concentration of the anestetic in the surrounding solution,

$\varphi_\nu(r) = \frac{J_0(\alpha_\nu r)}{\alpha_\nu R J_1(\alpha_\nu R)}$, J_0 and J_1 being the Bessel's functions, and α_ν the zeros of J_0,

R = the nerve radius,

D = the diffusion constant.

Thus, the concentrations in the centre of the nerve at times t are, terms of higher order being rejected:

$$c\ (t;0) = C\ (1—2\ \varphi_1\ (0)\ e^{-D\alpha_1^2 t})$$

where $\varphi_1\ (0) = 0.8$ and $\alpha_1^2 = \frac{5.76}{R^2}$

Total block occurs when $c(t;0) = C_m$, *i. e.*

$$C_m = C(1—1.6\ e^{-5.76DT/R^2}) \qquad (2)*$$

The expression (2) is used for the determination of C_m and D for local anesthetics from measurements of C, T, and R. For $C < 10C_m$, (2) holds within 1 %.

The pH of the inner of the nerve is not known exactly. If there were free exchange of ions between the surrounding fluid and the extrafibrillar, fluid-filled spaces of the nerve (as in the case of *Loligo* [57a]), these spaces would soon get the pH of the surrounding fluid. Further, at diffusion equilibrium, C would be the same in the surrounding fluid and in the extra-fibrillar spaces mentioned. But, if there were diffusion of uncharged molecules, B, only, the pH of the inner spaces of the nerve would not alter much, and, at diffusion equilibrium, only C of the free base (C_B) would be equal outside and inside the nerve. As will be shown, the latter is valid in the present case, although it is possible that BH^+ can partake in the diffusion *within* the nerve. The fact that, in the following, C and C_m refer to the total concentration outside the nerve does not influence on the calculations [*cf.* eq. (2)]. However, the more important minimum concentrations of free base, $(C_m)_B$, are calculated from the pK_s values [7] (Table 1). f_{BH^+} in physiological solutions is assumed to be 0.80 (calculated from the Debye-Hückel formula [68]).

RESULTS

With the methods presented above 22 alkylaminoacyl anilides (see Table 1) and procaine were investigated. From corresponding C and T values, listed in Table 3, $C/\frac{1}{T}$ curves were drawn, because at infinite time, *i. e.* $\frac{1}{T} = 0$,

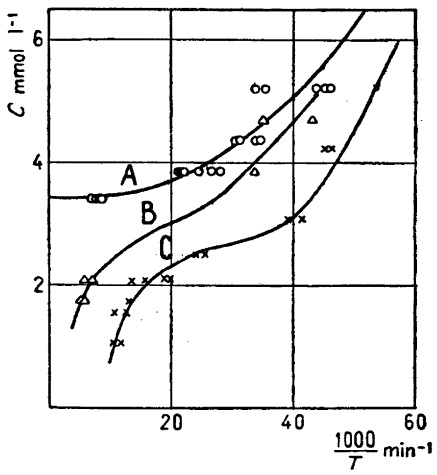


Fig. 3. $C/\frac{1}{T}$ *curves of compound N.*

A: Ringer without phosphate (expt. Nε) (= 0.01 C phosphate).
B: Ringer with 0.02 C phosphate.
C: 0.10 C phosphate.

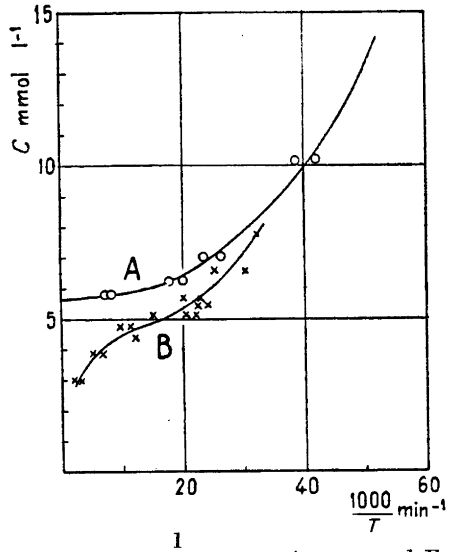


Fig. 4. $C/\frac{1}{T}$ *curves of compound F.*

A: 0.01 C phosphate.
B: 0.10 C phosphate.

this curve is horizontal and $C = C_m$ [eq. (2)]. Thus, C_m could be determined approximately (C'_m) through extrapolation of the curve to $\frac{1}{T} = 0$. Putting C'_m and R into eq. (2), a D value could be calculated for every C, T pair. The fact that in nearly all of the experiments, the C'_m values calculated are such that D is independent of C [*i. e.* the points $(C;\frac{1}{T})$ lie at random on both sides of the theoretical curve, Figs. 3A, 4A] is taken as evidence that within the limits of error, the experimental $C/\frac{1}{T}$ curves do not differ from the theoretical ones. Concentrations smaller than C'_m gave no anesthesia, even after long times (expts. A, C, J, K, Nε, Table 3). These experiments are important because they prove that the curve actually does not descend at small $\frac{1}{T}$ values as in the presence of higher phosphate concentrations (*cf.* p. 73; Figs. 3, 4). — It should be pointed out that the correctness of the theory cannot be proved merely by the present investigation; it can only be made probable.

If for each substance tested, the average of the D values obtained be called D', the true C_m and D values may be written $C'_m + \Delta$ and $D' + \delta$, resp. If these values are substituted into eq. (2) the expression

$$(C - C'_m)\left[\log\left(1 - \frac{C'_m}{C}\right) + 3288\ D'T - 0.204\right] = \Delta - \delta\ 3288\ T\ (C - C'_m) \qquad (3)$$

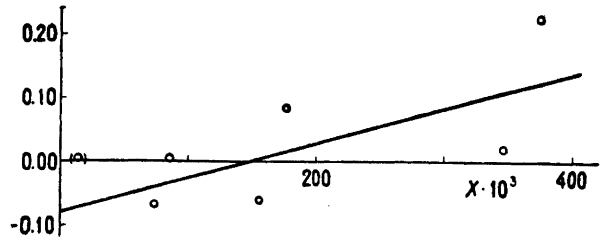


Fig. 5. Expt. F as example of correction curve. The points are obtained from original values $C'_m = 5.76$ *mmol* l^{-1}; $D' = 0.725 \cdot 10^{-5}$ cm^2 min^{-1} *put into eq. (3). From the slope and intercept of the most probable line* $-\delta = (+ 0.05 \pm 0.03)\ 10^{-5}$ *and* $\Delta = - 0.08 \pm 0.06$. *Hence,* $C_m = 5.64$ *mmol* l^{-1}; $D = 0.68 \cdot 10^{-5}$ cm^2 min^{-1}.

may be derived. Eq. (3) is of the type $x = a + by$. It is valid at small errors Δ and δ. The curves (3) were drawn for the substances investigated, and $+ \Delta$ and $- \delta$ were estimated from the intercept and the slope of the line, resp. Thus C'_m and D' could be corrected to more true values, C_m and D, at the same time as the uncertainty of these could be estimated. The C_m and D values are listed in Table 1. The mean errors of D are given; as a rule, these are about 30 % of the maximum errors obtained from the curves (3). Points at low concentrations give very uncertain D values: they were excluded from the calculations of D' and the mean error of D. In Table 1, (n) is the number of measurements used for these calculations.

During the experiments the pH of the test solutions decreased 0.00—0.01 (determined at the times, $T/2$), due to diffusion of B into the nerve. — The use of larger or, better, streaming test solutions could have removed this source of error; however, the supplies of purified substances were too limited. — It follows that C_B of the outer solution is diminished maximally about 2 % through a) diminished total concentration, b) decreased pH. This error of C_m, ΔC_m (pH), which is of a systematical character, as a rule dominates. The total error of C_m, ΔC_m (pH, D, T) depends further on uncertainty in D and T. ΔC_m (D) and $\Delta C_m(T)$ do not exceed 0.2 % and 0.5 %, resp.; only in bad cases the latter exceeds 2 %. Thus, with some higher exceptions (expts. H, J, L, R, V, W, X), the maximum errors, ΔC_m of Table 1, are given to $\pm$ 3 %.

INFLUENCE OF EXTERNAL FACTORS

a. The nature of the salt anions used (Cl^- and NO_3^-), within limits of error did not influence the properties of the curve (Table 2, expts. Nα and Nβ).

b. Within limits of error, the pH of the outer solution proved to be without influence on D, whereas C_m varies in such a way that the minimum con-

Table 1. Mol weights, dissociation constants (according to Löfgren and Östlund [7]), minimum concentrations, diffusion constants, and minimum times of local anesthetics.

Expt. no.	Anesthetic (BH^+X^-)		Synthesis Literature (1—6)	Mol. wt. of B	$pK_s = -\log \frac{a_{H^+} \cdot a_B}{a_{BH^+}}$ [from 7]	$C_m \pm \Delta C_m$ = total conc. at pH 7.39	$(C_m)_B$ = base conc.	$D \pm$ mean error (n), see text	T_m (at nerve radius R = 0.0275 cm) $= \frac{\log 1.6}{5.76 \log e} \cdot \frac{R^2}{D}$ min
	B of BH^+	X				mmol l^{-1}	mmol l^{-1}	10^{-5} cm^2 min^{-1}	
	I. $RCOCH_2N(C_2H_5)_2$; R =								
A	C_6H_5NH	NO_3	(1)	206.3	8.029	9.54 ±0.29	1.48	1.39 ±0.06 (5)	4.4
B	cyclohexyl NH	Cl	(6)	212.3	8.21	25.4 ±0.8	2.73	1.12 ±0.13 (5)	5.5
C	2-$CH_3C_6H_4NH$	NO_3	(1)	220.3	7.905	5.22 ±0.16	1.02	0.95 ±0.07 (5,8)***	6.5
D	2-$CH_3C_6H_4NCH_3$	Cl	(2)	234.3	8.74	50.8 ±1.5	1.75	0.50 ±0.03 (4)	12.3
E	3-$CH_3C_6H_4NH$	Cl	(2)	220.3	8.035	4.09 ±0.12	0.625	0.80 ±0.03 (9)	7.7
F	4-$CH_3C_6H_4NH$	Cl	(2)	220.3	8.055	5.65 ±0.17	0.832	0.68 ±0.02 (6)	9.1
G	2,3-$(CH_3)_2C_6H_3NH$	Cl	(2)	234.3	7.940	3.51 ±0.11	0.644	0.46 ±0.02 (6)	13.4
H	2,4-$(CH_3)_2C_6H_3NH$	NO_3	(2)	234.3	7.945	3.50 ±0.18	0.636	0.52 ±0.04 (4)	11.9
J	2,5-$(CH_3)_2C_6H_3NH$	NO_3	(2)	234.3	7.920	4.04 ±0.34	0.769	0.63 ±0.05 (8)*	9.8
K	3,4-$(CH_3)_2C_6H_3NH$	Cl	(2)	234.3	8.055	2.51 ±0.08	0.369	0.42 ±0.01 (9)	14.7
L	3,5-$(CH_3)_2C_6H_3NH$	Cl	(2)	234.3	8.015	2.07 ±0.12	0.329	0.47 ±0.02 (11)	13.1
M	2,6-$(CH_3)_2C_6H_3NH$	Cl,NO_3	(2)	234.3	7.855	3.79 ±0.11	0.815	1.00 ±0.06 (18)	6.2
N	2,4,6-$(CH_3)_3C_6H_2NH$ (mean values)	Cl,NO_3	(2)	248.4	7.900	3.44 ±0.06	0.680	0.89 ±0.02	6.9
	II. 2,6-derivatives others than M, N. 2,6-$(CH_3)_2C_6H_3NH$ COR_1; R_1 =								
P	$CH_2N(CH_3)_2$	Cl	(2)	206.3	7.355	11.1 ±0.3	5.14	1.9 ±0.1 (6)	3.2
Q	$CH_2N(n\text{-}C_3H_7)_2$	NO_3	(5)	262.4	7.68	0.77 ±0.02	0.224	0.43 ±0.02 (6)	14.3
R	CH_2NHCH_3	Cl	(5)	192.3	7.990	47 ±5	7.81	probably small	
S	$CH_2NHC_2H_5$	Cl	(5)	206.3	8.075	23.9 ±0.7	3.38	0.47 ±0.01 (8)	13.1
T	$CH_2NH\text{-}n\text{-}C_3H_7$	Cl	(5)	220.3	8.012	9.35 ±0.28	1.50	0.52 ±0.01 (10)	11.9
U	$CH_2NH\text{-}n\text{-}C_4H_9$	Cl	(4)	234.3	8.035	4.21 ±0.13	0.644	0.65 ±0.03 (7)	9.5
V	$CH_2NH\text{-}iso\text{-}C_4H_9$	Cl	(4)	234.3	7.830	4.1 ±0.4	0.912	1.16 ±0.20 (6)**	5.3
W	$CH(CH_3)N(C_2H_5)_2$	Cl	(3)	248.4	8.110	3.97 ±0.20	0.524	1.25 ±0.12 (4)**	4.9
X	$CH_2CH_2N(C_2H_5)_2$	Cl	(3)	248.4	8.96	36 ±4	0.761	0.30 ±0.05 (3)*	20.5
Y	*III.* Procaine	Cl		236.3	8.96 (From 67)	12.46 ±0.37	0.262	1.10 ±0.05 (9)	5.6

* Maximum error. ** Temp. error, see table 3. *** Eliminated points are regarded to some extent.

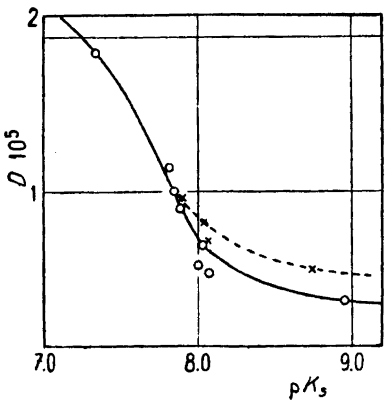


Fig. 6. D/pK_s curves of 2,6-xylidides (○) and toluidides (×).

Table 2. N-Diethylaminoaceto-2,4,6-trimethyl anilide (expt. no. N). Influence of pH, anion, and phosphate on minimum concentration and diffusion constant.

Expt. no.	Anion X^-	Conc. of phosphate buffer mol l^{-1}	pH	$C_m \pm \Delta C_m$ = total conc. mmol l^{-1}	$(C_m)_B \pm \Delta(C_m)_B$ = base conc. mmol l^{-1}	$D \pm$ mean error 10^{-5} cm^2 min^{-1}
α	Cl^-	0.01	7.39	3.41 ±0.10	0.674 ±0.020	0.95 ±0.10
β	NO_3^-	0.01	7.39	3.50 ±0.10	0.691 ±0.021	0.88 ±0.06
γ	Cl^-	0.01	6.96	8.07 ±0.24	0.678 ±0.020	0.92 ±0.02
δ	Cl^-	0.01	7.00	7.37 ±0.22	0.673 ±0.020	0.86 ±0.08
ε	Cl^-	none	7.39	3.41 ±0.10	0.674 ±0.020	0.83 ±0.03

centration of the free base, $(C_m)_B$, is constant (Table 2). This fact needs not evidence that B is the active principle of a local anesthetic (*cf.* Trevan and Boock [18]), but only that the anesthetic acts on the other side of a membrane permeable to B only. However, the fact that most general and many local anesthetics (*e. g.* alcohols [58, 59]) are unionizable molecules, makes it probable that B can anesthetize.

c. The small amounts of phosphate (0.01 moles per l) did not, within limits of error, influence the course of the curve, *cf.* Table 2, expts. Nα and Nε. In expt. Nε, where the anesthetic was dissolved in unbuffered Ringer solution, the anesthetic solution in the cup was renewed every 15 min during the experiment, so that pH should not sink appreciably. In fact the two C_m values are identical.

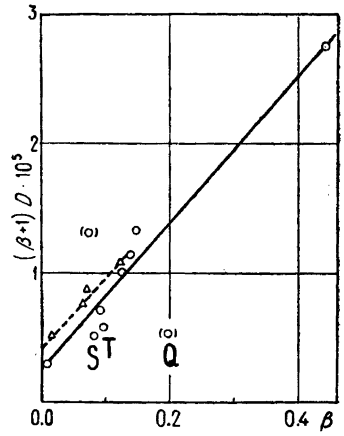


Fig. 7. Cf. Fig. 6. Curves from eq. (5). 2,6-xylidides (O) and toluidides (△). Expts. Q, S, and T (see text) are marked.

However, greater amounts of phosphate had a remarkably increasing effect on the anesthetizability of nerves (Figs. 3C, 4B). The sodium phosphate buffer used in these experiments was made according to Green [53]. It was isotonic with the Ringer and contained, per liter: 0.0213 moles of HPO_4^{2-}, 0.0787 moles of $H_2PO_4^-$, and $1.9 \cdot 10^{-3}$ moles of KCl. The 1×10^{-3} moles of Ca^{2+} required for a frog Ringer [46] could not be added, because part of it precipitated as $Ca_3(PO_4)_2$. From the solubility product of this substance, $1 \cdot 10^{-25}$, [60a] it follows that only ca. $2 \cdot 10^{-5}$ moles of Ca^{2+} can remain in solution.

In the calculation the phosphate ion activity, $a_{PO_4^{3-}}$, is determined from the expression $pH = pK_s + \log \frac{a_{PO_4^{3-}}}{c_{HPO_4^{2-}} \cdot f_{HPO_4^{2-}}}$, [17] where the 3rd dissociation constant of H_3PO_4, pK_s, is 12.32 [60b]. The activity coefficients of divalent ions, $f_{Ca^{2+}}$, and $f_{HPO_4^{2-}}$, are supposed to be ca. 0.33, calculated from the simplified form of Debye-Hückel's formula:

$$-\log f_i = 0.5\, z_i^2 \frac{1+\sqrt{I}}{\sqrt{I}}.$$ [17]

At more intermediate concentrations of phosphate (*e. g.* 0.02 moles per liter) the effect sets in later (Fig. 3B), and in the Ringer with 0.01-C phosphate *i. e.* the solution used in the head experiments, there is no influence on the narcotizability (*cf.* above), neither is Ca^{2+} precipitated at physiological concentrations. It is probable that the effect is due, at least partly, to precipitation or extraction of the cell Ca, which is necessary for irritability [61]. This explanation may also hold for some of the effects of other anions [24, 25]. Oxalate and citrate are known to prevent nerve activity [62a]. — The »phosphate anesthesia» is reversible.

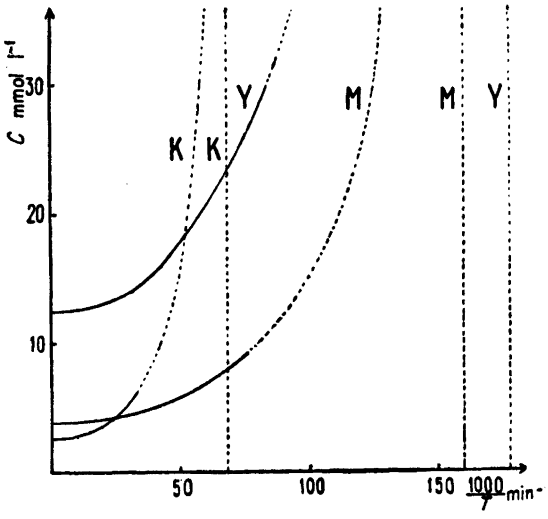


Fig. 8. Theoretical curves and asymptotes of xylocaine (M), its 3,4-isomer (K), and procaine (Y). The extrapolated parts are dotted.

d. Within a group of related substances (*e. g.* toluidides, or 2,6-xylidides) the compounds exhibit some kind of negative proportionality between D and pK_s (Table 1). Although the situation is too complicated to be analysed in detail, it is evident that the base, B, more easily diffuses through nerve tissue than does BH^+. This indicates what could be expected from histological data, namely, that only part of the cross section of the diffusion path, the fraction γ say, is open to diffusion of BH^+ (and, naturally, of B, occurrent in a concentration determined by pH and pK_s). The fraction $1-\gamma$, which is probably lipoidic, is permeated by B only. From D/pK_s diagrams (Fig. 6) it is seen that at high pK_s values, where only BH^+ diffuses, the curve tends to a lower limit, whereas at low pK_s values, where only B diffuses, it probably tends to an upper limit. If the real diffusion constants (D_{real}) are supposed to be identical in both fractions of the nerve, the effective diffusion constant of B will be D_{real}, that of BH^+ only $\gamma \cdot D_{real}$. Substituting differences for the derivates of Fick's diffusion law the expression

$$\Delta C = C\alpha^2 \left(D \Delta t - \tfrac{1}{a} \Delta x\right) \tag{4}$$

can be derived, where $C = f(x) \cdot g(t)$ is the concentration and α^2 is a constant.

From $\frac{\delta c}{\delta t} = D \cdot \frac{\delta^2 c}{\delta x^2}$ the expression $\frac{1}{D \cdot g} \cdot \frac{dg}{dt} = \frac{1}{f} \cdot \frac{d^2 f}{dx^2} = \alpha^2$ is obtained.

I. e. $\frac{dg}{dt} = \frac{\Delta g}{\Delta t} = D g \alpha^2$; $f = k \cdot e^{-\alpha x}$, and $\frac{df}{dx} = \frac{\Delta f}{\Delta x} = -\alpha f$.

Because $\frac{\Delta C}{C} = \frac{\Delta g}{g} + \frac{\Delta f}{f}$, eq. (4) is valid.

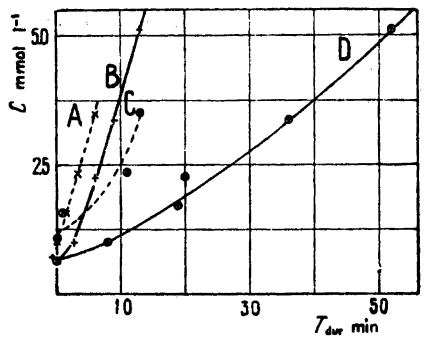


Fig. 9. Intracutaneous wheals.
A: Compound K.
B: Xylocaine.
C: Compound K, with epinephrine.
D: Xylocaine, with epinephrine.

The increase in concentration, ΔC, can be designed to consist of two parts, ΔC_B and ΔC_{BH+}. According to (4)

$$\Delta C = \Delta C_B + \Delta C_{BH+} = \alpha^2 [(C_B + \gamma C_{BH+}) D_{real} \Delta t - (C_B + C_{BH+}) \tfrac{1}{\alpha} \Delta x],$$

which can be expressed $\Delta C = \alpha^2 (C D \Delta t - C \tfrac{1}{\alpha} \Delta x)$.

$$\therefore C D = (C_B + \gamma C_{HB+}) D_{real}; \quad i.e. \frac{D}{D_{real}} = \frac{\beta + \gamma}{\beta + 1}, \tag{5}$$

where $\beta = C_B/C_{BH+}$ at the pH of the nerve, which is here assumed to be 7.00 (*cf.* Heilbrunn [62b]). If $(\beta + 1)$ D is plotted against β (Fig. 7) a straight line can be obtained, the slope of which is D_{real} and the intercept of which is $\gamma \cdot D_{real}$. D_{real} of 2,6-xylidides is about $6 \cdot 10^{-5}$ cm^2 min^{-1}, *i. e.* nearly the same as D of solutes in gels [60c] and slightly smaller than D of correspondingly large molecules in water [60d] or organic solvents [60e] (α-bromonaphthaline!). This strongly supports the theory upon which the present investigation is based. — The »hydrophilic» nerve fraction, γ, is only about 0.04.

It should be noted that different groups of anesthetics show different ability to diffuse into the nerve (*cf.* Table 1), a phenomenon which could be ascribed to specific properties. The high D of procaine when compared with its high pK_s is notable. On the other hand, high molecular weight (compound Q) and low lipid solubility (*e. g.* compounds S and T) are known to diminish diffusivity through biological membranes [63, 57b]. *Cf.* Fig. 7.

Anyhow, it is proved that D depends on pK_s. This fact in connection with the fact that D is not affected by an altered pH of the outer solution (point b. above) proves that the pH of the nerve is not altered appreciably during an experiment. Otherwise β, and, followingly, D should be altered.

Table 3. Experimental C and T values; the latter are recalculated to the nerve radius R = 0.0275 cm.

Expt.	C mmol l^{-1}	T min
A	16.7	15
	»	12
	11.1	22
	»	23
	10.0	37 ½
	9.00	>200
	7.41	>200
B	76.8	8
	»	8 ½
	57.5	13 ½
	28.7	48 ½
	»	29 ½
	25.9	82
C	6.95	19
	»	27 ½
	»	29
	6.39	34
	5.93	37
	»	40
	5.31	69 ½
	»	195
	5.29	>200
D	78.1	42
	»	48
	65.3	47
	»	48
	51.2	150
	»	203
E	9.19	19 ½
	»	20
	7.20	21
	»	21 ½
	5.45	32 ½
	5.41	27
	»	27 ½
	4.67	41
	»	42 ½
	4.28	84
	»	>120
F	10.16	24
	»	26
	7.01	38
	»	43
	6.23	50
	»	56
	5.80	120
	»	130
G	6.20	32 ½
	»	43
	5.13	42 ½
	»	45
	4.43	60
	»	64
	3.69	76 ½
	»	150
H	5.38	38
	»	43 ½
	4.27	46
	»	58
	3.70	128
	3.36	240
J	6.22	26 ½
	»	35 ½
	5.85	30
	»	34
	5.05	34
	»	36
	»	35 ½
	4.47	63
	»	200
	4.10	>200
K	5.43	35 ½
	»	36
	4.06	43 ½
	»	43 ½
	3.53	47 ½
	»	51 ½
	»	55 ½
	2.93	81 ½
	»	81 ½
	2.40	>200
L	6.27	22 ½
	»	24
	»	24 ½
	4.61	26 ½
	»	29
	3.69	34
	»	39
	2.95	47
	»	57
	2.33	77
	»	83
	1.99	140
M	8.32	12
	»	15 ½
	»	17
	6.40	(13 ½)
	»	16
	6.36	11 ½
	»	13 ½
	»	16 ½
	»	18 ½
	5.52	15 ½
	»	22 ½
	5.16	(16 ½)
	»	24 ½
	5.12	27
	»	29
	4.27	42
	»	45 ½
	4.14	40
	»	94
	3.95	53 ½
Na	4.74	21
	»	23 ½
	»	27 ½
	3.86	27 ½
	»	28

Expt.	C mmol l^{-1}	T min
	3.86	53
	3.42	165
	»	170
	»	250
β	5.40	23
	4.50	27
	»	29 ½
	»	32
	3.98	53
	»	75
	3.53	131
	»	160
γ	10.36	28
	»	29 ½
	8.78	43
	»	45
	8.35	57 ½
	»	72
δ	10.42	26
	»	29
	9.30	29 ½
	»	(42)
	8.36	40
	»	(60)
	7.87	55 ½
	»	60
	»	61
	»	66
ε	5.25	22
	»	22
	»	22 ½
	»	28 ½
	»	30
	4.42	29
	»	29 ½
	»	32
	»	32 ½
	3.89	35 ½
	»	38
	»	41
	»	45 ½

Expt.	C mmol l^{-1}	T min
	3.89	46
	»	46
	3.42	120
	»	123
	»	125
	»	137
	2.50	>210
	»	>210
P	28.2	8⅓
	»	7
	18.9	7⅔
	»	9⅓
	12.4	18
	»	18 ½
	10.7	125
Q	1.96	27
	»	30
	1.26	43 ½
	»	44 ½
	1.01	52
	»	65
R	53.6	87
	39.0	>300
S	50.8	30
	»	38
	41.2	32
	»	47
	32.5	43 ½
	»	51
	28.8	71
	»	71
T	28.7	21
	»	21 ½
	22.2	24 ½
	»	28
	18.5	31
	»	31 ½
	15.4	34
	»	37
	12.9	(60)

Expt.	C mmol l^{-1}	T min
	9.81	82
	»	85
U	7.50	22
	»	27
	6.46	30
	»	30 ½
	5.54	38
	»	45
	4.69	65
	4.25	132
V	8.71	10
	»	11 ½
	5.79	17 ½
	»	17 ½
	»	24 ½
	4.84	(19 ½)*
	»	(21)*
	»	37
W	6.88	12*
	»	13*
	4.60	24 ½
	»	25
	4.07	30*
	»	76
X	59.2	61
	42.6	86
	»	134
	22.0	>200
Y	29.7	11
	»	14 ½
	20.6	13 ½
	»	15 ½
	14.8	30
	»	30 ½
	»	31
	14.1	32 ½
	»	32 ½
	12.8	59
	»	64

* Temperature erroneously 17° C.

SOME CONNECTIONS WITH CLINICAL ANESTHESIA

a. From the C, T relation found experimentally (Figs. 3, 4, 8) and derived mathematically [eq. (2)] it follows that there is, at a given nerve radius, a shortest time (T_m), within which anestesia can be produced. T_m is proportional to $\frac{R^2}{D}$(values, see Table 1). In clinical conduction anesthesia, the rapid action of good anesthetics like xylocaine [2–5] and novocaine (Table 1, compounds M and Y) is related to the quantity, T_m. — Further, it should be noted, that it is impossible to compare the activities of a couple of anesthetics by means of their action time values at only one concentration, because the curves may intersect at some point (*cf.* Fig. 8) below which one and above which the other compound is the better anesthetic, *i. e.* has the shortest T.

b. It should be pointed out once more that, although there may be some connections of interest, the present methods to characterize anesthetics does not try to evaluate their pharmacodynamical properties. For a study of the latter the ability to narcotize sensory nerve fibers must catch the greatest interest. Especially as the ratio of motor paralysis to sensory paralysis may not always be the same [34, 64, 65], although C_m in the two cases is of the same order of magnitude. Now, for a few substances, C_m was determined in the case of intracutaneous wheals in man [58, 66]. 0.4 ml of anesthetic solutions (containing 0.85 % of NaCl and with pH = 7.4) were injected in the skin of the inside of the forearm, and the duration, T_{dur}, of the anesthesia, which occurred instantaneously, was measured (pin prick). The T_{dur}/C curves of xylocaine and its 3,4-isomer are seen in Fig. 9. Here the C_m ratio is the inverse of that found in motor paralysis. Further it is clear that T_{dur} should diminish with increasing ability of the substance to diffuse away from the point of action. If this diffusion were related to the diffusion into nerves described above, xylocaine would be expected to exert the shorter duration, which is not the case (Fig. 9). The longer remaining of xylocaine might be related to a greater vasoconstricting effect of this substance, but as the addition of epinephrine (0.002 %) does not reverse the T_{dur} ratio (Fig. 9), specific adsorption may play a role. Also in the case of D, specific properties could be traced to the 2,6-derivatives (p. 74—75).

SUMMARY

1. A method is described to determine, with high accuracy, the minimum concentration for anesthesia (C_m) of the motoric fibers of the frog sciatic nerve.

2. The action times (T) at different concentrations (C) of local anesthetics

(22 alkylaminoacyl anilides and procaine) were determined. The compounds were applied in buffered Ringer at pH 7.39.

3. Applying the theory of heat conduction in an infinitely long cylinder, C could be expressed as a function of C_m, T, D (*i. e.* diffusion constant of the anesthetic in nerve), and R (*i. e.* nerve radius). From measurements of C, T, and R the C_m of an anesthetic could be determined.

4. The validity of the theory was made probable by a. conformity of the empirical curve with the theoretical one b. agreement of known diffusion constants with the D values calculated; the latter are in fact depending on the diss. constants of the compounds, only the free base diffusing through all parts of the nerve.

5. C_m depended on the pH of the test solution, the minimum concentration of the free base being constant.

6. Phosphate at concentrations able to precipitate cell Ca increased the anesthetic activity.

This investigation was supported by *Statens Medicinska Forskningsråd.* Further, I wish to express my gratitude to Cand. Med. G. Molander for valuable assistance, to Prof. K. Myrbäck for valuable discussions, and to Mr J. Mosley, who read the manuscript.

LITERATURE

1. Erdtman, H., and Löfgren, N. *Svensk Kem. Tid.* **49** (1937) 163.
2. Löfgren, N. *Arkiv Kemi, Mineral. Geol.* **22 A** (1946) no. 18.
3. Löfgren, N., and Lundquist, B. *Svensk Kem. Tid.* **58** (1946) 206.
4. Löfgren, N., and Fischer, I. *Ib.* 219.
5. Löfgren, N., and Widmark, G. *Ib.* 323.
6. Löfgren, N., and Takman, B. (unpubl.).
7. Löfgren, N., and Östlund, G. (unpubl.).
8. Lillie, R. S. *Biol. Bull.* **30** (1916) 311.
9. Overton, E. *Studien über die Narkose* Jena (1901).
10. Meyer, K. H., and Hemmi, H. *Biochem. Z.* **277** (1935) 39.
11. Levan, A., and Östergren, G. *Hereditas* **29** (1943) 381; Östergren, G. *Hereditas* **30** (1944) 429.
12. See also Cone, N. M., Forman, S. E., and Krantz, J. C. Jr. *Proc. Soc. Exptl. Biol. Med.* **48** (1941) 461.
13. *Cf.* Rohman, C., and Scheurle, B. *Arch. Pharm.* **274** (1936) 225, 236; Rohman, C., and Koch, A. *Ib.* **276** (1938) 189.
14. Girouard, M., and Robillard, E. *Rev. can. biol.* 4 (1945) a: 146; b: 157.
15. The pharmacological methods are reviewed by Ther, L. *Süddeut. Apoth. Ztg.* **81** (1941) 419, 449, 458.
16. Meidinger, F. *Compt. rend. soc. biol.* **139** (1945) 907.
17. See fundamental text-books of physical chemistry.
18. Trevan, J. W., and Boock, E. *Brit. J. Exptl. Path.* **8** (1927) 307.

19. Gros, O. *Arch. exptl. Path. Pharmakol.* **63** (1910) 80.
20. Protz, G. *Arch. exptl. Path. Pharmakol.* **86** (1920) 238.
21. Gardner, J. H., and Semb, J. *J. Pharmacol. Exptl. Therap.* **54** (1935) 309.
22. Osterhout, W. J. V. *J. Gen. Physiol.* **8** (1925) 131.
23. Poijärvi, L. A. P. *Acta Botan. Fennica* **4** (1928) 1.
24. Régnier, J., and Quevauviller, A. *Compt. rend. soc. biol.* **122** (1936) 251; *cf.* **125** (1937) 627.
25. Quevauviller, A. *Compt. rend. soc. biol.* **137** (1943) 630.
26. Régnier, J., and David, R. *Anesthésie et analgésie* **4** (1938) 483.
27. Beutner, R. *Proc. Soc. Exptl. Biol. Med.* **42** (1939) 380.
28. Kaempf, J. *Schmerz, Narkose-Anaesthesie* **6** (1934) 14, 80.
29. *Cf.* Boothe, R. G. *Illinois Dental J.* **10** (1941) 345 (from *C. A.* **35** (1941) 8206).
30. Gros, O. *Arch. exptl. Path. Pharmakol.* **62** (1910) 380.
31. Sollmann, T. *J. Pharmacol. Exptl. Therap.* **10** (1917/18) 379.
32. Gros, O., and Kochmann, M. *Arch. exptl. Path. Pharmakol.* **98** (1923) 129.
33. Bennett, A. L., Wagner, J. C., and McIntyre, A. R. *J. Pharmacol. Exptl. Therap.* **75** (1942) 125.
34. Boehminghaus, H., and Kochmann, M. *Arch. exptl. Path. Pharmakol.* **141** (1929) 238; Kochmann, M., and Lyding, H. *Ib.* 246.
35. Clark, A. J. *Mode of Action of Drugs on Cells* London (1933).
36. Miescher, K. *Helv. Med. Acta* **7** Suppl. VI (1940/41) 126.
37. Ostwald, Wo. *Pflüger's Arch. ges. Physiol.* **120** (1907) 19.
38. Laubender, W. *Arch. exptl. Path. Pharmakol.* **137** (1928) 25.
39. Grammacioni, B. *Arch. intern. pharmacodynamie* **40** (1931) 357.
40. Rabbeno, A., and Cavalli, F. *Arch. intern. pharmacodynamie* **61** (1939) 11.
41. Scheiner, H. *Compt. rend. soc. biol.* **139** (1945) 235.
42. Adams, R., Rideal, E. K., Burnett, W. B., Jenkins, R. L., and Dreger, E. E. *J. Am. Chem. Soc.* **48** (1926) 1758.
43. Bülbring, E., and Wajda, I. *J. Pharmacol. Exptl. Therap.* **85** (1945) 78.
44. Macovski, E. and E. *Arch. exptl. Path. Pharmakol.* **192** (1939) 321, **185** (1937) 309.
45. Abderhalden, E. *Physiologisches Praktikum* II. 5th ed. Dresden and Leipzig (1942) p. 62—65.
46. Laubender, W., and Saum, M. *Arch. exptl. Path. Pharmakol.* **171** (1933) 619.
47. Danielli, J. F. *J. Physiol. London* **96** (1939) 65.
48. McCombs, R. K., and Walz, F. C. *Rev. Sci. Instruments* **16** (1945) 249.
49. Lapicque, L. *L'excitabilité en fonct. du temps* Paris (1926). See p. 222 ff.
50. Schaefer, H. *Elektrophysiologie* 1. Wien (1940); Ann Arbor, Mich. (1944). a: p. 104; b: p. 417.
51. Wiersma, C. A. G. *Proc. Koninkl. Akad. Wetenschap.* **33** (1930) 180.
52. Hitchcock, D. I., and Taylor, A. C. *J. Am. Chem. Soc.* **59** (1937) 1812.
53. Green, A. A. *J. Am. Chem. Soc.* **55** (1933) 2331.
54. *Cf.* Winterstein, H., and Derman, H. *Arch. intern. pharmacodynamie* **66** (1941) 293.
55. Kato, G. *Cold Spring Harbor Symposia Quant. Biol.* **4** (1936) 202.
56. Hill, A. V. *Proc. Roy. Soc. London* **B 104** (1928) 39; see p. 70.
57. Höber, R. *Physical Chemistry of Cells and Tissues* London (1945). a: See p. 257 ff. b: 229 ff.
58. Barbour, H. G. *Science* **51** (1920) 497.
59. Berger, F. M., and Bradley, W. *Brit. J. Pharmacol.* **1** (1946) 265.

60. Landolt-Börnstein. *Physikalisch-chemische Tabellen.* a: Eg IIb, 1109; b: *Ib.* 1080; c: Eg IIIa, 235; d: *Ib.* 228, Hw I, 246; e: Eg IIIa, 231—233.
61. Heilbrunn, L. V. *Biol. Bull.* **66** (1934) 264.
62. Heilbrunn, L. V. *An Outline of General Physiology* Philadelphia and London (1943). — a: p. 472; b: p. 54.
63. Collander, R., and Bärlund, H. *Acta Botan. Fennica* **11** (1933); Bärlund, H. *Ib.* **5** (1929).
64. Santesson, C. G. *Upsala Läkareförens. Förh.* **11** Suppl. (Festschrift Hammarsten) (1906) XV.
65. Fromherz, K. *Arch. exptl. Path. Pharmakol.* **76** (1914) 257.
66. Hoffmann, A., and Kochmann, M. *Bruns' Beitr. klin. Chir.* **91** (1914) 489.
67. Eisenbrand, J., and Picher, H. *Arch. Pharm.* **276** (1938) 1.
68. Kielland, J. *J. Am. Chem. Soc.* **59** (1937) 1675.

Received December 29, 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 82—87

Die Konstitution der Harzphenole und ihre biogenetischen Zusammenhänge

XI.* Die Konfiguration des *Epi*-pinoresinols

JARL GRIPENBERG

Organisk-kemiska institutionen, Kungl. Tekniska Högskolan, Stockholm, Schweden

Pinoresinol ist symmetrisch gebaut, und sein Dimethyläther besitzt die Struktur I oder III. Dies wurde von Erdtman [1] gezeigt durch Äthylierung des Monomethyläthers und Methylierung des Monoäthyläthers des Pinoresinols, wobei identische Methyl-äthyläther erhalten wurden. Weiterhin gaben die Nitrierung des Monobrompinoresinol-dimethyläthers sowie die Bromierung des Mononitropinoresinol-dimethyläthers identische Bromnitroderivate des Pinoresinol-dimethyläthers [2], und die Auffassung vom symmetrischen Bau des Pinoresinols erscheint damit endgültig gesichert.

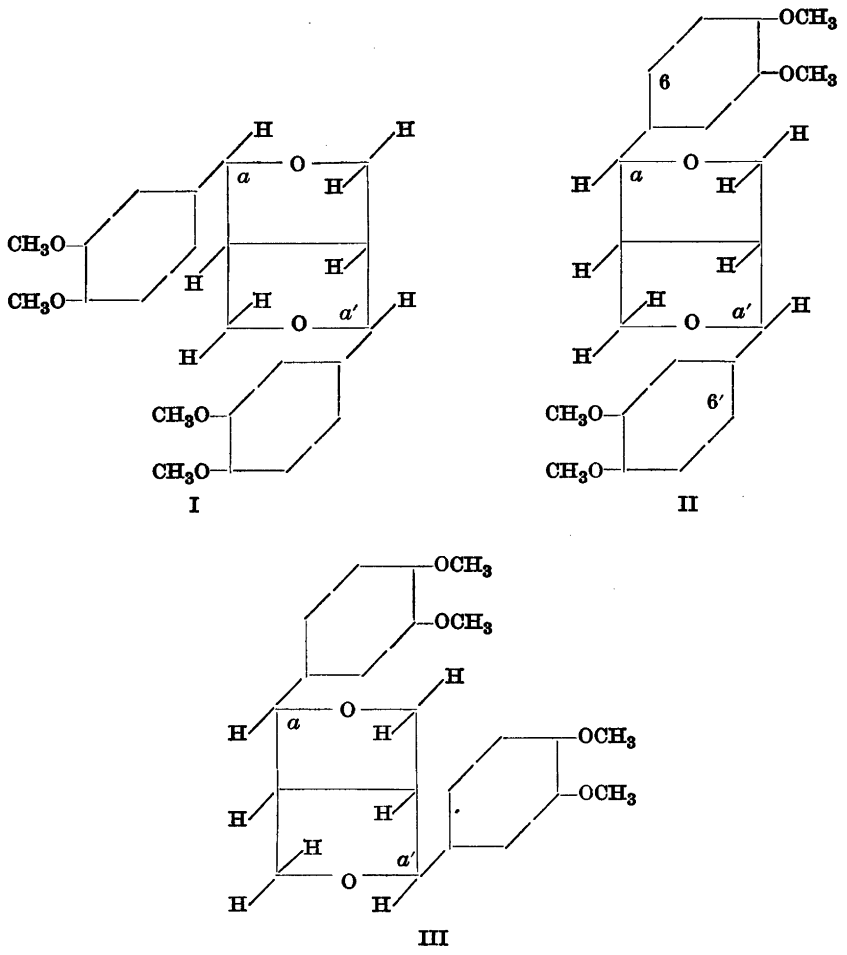
Kaku und Ri [3] erhielten durch Einwirkung von Säuren auf Pinoresinol-dimethyläther einen *Epi*-pinoresinol-dimethyläther vom Smp. 133—134°, $[\alpha]_D$ + 145,5°. Dieses Isomerisierungsprodukt konnte von denselben Autoren durch Säuren wieder in Pinoresinol-dimethyläther zurückverwandelt werden, sodass sicher nur eine Epimerisierung und keine tiefergreifende Umlagerung stattgefunden hat.

Erdtman erhielt bei der Einwirkung von Säuren auf Pinoresinol und darauffolgende Methylierung zwei Isomere des Pinoresinol-dimethyläthers (Smp. 131—133°, $[\alpha]_D$ + 140° bzw. Smp. 133—135°, $[\alpha]_D$ + 182°). Das erstgenannte ist identisch mit dem *Epi*-pinoresinol-dimethyläther von Kaku und Ri [4].

Es erhebt sich nun die Frage, ob *Epi*-pinoresinol nur durch Waldensche Umlagerung am Kohlenstoff *a* der Formel I (oder III) oder durch Waldensche Umlagerung an den beiden Kohlenstoffatomen *a* und *a'* entstanden ist.

---

* X. Mitt. *Acta Chem. Scand.* 1 (1947) 71.



I II III

Im ersteren Fall muss *Epi*-pinoresinol sterisch unsymmetrisch gebaut sein (Formel II). Eine doppelte Waldensche Umkehrung würde zu der sterisch symmetrischen Konfiguration III (oder I) führen.

Diese Frage ist nun durch Anwendung der früher beschriebenen Methode [2], nämlich Monobromierung und darauf folgender Nitrierung bzw. Mononitrierung und nachfolgender Bromierung, beantwortet worden. Bei Mononitrierung des *Epi*-pinoresinol-dimethyläthers entstehen zwei verschiedene Mononitroderivate, das eine vom Smp. 143—144°, das andere, welches in sehr kleiner Menge erhalten wurde, vom Smp. 129—130° (wohl nicht ganz rein). Sie werden vorläufig als α- bzw. β-Nitro-*epi*-pinoresinol-dimethyläther bezeichnet.

Das α-Nitroderivat gibt bei der Bromierung einen Bromnitro-*epi*-pinoresinol-dimethyläther vom Smp. 157—157,5° (143—145°), während das β-Nitroderivat einen Bromnitro-*epi*-pinoresinol-dimethyläther vom Smp. 180—180,5° liefert.

Bei der Bromierung von *Epi*-pinoresinol-dimethyläther konnte indessen nur ein einziges Monobromderivat gefasst werden. Ganz wie bei der Monobromierung des Pinoresinol-dimethyläthers entsteht bei der Monobromierung des *Epi*-pinoresinol-dimethyläthers stets eine beträchtliche Menge öliger Produkte, die nicht zur Kristallisation gebracht werden können. Dies erschwert die Aufarbeitung, und es ist möglich, dass sich in diesen öligen Anteilen das zweite Monobromderivat verbirgt. Bei der Epimerisierung des Monobrompinoresinol-dimethyläthers kommt man zu derselben Substanz wie bei der Bromierung des *Epi*-pinoresinol-dimethyläthers. Auch in diesem Falle konnte ein zweites Monobromderivat nicht erhalten werden. Der Monobrom-*epi*-pinoresinol-dimethyläther gibt bei der Nitrierung einen Nitrobrom-*epi*-pinoresinol-dimethyläther vom Smp. 181—182°, der mit dem Bromnitro-*epi*-pinoresinol-dimethyläther vom Smp. 180—180,5° keine Schmelzpunktsdepression zeigt. Im Monobrom-*epi*-pinoresinol-dimethyläther befindet sich somit das Bromatom in derselben Stellung wie die Nitrogruppe im α-Nitro-*epi*-pinoresinol-dimethyläther, und die Verbindung wird deshalb entsprechend als α-Bromderivat bezeichnet.

Da es somit zwei verschiedene Mononitroderivate und zwei verschiedene Bromnitroderivate des *Epi*-pinoresinol-dimethyläthers gibt, muss dieser die unsymmetrische Konfiguration II besitzen. In welcher der beiden möglichen Positionen 6 oder 6′ die α-Derivate ihre Substituenten tragen, und in welcher die β-Derivate, lässt sich nicht sagen. Ebensowenig lässt sich sagen, welche der beiden symmetrischen Konfigurationen I oder III dem Pinoresinol zukommt. Wir hoffen aber darüber später berichten zu können.

Was die Konfiguration der übrigen Lignane mit derselben Struktur des Mittelstückes betrifft, so ist angenommen worden [5], dass das Asarinin dieselbe Konfiguration besitzt wie das Pinoresinol. Ob dem Sesamin die andere symmetrische Konfiguration oder die dem Epipinoresinol entsprechende unsymmetrische Konfiguration zukommt, soll nun mit der oben beschriebenen Methode untersucht werden.

Die Frage der Konfiguration des Forsythigenols und derjenigem des mit ihm wahrscheinlich identischen Phillygenols [6] steht in naher Beziehung zu der des Pinoresinols und seiner Epimerisierungsprodukte, da der Methyläther des Forsythigenols möglicherweise mit dem Methyläther des zweiten theoretisch denkbaren Epimerisierungsproduktes des Pinoresinols identisch ist, d. h. also die zweite symmetrische Form darstellt. Die Literaturangaben über

Forsythigenol und Phillygenol und ihrer Derivate [6-8] sind indessen so widersprechend, dass man daraus keine sicheren Schlussfolgerungen ziehen kann. Versuche, diese Fragen zu entscheiden, sind im Gange.

VERSUCHSTEIL

Epi-pinoresinol-dimethyläther: 20 g Pinoresinol-dimethyläther wurden in 100 ml Eisessig gelöst und 1 ml konc. Schwefelsäure zugesetzt. Nach einer Woche bei gewöhnlicher Temperatur wurde das Gemisch in Wasser gegossen und die ausgeschiedene feinpulvrige Masse aus Methanol fraktionierend kristallisiert. So wurden 4 g reiner *Epi*-pinoresinol-dimethyläther erhalten. Die anderen Fraktionen gaben nur dieselbe Substanz sowie unveränderten Pinoresinol-dimethyläther. Die restlose Trennung dieser beiden Stoffe ist jedoch sehr mühsam, und es lohnt sich besser, das Gemisch erneut mit Säure zu behandeln.

Der *Epi*-pinoresinol-dimethyläther hatte Smp. 130—131°, $[\alpha]_D^{18}$ + 141° ± 1° (α = + 1,01° ± 0,01°, l = 0,55 dm, c = 1,305 in Chloroform).

Nitrierung: 2 g *Epi*-pinoresinol-dimethyläther wurden in 5 ml Essigsäure und 5 ml Essigsäureanhydrid gelöst und bei — 10° mit einer Lösung von 0,35 ml konc. HNO_3 (sp. Gew. 1,41) in 3 ml Essigsäureanhydrid versetzt. Nach einer Stunde wurde Wasser bis zur Trübung zugesetzt, wobei nach einiger Zeit 0,15 g α-Nitro-*epi*-pinoresinol-dimethyläther ausfiel. Zur Mutterlauge wurde mehr Wasser gefügt und die ausgefallene, ein wenig schmierige Masse aus Alkohol fraktionierend kristalliert. Hierbei wurden gewonnen: weitere 0,06 g α-Nitro-*epi*-pinoresinol-dimethyläther und 0,09 g β-Nitro-*epi*-pinoresinol-dimethyläther sowie 0,13 g Dinitro-*epi*-pinoresinol-dimethyläther (Smp. 180°) und 0,27 g unveränderter *Epi*-pinoresinol-dimethyläther. Der Rest war ein teilweise schmieriges Gemisch, aus welchem keine reine Substanz erhalten werden konnte.

α-Nitro-epi-pinoresinol-dimethyläther, aus Alkohol mehrmals umkristallisiert, Smp. 140,5—141°, $[\alpha]_D^{21}$ + 41° ± 2° (α = + 0,23° ± 0,01°, l = 0,55 dm, c = 1,01 in Chloroform).

$C_{22}H_{25}O_8N$ (431,3) Ber. N 3,25 Gef. N 3,43

β-Nitro-epi-pinoresinol-dimethyläther, aus Methanol kristallisiert, Smp. 129—130°, $[\alpha]_D^{20}$ + 114° ± 2° (α = + 0,63° ± 0,01°, l = 0,55 dm, c = 1,005 in Chloroform).

$C_{22}H_{25}O_8N$ (431,3) Ber. N 3,25 Gef. N 3,81

Bromierung des α-Nitro-epi-pinoresinol-dimethyläthers: 0,13 g des α-Nitroderivates wurden in 2 ml Essigsäure gelöst, mit einigen Tropfen Pyridin versetzt und mit einer Lösung von 0,015 ml Brom in 1 ml Essigsäure bromiert. Das Bromierungsprodukt wurde mit Wasser ausgefällt (0,13 g) und aus Alkohol-Chloroform kristallisiert. Es schmilzt bei 143—145°, erstarrt dann aufs neue, um bei 157—157,5° wieder zu schmelzen. $[\alpha]_D^{21}$ + 78° ± 2° (α = + 0,42° ± 0,01°, l = 0,55 dm, c = 0,98 in Chloroform).

$C_{22}H_{24}O_8NBr$ (510,2) Ber. N 2,75 Br 15,69
Gef. » 2,87 » 15,64

Bromierung des β-Nitro-epi-pinoresinol-dimethyläthers: 10 mg des β-Nitroderivates wurden in wenig Chloroform gelöst und mit einer verdünnten Bromlösung in Chloroform bis zur bleibenden Rotfärbung versetzt. Das Chloroform und überschüssiges Brom wurden verdampft und der Rückstand aus Äthylacetat-Methanol umkristallisiert. So wurden etwa 5 mg Kristalle erhalten vom Smp. 180—180,5°. Die Substanz gab mit dem später beschriebenen Nitrobromderivate von Smp. 181—182° keine Schmelzpunktsdepression.

α-Brom-epi-pinoresinol-dimethyläther: a) 5 g *Epi*-pinoresinol-dimethyläther wurden in 40 ml Essigsäure und 7 ml Pyridin gelöst und mit 1,75 g Brom in 30 ml Essigsäure bromiert. Nach einer Stunde wurde in Wasser gegossen. Der Niederschlag wurde aus Methanol fraktionierend kristallisiert. Dabei wurden erhalten: 1,3 g α-Brom-*epi*-pinoresinol-dimethyläther, nebst 0,7 g Dibrom-*epi*-pinoresinol-dimethyläther (Smp. 160—161°) und 1,7 g unverändertem Ausgangsmaterial. Das Rest war ein Öl, aus dem nichts kristallisiertes erhalten werden konnte. Der α-Brom-*epi*-pinoresinol-dimethyläther wurde aus Alkohol umkristallisiert. Smp. 143—144°, $[\alpha]_D^{21}$ + 51° ± 2° (α = + 0,32° ± 0,01°, l = 0,55 dm, c = 1,15 in Chloroform).

$C_{22}H_{25}O_6Br$ (465,3) Ber. Br 17,19 Gef. Br 17,42

b) 1 g Monobrompinoresinol-dimethyläther wurde in 10 ml Essigsäure gelöst und 0,1 ml konc. H_2SO_4 zugesetzt. Nach 40 Stunden wurde die Lösung in Wasser gegossen und der Niederschlag aus Alkohol fraktionierend kristallisiert. Es wurden erhalten: 0,7 g unverändertes Ausgangsmaterial und 0,17 g Monobrom-*epi*-pinoresinol-dimethyläther, der nach nochmaligen Umkristallisieren aus Alkohol bei 143—144° schmolz und mit dem nach a) dargestellten α-Brom-*epi*-pinoresinol-dimethyläther keine Depression gab. $[\alpha]_D^{21}$ + 50° ± 2° (α = + 0,25° ± 0,01°, l = 0,55 dm, c = 0,91 in Chloroform).

Nitrierung des α-Brom-epi-pinoresinol-dimethyläthers: 0,25 g α-Brom-*epi*-pinoresinol-dimethyläther wurden in 1 ml Essigsäure und 1 ml Essigsäureanhydrid gelöst und bei — 15° mit einem Gemisch von 0,05 ml konc. HNO_3 (sp. Gew. 1,41) und 1 ml Essigsäureanhydrid nitriert. Bei Zusatz von Wasser kristallisierten 0,18 g Nitrobrom-*epi*-pinoresinol-dimethyläther aus. Smp. 181—182° aus Alkohol-Chloroform. $[\alpha]_D^{21}$ + 84° ± 2° (α = + 0,53° ± 0,01°; l = 0,55 dm, c = 1,15 in Chloroform).

$C_{22}H_{24}O_8NBr$ (510,2) Ber. N 2,75 Br 15,69
Gef. » 2,71 » 16,38

ZUSAMMENFASSUNG

Es ist gezeigt worden dass *Epi*-pinoresinol eine unsymmetrische Konfiguration besitzt.

Der Technischen Hochschule Finnlands danke ich für ein Stipendium.

LITERATUR

1. Erdtman, H. *Svensk Kem. Tid.* **48** (1936) 236.
2. Gripenberg, J. *Suomen Kemistilehti* **19B** (1946) 138.
3. Kaku, T., und Ri, H. *J. Pharm. Soc. Japan* **57** (1937) 289.
4. Erdtman, H. *Svensk Kem. Tid.* **50** (1938) 161.
5. Erdtman, H. *Svensk Kem. Tid.* **48** (1936) 280.
6. Kunimine, S., und Susuki, S. *J. Pharm. Soc. Japan* **58** (1938) 25.
7. Kunimine, S., und Wada, S. *J. Pharm. Soc. Japan* **58** (1938) 182.
8. Kaku, T., Ri, H., und Hara, N. *J. Pharm. Soc. Japan* **58** (1938) 248.

Eingegangen am 20. Januar 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 88—94

Short Communications

The Formulae of Sodium and Ammonium Paramolybdate

INGVAR LINDQVIST

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

In a recent critical review on the polymolybdates, Carpéni[1] reports two possible mole ratios M_2O:MoO_3 for the paramolybdates without deciding in favour of either. In the following these mole ratios are given the symbols (5 : 12) and (3 : 7). In connection with an X-ray investigation of the polymolybdates, polytungstates and polyvanadates, which has been started in this institute, it was found necessary to take up the problem of the mole ratio of the paramolybdates.

Our efforts were confined to the sodium and ammonium paramolybdates, which possess very different amounts of water of crystallization. These compounds were discovered in 1853 by Zenker[2] and in 1848 by Svanberg and Struve[3] and were by them given the mole ratios (4 : 9) and (2 : 5). Later the (3 : 7) formulae $3Na_2O \cdot 7MoO_3 \cdot 22H_2O$ and $3(NH_4)_2O \cdot 7MoO_3 \cdot 4H_2O$ were ascribed to these salts by Delafontaine[4,5] and Ullik[6]. In 1916, however, Rosenheim[7] carried out analyses leading to the (5 : 12) formulae $5Na_2O \cdot 12MoO_3 \cdot 38H_2O$ and $5(NH_4)_2O \cdot 12MoO_3 \cdot 7H_2O$, which were then widely accepted (*cf.* Gmelin[8]). In a review, Jander[9] supported these later formulae, obviously because they were required by his theory of the ion aggregation in molybdate solutions. No analyses were carried out by Jander. In recent years Byé[10] and Guiter[11,12], have again reported (3 : 7) formulae, but none of them has tried to make a complete analysis with modern and well established methods. As a consequence of these diverging results Carpéni[1] has not decided in favour of either formula. He does not, however, mention the important X-ray investigation on the ammonium paramolybdate by Sturdivant[13], which seems to us quite deciding in this question. As the corresponding paratungstates are generally given (5 : 12) formulae, one can easily understand the actual confusion in this field. Under these circumstances we have found a thorough analysis desirable.

*Sodium paramolybdate.** This compound was formed by isothermal evaporation at 20° from a solution of 9.68 g $Na_2MoO_4 \cdot 2H_2O$ in 21.5 ml 1.98—C HCl and 20 ml H_2O. We obtained well defined crystals which were shown to be free from chloride ions. These crystals were quickly dried between filter papers, weighed, and heated in a platinum crucible in order to determine the water content. The residue was weighed and then dissolved in water. Part of the solution was poured through a cadmium reducer and molybdenum was determined[14] by titration with $KMnO_4$. Another part was drawn through a column of »Amberlite IR 100 Ion Exchanger», which had previously been saturated with ammonium ions[15]. The solution containing the molybdate ions was discarded. The ion exchanger was then washed with 5 % HCl until we did not get any sodium flame

* In the analytical work, I was assisted by Mr Olof Karlsson, whom I here wish to thank for his laborious work.

from the washing solution. In this solution, sodium was determined as $(UO_2)_3NaMg(CH_3CO_2)_9 \cdot 8H_2O$ by a method given by Nydahl [16] **. This method was first established by analyses of solutions containing known amounts of sodium and molybdenum. The results are all given in Table 1.

Table 1. Sodium paramolybdate.

1. *Check on the method.*

Weighed amounts		Measured amounts	
Na_2O	MoO_3	Na_2O	MoO_3
15.44 mg	83.68 mg	15.51 mg	84.12 mg
9.380 »	86.40 »	9.428 »	86.78 »

2. *Analyses.*

	Weight percentages		
	Na_2O	MoO_3	H_2O
(5 : 12) calc.	11.39	63.47	25.14
(3 : 7) »	11.69	63.38	24.92
Analysis 1	11.77	63.40	24.84
» 2	11.78	63.23	25.01

Ammonium paramolybdate. This compound was recrystallized from solutions of commercial ammonium molybdate. Molybdenum was reduced as above and titrated with $KMnO_4$. NH_3 was determined by Kjeldahl-distillation into 0.1—C HCl and back titration with 0.1—C NaOH with methyl red as indicator. From the sum of NH_3 and H_2O lost by heating, H_2O could then be calculated. The results are given in Table 2.

Results. The (5 : 12) formulae can at once be excluded, but the compositions are in agreement with the (3 : 7) formulae. We hope that our X-ray investigations will make it possible for us to decide that the atomic arrangements give the formulae $3Na_2O \cdot 7MoO_3 \cdot 22H_2O$ and $3(NH_4)_2O \cdot 7MoO_3 \cdot 4H_2O$. Of course we are also going to investigate the formulae of paratungstates. In any case the formulae $5Na_2O \cdot 12MoO_3 \cdot 38H_2O$ and $5(NH_4)_2O \cdot 12MoO_3 \cdot 7H_2O$ are wrong and so are the theories of Jander as far as they are based on the existence of such compounds.

Table 2. Ammonium paramolybdate.

	Weight percentages		
	$(NH_4)_2O$	MoO_3	H_2O
(5 : 12) calc.	12.32	81.72	5.96
(3 : 7) »	12.64	81.52	5.84
Analysis 1	12.55	81.43	5.89
» 2	12.55	81.40	5.80

This investigation has been supported by a grant, which is here gratefully acknowledged, from *Statens Naturvetenskapliga Forskningsråd*. Finally I wish to thank Dr F. Nydahl for valuable discussions of the analytical methods.

1. Carpéni, G. *Bull. Soc. Chim.* **14** (1947) 492.
2. Zenker, F. E. *J. prakt. Chem.* **58** (1853) 487.
3. Svanberg, L., and Struve, H. *J. prakt. Chem.* **44** (1848) 282.
4. Delafontaine, M. *J. prakt. Chem.* **95** (1865) 141.
5. Delafontaine, M. *Arch. phys. nat.* [2] **23** (1865) 17.
6. Ullik, F. *Ann.* **144** (1867) 219.
7. Rosenheim, A. *Z. anorg. Chem.* **96** (1916) 141.
8. Gmelin, *Handbuch der anorganischen Chemie* 8. Auflage 53:Mo. 222, 254.
9. Jander, G. *Z. anorg. Chem.* **194** (1930) 383.
10. Byé, J. *Bull. Soc. Chim.* **10** (1943) 239.
11. Guiter, H. *Bull. Soc. Chim.* **9** (1942) 622.
12. Guiter, H. *Bull. Soc. Chim.* **12** (1945) 74.
13. Sturdivant, J. H. *J. Am. Chem. Soc.* **59** (1937) 630.
14. Nydahl, F. *Lantbruks-Högskol. Ann.* **10** (1942) 120.
15. Runeberg, G., and Samuelsson, O. *Svensk Kem. Tid.* **57** (1945) 250.
16. Nydahl, F. *Lantbruks-Högskol. Ann.* **6** (1937) 45.

Received April 5, 1948.

** *Cf.* Schoorl *Rec. Trav. Chim. Pays-Bas* **59** (1940) 305—313.

The Crystallization of Liver Esterase

M. SAFWAT MOHAMED*

Biokemiska Institutet, Stockholms Högskola, Stockholm, Sweden

Many investigators have attempted to prepare and purify liver esterase [1–7]. Baker and King [8] developed a procedure by which the esterase activity per milligram of total solid was increased twelvefold.

In the course of attempting to further purify liver arginase tests were systematically conducted for the presence of several enzymes. It was found that certain fractions obtained were extremely rich in esterase activity. The crystallization of the enzyme from these fractions was finally attained.

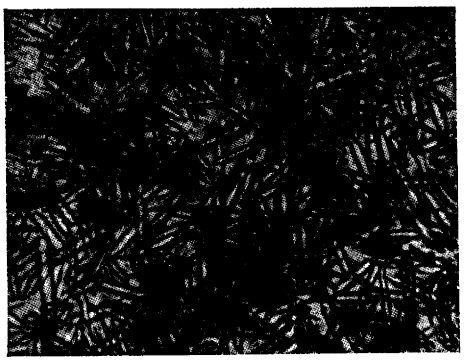
Method of assay. Horse liver was used as the source for the enzyme. The liver was obtained from the slaughter house, ground and treated three times with several volumes (4—5) of acetone and then it was spread on filter paper and left to dry at room temperature. The dry material was stored at 3° C. No loss of esterase activity was noticed on storage at this temperature for 3 months.

Ethyl butyrate was used as substrate for the assay. The procedure consisted of mixing 1.75 g of ethyl butyrate in 100 ml Erlenmeyer flask with 50 ml of H_2O. The mixture was incubated at 40° C for 5 minutes and then 1 ml of liver powder suspension, extract, or dilute solution of crystalline enzyme was added. A control determination using the boiled extract (5 minutes) was always made. Another control (0 time incubation) was also made at the beginning of each experiment to determine the initial amount of alkali necessary for neutralization of the mixture. After 30 minutes incubation the solutions were titrated with 0.1 *N* alkali using phenolphthalein as an indicator.

Preparation of crystalline liver esterase. 300 g of the acetone dried liver is extracted with 3 l of 2.5 % solution of sodium acetate. The extraction is carried out at room temperature for two hours with frequent shakings. The pH is adjusted to 7.2 and the mixture is held at 55° C for 5 minutes in a water bath. It is cooled rapidly under running cold water and then centrifuged. The supernatent is stored at 3° C for several hours and then treated with 0.75 volumes of cold acetone (3—5). The mixture is left in the cold room over night. The pink flocculent precipitate that has settled is separated by centrifugation. To the clear supernatent is added 0.45 volumes of cold acetone. A light cream colored quite flocculent precipitate is formed. This is left at 3° C for 10 hours after which it is centrifuged and the precipitate is taken up in 1/10 the original volume of water. The pH is again adjusted to 7.2 and the mixture is centrifuged, discarding the precipitate. The supernatent fluid is heated at 65° C in a water bath for 2 minutes and cooled rapidly. A clear extract is obtained by centrifugation. This is then dialyzed at 3° C against a cold mixture of acetone and water (55 % acetone) for 15 hours. The recovered precipitate is suspended in water (1/30 of the original volume) and then centrifuged discarding the insoluble precipitate. Solid ammonium sulfate is added to the clear extract to render it 60 % saturated. The precipitate formed at room temperature is removed after 10 hours and discarded. It consists of amorphous proteins and the structures claimed by Bach [5] to be crystalline arginase. The partially turbid extract is left standing and in a few hours crystals of esterase begin to appear. This process can be hastened by making the extract 65 % saturated with respect to ammonium sulfate.

---

* On a leave of absence from Farouk Ist University, Alexandria, Egypt.



Figur 1. Crystalline horse liver esterase (× 450).

Recrystallization. The crystalline precipitate is separated and taken up in 1/40 of the original volume of extract. Enough ammonium sulfate is added to make it 60—65 % saturated, and the mixture is left standing at room temperature. Crystallization starts slowly but a good crop of crystals is obtained in 24 hours. The crystals (Fig. 1) have needle shape. 1 ml of crystalline enzyme containing 0.1 mg nitrogen when allowed to act on 1.75 g of ethyl butyrate will produce anough acid to neutralize 35.0 ml of 0.01 *N* sodium hydroxide in one half hour.

Work is in progress to study the purity of the preparation electrophoretically and by solubility methods. Investigations on the kinetics, specificity and activation inhibition reactions are also being carried out and will be communicated in more detail in a later publication.

The author desires to express his appreciation and gratitude to Prof. K. Myrbäck for inviting him to work in his laboratories and for his continued zeal and interest.

1. Dakin, H. D. *J. Physiol.* 32 (1904) 199.
2. Prierice, G. *J. Biol. Chem.* 16 (1913) 1.
3. Willstätter, R., and Benz, M. *Ann.* 358 (1907—08) 267.
4. Willstätter, R., and Memmen, F. *Z. physiol. Chem.* 129 (1923) 1.
5. Bamann, E. *Ber.* 62 *B* (1929) 1538.
6. Bamann, E., and Schneller, H. *Z. physiol. Chem.* 188 (1930) 251.
7. Kraut, H., and Rubenhauer, H. *Z. physiol. Chem.* 173 (1928) 103.
8. Baker, Z., and King, C. G. *J. Am. Chem. Soc.* 57 (1935) 358.

Received May 4, 1948.

Sulphanilylderivatives of Heterocyclic Amines. XI.* Derivatives of Quinoxaline

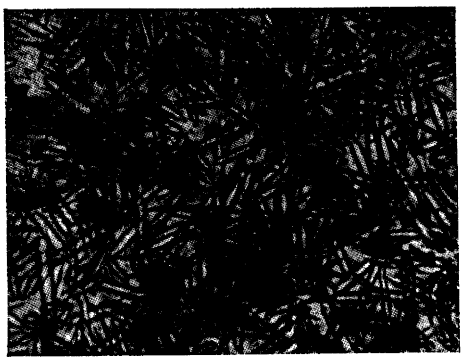
KAI ARNE JENSEN

Chemical Laboratory of the University of Copenhagen, Denmark

In connection with researches on the bacteriostatic effect of isomeric N^1-heterocyclic derivatives of sulphanilamide [1] it was desired to prepare the three isomeric sulphaquinoxalines. It was attempted to prepare 2-amino-quinoxaline from *o*-phenylenediamine plus glyoxylic nitrile or dichloroacetonitrile or from quinoxaline and sodium amide, but with negative results. While these experiments were still in progress, Weijlard, Tishler and Erickson published a paper [2] in which the synthesis of 2-sulphaquinoxaline and 2-aminoquinoxaline, starting from alloxazine, was announced. 2-Sulphaquinoxaline was found to be as effective as sulphadiazine in experimental pneumococcus infections in mice.

The two remaining isomers, 5- and 6-sulphaquinoxaline, have now been prepared by treating the appropriate aminoquinoxalines with acetylsulphanilyl chloride in the presence of pyridine and hydrolyzing the resulting acetyl derivatives. The two aminoquinoxalines required were prepared from 1,2,4- and 1,2,3-triaminobenzene and glyoxal. The 6-aminoquinoxaline

* No. X in this series appeared in *Dansk Tids. Farm.* 17 (1943) 189.



Figur 1. Crystalline horse liver esterase (× 450).

Recrystallization. The crystalline precipitate is separated and taken up in 1/40 of the original volume of extract. Enough ammonium sulfate is added to make it 60—65 % saturated, and the mixture is left standing at room temperature. Crystallization starts slowly but a good crop of crystals is obtained in 24 hours. The crystals (Fig. 1) have needle shape. 1 ml of crystalline enzyme containing 0.1 mg nitrogen when allowed to act on 1.75 g of ethyl butyrate will produce anough acid to neutralize 35.0 ml of 0.01 *N* sodium hydroxide in one half hour.

Work is in progress to study the purity of the preparation electrophoretically and by solubility methods. Investigations on the kinetics, specificity and activation inhibition reactions are also being carried out and will be communicated in more detail in a later publication.

The author desires to express his appreciation and gratitude to Prof. K. Myrbäck for inviting him to work in his laboratories and for his continued zeal and interest.

1. Dakin, H. D. *J. Physiol.* 32 (1904) 199.
2. Prierice, G. *J. Biol. Chem.* 16 (1913) 1.
3. Willstätter, R., and Benz, M. *Ann.* 358 (1907—08) 267.
4. Willstätter, R., and Memmen, F. *Z. physiol. Chem.* 129 (1923) 1.
5. Bamann, E. *Ber.* 62 *B* (1929) 1538.
6. Bamann, E., and Schneller, H. *Z. physiol. Chem.* 188 (1930) 251.
7. Kraut, H., and Rubenhauer, H. *Z. physiol. Chem.* 173 (1928) 103.
8. Baker, Z., and King, C. G. *J. Am. Chem. Soc.* 57 (1935) 358.

Received May 4, 1948.

Sulphanilylderivatives of Heterocyclic Amines. XI.* Derivatives of Quinoxaline

KAI ARNE JENSEN

Chemical Laboratory of the University of Copenhagen, Denmark

In connection with researches on the bacteriostatic effect of isomeric N^1-heterocyclic derivatives of sulphanilamide [1] it was desired to prepare the three isomeric sulphaquinoxalines. It was attempted to prepare 2-amino-quinoxaline from *o*-phenylenediamine plus glyoxylic nitrile or dichloroacetonitrile or from quinoxaline and sodium amide, but with negative results. While these experiments were still in progress, Weijlard, Tishler and Erickson published a paper [2] in which the synthesis of 2-sulphaquinoxaline and 2-aminoquinoxaline, starting from alloxazine, was announced. 2-Sulphaquinoxaline was found to be as effective as sulphadiazine in experimental pneumococcus infections in mice.

The two remaining isomers, 5- and 6-sulphaquinoxaline, have now been prepared by treating the appropriate aminoquinoxalines with acetylsulphanilyl chloride in the presence of pyridine and hydrolyzing the resulting acetyl derivatives. The two aminoquinoxalines required were prepared from 1,2,4- and 1,2,3-triaminobenzene and glyoxal. The 6-aminoquinoxaline

* No. X in this series appeared in *Dansk Tids. Farm.* 17 (1943) 189.

has been described previously [3] while 5-aminoquinoxaline is a new compound.

The two new sulphaquinoxalines form beautiful pale yellow crystals and are more soluble in water than most other sulphonamides. Their bacteriostatic activity against pneumococci (type I) is of the same order as that of sulphathiazole. Thus, analogous to what was found for the sulphaquinolines [4] the bacteriostatic activity is practically independent of the position of the sulphanilamido group.

6-Aminoquinoxaline was prepared from 1,2,4-triaminobenzene and glyoxal-sodium-hydrogensulfite [3]. Recrystallized from ether. M. p. 158—159°.

5-Aminoquinoxaline. The required 1,2,3-triaminobenzene was prepared by decarboxylation of 3,4,5-triaminobenzoic acid [5]. To an aqueous solution of 1,2,3-triaminobenzene (0.60 g) was added glyoxal-sodium-hydrogensulfite (1.40 g), and the mixture was heated for one hour at 50°. After cooling the solution was made strongly alkaline and extracted several times with chloroform. The chloroform solution was evaporated to dryness and the residue recrystallized from water. Yield 0.40 g or 55 % of the theoretical. From water 5-aminoquinoxaline crystallizes in chrome-yellow needles containing one mole of water.

$C_8H_7N_3,H_2O$ (163.2) Calc. N 25.74
Found » 25 87

The compound melts at 90.5°, loosing its water. By sublimation *in vacuo* over P_2O_5 the anhydrous compound is obtained as yellow-orange crystals, melting at 91°.

$C_8H_7N_3$ (145.2) Calc. N 28.94
Found » 28.71

The anhydrous compound is hygroscopic and exposed to air it is soon transformed in the hydrate. It is easily soluble in water and in most organic solvents.

6-Sulphanilamidoquinoxaline. 6-Aminoquinoxaline (1 g) was dissolved in dry pyridine (5 ml) and pure acetylsulphanilyl chloride (1.6 g) was added with stirring. When all had dissolved the solution was heated to boiling point and then left cooling. By addition of 50 ml of water an oily product separated, which by addition of hydrochloric acid and rubbing soon became crystalline. The crude acetyl compound was filtered, washed with water, suspended in 20 ml of 2 N-hydrochloric acid and refluxed one hour; the product gradually dissolved. The hot solution was treated with activated carbon, filtered, neutralized with sodium hydroxide and chilled. The pale yellow crystals were filtred, washed with water and recrystallized from water containing 10—20 % ethanol. The purified product form very pale yellow needles with m. p. 231.5—232° (corr.). Yield 0.60 g~30 %. The compound contains half a mole of water of crystallization, which is given off at 198—200° or at room temperature over P_2O_5.

$C_{14}H_{12}O_2N_4S, \frac{1}{2}H_2O$ (309.3) Calc. N 18.10
Found » 18.18
$C_{14}H_{12}O_2N_4S$ (300.3) Calc. N 18.65
Found » 18.71

The compound is slightly soluble in hot water, very soluble in ethanol.

5-Sulphanilamidoquinoxaline. This compound was prepared from 5-aminoquinoxaline in exactly the same way as the 6-derivative. It crystallizes, however, from the aqueous solution without water of crystallization:

$C_{14}H_{12}I_2N_4S$ (300.3) Calc. N 18.65
Found » 18.69

The compound form pale yellow plates with m. p. 168.5—169.5° (corr.).

The author is indebted to Mr. M. Solstad for preparing 1,2,3-triaminobenzene and 5-aminoquinoxaline and to Dr. Kai Schmith for carrying out the bacteriological tests.

1. Jensen, K. A., and Schmith, K. *Z. Immunitätsforsch.* **102** (1942) 261; **105** (1944) 40-

2. Weijlard, J., Tishler, M., and Erickson, A. E. *J. Am. Chem. Soc.* **62** (1944) 1957.
3. Hinsberg, O. *Ann.* **237** (1887) 345.
4. Jensen, K. A., and Lundquist, F. *Dansk Tids. Farm.* **14** (1940) 208.
5. Salkowski, H. *Ann.* **163** (1872) 23.

Received May 27, 1948.

Über die Ursache der Farbreaktionen des Holzes

E. ADLER, K. J. BJÖRKQVIST UND S. HÄGGROTH

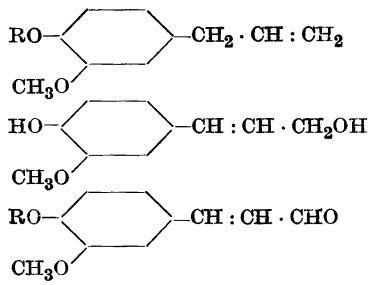
Holzchemische Abteilung des Schwedischen Instituts für Holzforschung, Stockholm, Schweden

Seit der Entdeckung der Farbreaktionen verholzter Gewebe mit Anilin oder Phenol-Salzsäure (Runge 1834) sind zahlreiche Versuche unternommen worden, den Träger dieser Reaktionen zu charakterisieren, ohne dass eine klare Entscheidung erzielt worden ist [1]. Unsere Ergebnisse stützten zunächst die auch von manchen anderen Autoren früher vertretene Auffassung, wonach die Reaktionen auf das Lignin selbst und nicht etwa (ausschliesslich) auf accessorische Bestandteile des Holzes zurückzuführen sind. Eine Identifizierung der dabei in Reaktion tretenden Gruppen könnte daher von Bedeutung für die Kenntnis der Konstitution des Lignins sein.

Bisher schien es aber, als ob die »Holzreaktionen» weitgehend unspezifisch seien. So wurde z. B. behauptet, dass sowohl Eugenol (I) und dessen Methyläther (II) [2] wie Coniferylalkohol (III) [3] und Coniferylaldehyd (IV) [4] die für Holz typischen Farbreaktionen mit Phenolen und aromatischen Aminen liefern. Eine nähere Untersuchung von Präparaten von I und II zeigte aber, dass die Farbreaktionen nicht von den reinen Substanzen gegeben werden, sondern durch aldehydische Verunreinigungen verursacht sind. Die Reaktionen waren nämlich nach Behandeln der Präparate mit Hydroxylamin oder Dimedon oder nach sehr sorgfältiger Destillation negativ. Aus einem Handelspräparat von Eugenolmethyläther (II) wurden Veratrumaldehyd sowie ein weiterer, vorher unbekannter Aldehyd in Form ihrer 2,4-Dinitrophenylhydrazone isoliert. Der letztere erwies sich als identisch mit Coniferylaldehyd-methyläther (V), den wir aus Veratrumaldehyd und Acetaldehyd synthetisierten (Schmp. 86—87°). Es zeigte sich, dass dieser Aldehyd ebenso wie der freie Coniferylaldehyd (IV) die »Holzreaktionen» in typischer Weise gibt.

Wir überzeugten uns ferner davon, dass durch Emulsinspaltung von Coniferin * frisch dargestellter Coniferylalkohol (III) die Holzreaktionen nicht liefert. Auch zahlreiche andere Substanzen, die irgendwie in Beziehung zu Lignin bzw. den untersuchten Modellsubstanzen stehen, wurden geprüft, mit negativem Ergebnis. Die »Holzreaktionen» sind somit spezifisch für die Coniferylaldehydstruktur.

Extrahierbares, sog. »natives» Lignin nach Brauns [5] wurde in alkoholischer Lö-



I. R = H II. R = CH_3

III.

IV. R = H V. R = CH_3

* Für die freundliche Überlassung dieser Substanz sind wir Herrn Prof. B. Holmberg zu grossem Dank verpflichtet.

2. Weijlard, J., Tishler, M., and Erickson, A. E. *J. Am. Chem. Soc.* **62** (1944) 1957.
3. Hinsberg, O. *Ann.* **237** (1887) 345.
4. Jensen, K. A., and Lundquist, F. *Dansk Tids. Farm.* **14** (1940) 208.
5. Salkowski, H. *Ann.* **163** (1872) 23.

Received May 27, 1948.

Über die Ursache der Farbreaktionen des Holzes

E. ADLER, K. J. BJÖRKQVIST UND S. HÄGGROTH

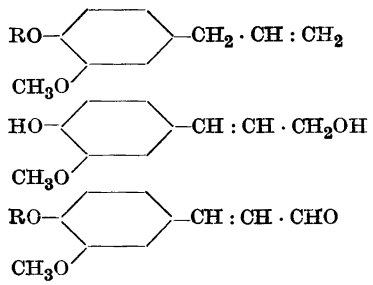
Holzchemische Abteilung des Schwedischen Instituts für Holzforschung, Stockholm, Schweden

Seit der Entdeckung der Farbreaktionen verholzter Gewebe mit Anilin oder Phenol-Salzsäure (Runge 1834) sind zahlreiche Versuche unternommen worden, den Träger dieser Reaktionen zu charakterisieren, ohne dass eine klare Entscheidung erzielt worden ist [1]. Unsere Ergebnisse stützten zunächst die auch von manchen anderen Autoren früher vertretene Auffassung, wonach die Reaktionen auf das Lignin selbst und nicht etwa (ausschliesslich) auf accessorische Bestandteile des Holzes zurückzuführen sind. Eine Identifizierung der dabei in Reaktion tretenden Gruppen könnte daher von Bedeutung für die Kenntnis der Konstitution des Lignins sein.

Bisher schien es aber, als ob die »Holzreaktionen» weitgehend unspezifisch seien. So wurde z. B. behauptet, dass sowohl Eugenol (I) und dessen Methyläther (II) [2] wie Coniferylalkohol (III) [3] und Coniferylaldehyd (IV) [4] die für Holz typischen Farbreaktionen mit Phenolen und aromatischen Aminen liefern. Eine nähere Untersuchung von Präparaten von I und II zeigte aber, dass die Farbreaktionen nicht von den reinen Substanzen gegeben werden, sondern durch aldehydische Verunreinigungen verursacht sind. Die Reaktionen waren nämlich nach Behandeln der Präparate mit Hydroxylamin oder Dimedon oder nach sehr sorgfältiger Destillation negativ. Aus einem Handelspräparat von Eugenolmethyläther (II) wurden Veratrumaldehyd sowie ein weiterer, vorher unbekannter Aldehyd in Form ihrer 2,4-Dinitrophenylhydrazone isoliert. Der letztere erwies sich als identisch mit Coniferylaldehyd-methyläther (V), den wir aus Veratrumaldehyd und Acetaldehyd synthetisierten (Schmp. 86—87°). Es zeigte sich, dass dieser Aldehyd ebenso wie der freie Coniferylaldehyd (IV) die »Holzreaktionen» in typischer Weise gibt.

Wir überzeugten uns ferner davon, dass durch Emulsinspaltung von Coniferin * frisch dargestellter Coniferylalkohol (III) die Holzreaktionen nicht liefert. Auch zahlreiche andere Substanzen, die irgendwie in Beziehung zu Lignin bzw. den untersuchten Modellsubstanzen stehen, wurden geprüft, mit negativem Ergebnis. Die »Holzreaktionen» sind somit spezifisch für die Coniferylaldehydstruktur.

Extrahierbares, sog. »natives» Lignin nach Brauns [5] wurde in alkoholischer Lö-



I. R = H II. R = CH_3

III.

IV. R = H V. R = CH_3

* Für die freundliche Überlassung dieser Substanz sind wir Herrn Prof. B. Holmberg zu grossem Dank verpflichtet.

sung mit den Modellsubstanzen IV und V verglichen. Es zeigte sich, dass die Absorptionskurven der durch Phloroglucin-HCl hervorgerufenen Farbstoffe einander sehr ähnlich sind; dasselbe gilt für die mit aromatischen Aminen erhaltenen Farbreaktionen. Die Aldehyde IV und V geben auch spezifisch das für Holz charakteristische Farbenspiel (→ gelb → smaragdgrün) mit konz. Salzsäure. Aus diesen Befunden lässt sich schliessen, dass in das Ligninmolekül Coniferylaldehydreste eingebaut sind.

Bei der Sulfitkochung von Fichtenholz wird die ungesättigte Seitenkette dieser Reste blockiert, denn Lignosulfonsäuren geben die Farbreaktionen nicht oder nur sehr schwach. Wir fanden jedoch, dass die Farbreaktionen stark positiv werden, wenn die Lignosulfonsäure während einiger Minuten bei gewöhnlicher Temperatur mit Alkali behandelt wird. Dieser Effekt ist damit zu erklären, dass in der Lignosulfonsäure Seitenketten vom Typus —CH(SO_3H)—CH_2—CHO (VI) enthalten sind [6]; diese spalten nämlich, wie von der Zimtaldehyd-hydrosulfonsäure her bekannt ist [7] und an den entsprechenden Sulfonsäuren von IV und V bestätigt wurde, mit Alkali Sulfit ab unter Regenerierung der ungesättigten Seitenkette.

Die Coniferylaldehydgruppen im Lignin bzw. deren Sulfonsäurederivate (VI) in den Lignosulfonsäuren machen nur einen geringen Anteil des Gesamtmoleküls aus; man könnte daran denken, sie als Endgruppen zu betrachten.

In kommenden ausführlichen Mitteilungen werden wir u. a. über weitere Modellversuche sowie näheres über die quantitativen Verhältnisse berichten.

1. Vgl. Hägglund, E. *Holzchemie* Leipzig (1939) S. 128; Wise, L. E. *Wood chemistry* New York (1946) S. 276.
2. Podbreznik, F. *Pulp & Paper Mag. Can.* **27** (1929) 229.
3. Kürschner, K. *Technol. u. Chem. Papier- u. Zellstoff-Fabr.* **26** (1929) 61.
4. Klason, P. *Ber.* **53** (1920) 706; Wiechert, K. *Papier-Fabr.* **37** Tl 1 (1939) 17.
5. Brauns, F. E. *J. Am. Chem. Soc.* **61** (1939) 2120.
6. Vgl. hierzu auch Kratzl, K. *Monatsh.* **78** (1948) 173.
7. Tiemann, F. *Ber.* **31** (1898) 3297.

Eingegangen am 18. Juni 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 95—96

New Books

Lars Gunnar Sillén, Paul W. Lange and C. Olof Gabrielsson. *Fysikalisk-kemiska räkneuppgifter.* Hugo Gebers Förlag, Uppsala, 1948. 300 pp. 17 Swedish crowns.

In teaching physical chemistry the working of numerical problems plays a very essential rôle. Before the student has obtained a training in applying the physico-chemical laws to definite problems, the greater part of his knowledge is sterile. He will obtain an idea of the potentialities as well as the limitations of the laws only when he is trained in their application. And this training ought also to give him the self-confidence which is necessary when he has to attack the problems which later occur in his own scientific work.

The collection of numerical physico-chemical problems, which has now appeared, will certainly prove extremely valuable in furnishing both the teacher and the students with appropriate exercises. No less than 614 problems are given, many of them covering fields of research which have not earlier been represented in collections of problems. Nearly all of them have been selected from the literature and thus obtain a touch of reality and usefulness which is quite stimulating. It is true that this method has been practised in earlier collections of problems but hardly to such an extent as here. The same applies to the mode of giving complete references. A student who has access to a library can, therefore, check problems of special interest and obtain a very valuable idea of the basis of the work and the experimental conditions.

The standard of the problems is on the level with the requirements for the higher classes in the Swedish degrees »filosofie kandidat» and »filosofie magister». Many of them have already served as examination questions.

The problems have been arranged in 11 different chapters. Each chapter begins with a recapitulation of the more important laws in the field in question, which will certainly serve as a useful means of checking the knowledge before beginning with the problems. In connection with this introduction the treatment of certain typical and illustrative problems is shown. In the actual collection of problems, which follows the introduction, no hints facilitate the work. Answers are given to all problems.

The printing and paper of the book are excellent and the general appearance quite attractive (with the exception of the wrapper). Some practical typographical features have been introduced, amongst which the space-saving setting of the small tables within the problems ought to be mentioned.

The reviewer is convinced that this book will serve a very useful purpose in all physico-chemical teaching on the university level, where a book in Swedish can be read. *G. Hägg*

Festskrift tillägnad J. Arvid Hedvall. Edited by Erik Hemlin. Gumperts AB, Gothenburg, 1948. 659 pp. 30 Swedish crowns.

To celibrate Professor Hedvalls' sixtieth birthday, a number of his friends have

dedicated to him a festival publication, containing a large number of contributions from different authors.

An objection often raised against such jubilee volumes is that they act as cemeteries, where pieces of valuable research work are buried in a form almost inaccessible to the majority of future scientists. For example, one of the foundations of the modern methods of studying complex equilibria is a paper by Bodländer in the »Festschrift für R. Dedekind, Braunschweig, 1901», which is often quoted but which I, for instance, have never managed to get hold of.

This objection does not apply to the present volume. Most of the contributions are not of the original, rather dry type that one would rather have seen in a scientific journal. On the contrary, the stress seems to have been put on readability. An unusually large number of the papers are entertaining surveys on various topics that might be read with pleasure by any chemist.

The book begins with a long list of congratulants, which could easily have been made still longer, had only more of Hedvalls friends and admirers been asked. It ends with an impressive list of Professor Hedvall's own publications. Between these two lists there are 49 papers by in all 61 authors. The language is Swedish in 28, English in 11, and German in 10. About a third of them are directly connected with the fields of science in which Hedvall is famous: reactions in the solid state, and the chemistry of oxides, and especially silicates. The rest are on a wide variety of subjects.

He who understands Swedish can read here surveys of various technical subjects, such as acid-proof materials, sugar from wood, synthetic rubber, and modern ion exchangers; or on the history of chemical symbols, on patents as a part of scientific literature, and on the latest changes in the periodic system of elements. But if he prefers, he can also enjoy articles on Chinese poets from the T'ang period, or on the correlation between winter temperature and the frequency of *Glaucium flavum* Cr. on Store Vassholmen in the archipelago of Fjällbacka. The latter article, of course, is written by our famous chemist The Svedberg.

Among the Swedish scientists contributing in one of the international languages we find de Hevesy on the preparation of radioactive tracers, Hössjer on the foundations of electrodynamics, von Euler on plasticizers, and Enkvist and Hägglund on sulphur lignins.

A number of prominent scientists from abroad have also honoured Hedvall by sending articles, generally on subjects related to Hedvall's own work. Bragg writes on atomic rearrangement in metals, Cohn and Kolthoff on the thermal aging of silica, Fricke on active solids, Hahn on radioactive methods for studying surfaces, Hüttig and Dreithaler on experiments on dehydration, Niggli and Brandenberger on solid state reactions in minerals, Schwab and Pesmatjoglou on the poisoning of nickel catalysts, Vogel and Fülling on equilibria Fe-FeS-FeO, and Zawadzki on the action of gaseous oxides on metal oxides. Walden has given an essay on the oscillations between the speculative and the experimental trend in scientific thinking, containing some rather amusing samples of speculative work.

The examples given above may suffice to show that Hedvall's friends have honoured him by composing not only a heavy volume to put on a shelf but a book to be read. If there is anyone claiming to be an inorganic or physical chemist, or a chemical engineer, who would not read with great interest at least ten of the articles in this volume, then there must be something wrong both with his interest in his own science and with the breadth of his general outlook.

Lars Gunnar Sillén

ACTA CHEMICA SCANDINAVICA 2 (1948) 97—108

Effect of Oxidation-Reduction Potential on the Stability of Ascorbic Acid in Milk and in Fermented Milk

PAAVO ROINE*

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

It is a well-known fact that ascorbic acid is easily destroyed through oxidative processes. Destruction is brought about through autoxidation as well as through catalytic oxidation caused by heavy metals or different enzyme systems. In investigations on the oxidation of ascorbic acid in various biological materials, different substances or mechanisms have been found which have an inhibitory effect on this reaction. Such mechanisms are known to occur above all in animal tissues and fluids [1—4] but vegetable materials, particularly those containing appreciable quantities of ascorbic acid, also, have protective properties [5—9]. The nature of the protective mechanisms has until now remained obscure, although several substances — glutathione, cysteine and some other amino acids, proteins, etc. — as well as enzymatic systems have been assumed to be responsible for the protection of the oxidation. Surprisingly little attention has been paid to the effect of the oxidation-reduction potential, although the equilibrium ascorbic acid — dehydroascorbic acid is theoretically wholly dependent on the electrode potential of the medium. In a medium of high potential, ascorbic acid is oxidized to dehydroascorbic acid, which, being unstable, gradually disappears, thus causing losses in the vitamin C potency of the system. The effect of the oxidation-reduction potential is important not only from the theoretical point of view but also from the practical, because the potentials of various foodstuffs are greatly different.

The behaviour of ascorbic acid in milk and particularly its role in the development of the »oxidized» flavors has been an object of much research

* The author wishes to express his sincere gratitude to Mr. Ahti Harjanne for his valuable assistance throughout the investigation. Thanks are also due to Dipl.eng. Raakel Porvari, who assisted in some experiments.

work. It should be here stated only that ascorbic acid in milk has been found to be comparatively unstable. In addition to its photo-chemical oxidation by daylight [10–12] it is slowly oxidized also in the dark and at low temperatures [13]. In the commercial handling of milk, when milk is exposured to light, contaminated with metals, etc., the losses of ascorbic acid are very high. This fact has a great practical significance because milk is, in those countries where it is amply consumed, an important source of ascorbic acid.

There seem to exist no investigations of the stability of ascorbic acid in different kinds of fermented milks, although these milk products have a large use in many countries. For example, in Finland buttermilks, either natural churned buttermilk or different kinds of cultured buttermilks made from whole or skim milk, are in common use. Bacterial cultures are nearly always strongly reducing systems, often lowering the oxidation-reduction potential of the medium to a very low level, and this holds true also for fermented milk. It was therefore interesting to know whether ascorbic acid is protected against oxidation better in fermented milk than in ordinary milk and, if so, whether the effect is actually due to the low oxidation-reduction potential or only to the acidity of the fermented milk. In order to get information on this problem the experiments reported herein were performed.

EXPERIMENTAL

Experiments with *Lactobacillus acidophilus* grown in an artificial medium

Esselen and Fuller [14] and Esselen [15] have found that certain bacteria, particularly members of the coliform group, are able to inhibit the oxidation of ascorbic acid in culture media. Some strains of *Escherichia coli* were even found to be able to reduce dehydroascorbic acid to ascorbic acid. Concerning the behaviour of the lactic acid bacteria there are in literature conflicting reports. In the experiments of Esselen [15] *Streptococcus lactis* was found to exert a weak, and *Lactobacillus acidophilus* a strong inhibitory action on the oxidation of ascorbic acid. Tkachenko [16] stated that *L. bulgaricus*, *L. acidophilus*, and *L. leichmannii* were able to reduce dehydroascorbic acid into ascorbic acid, while Harowitz-Wlasowa and Buchman [17], on the other hand, found the same bacteria to exert a destructive action on ascorbic acid.

An experiment made to determine the stability of ascorbic acid in cultures of *L. acidophilus* is given in the following.

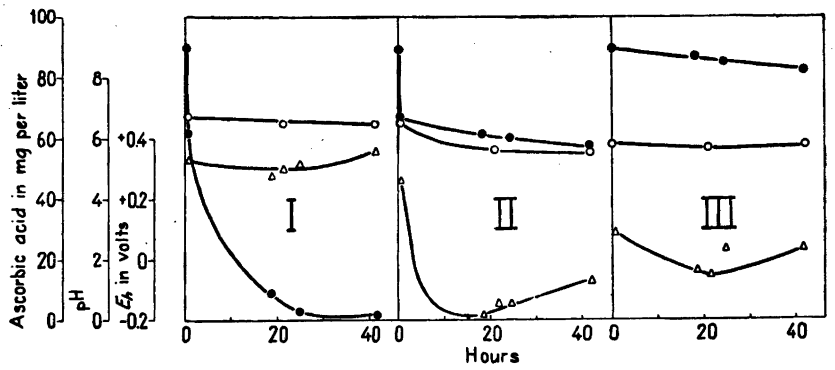


Fig. 1. Stability of added ascorbic acid in cultures of Lactobacillus acidophilus. I. Sterile medium. II. Culture inoculated simultaneously with the addition of ascorbic acid. III. Culture inoculated the day before the addition of ascorbic acid.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

The nutrient medium had the following composition:

NaCl	2 g
K_2HPO_4	2 »
$MgSO_4 \cdot 7\ H_2O$	1 »
Glucose	10 »
Lactose	10 »
Peptone	20 »
Tap water	1 l

200 ml of this medium together with 2 g of $CaCO_3$ were put in 250 ml Erlenmeyer flasks and sterilized in autoclave. One of the flasks was inoculated with *L. acidophilus*, allowed to stand for 20 hours at 37° C, whereupon 20 mg ascorbic acid (Merck), dissolved in water and sterilized with »Jena G 5 auf 3» bacterial filter, were added. In the second flask ascorbic acid was added immediately after the inoculation. The third flask was kept sterile and only ascorbic acid was added to it. In addition the experiment included two control flasks without ascorbic acid, one of which was inoculated and the other kept sterile. The flasks were incubated at 37° C and samples were taken at the beginning of the experiment and then afterward at certain intervals.

The pH and E_h measurements were made by potentiometric methods. All the E_h values were calculated for pH = 6. Ascorbic acid was determined by 2,6-dichlorophenol indophenol titration after the solution had been made acid with trichloroacetic acid, and centrifuged. The results are given in Fig. 1. In addition it should be mentioned that no substances able to reduce dichlorophenol indophenol were formed in the inoculated control without ascorbic acid.

It can be seen from Fig. 1 that cultures of *L. acidophilus* have a distinct protective effect upon the oxidation of ascorbic acid. While in the sterile medium (I) ascorbic acid was completely destroyed in 25 hours, the destruction range was only 33 % in culture II, which had been inoculated simultaneously with the addition of ascorbic acid. In culture III, where ascorbic acid was added into a medium inoculated the day before, the destruction was insignificant. The E_h curves show the dependence of the destruction upon the oxidation-reduction potential. In the sterile medium with high potential, the oxidation has occured very rapidly, while in culture III the low potential has almost completely inhibited the oxidation. In culture II oxidation has taken place during the beginning of the experiment while the oxidation-reduction potential was still high; the rapid fall of the potential, caused by bacterial growth, has, however, inhibited further oxidation. The slight changes of pH during the experiment have no effect on the oxidation; as a separate experiment showed, the rate of oxidation of ascorbic acid in the above conditions was practically independent of the pH in the interval between 4 and 7.

Experiments with milk

The *milk* used in the experiments was milked into glass vessels and kept in dark flasks until the experiment was started (usually 6 to 8 hours later).

Determination of ascorbic acid was performed according to Lunde [18]. 10 ml of milk were precipitated with 6 ml of 20 % trichloroacetic acid. After centrifuging or filtering, an aliquot part was titrated with 0.001 *N* solution of 2,6-dichlorophenol indophenol.

Total vitamin C was determined according to Gunsalus and Hand [19]. 10 ml of milk was mixed with 1 ml of resting suspension of *Escherichia coli* and allowed to stand at 40° C in order to reduce dehydroascorbic acid to ascorbic acid. After 15 minutes 6 ml of 20 % trichloroacetic acid were added, filtered, and titrated with 2,6-dichlorophenol indophenol. With fermented milks the pH was adjusted to 6.2—6.6 before the incubation.

In order to discover the *effect of pH on the stability of ascorbic acid in milk* some experiments were performed in which the pH of milk was adjusted to desired values by addition of acids.

Because lactic acid seemed to accelerate the oxidation of ascorbic acid, an experiment was made in which in addition to lactic acid (P. G. VI, Schering-Kahlbaum) the effect of metaphosphoric acid (glacial, sticks, Schering) and sulphuric acid (for forensic purposes, Merck) also were tested. It was stated that lactic and metaphosphoric acids distinctly accelerated the destruction of ascorbic acid as compared with a milk sample to which no acids were added. Sulphuric acid was without effect. The destructive action of lactic and metaphosphoric acids was probably due to their content of heavy metals; the copper content of the lactic acid used was according to determination 1.0 μg per ml.

To eliminate the effect of heavy metals, lactic acid was distilled twice *in vacuo* whereby its copper content fell to a value below 0.02 μg per ml. With this acid the pH of three 40 ml milk samples in large test tubes was adjusted to 4.2, 5.2, and 6.0, respectively. A

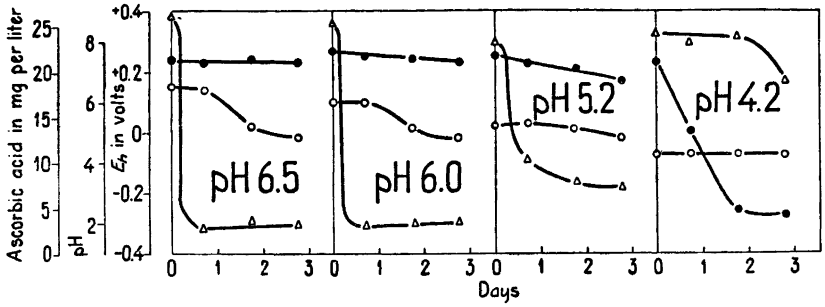


Fig. 2. Effects of pH and E_h on the stability of ascorbic acid in milk.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

fourth, a control tube, contained ordinary milk (pH = 6.5). The tubes were equipped with electrodes for the E_h measurements and allowed to stand in the dark at room temperature (about 20° C). At certain intervals samples for pH and ascorbic acid determinations were taken. The results are given in Fig. 2.

It can be seen from Fig. 2 that the stability of ascorbic acid has been practically independent of the pH of milk. In samples with starting pH of 6.5 or 6.0 hardly any destruction of ascorbic acid has taken place and even in the 5.2-sample destruction has been very slight. At the lowest pH, 4.2, ascorbic acid has been rapidly destroyed, but this is evidently not owing to the acidity but to the high oxidation-reduction potential of that sample. The E_h curves show distinctly the dependence of the stability upon the oxidation-reduction potential. In 6.5- and 6.0-samples there is an abrupt fall in the curves owing to the bacterial growth activities, and it is seen that ascorbic acid has been almost completely protected from oxidation. Even in the 5.2-sample a rapid fall in the E_h curve and a corresponding great stability of ascorbic acid can be noticed. In the 4.2-sample the bacteria have no longer been able to grow. The potential has therefore remained at the original high level, with the result that ascorbic acid has been largely destroyed. These results show that the stability of ascorbic acid in milk is not dependent on the acidity, but on the oxidation-reduction potential of milk.

Some of the typical experiments concerning the *stability of ascorbic acid in milk and in fermented milk* will be given in the following.

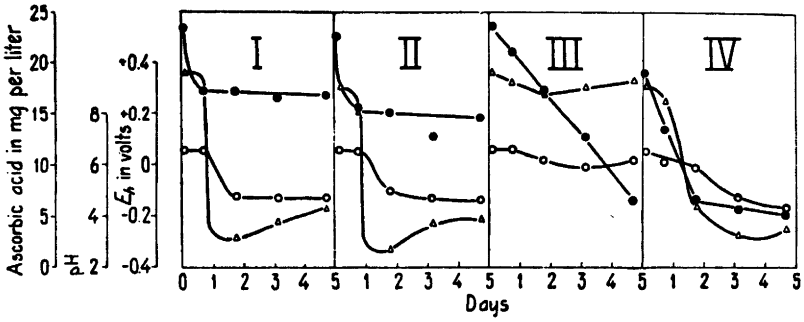


Fig. 3. Stability of ascorbic acid in milk. I. Milk stored at 20° C. II. Skim milk stored at 20° C. III. Milk stored at 1—3° C. IV. Pasteurized milk stored at 20° C.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

The following samples were included in the experiment:

I. Milk.
II. Skim milk.
III. Milk, kept in an ice-box (1—3° C).
IV. Milk, pasteurized at 72° C for 30 seconds.
V. Milk, fermented with a starter (the ordinary butter culture).
VI. Milk, fermented with *Lactobacillus acidophilus.*
VII. Milk, fermented with a ropiness-causing culture of *Streptococcus lactis.*

140 ml of milk were placed in cylinder-shaped 250 ml flasks, wrapped in thick brown paper and equipped with electrodes for the E_h measurements. All the flasks except III were kept at room temperature. Samples for pH and ascorbic acid determinations were taken at certain intervals, the first 1 ½ hours after the inoculation of flasks V—VII. The results are given in Fig. 3 and Fig. 4.

It can be seen from Fig. 3 that a remarkable fall in the ascorbic acid content of all the samples during the beginning of the experiment has taken place. In cases I and II, milk and skim milk stored at room temperature, the bacterial growth has quickly and abruptly lowered the oxidation-reduction potential, and subsequently only a slight decrease in ascorbic acid can be noticed. In pasteurized milk the fall of E_h has been weaker and much more ascorbic acid has been destroyed. In case III where milk was stored in an ice-box, the E_h has remained high during the whole period of the experiment, and a continuous, rapid fall in ascorbic acid has occurred.

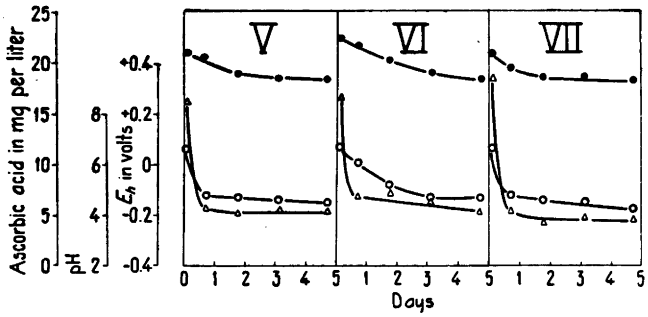


Fig. 4. Stability of ascorbic acid in fermented milks. V. Fermented with starter. VI. Fermented with Lactobacillus acidophilus. VII. Fermented with Streptococcus lactis. All stored at 20° C.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

Fig. 4 shows that in all fermented milks the stability of ascorbic acid has been fairly good. Owing to the inoculation with bacteria, the potential of these samples has begun to fall at the very beginning of the experiment and only slight losses of ascorbic acid have occurred.

Garret, Arnold, and Hartman [20] have stated that factors associated with the individual cow have a marked influence on the stability of ascorbic acid in milk. This fact could be noticed also in the present investigation. The milk used in different experiments was usually taken from one cow only, and marked variations in the stability of ascorbic acid was observed. As the E_h measurements showed (compare, *e. g.*, the E_h curves of milk in Fig. 2 and Fig. 3, respectively) the abrupt fall of the potential occurred sometimes earlier, sometimes later, probably depending on the extent of the bacterial contamination of milk. This observation gives one possible explanation for the variations in the stability of ascorbic acid in different milk samples and in the milk of different cows. Variations in the air content of milk offer another explanation. It is probable that relatively more air is mixed in the milk of a cow with low milk production than in that of a cow with high production.

In the experiments described above attention was paid to the changes in the amounts of reduced ascorbic acid only, the amounts of dehydroascorbic acid not being determined. In some experiments, however, also the *total vitamin C* was determined, and its changes during the storage of milk and fermented milks were investigated. The results of such an experiment, made with pasteurized milk (62—63° C, 30 minutes), are given in Fig. 5.

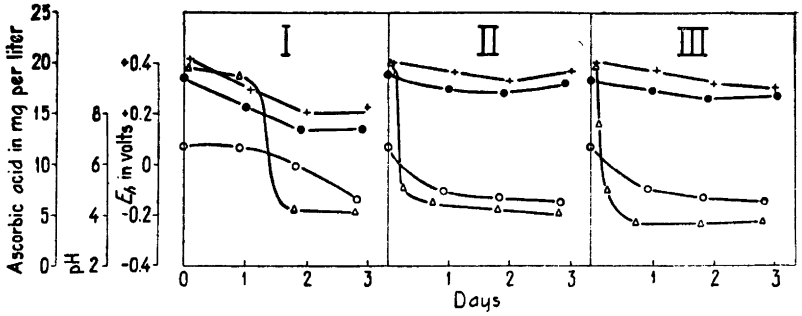


Fig. 5. Stability of total vitamin C and of ascorbic acid in milk and in fermented milks. I. Pasteurized milk. II. Pasteurized milk fermented with starter. III. Pasteurized milk fermented with Streptococcus lactis. All stored at 20° C.

+——+ *Total vitamin C*
●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

Although the differences are not very great it can be distinctly observed also in this experiment that the reduced ascorbic acid has disappeared more rapidly in ordinary milk than in fermented milks. In addition it is observable that the course of the curve representing total vitamin C and of that representing reduced ascorbic acid, are quite parallel. Accordingly, the dehydroascorbic acid formed by the oxidation of ascorbic acid is rapidly destroyed (*cf.* Hand[13]) and a decrease observed in the amount of reduced ascorbic acid means thus also that a decrease in the total vitamin C potency of milk has taken place.

In order to know whether the fermented milks are able only to inhibit the oxidation of ascorbic acid, or whether they can also reduce dehydroascorbic acid to ascorbic acid, experiments were arranged where dehydroascorbic acid (prepared by iodine oxidation according to Stewart and Sharp[21]) was added to fermented milks. Fig. 6 shows the course of an experiment with milk fermented by a ropiness-causing culture of *Streptococcus lactis*. It is seen that the amount of dehydroascorbic acid has fallen rapidly, while that of ascorbic acid has continuously increased. Exactly the same picture was obtained from milk fermented with ordinary starter. In uninoculated milk, the added dehydroascorbic acid disappeared more rapidly still, but no reduction to ascorbic acid could be noticed.

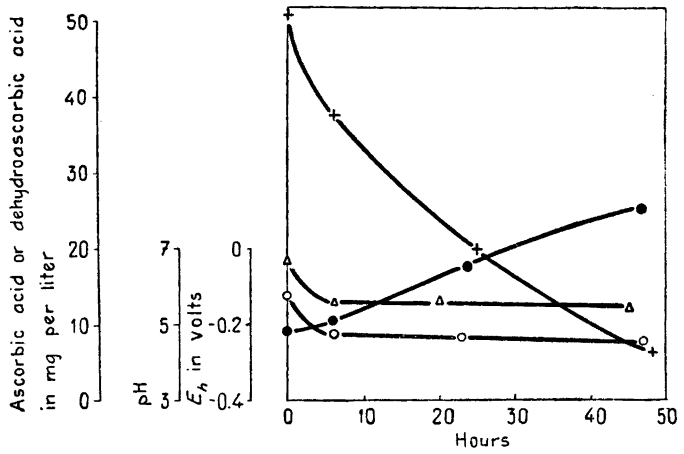


Fig. 6. Reduction of added dehydroascorbic acid in fermented milk. (Stored at 20° C).

●——● *Ascorbic acid*
\+ —— + *Dehydroascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

DISCUSSION

The oxidation of ascorbic acid is a problem of much interest both from the theoretical and the practical points of view. Numerous investigations have been made concerning the mechanism of oxidation as well as concerning the stability of ascorbic acid in different foodstuffs and under different conditions. It has been shown that foods contain heavy metals and enzymes which are able to oxidize ascorbic acid and, on the other hand, substances or systems which tend to inhibit this oxidation. Since ascorbic acid is very stable in strongly acid solutions, it has been commonly assumed that also the acidity of foodstuffs has a marked influence on the stability, ascorbic acid being more stable in acid than in neutral materials. Very little attention has been paid to the direct importance of the oxidation-reduction potential, although great variations occur in the potentials of different foodstuffs.

In the experiments described above the influence of the acidity, as well as that of the oxidation-reduction potential on the stability of ascorbic acid in milk and fermented milks was investigated. Some experiments were made also with cultures of *Lactobacillus acidophilus* in an artificial medium. It was shown that the stability of ascorbic acid was neither in milk nor in the culture medium used dependent on the acidity in the pH range tested, *i. e.*, between

pH 4 and 7. In general, it seems probable that the differences of acidity occurring in foodstuffs have no influence or only a slight one on the stability of ascorbic acid.

The oxidation-reduction potential, on the other hand, was shown to have a distinct effect on the stability of ascorbic acid. While ascorbic acid was rapidly destroyed in a sterile culture medium, only very slight destruction could be noticed when it was added into a culture of *L. acidophilus*, in which the bacterial growth had brought down the oxidation-reduction potential. In fresh milk with its high initial potential a rapid fall in the ascorbic acid content, and in the total vitamin C content as well, was shown to take place, until bacterial growth had brought the oxidation-reduction potential to a low level. In fermented milks, on the other hand, the decrease in the ascorbic acid content was only very slight. Dehydroascorbic acid when added to fermented milks was to a large extent reduced to ascorbic acid.

It is probable that the effect of the oxidation-reduction potential on the stability of ascorbic acid similar to that observed in milk and in fermented milk has importance also in other foodstuffs. Furthermore it is possible that the level of the oxidation-reduction potential of foods is significant also for the stability of other readily oxidizable food constituents, such as vitamin A, carotene, vitamin E, »essential fatty acids,» etc. — There are great differences in the oxidation-reduction potentials of different foodstuffs. The E_h values of certain fruits and vegetables, for example, are known to be widely differing (*cf.* Jørgensen [22]). On the other hand, it is well known that great variations occur in the ascorbic acid contents of different vegetable foodstuffs. It will be an interesting problem to investigate the stability of ascorbic acid and other easily oxidizable and reducible constituents in such foodstuffs in the preparation of which fermentation processes take place, as bread, butter, cheese, salted meat, sauerkraut, beer, etc.

From the practical point of view it would be important to know, first, the fate of »reduced» foodstuffs in the alimentary canal, and, second, how to choose and prepare such foodstuffs, the constituents of which are in a »reduced» state. The oxidation-reduction potentials of the different parts of the alimentary canal are to date very little known. In the mouth the conditions evidently are suitable for oxidations, due to the presence of air, but in the stomach the potential is probably already lower. In the intestines with vigorous bacterial growth strongly reducing conditions evidently prevail. Noticeable oxidation of food constituents could be expected to take place above all in the mouth and perhaps still in the stomach. As far as ascorbic acid is concerned it has been shown by Jenkins [23], however, that only small irreversible losses may occur during mastication. According to Wacholder [24], saliva,

gastric juice, and the digestive juice of the duodenum contain some protective agents which inhibit the oxidation of ascorbic acid and dehydroascorbic acid, until absorption takes place in the duodenum. As for ascorbic acid, at least, there thus seems to exist no great danger of destruction once the food has come into the mouth.

Treatment of foodstuffs in such a way as to avoid the oxidative processes as much as possible, is a problem on which an enormous number of investigations has been performed. Efforts have been made to find out methods by the use of which the losses of valuable food constituents as well as the development of objectionable flavors could be avoided. Important progress in this direction could be effected by an increased use of such food products as have natural reducing properties. Such products are, *e. g.*, fermented milks produced in appropriate ways. Ordinary milk stored in the cold often takes on, as result of oxidative processes, objectionable flavors which make the milk distasteful to the human palate. In a reducing environment the off flavors do not form readily (*cf.* Greenbank [25]); the lowering of the E_h can even remove slight flavors already developed. Because bacterial growth rapidly lowers the oxidation-reduction potential of milk, the flavors do not develop when milk is fermented. On the contrary, fermented milks often take on flavors which make them very pleasing to many people. Fermented milks are commonly used in Finland and the other Scandinavian countries and in many other countries as well, and they are known to be valuable therapeutic foodstuffs. In the light of newer knowledge an increased use of fermented milks can well be recommended. The explanation of the different aspects which have significance for the use of fermented milks in nutrition, requires, however, much research work, including nutrition experiments both with animals and human beings.

SUMMARY

The main part of the experiments described deal with the effect of the oxidation-reduction potential on the stability of ascorbic acid in milk and in fermented milks. Some experiments were made also with cultures of *Lactobacillus acidophilus* in an artificial medium.

Ascorbic acid was found to be very stable when it was added into a culture of *L. acidophilus*, where the bacterial growth had brought down the oxidation-reduction potential. In the sterile medium with its high potential, on the other hand, ascorbic acid was rapidly destroyed. The stability was not dependent upon the pH within the range from 4 to 7.

Acidifying of milk with lactic acid down to pH 6.0, 5.2, or 4.2, respectively, did not increase the stability of ascorbic acid. When fresh milk was stored at

room temperature its ascorbic acid content, as well as the total vitamin C content decreased at first rapidly. The fall ceased, however, after bacterial growth had abruptly lowered the oxidation-reduction potential. When milk was stored in cold (1—3° C) the potential remained high during several days and the ascorbic acid content fell continuously.

The oxidation-reduction potentials of milks inoculated with a starter, *L. acidophilus*, or *Str. lactis*, respectively, fell rapidly. The losses of ascorbic acid in these fermented milks during storage at room temperature were very small.

Dehydroascorbic acid, added to fermented milks was to a large extent reduced to ascorbic acid.

The effect of the oxidation-reduction potential of foods on the stability of ascorbic acid and other easily oxidizable constituents is discussed. Attention is paid also to the fate of »reduced» foodstuffs in the alimentary canal as well as to an increased use of such foods, *e. g.*, fermented milks, in nutrition.

REFERENCES

1. De Caro, L., and Giani, M. *Z. physiol. Chem.* **228** (1934) 13.
2. Mawson, C. A. *Biochem. J.* **29** (1935) 569.
3. Kellie, A. E., and Zilva, S. S. *Biochem. J.* **29** (1935) 1028.
4. Barron, E. S. G., Barron, A. G., and Klemperer, F. *J. Biol. Chem.* **116** (1936) 563.
5. Zilva, S. S. *Biochem. J.* **22** (1928) 779.
6. v. Hausen, S. *Ann. Acad. Sci. Fenn.*, Ser. A. Tom. XLVI (1936) no. 3.
7. Strohecker, R., Busse-Sundermann, A., and Buchholz, E. *Z. Untersuch. Lebensm.* **82** (1941) 113.
8. Wacholder, K. *Biochem. Z.* **312** (1942) 394.
9. Somogyi, J. C. *Z. Vitaminforsch.* **16** (1945) 134.
10. Kon, S. K., and Watson, M. B. *Biochem. J.* **30** (1936) 2273.
11. Hand, D. B., Guthrie, E. S., and Sharp, P. F. *Science* **87** (1938) 439.
12. Kroker, F. *Milchw. Forsch.* **19** (1938) 318.
13. Hand, D. B. *J. Dairy Sci.* **26** (1943) 7.
14. Esselen, W. B., Jr., and Fuller, J. E. *J. Bact.* **37** (1939) 501.
15. Esselen, W. B., Jr. *Food Research* **4** (1939) 329.
16. Tkachenko, E. S. *Biokhimiya* **1** (1936) 295. Ref. *C. A.* **31** (1937) 7461.
17. Harowitz-Wlasowa, L., and Buchman, N. *Voprosy Pitaniya* **7** (1938) 66. Ref. *Z. Untersuch. Lebensm.* **80** (1940) 110.
18. Lunde, G. *Vitamine in frischen und konservierten Nahrungsmitteln* 2nd ed., Berlin (1943).
19. Gunsalus, I. C., and Hand, D. B. *J. Biol. Chem.* **141** (1941) 853.
20. Garret, O. F., Arnold, R. B., and Hartman, G. H. *J. Dairy Sci.* **23** (1940) 47.
21. Stewart, A. P. Jr., and Sharp, P. F. *Ind. Eng. Chem. Anal. Ed.* **17** (1945) 373.
22. Jørgensen, H. *Redox-Maalinger* København (1945).
23. Jenkins, G. N. *Biochem. J.* **40** (1946) 415.
24. Wacholder, K. *Vitamine u. Hormone* **3** (1942) 1. Ref. *C. A.* **38** (1944) 6354.
25. Greenbank, G. R. *J. Dairy Sci.* **23** (1940) 725.

Received March 31, 1947.

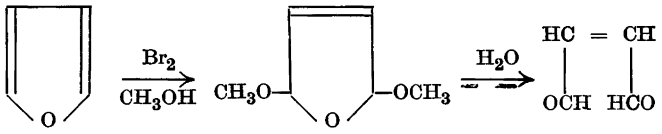
ACTA CHEMICA SCANDINAVICA 2 (1948) 109—115

The Alkoxylation of Simple Furans and Related Reactions

NIELS CLAUSON-KAAS, FRANZ LIMBORG and
JØRGEN FAKSTORP

Chemical Laboratory of the University, Copenhagen, Denmark

By the addition of alkoxy groups to the α-carbons of furans, stable 2,5-dialkoxy-2,5-dihydrofurans are obtained [1]. These addition products may be hydrolyzed under very mild conditions to the corresponding unsaturated 1,4-dicarbonyl compounds, which often are unstable and therefore difficult of accession. The procedure can be illustrated by the methoxylation of furan.



In this article we report some experiments concerning the general applicability of the alkoxylation method. In conclusion, other methods for the preparation of dihydrofurans by oxidation of furans are discussed.

ALKOXYLATION

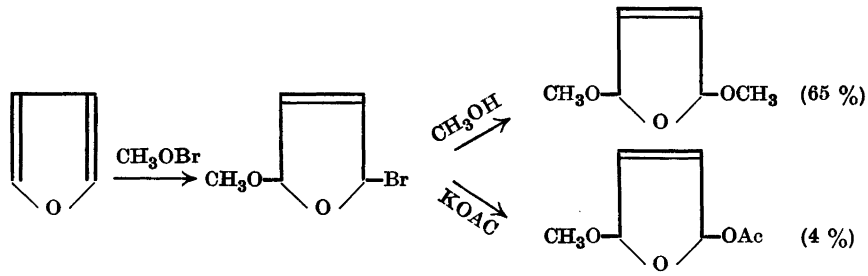
Alkoxylation of furans is accomplished with bromine in alkohol at low temperature in the presence of potassium acetate, which neutralizes the hydrogen bromide formed by the reaction. Furan [1], silvan [2], furfuryl alcohol [3], furfuryl acetate [2], furfural [3] and furfural diacetate [4] have been methoxylated by this or a similar method. Furan has also been ethoxylated in the same way [1].

We have tried to methoxylate some furans with electronegative substituents in the α-positions, *viz.* furoic acid, ethyl furoate, ethyl furylacrylate, acetylfuran and 2,5-dibromofuran. None of the compounds yielded the corresponding dimethoxy derivatives. It therefore seems fairly safe to assume,

that *α-substituted furans with substituents, which are known to decrease the reactivity of unsaturated systems towards the usual double bond reagents, as a rule do not yield addition products with bromine in methanol.* The only furan derivative, with an electronegative α-substituent, which has been methoxylated, is furfural [3]. But in this case dimethoxydihydrofurfural dimethyl-acetal was formed, so that probably acetalization or semi-acetalization has taken place prior to methoxylation.

At present nothing is known about the applicability of the alkoxylation method on β-substituted furans.

It has previously been observed that a small amount of a higher boiling compound is obtained by the preparation of dimethoxydihydrofuran. This has now been identified as 2-methoxy-5-acetoxy-2,5-dihydrofuran. The structure was proved by analysis, methoxy, acetoxy and molecular weight determination, and by hydrolysis to malealdehyde. The formation of methoxy-acetoxy-dihydrofuran together with dimethoxydihydrofuran may be explained as follows:



We have also investigated the alkoxylation of furan with higher alcohols, *viz.* isopropyl, *n*-butyl and isoamyl alcohol. The reaction mixture was not poured into a solution of calcium chloride, but sodium alcoholate was added to neutralize the acetic acid formed by the reaction, after which the mixture was distilled through a column. In this way the major part of the alcohols could be recovered. The acetals were formed in good yields. They are stable liquids, which can be distilled in vacuum. The structure was confirmed by hydrolysis to malealdehyde.

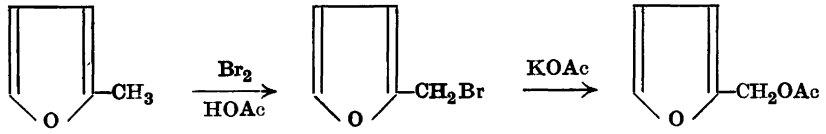
Dibutoxy- and diisoamyloxy-dihydrofuran have a pleasant odor, faintly resembling that of diisoamyl ether.

The molar refraction of the various dihydrofurans was determined, and a small negative exaltation of approximately the same size (0.4—0.6) was found for all compounds. This is in agreement with the proposed similarity of structure.

RELATED REACTIONS

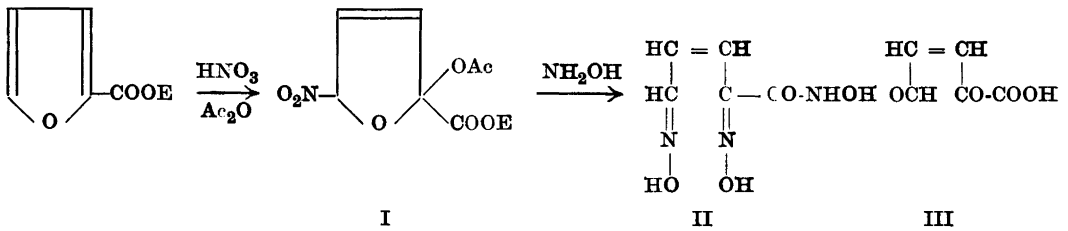
Acetoxylation. Addition of acetoxy groups to furan has been performed with lead tetraacetate, [1] and with bromine in acetic acid in the presence of potassium acetate [5]. No other furans have hitherto been acetoxylated.

It was to be expected that acetoxylation would be subject to the same limitation as alkoxylation. In agreement herewith, we were not able to add acetoxy groups to furoic acid or ethyl furoate by the above methods. But this is obviously not the only limitation of the methods, as silvan could not be acetoxylated with bromine in acetic acid either. The reaction product decomposed suddenly to a black mass when a distillation was attempted (at 1 mm). We believe that decomposition is due to the formation of furfuryl bromide. This suggestion was further strengthened by the isolation from the reaction mixture of a small amount of furfuryl acetate.



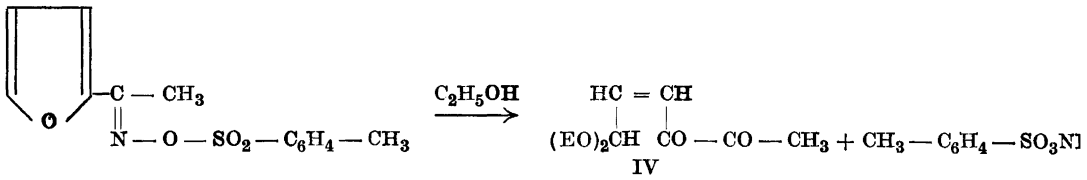
Buu-Hoï and Lecocq found, that silvan reacts with bromo succinimide in the same way [6].

Marquis' method. Marquis found [7], that a nitro and an acetoxy group are added to furan and ethyl furoate by the action of nitric acid in acetic anhydride. The procedure has later been extended to other furans. The resulting nitroacetates may in some cases be hydrolyzed to the corresponding unsaturated 1,4-dicarbonyl compounds, but these can, as a rule, be obtained much easier from dialkoxydihydrofurans, because the nitroacetates often are labile and difficult to hydrolyze. They also very easily split off acetic acid with the formation of an α-nitrofuran. However, Marquis' method is of importance for the preparation of unsaturated 1.4-dicarbonyl compounds from furans because *at present it is the only general way of obtaining 1,4-addition products of simple furans with an electronegative α-substituent. E. g.*, oxidation of ethyl furoate and addition of hydroxylamine to the nitroacetate (I) yields a derivative (II) of the acid III (Marquis [7]). This compound cannot be obtained by any other method.



I II III

Oxidation with hydroxylamine. It has recently been observed [8], that *p*-toluenesulphonyl 2-acetofuran-oxime reacts with alcohol to form the acetal IV and toluenesulphonic acidic ammonium. No details of the reaction mechanism are known, but the net result of the reaction is that hydroxylamine



IV

has oxidized acetofuran to the corresponding carbonyl compound. It is possible that the method is of general applicability for the oxidation of furans with a carbonyl group in the α-position.

EXPERIMENTAL

2,5-Dimethoxy-2,5-dihydrofuran and 2-methoxy-5-acetoxy-2,5-dihydrofuran

36 ml of freshly distilled furan (0.5 mole) and 100 g of anhydrous potassium acetate (1 mole) are dissolved in 600 ml of methanol. The mixture is cooled in an ice-salt bath and a solution of 25 ml of bromine in 500 ml of methanol is added under efficient stirring. The temperature must be kept below —7°. After all the bromine has been added (1 hour) stirring is continued for 5—10 minutes. Then the reaction mixture is poured into 1500 ml of a cooled, saturated, solution of calcium chloride. The acetal is extracted with 2000 ml of ether, and the etheral layer shaken with 250 ml of a saturated solution of potassium carbonate and dried over 100 + 500 g of solid potassium carbonate. The ether is removed on the steam bath through a Vigreux column and the residue distilled in vacuum. Distillation begins at 54°/13 mm and the fraction boiling at 54—56° is collected. It consists of dimethoxydihydrofuran, yield 42 g = 65 %. Distillation is continued, whereby about 4 g of higher boiling material is obtained (b. p. 56—106°). Redistillation of this fraction yields 3.2 g of a colourless liquid (b.p.$_{13}$ 96—99°) which is analytically pure methoxy-acetoxy-dihydrofuran. The compound turns yellow on prolonged standing but is otherwise stable.

$C_7H_{10}O_4$	Calc.	C	53.18	H	6.38	CH_3O	19.6	CH_3COO	37.4	Mol. wt.	158.2
	Found	»	53.63	»	6.22	»	19.5	»	35.9	» »	176

We have tried to carry out the synthesis of dimethoxydihydrofuran by adding sodium methoxide to the reaction mixture as described for the preparation of higher alkoxy derivatives, and also by substituting potassium acetate by pyridine. In both cases good yields of dimethoxydihydrofuran were obtained, but the method reported above was found to be more convenient.

Hydrolysis of methoxy-acetoxy-dihydrofuran; identification of malealdehyde. 189 mg of methoxy-acetoxy-dihydrofuran were boiled with 3 ml of 0.01 N hydrochloric acid and 2 ml of alcohol for one minute. After cooling, a solution of 0.3 ml of phenylhydrazine in diluted acetic acid (10 ml) was added and the bis-phenyl hydrazone of malealdehyde isolated in the usual manner [1]. Yield of crude product 209 mg = 66 %. Yield after recrystallization from acetone-benzene 151 mg = 48 %. M. p. 168—69° (Kofler stage, corr.), previously found [9] 171°.

$C_{16}H_{16}N_4$ (264) Calc. N 21.21 Found N 21.13

Higher dialkoxy-dihydrofurans

7.25 ml of furan (0.1 mole) and 20 g of potassium acetate (0.2 mole) are dissolved, or suspended, in 130 ml of the alcohol, (technical grade) and 5 ml of bromine in 100 ml of the alcohol are added at —7° during 20—30 minutes, as described above. The reaction mixture is heated to room temperature and allowed to stand for 30 minutes. Then a solution of sodium alkoxide from 4.60 g of sodium (0.2 mole) is added at 0° under stirring and efficient cooling. After 15 minutes 300 ml of ether are added, and the precipitated potassium bromide and sodium acetate filtered off and washed with 50 ml of ether. The ether is removed on the steam bath, and the residue distilled in vacuum through a Widmer column. Hereby the major part of the alcohol is recovered and may be employed in a subsequent run. The remaining acetal is distilled in vacuum without the column. It is sometimes necessary to remove traces of sodium acetate prior to distillation by renewed precipitation with ether.

Di-n-butoxy-dihydrofuran. B. $p._{15}$ 127—128°, yield 16.0 g = 75 %.

$C_{12}H_{22}O_3$	Calc. C 67.26	H 10.35	Mol. wt.	214.3
	Found » 66.59	» 10.17	» »	190

188 mg of dibutoxy-dihydrofuran were boiled with 2 ml of 0.01 N sulfuric acid and 2 ml of alcohol for 2 minutes, and the bis-phenylhydrazone of malealdehyde prepared in the usual manner. Yield of crude product 164 mg = 71 %. Yield after recrystallization 94 mg = 41 %, m. p. 170—172°.

$C_{16}H_{16}N_4$ Calc. N 21.21 Found N 21.34

Diisoamyloxy-dihydrofuran. B. $p._{0.6}$ 101—103°, yield 15.5 g = 64 %.

$C_{14}H_{26}O_3$	Calc. C 69.39	H 10.82	Mol. wt.	242.3
	Found » 69.53	» 10.54	» »	230

169 mg of diisoamyloxy-dihydrofuran were boiled with 1 ml of N sulfuric acid and 1 ml of alcohol for one minute and the bis-phenylhydrazone of malealdehyde prepared in the usual manner. Yield of crude product 114 mg = 62 %. Yield after recrystallization 56 mg = 31 %, m. p. 169—170°.

$C_{16}H_{16}N_4$ (264) Calc. N 21.21 Found N 21.32

Diisopropoxy-dihydrofuran. In the preparation of this acetal, it was necessary to add the bromine in a solution of carbon tetrachloride because of the ease with which isopropyl alcohol is oxidized by bromine. Furthermore, the acetic acid was removed by shaking the reaction mixture with 300 ml of ether and two portions of potassium carbonate (each 50 g) for 10 and 30 minutes, respectively. B. $p._{15}$ 85—90°, yield 13.0 g = 70 %.

$C_{10}H_{18}O_4$	Calc.	C 64.51	H 9.74	Mol. wt. 186.2
	Found	» 64.98	» 9.73	» » 188

Diisopropoxy-dihydrofuran turns yellow after some days. This may, however, be due to a small amount of an impurity.

209 mg of the acetal were hydrolyzed as described for the butoxy derivative. Yield of crude malealdehyde-bisphenylhydrazone 150 mg = 51 %. Yield after recrystallization 117 mg = 40 %, m. p. 169—170°.

$C_{16}H_{16}N_4$ (264) Calc. N 21.21 Found N 21.25

Molar refractions of dialkoxydihydrofurans

2 ml of the dialkoxydihydrofuran were redistilled carefully at 12 mm. The middle fraction (about 1 ml) was collected and employed for the determination of the density, and the refractive index. The results are tabulated below.

Dihydrofuran	B.$p._{12}$	d_4^{20}	n_D^{20}	M_D	M_D calc.	Δ
Dimethoxy-	51°	1.0713	1.4328	31.56	32.17	— 0.61
Diethoxy-	71— 73°	1.0019	1.4309	40.86	41.41	— 0.55
Diisopropoxy-	83— 84°	0.9539	1.4284	50.27	50.64	— 0.37
Di-*n*-butoxy-	118—120°	0.9436	1.4371	59.51	59.88	— 0.37
Diisoamyloxy-	130—131°	0.9267	1.4380	68.67	69.11	— 0.44

SUMMARY

The general applicability of the alkoxylation method in the furan series has been investigated. 2,5-Diisopropoxy-, 2,5-di-*n*-butoxy- and 2,5-diisoamyloxy-2,5-dihydrofuran were prepared. 2-methoxy-5-acetoxy-2,5-dihydrofuran was isolated as a by-product from the methoxylation of furan. Other methods for the preparation of dihydrofurans by oxidation of furans have been discussed.

We are indebted to Mr. O. Rosenlund Hansen and Miss Muriel Schmidt for the microanalyses.

During the investigation we have received financial aid from *Det teknisk-videnskabelige Forskningsraad* (N. Clauson-Kaas and J. Fakstorp) and from *Kemisk Værk Køge A/S*, Copenhagen (F. Limborg).

REFERENCES

1. Clauson-Kaas, N. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* 24 (1947) (6).
2. Clauson-Kaas, N., and Limborg, F. *Acta Chem. Scand.* **1** (1947) 619.
3. Meinel, K. *Ann.* **516** (1935) 231.
4. Clauson-Kaas, N., and Fakstorp, J. *Acta Chem. Scand.* **1** (1947) 415.
5. Clauson-Kaas, N. *Acta Chem. Scand.* **1** (1947) 379.
6. Buu-Hoï, Ng., and Lecocq, J. *Compt. rend.* **222** (1946) 1441.
7. Marquis, R. *Ann. chim.* (8) **4** (1905) 196.
8. Vargha, L., Ramonczai, J. and Bite, P. *Nature* **159** (1947) 744.
9. Clauson-Kaas, N., and Fakstorp, J. *Acta Chem. Scand.* **1** (1947) 210.

Received January 9, 1948.

ACTA CHEMICA SCANDINAVICA **2** (1948) 116—126

Can Ionic Concentrations in Diethyl Ether be Determined by Emf Measurements?

ULLA BERGLUND and LARS GUNNAR SILLÉN

Institute of Inorganic and Physical Chemistry, University of Stockholm, Stockholm, Sweden

The present investigation was started in order to find methods for studying the equilibria in solutions of organomagnesium compounds RMgX (R=alkyl or aryl radical, X = halogen) in diethyl ether, so-called Grignard solutions. As is well known, such solutions have found extensive use in preparative organic chemistry.

It was shown by Kondyrew [1,2] that Grignard solutions are conductors of electricity and thus contain ions; Kondyrew measured their conductivity at different concentrations. Evans and various co-workers (see *e. g.* Evans and Pearson [3]) have studied also the changes in concentrations and the electrode products during electrolysis and have tried to explain their results by assuming the solutions to contain ions such as RMg^+, $RMgX_2^-$, and $R_2MgX_2^{2-}$.

Schlenk and Schlenk [4] have tried to measure the equilibria $2\ RMgX \rightleftharpoons MgX_2 + MgR_2$ by precipitating MgX_2 and RMgX with dioxane. However, certain sources of error in this method have been pointed out by Probert [5] and by Noller and White [6] (*cf.* Noller and Raney [7]).

It was (and is) our intention to study the Grignard solutions by the same methods that have proved so useful in investigating complex formation in aqueous solutions, namely by measuring the free concentration of individual ionic species by emf methods. However, as far as we know, nobody has ever before been able to measure ionic concentrations by emf methods in a solvent with such a low dielectric constant as diethyl ether, and we were quite aware that our task would be a difficult one.

In an *aqueous* solution, the concentration of an ionic species such as Ag^+ can be measured comparatively easily by means of concentration cells of the type

$$-\ Ag|Ag^+\ (c_1)\ ||\ Ag^+\ (c_2)|Ag\ +$$

($\|$ = salt bridge for eliminating the liquid junction potential, E_d). The emf E of such a cell is given by Nernst's equation in its modern form

$$E\ (-E_d) = RTF^{-1}\ln\left\{Ag^+\right\}_2\left\{Ag^+\right\}_1^{-1} = RTF^{-1}\ln\frac{f_2c_2}{f_1c_1}$$

If one works with dilute solutions, the activity factors f_1 and f_2 can be estimated with the aid of some approximative formula, and then, if c_2 is known, c_1 can be calculated from the measured emf. It is, of course, of no importance whether the potential difference between the two electrodes concerned is measured directly in a single cell or if they are both measured against the same reference electrode. — In an analogous way, the concentration of Cl^- can be determined by means of Ag, AgCl electrodes.

The higher the ionic strength of a solution, the less reliable are the approximate formulae for activity factors, especially if polyvalent ions are considered. For this reason it is in many cases advantageous to use a device first proposed by Bodländer (according to Grossmann [8]). To both the solutions to be compared a *neutral salt, e. g.* KNO_3 or $NaClO_4$, is added in such amounts as to make the ionic strengths of the solutions equal and rather large. In this way the influence of the liquid junction potential is made negligible, and, above all, the activity factors f_1 and f_2 are made equal so that we can use the simple form of Nernst's formula

$$E = RTF^{-1}\ln\frac{c_2}{c_1}$$

In *diethyl ether*, the dielectric constant is only 4.4 as compared with about 80 in water. For this reason the interionic forces are much stronger than in aqueous solutions, the ions aggregate in clusters, the activity factors decrease very rapidly with increasing concentration, and no approximate formulae for the activity factors of individual ionic species can be used in the range of concentration suitable for practical work (*cf.* McInnes [9] and Kortüm [10]).

Thus it would certainly not be possible to calculate the ionic concentrations of *e. g.* Mg^{2+} and Br^- from the emfs that might be obtained using cells with pure Grignard solutions. However, we decided to try whether it would be possible to fix the activity factors by adding a high concentration of a suitable neutral salt. A salt that seemed promising is *lithium perchlorate*, since Willard and Smith [11] have shown that the solubility of anhydrous $LiClO_4$ is as high as 53.21 % by weight in diethyl ether at 25° C (whereas that of $LiClO_4(H_2O)_3$ is only 0.196 %).

The preparation of anhydrous $LiClO_4$ involved considerable difficulties which were overcome as described below. Preliminary experiments seemed to show that $LiClO_4$ in ether solution does not react with Mg metal or Mg amalgam; at least no Cl^- could be detected in the solution after prolonged contact. Solutions of C_2H_5MgBr and $LiClO_4$ did not seem to react on mixing. Thus there seemed to be no difficulties arising from unwanted reactions.

MATERIALS

The *diethyl ether* used has been distilled over Na metal and is stored in contact with Na.

The *lithium perchlorate* is prepared in the 1 liter Pyrex bulb shown to the left in Fig. 1. First, solid lithium carbonate, *pro analysi* or purest available make, is neutralized with a slight excess of 70 % perchloric acid *pro analysi*. The bulb is then brought into a large cylindrical electric oven which is heated to 140° C. By applying a water pump to the tube at the extreme left of Fig. 1 (this tube is heated by an electric coil in order to prevent the condensation of water), a current of dry air filtered through cotton is passed through the $LiClO_4$ solution.

The temperature of the oven is slowly increased to 200° C and kept there till the volume of the liquid has decreased to one third of the original volume. Then the solution is allowed to cool to about 140° C, the left three-way stopcock in Fig. 1 is turned, and distilled water is pressed into the bulb until the volume is two-thirds of the original one. The current of air is again turned on, and the temperature of the oven is slowly increased to 300° C and kept there for about 12 hours (the addition of water is made in order to remove remaining traces of $HClO_4$).

After the temperature has attained 300° C, the right three-way stopcock in Fig. 1 is turned so that the air is dried. As is seen from the simplified Fig. 1, the air is first filtered through cotton for removing particles of dust, then passed through a washing bottle with 40 % NaOH, two washing bottles both containing 50 % H_3PO_4 saturated with $KMnO_4$, a desiccating tower with NaOH pellets and one with P_2O_5 on glass wool, a U-tube with $Mg(ClO_4)_2$ and one with blue gel and cotton. This device for filtering the air, which is essential for obtaining a pure product, is principally the same as that described by Richards and Cox [12].

On cooling, the contents of the bulb solidifies; the solid can be assumed to consist of anhydrous $LiClO_4$ with a certain admixture of $LiClO_4(H_2O)_3$. The anhydrous salt is extracted by reflux boiling with ether on a water bath for 12 hours.

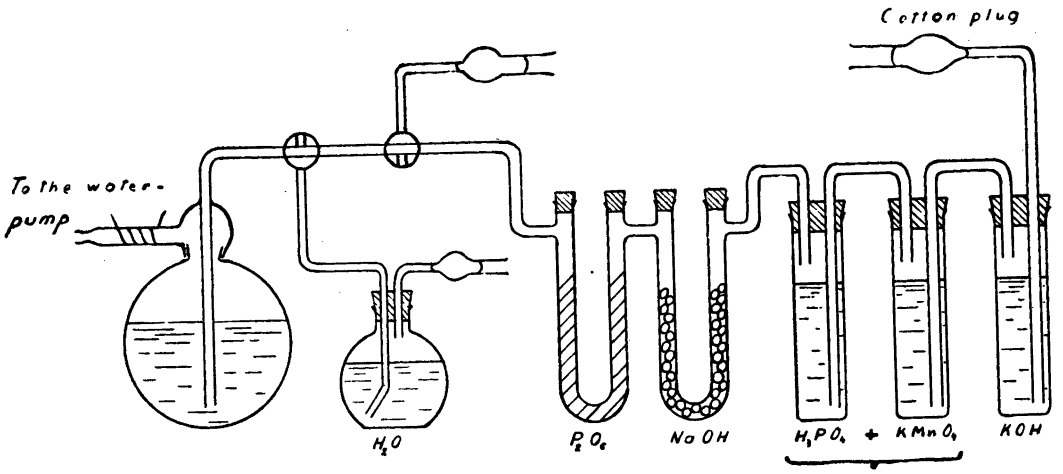


Fig. 1. Apparatus for preparing anhydrous $LiClO_4$. The picture is very much simplified in comparison with the present large-scale apparatus. Instead of test tubes, washing bottles are used for KOH and $H_3PO_4 + KMnO_4$, and instead of U-tubes, desiccating towers are used for P_2O_5 and NaOH. The U-tubes with $Mg(ClO_4)_2$ and blue gel have been left out.

The ether solution, which contains about 35 % $LiClO_4$, is used as stock solution. In our first experiments, ether was usually driven off till crystals of $LiClO_4$ appeared. (The boiling point of the saturated solution was found to be about 72° C.) In this way solutions containing 50—52 % $LiClO_4$ were obtained, which agrees well with 53.21 % given as the solubility at 25° C by Willard and Smith [11].

Lithium bromide, Merck *pro analysi*, was dried at 200° C and extracted with ether in the same apparatus as $LiClO_4$.

Magnesium perchlorate, Merck *pro analysi*, was dried just below the melting point of anhydrous $Mg(ClO_4)_2$ at 250° C, and extracted with ether in the same way as $LiClO_4$. In this way solutions were obtained containing as much as 20—25 % $Mg(ClO_4)_2$, whereas Willard and Smith [11] give the solubility at 25° C as 0.29 %.

Magnesium was available in the form of rods (Merck *pro analysi*), ribbon (pro analysi), granules (for Grignard syntheses) and powder (purum).

The emfs were measured with a Radiometer valve potentiometer with an accuracy of about $\pm$ 0.1 mV.

Liquid *magnesium amalgam* (with about 0.3 % Mg, *cf.* Hansen [13]) is prepared by bringing Mg powder and Hg into a glass tube, provided with stop-cocks at both ends, evacuating, and keeping the tube in an oven at 110° C

for 12 hours. The liquid amalgam is filtered through glass wool, still in vacuum, in order to remove solid amalgam (Hg_2Mg [13]) and traces of MgO. The use of stopcocks is possible thanks to Dow-Corning silicone stopcock grease, which maintains the vacuum at 110° C without making the stopcock immobile at room temperature. Otherwise the method is similar to that used by Sillén and Liljeqvist [14] for preparing zinc amalgam.

We tried first to use the same method as used by Granér and Sillén [15] for preparing bismuth amalgam, namely heating the metal-Hg mixture to 110° C under a cover of paraffin oil (alphachloronaphtalene was also tried). In this way also, pure magnesium amalgam was obtained, but between the amalgam and the oil there was a disagreeable black film, which may have consisted of MgO,Hg, and decomposition products of the paraffin oil. For this reason the vacuum method was preferred.

The *nitrogen* gas used was made free from oxygen by passing it over activated copper (Meyer and Ronge [16]), and dried by bubbling through a glass filter in conc. H_2SO_4. For most purposes it was afterwards bubbled through diethyl ether or through a suitable solution of $LiClO_4$ in ether in order to give it a certain partial pressure of ether vapour.

APPARATUS

The cell is shown schematically in Fig. 2, for the case with Ag,AgBr electrodes; with amalgam electrodes the same glass vessels were used, with slight modifications. The counter electrode vessel (left) is connected to the titration vessel (right) by means of a salt bridge. As is seen from the figure, the five necks of the titration vessel (a 200 ml Pyrex bulb) are provided with an electrode, a burette, the end of the salt bridge, a stirrer with a Hg lock, and a thermometer.

In order to lessen the diffusion, which is much more prominent in ether solution than in water, the end of the salt bridge is drawn out to a bent tip. In the salt bridge there is also a stopcock with a Hg lock; this stopcock is kept open only for the short intervals of time when the emf is being measured.

The stopcocks are greased with silicone, which does not react with and dissolves only slightly in the solutions.

The cell is immersed in a paraffin oil thermostat at 25.0 ± 0.1° C. The resistance of the cell is usually 0.5—1.0 MΩ.

PROCEDURE

It is important that the solutions do not change their concentrations by evaporation or by taking up moisture from the air; in the latter case $LiClO_4(H_2O)_3$ precipitates.

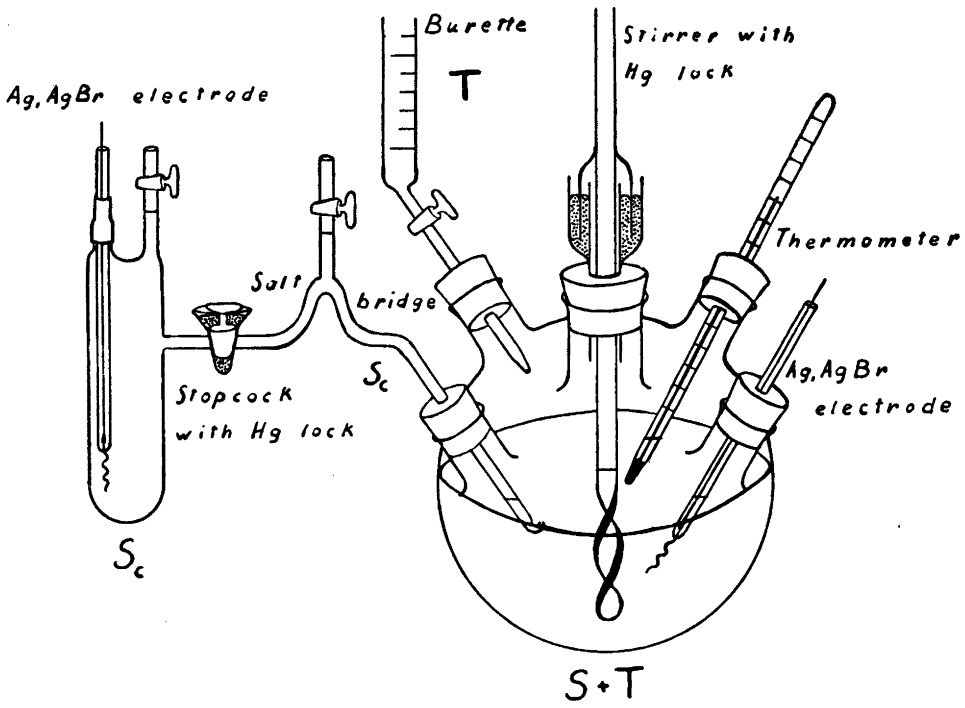


Fig. 2. Cell for emf measurements (schematic).

The solutions were always transferred from one vessel to another by pressure, using dry nitrogen gas, saturated with ether vapour. All air inlets of the different containers were provided with tubes with anhydrous $Mg(ClO_4)_2$.

Stock solutions were prepared of $LiClO_4$, LiBr, and $Mg(ClO_4)_2$ in ether. The stock solutions were placed into burettes, and calculated amounts of them were introduced into a measuring flask, which was filled to the mark with pure ether (a considerable volume contraction was observed).

About two hours before the beginning of a titration, the solutions were transferred to bubbling tubes (Sillén [17], Fig. 2) where a slow current of nitrogen, saturated with ether vapour, was passed through them for about 90 minutes in order to remove traces of dissolved oxygen. The change of volume during this operation proved to be negligible.

The solution was then introduced into a burette and from this to the electrode vessels. All vessels and tubes in the path of the solution had previously been rinsed free from air with nitrogen gas.

The volume of the original solution, S, in the titration vessel was measured accurately; it was usually made to be 100.0 ml. The counter electrode vessel and the salt bridge were filled with another solution, S_c, and placed in position. A third solution, T, was added from the burette in Fig. 1 during the titration.

Of course the fire and explosion hazards were considered. Peroxide tests with benzidine on the ether solutions have hitherto given negative results, even after storing for a long time. If intentionally ignited the concentrated solution of $LiClO_4$ in ether burns rapidly with a beautiful red flame, but does not detonate. It was possible to make the solution detonate by applying a primer, but the detonation was rather a weak one.

ANALYSIS

For determining *lithium perchlorate*, 10—15 ml water was added to a known volume, usually 3.00 ml, of the stock solution. The sample was evaporated to dryness, first on a water bath and then over a small flame. Water and excess of H_2SO_4 was added. The sample was again evaporated to dryness, ignited to dull red heat, and weighed as Li_2SO_4.

Lithium bromide was determined by Volhard titration, after adding excess of water to a known volume of ether solution. The ether was usually evaporated before titrating. — For control, the Br^- analysis was applied to the solutions S_c, S and T as well as to the stock solution.

Magnesium perchlorate was transformed to $MgSO_4$ by the method described for $LiClO_4$. The sulfate was ignited to 600° C and weighed.

EXPERIMENTS

The first cells studied were of the type

$$Mg_xHg|Mg(ClO_4)_2(c_1),LiClO_4(2\ C)|Mg(ClO_4)_2(c_2),LiClO_4\ (2\ C)|Mg_xHg$$

The stirrers were made to serve as electrodes also. A stirrer consisted of a long glass tube, the lower end of which was sealed and pierced by five Pt needles, which dipped into the pool of Mg amalgam on the bottom of the titration vessel. Electrical contact between the Mg amalgam and the potentiometer was obtained by filling the stirrer tube with mercury and dipping into it a copper wire connected with the potentiometer. Sometimes contact was also obtained by means of a capillary filled with mercury which was dipped into the amalgam. When the stirrer was rotated, the thin film of

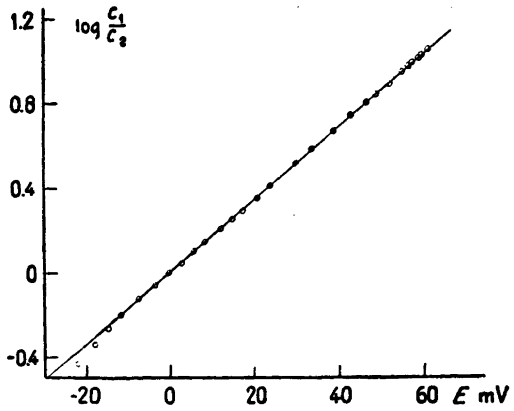


Fig. 3. Plot of E_{obs} against log (c_1/c_2) for cells + Ag,AgBr/LiBr (c_2)/LiBr (c_1)/AgBr, Ag —. Values for three different titrations are given (Tab. 3): those of the first and third titration are given as circles, those of the second as dots. Straight line: calculated E according to Nernst's equation $E_{calc} = 59.16 \log(c_1/c_2)$.

oxide that tends to form on the amalgam surface was removed, and a clean surface was exposed.

With cells of this type it proved difficult to obtain reproducible emfs, and in any case it was very inconvenient to have a stirrer in the counter electrode vessel. It was decided to try to find another type of electrode that would be more easily handled, to study the behaviour of this electrode, and then to study cells with a Mg or Mg amalgam electrode in the titration vessel and with the new electrode as counter electrode.

We prepared a cell Hg,Hg_2Cl_2|LiCl in ether|AgCl,Ag, which proved after a few hours to give a steady emf. Thus we concluded that both the calomel and the Ag,AgCl electrode can be employed in ether solutions. Since we intended to work with bromides we decided to try the Ag,AgBr electrode.

A number of Ag,AgBr electrodes were prepared by the same method as that given for Ag,AgCl electrodes by Brown [18]. After washing, the water was removed by prolonged soaking, first in ethanol and then in ether.

These electrodes were immersed in ether solutions of LiBr and proved to acquire a steady potential very quickly. When inserted in the same solution they gave same potential within the limits of error.

The following cell was studied

$$+ \text{Ag,AgBr}|\text{LiBr}(c_2)|\text{LiBr}(c_1)|\text{Ag,AgBr} -$$

where both solutions contained 2 C $LiClO_4$. The right solution, in the titration vessel, was obtained by adding v ml of T to (usually 100 ml of) S. The left solution, S_c, in the counter electrode vessel, had a constant concentration c_2.

Table 1 and Fig. 3 give the measured emfs and those calculated by means of Nernst's formula

$$E_{calc} = RTF^{-1} \ln \frac{c_1}{c_2} = 59{,}16 \log \frac{c_1}{c_2} \text{ mV}$$

The agreement is seen to be rather good, even at the highest c_1 used, 25 mC. At the very lowest c_1, about 1 mC, small deviations are observed, which may have been caused by diffusion from S_c.

DISCUSSION

The very high solubility of anhydrous $LiClO_4$ and $Mg(ClO_4)_2$ in ether is remarkable. One might imagine that the small cations Li^+ and Mg^{2+} surround themselves with a cover of $(C_2H_5)_2O$ dipoles, which should greatly lessen the electrostatic attraction between the ions. This view, however, cannot apply to the saturated solution of $LiClO_4$, where there is only 1.26 $(C_2H_5)_2O$ for every Li^+. In this case some sort of cluster or lattice formation seems probable, which is also indicated by the high viscosity. It is also remarkable that small quantities of water added to these solutions are immediately removed by the precipitation of $LiClO_4(H_2O)_3$.

The experiments seem to prove that Nernst's equation applies to an ionic species such as Br^-, added in small amounts to a concentrated solution of $LiClO_4$ in ether. Thus the activity coefficient is not appreciably changed by small shifts in the Br^- concentration. This indicates that the external forces acting on a Br^- ion, which must be imagined as taking part in incessantly changing clusters of Li^+, ClO_4^- and $(C_2H_5)_2O$, are determined chiefly by the large concentration of $LiClO_4$.

SUMMARY

In cells of the type—Ag,AgBr|LiBr(c_1) in ether|LiBr(c_2) in ether|AgBr, Ag +, the emf obeys Nernst's law: $E = RTF^{-1}\ln(c_1c_2^{-1})$, if both solutions contain large and equal concentrations of $LiClO_4$. By adding $LiClO_4$ it will thus be possible to measure ionic concentrations in diethyl ether. This principle will be used in an investigation of so-called Grignard solutions.

The solubility of anhydrous $Mg(ClO_4)_2$ in ether is much greater than has hitherto been stated.

Table 1. Titrations with cells + Ag,AgBr/c_2 mC LiBr/c_1 mC LiBr/AgBr, Ag —.

a) S = 2 C $LiClO_4$ (100.0 ml)
T = 2 C $LiClO_4$, 25.4_2 mC LiBr (v ml)
S_c = 2 C $LiClO_4$, 2.65 mC LiBr ($= c_2$)

v ml	c_1 mC	$\log \frac{c_1}{c_2}$	E_{calc} mV	E_{obs} mV
4.0	0.978	— 0.431	— 25.5	— 22.2
5.0	1.213	— 0.339	— 20.1	— 18.0
6.0	1.442	— 0.264	— 15.6	— 14.8
7.0	1.666	— 0.202	— 11.9	— 10.8
8.5	1.995	— 0.123	— 7.3	— 7.5
10.0	2.315	— 0.059	— 3.5	— 3.5
11.5	2.627	— 0.004	— 0.2	+ 0.2
13.0	2.93	+ 0.044	+ 2.6	3.0
15.0	3.32	0.098	5.8	5.9
17.0	3.70	0.145	8.6	8.4
20.0	4.24	0.204	12.1	12.3
22.5	4.68	0.247	14.6	15.1
25.0	5.09	0.284	16.8	17.5
30.0	5.86	0.345	20.4	21.0
35.0	6.60	0.396	23.5	24.0

b) S = 2 C $LiClO_4$, 2.65 mC LiBr (92.2 ml)
T = 2 C $LiClO_4$, 98.7 mC LiBr (v ml)
S_c = 2 C $LiClO_4$, 2.65 mC LiBr ($= c_2$)

v ml	c_1 mC	$\log \frac{c_1}{c_2}$	E_{calc} mV	E_{obs} mV
4.0	6.64	0.399	23.6	23.8
6.0	8.51	0.507	30.0	30.0
7.5	9.87	0.571	33.8	33.8
10.0	12.05	0.658	38.9	38.9
12.6	14.20	0.729	43.1	42.9
15.0	16.09	0.782	46.3	46.0

c) S = 2 C $LiClO_4$, 14.10 mC LiBr (100.0 ml)
T = 2 C $LiClO_4$, 47.47 mC LiBr (v ml)
S_c = 2 C $LiClO_4$, 2.30 mC LiBr ($= c_2$)

v ml	c_1 mC	$\log \frac{c_1}{c_2}$	E_{calc} mV	E_{obs} mV
4.0	15.39	0.825	48.8	49.0
10.0	17.14	0.872	51.6	52.1
15.0	18.45	0.904	53.5	53.5
20.0	19.66	0.932	55.1	55.0
25.0	20.76	0.956	56.5	56.5
30.0	21.81	0.977	57.8	57.4
35.0	22.75	0.995	58.9	59.0
40.0	23.63	1.012	59.9	59.6
49.0	25.07	1.037	61.4	61.2

This work has been supported by a grant from *Statens Naturvetenskapliga Forskningsråd*. We wish to thank Professor Karl Myrbäck for the interest he has shown in this investigation and for aiding us in many ways. Mr Lars Evers has devoted a large amount of skilful work on constructing and operating the apparatus for preparing large amounts of the inorganic salts needed. Mr Erik Ekedahl and Miss Karin Solders have given us valuable aid. This work will be continued by Miss Karin Solders.

REFERENCES

1. Kondyrew, N. W., and Manojew, D. P. *Ber.* **58** (1925) 464.
2. Kondyrew, N. W., and Ssusi, A. K. *Ber.* **62** (1929) 1856.
3. Evans, W. V., and Pearson, R. *J. Am. Chem. Soc.* **64** (1942) 2865.
4. Schlenk, W., and Schlenk, W. jr. *Ber.* **62** (1929) 920.
5. Probert, Miss. Master's Thesis, Stanford University (1933) (quoted by 6).
6. Noller, C. R., and White, W. R. *J. Am. Chem. Soc.* **59** (1937) 1354.
7. Noller, C. R., and Raney, D. C. *J. Am. Chem. Soc.* **62** (1940) 1749.
8. Grossmann, H. *Z. anorg. Chem.* **43** (1905) 362.
9. McInnes, D. A. *The Principles of Electrochemistry* New York (1939) chapter 19.
10. Kortüm, G. *Elektrolytlösungen* Leipzig (1941) 251—263.
11. Willard, H. H., and Smith, G. F. *J. Am. Chem. Soc.* **45** (1923) 286.
12. Richards, Th. W., and Cox, M. W. *J. Am. Chem. Soc.* **36** (1914) 819.
13. Hansen, M. *Der Aufbau der Zweistofflegierungen* Berlin (1936) 786—790.
14. Sillén, L. G., and Liljeqvist, B. *Svensk Kem. Tid.* **56** (1944) 85.
15. Granér, F., and Sillén, L. G. *Acta Chem. Scand.* **1** (1948) 631.
16. Meyer, F. R., and Ronge, G. *Z. angew. Chem.* **52** (1939) 637.
17. Sillén, L. G. *Svensk Kem. Tid.* **58** (1946) 52.
18. Brown, U. S. *J. Am. Chem. Soc.* **56** (1934) 646.

Received January 13, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 127—139

On the Formation of Amino Acids and Amides in Green Plants

NIILO RAUTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The synthesis of amino acids and proteins in green plants during the assimilation of different nitrogen compounds forms one of the most important links in the chain of the nitrogen cycle in nature. This phenomenon is very slow under normal conditions owing to many limiting factors. In experimental conditions we have, however, the opportunity of eliminating these limiting factors and arranging the experiments so that all processes in cells occur very intensively. We can then follow with chemical analyses the formation and transformation of different intermediate compounds. In this connection, however, it must be emphasized, that obtaining a detailed picture of the mechanism of these processes demands that the experimental time even in plant experiments must be very short, only some hours.

The work reported here was undertaken to investigate more intimately and quantitatively the mechanism and different phases of amino acid synthesis in green plants.

One of the central problems in the study of nitrogen assimilation in the living cells in general is the formation of the primary amino acids. The investigations of recent years emphasize the primary and central position of aminodicarboxylic acids, glutamic and aspartic acids, in this process. In this work particular attention thus has been paid to these amino acids and also to alanine which can easily be formed from the aminodicarboxylic acids, for example through the transamination reaction.

EXPERIMENTAL

All experiments were carried out with pea plant *(Pisum sativum sp.)*. This because of its fairly rapid growth and lively nitrogen metabolism. Moreover, it can develope inoculated with the specific nodule organisms without any combined nitrogen. It is

therefore possible to obtain plant material which does not contain a disturbing amount of inorganic (ammonia or nitrate) nitrogen. For practical reasons all the plant material was grown in the greenhouse in water culture inoculated with an effective *Rhizobium* strain. After a growth period varying from 4 to 5 weeks when the plants were in a very high degree of metabolic activity they were harvested for experiments. For this purpose they were cut just above the seed and the tops were placed in glass beakers containing a neutral aqueous solution of the nitrogen compound to be tested. The beakers were placed in a very intensive electric light. The suction due to transpiration caused a rapid rise of liquid into the plants and the transformation of the nitrogen taken up began immediately. During the experiment the chemical changes occurring as a result of the nitrogen uptake were followed at intervals of some hours with analyses. The whole experimental time was at the most 24 hours. Signs of wilting were observed only by feeding with ammonium salt. Thus it seems probable that the synthetical processes will take place as do the corresponding processes in the normal intact plant.

Analytical methods

For the analyses the cell sap was expressed from an equal number of plants at different points of the experiment. When *ammonia and the organic nitrogen fractions* were analysed the sap was deproteinized with trichloracetic acid. When again *nitrate* and *nitrite* were analysed lead acetate was used.

The analyses of the different nitrogen fractions were performed by the following methods. *Total organic nitrogen* was determined by the Kjeldahl micro method. If there was ammonia present the amount of ammonia nitrogen was subtracted from the Kjeldahl nitrogen. *Amino nitrogen* was determined after amide hydrolysis and removal of ammonia by the van Slyke volumetric method. *Ammonia nitrogen* was distilled according to Pucher *et al* [1]. *Total amide nitrogen* was likevise determined according to Pucher *et al.* *Asparagine* and *glutamine* were differentiated according to Schwab [2]. *Total nitrogen of amides* was calculated by multiplication of the corresponding amido nitrogen by two. *Aminodicarboxylic acids* were precipitated according to the principle of Foreman. The precipitate was dissolved in sulphuric acid. From this solution *aspartic acid* was determined by the malic acid method of Pucher *et al.*[3] adapted by Arhimo [4] to aspartic acid. Prior to the determination, the aliquot was extracted with ether. *Glutamic acid nitrogen* was calculated by subtracting the aspartic acid nitrogen from the total Foreman nitrogen. *Alanine* was determined by the method of Roine and Rautanen [5] after it had been oxidized with ninhydrin according to Virtanen and Rautanen [6]. *Nitrate* determination was carried out as described by Burström [7]. *Nitrite* was determined according to Jendrassik and Falcsik-Szabo [8]. *Malic acid* was extracted with ether in a Partheil-Rose extractor and then determined according to Pucher *et al.*[3]

Results

All results are given in milligrams nitrogen per 10 ml of original plant sap.

The first subject of this work was to follow the utilization of inorganic nitrogen compounds (ammonia and nitrate). In feeding solutions, ammonium

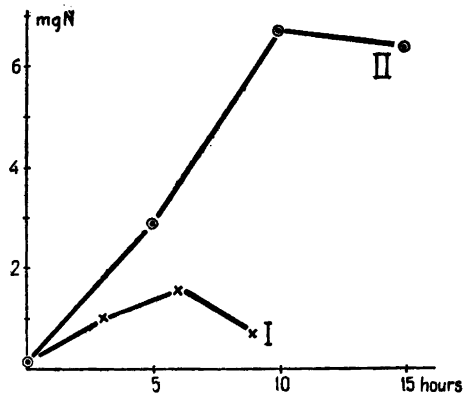


Fig. 1. The rate of the accumulation and disappearance of ammonia and nitrate nitrogen in plant sap during and after the uptake of corresponding nitrogen compound.

Curve I: Ammonia N.
» II: Nitrate N.

sulphate and calcium nitrate were used in concentration of 0.9 mg N per 1 ml of solution.

The objective in a preliminary experiment was to follow the rise and accumulation of ammonia and nitrate nitrogen in plants, as well as to state how rapidly they will be used for synthesis of organic nitrogen. For this purpose the accumulation of the corresponding nitrogen in the plants was followed with analyses during 6 hours in ammonia solution and during 10 hours in nitrate solution. Then the ammonia plants were transferred for 3 hours and the nitrate plants for 5 hours to water and the disappearance of the corresponding nitrogen was determined. Results are revealed in Fig. 1.

Although the results owing to the different times of the experiments are not quite comparable with each other it can be noted that ammonia nitrogen accumulates in plants much more slowly and disappears more rapidly than nitrate nitrogen. This fact indicates that at least the primary utilization of ammonia nitrogen is more rapid than the utilization of nitrate nitrogen.

The first stage of the utilization of nitrate is reduction. This process can occur in the roots as well as in the green parts of plants (*cf.* Dittrich [9], Burström [10]). The course of the reduction in plants was estimated during the uptake of nitrate by analyzing nitrate and its reduction products nitrite and ammonia. A control experiment was also made in which the plants were placed in water without any nitrogen sources. In them only ammonia was determined. Results are given in Table 1.

On the basis of the data in Table 1 it can be stated that during the continuous accumulation of nitrate only very little nitrite appears. The amount of nitrite after having reached rapidly its maximum concentration decreases a little or remains on the same level. Although the amount of ammonia even

Table 1. Amounts of nitrate and its reduction products in nitrate and control plants at different points of experiment.

		Hours			
		0	5	10	15
Nitrate plants:	Nitrate N	0.20	1.36	1.92	2.31
	Nitrite N	0.0003	0.0021	0.0019	0.0018
	Ammonia N	0.062	0.167	0.194	0.090
Control plants:	Ammonia N	0.062	0.055	0.075	0.068

in the nitrate plants is relatively small it can, however, be stated that some ammonia has been formed in nitrate plants but not in control plants. The appearance of ammonia in nitrate plants is of a temporary nature because it falls after some hours to the initial low level.

The possibility that hydroxylamine can act as an active intermediate compound in nitrate and ammonia assimilation was first suggested in 1884 by Meyer and Schulze [11]. Later the formation of oxime nitrogen as well in the assimilation of atmospheric nitrogen (Endres [12] with *Azotobacter*, Virtanen and Laine [13] in root nodules) as in the assimilation of nitrate nitrogen (Endres [14] with *Azotobacter*, Virtanen and Arhimo [15] in plants, Virtanen and Csáky [16] with yeast) has been established.

For the estimation of hydroxylamine and oxime, extraction with trichloracetic acid was used. It is impossible, however, to obtain colourless solutions from the green parts of plants when this agent is used. Hydroxylamine and oxime were therefore tested only in root extracts. *Hydroxylamine* was determined by the technique of Blom [17] and *oxime* according to Endres [12].

Four experiments were made in which the intact plants were kept in nitrate solution from 3 to 11 hours. All the hydroxylamine tests gave negative results. In two experiments could be noted a reaction of oxime which can be interpreted as positive.

The first task when beginning to follow the syntheses of organic nitrogen compounds due to the external supply of nitrogen was to determine the changes occurring in plant sap without any uptake of nitrogen. For this purpose the plants were placed in water and at certain intervals samples were taken for analysis. Results are given in Table 2.

It is evident that the composition of organic nitrogen fractions changes to a certain degree during the experimental time. The greatest variations can be observed in the fractions of glutamic acid and glutamine. Moreover, the inequality of the results is chiefly caused by the unhomogeneity of the analyzed plant samples.

Table 2. Amounts of organic nitrogen compounds during the experiment without any uptake of nitrogen.

	Hours			
	0	5	10	14
Ammonia N	0.12	0.05	0.12	0.07
Glutamic acid N	3.19	2.86	3.55	3.74
Aspartic acid N	1.41	1.39	1.33	1.33
Total N of glutamine	0.28	0.28	0.40	0.24
Total N of asparagine	7.86	7.52	8.00	7.82
Total N of both amides	8.14	7.80	8.40	8.06

During the uptake of nitrate the analyses gave the results shown in Table 3. The differences which can be observed in this case are of the same order as in the control experiment described above. The feeding of nitrate nitrogen has thus not caused any significant changes in the analyzed organic fractions of plant sap.

On the other hand, by feeding with ammonia nitrogen quite a different picture of the distribution of the soluble nitrogen compounds was observed. In order to give a clear picture of the relations existing between the alterations of different compounds the results are illustrated graphically in Fig. 2.

Immediately after the uptake of ammonia had begun intensive synthetical processes could be noted. It will be seen from the curves that glutamic acid and its amide, glutamine, are accumulated abundantly. The amount of aspartic acid increases too but its increase is much smaller and can be observed only in the form of the amide, asparagine. The accumulation of alanine is very slight and its amount decreases later. The pea plant used in these experiments is a typical asparagine plant (*cf.* Schwab [2]) which is seen from the relation of both amides. In this case for example over 95 % of the total amount of amide nitrogen was in the beginning asparagine nitrogen, the amide synthesized first to a greater extent during the uptake of ammonia was, how-

Table 3. Amounts of organic nitrogen compounds during the uptake of nitrate.

	Hours			
	0	5	10	15
Ammonia N	0.03	0.09	0.09	0.07
Glutamic acid N	4.02	3.61	3.59	5.17
Aspartic acid N	0.98	0.98	1.04	0.95
Total N of both amides	4.58	4.06	4.06	3.44

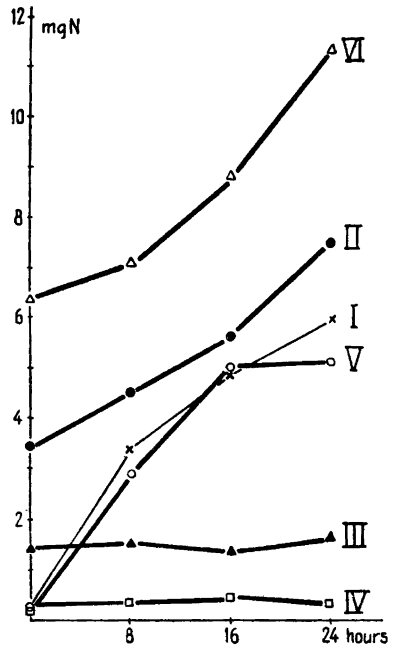


Fig. 2. Amounts of organic nitrogen compounds during the uptake of ammonia.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

ever, glutamine. The increase of asparagine takes place slowly in the beginning but continues, even accelerating, although the formation of glutamine has already come to a standstill. It can be seen from the curves of both amides that there is a correlation between them.

On the basis of the data given in Fig. 2 the amount of dicarboxylic acid nitrogen, the total nitrogen of amides, and alanine nitrogen are summed up at the different points of the experiment and illustrated graphically in Fig. 3. In the same graph is also illustrated the total nitrogen of plant sap at the corresponding moments.

It can clearly be seen from these curves that the increase of the fractions summed up above suffices to cover completely the increase of total nitrogen of plant sap. The accumulation of alanine is, however, so small that it is of no great importance. The inconsistency that the absolute values of total nitrogen are smaller than the sum of the partial fractions is apparently due to the small errors in analyzing of the many partial fractions. In spite of these errors it is evident that the plant sap may contain at most quite minute amounts of other nitrogen compounds. All these constituents accumulated in plant tissue during the primary stage of the ammonia assimilation are directly or indirectly dependant on each other.

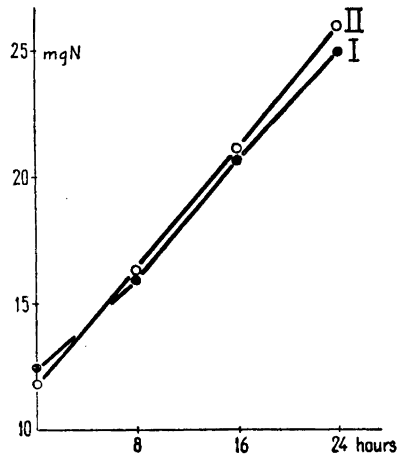


Fig. 3. Total organic nitrogen, and the sum of the nitrogen of different organic fractions during the uptake of ammonia.

Curve I: Total organic N.
» II: Sum of fractions of glutamic acid N, aspartic acid N, total N of amides, and alanine N.

The final aim of the nitrogen assimilation is to build up the cell protein. The amino acids are used to this protein synthesis. The disappearance of the amino nitrogen from the cell sap may be considered as a measure of the protein synthesis. To elucidate this fact an experiment was arranged in which the plants were first placed in ammonia solution for 14 hours and then transferred to water for 5 hours. The changes in ammonia nitrogen and total amino nitrogen were followed. When calculating the results, it was taken into consideration that the organic nitrogen of amido groups was distilled off before the determination of amino nitrogen according to van Slyke. The results of this experiment are given in Table 4.

Table 4. Variations of amounts of ammonia and total amino nitrogen during and after the uptake of ammonia. Plants were kept first 14 hours in ammonia solution and then 5 hours in water.

	Hours		
	0	14	19
Ammonia N	0.31	3.31	1.59
Total amino N	9.11	12.36	11.80

When the external supply of ammonia was interrupted the amino nitrogen of sap diminished although the sap still contained considerable amounts of ammonia from which it continuously synthesized new amino nitrogen. The protein synthesis thus takes place simultaneously with the processes described above. According to this it is very interesting to note that during the intensive

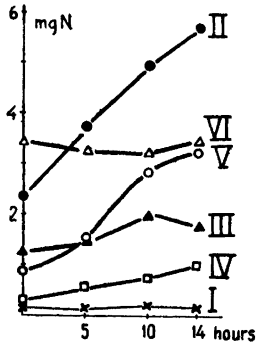


Fig. 4. Amounts of organic nitrogen compounds during the uptake of glutamic acid.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

formation of amino acids and protein, dicarboxylic acid and amido nitrogen almost exclusively were accumulated in plant sap. In this work therefore when continuing the experiments, attention was paid to the more minute investigation of the relations between aminodicarboxylic acids and amides. For this purpose the plants were fed with glutamic and aspartic acids and alanine. The concentration of the feeding solutions was in every experiment 0.5 mg N per 1 ml of solution. The analytical data of these experiments are illustrated in Figs. 4, 5, and 6.

It will be seen from the curves that during the uptake of glutamic and aspartic acid the accumulation of corresponding aminodicarboxylic acids in plant sap is very slight. This is due to the very rapid conversion of these acids not only into each other but into amides and alanine. On the other hand, the increasing of alanine in plant sap when feeding with this amino acid can be clearly noted at least at the beginning. In this case only the amount of glutamic acid at the first phase increases a little but the total amount of amides decreases during the whole experiment. The appearance of free ammonia is in every case very small and its amount does not increase during the experi-

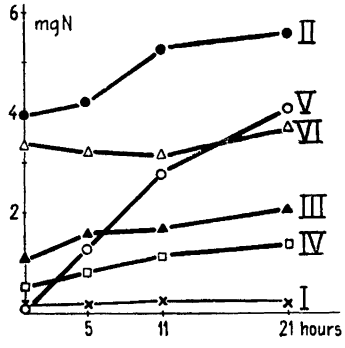


Fig. 5. Amounts of organic nitrogen compounds during the uptake of aspartic acid.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

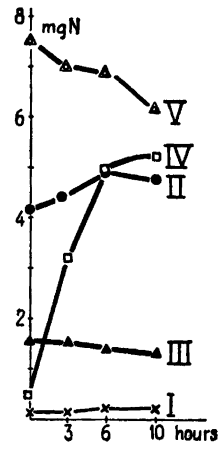


Fig. 6. Amounts of organic nitrogen compounds during the uptake of alanine.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of both amides.

ment. The splitting of amino group as ammonia must, however, have occurred because such considerable amounts of amides are formed. It is of particular interest to note that the amide primarily formed both from glutamic as well as aspartic acid is glutamine.

These experiments make evident that aspartic acid also is in fact a very active compound, though in the experiments with ammonia it seemed to be more stable than glutamic acid. Thus we can suppose that it could not accumulate in the earlier experiments because it changes so rapidly. Consequently, the question of the primary amino acid is very difficult to solve, on the basis of these experiments alone.

The key position of aminodicarboxylic acids and alanine in amino acid synthesis is partly explained by the central importance of the corresponding α-keto acids in the carbohydrate metabolism. They are members of the tricarboxylic acid cycle of Krebs [18]. Malic acid which is so characteristic of plants is also included in this cycle. All these compounds of the cycle are closely connected with each other as well as with the amino acid and protein metabolism. In order to obtain some information of the proportions between these carbohydrate derivatives and the nitrogen metabolism, only malic acid as a representative of carbohydrate derivatives was analyzed in experiments described in this paper, because the accurate quantitative determination of this compound is very easy. The amount of malic acid (given in mg per 10 ml of the original plant sap) at different points of experiments subtracted by the amount at zero point is illustrated in Fig. 7.

When the plants did not receive any nitrogen (control) a large quantity of malic acid was accumulated in plant tissue. The feeding of organic nitro-

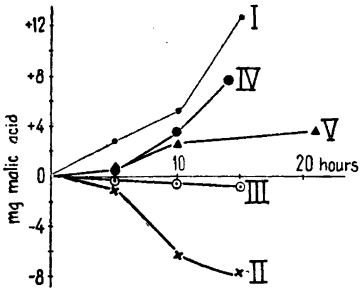


Fig. 7. Changes in the amounts of malic acid when plants were fed with different nitrogen compounds.

Curve I: Without any nitrogen source (control).
» II: With ammonia.
» III: » nitrate.
» IV: » glutamic acid.
» V: » aspartic acid.

gen in form of glutamic and aspartic acid also caused the increase in the amount of malic acid. On the other hand, when supplying inorganic (ammonia and nitrate) nitrogen, the disappearance of malic acid was observed. These findings are of some interest particularly because they show that even the carbon chain included in the organic nitrogen compounds is of importance in the assimilation of organic nitrogen.

DISCUSSION

According to our present knowledge ammonia is the central inorganic nitrogen compound in the nitrogen assimilation in the living cells in general. The temporary increase of ammonia in green plants even during the assimilation of nitrate, supports this view and at least that part of nitrate which has been reduced to ammonia is assimilated in this way. There may, however, be the possibility that even some other of the reduction products of nitrate *e. g.* hydroxylamine, can act as an active intermediate. The observations concerning the presence of hydroxylamine and its condensation product with keto bodies, oxime, during the nitrate assimilation make the existence of this possibility evident. The nature of this oxime and its real importance in nitrate assimilation are, however, quite obscure. According to Virtanen [19] it is possible that although in the fixation of atmospheric nitrogen by leguminous plants a formation of oxime can be noted this may be the result of a by-reaction, and the main reaction leads over ammonia.

On the basis of the results of this work the other primary organic nitrogen compounds which are synthesized during the nitrate assimilation are also of indefinite nature. So far as nitrate is reduced to ammonia the compounds which have been primarily synthesized are analogous with the compounds which are formed by the utilization of ammonia. The primary utilization of nitrate nitrogen is in any case much slower than the utilization of ammonia

nitrogen and on account of this, ammonia can not accumulate in plant sap to so great extent as nitrate. In agreement with this Virtanen and Linkola [20] have shown that in sterile cultures the intact plants have taken ammonia nitrogen more rapidly than nitrate nitrogen.

During the intensive uptake of ammonia there can be observed a very rapid and abundant accumulation of organic nitrogen in plant sap. This increase of the soluble nitrogen is formed exclusively by aminodicarboxylic acids, their amides, and alanine. It seems safe to conclude that these amino acids have a central position in the assimilation of different nitrogen compounds. The object of the formation of these amino compounds is to change rapidly the inorganic ammonia nitrogen into the organic form.

On account of the very easy interconversion of these amino acids it is difficult to solve the question of the primary amino acid. The synthesis of the other amino acids takes place more slowly and their synthesis is the limiting factor of the protein formation and thus only the above amino acids can accumulate in cells.

These results are in general agreement with the observations of Roine [21] made with yeast.

The amide problem which has had such a prominent position in the study of nitrogen metabolism in plants, comes out in this work in a quite new light. This question cannot be explained as a separate one but only in connection with the other problems of nitrogen metabolism because it is intimately connected with these primary processes of ammonia assimilation. In every case in which the amide formation could be observed independent of the source of nitrogen, the amide synthesized first was glutamine. The more stable amide to which also glutamine later was transformed was asparagine. When glutamic or aspartic acids were the sources of nitrogen there could be noted the synthesis of amides without formation of free ammonia. On the basis of this it is evident that even for plant cell it is in general very important to prevent the accumulation of ammonia. In this connection it is therefore necessary to emphasize that there seems to be no difference in principle between the mechanism of the amide formation and their importance on the one hand in plant and, on the other hand, in animal organisms. The quantitatively more important role of amides in plants is based on the peculiar nitrogen economy of these organisms in general.

The formation and transformation of all the nitrogen compounds to which attention has been paid in this work take place directly or indirectly through the wellknown reactions by which the amino or amido groups generally are formed in living cells. In these processes the metabolism of the carbon chains from which the amino acids are formed has generally been overlooked. As

we now know only the keto acids corresponding glutamic acid, aspartic acid, and alanine are members of the tricarboxylic acid cycle of Krebs, which is of so great importance in the carbohydrate metabolism. This fact explains partly the importance as well as the easy interconversion of these amino acids. The synthesis of the other amino acids necessary for the protein synthesis, on the other hand, and the final aim of the nitrogen assimilation, the protein formation, are still quite hypothetical.

SUMMARY

In this paper some observations concerning the formation of organic nitrogen compounds in green plants during the assimilation of inorganic and organic nitrogen compounds have been reported.

It was shown that nitrate is rapidly reduced to ammonia and thus can be assimilated in the same way as ammonia. On the other hand, there is the possibility that the nitrate assimilation can occur through hydroxylamine and oxime but this process is as yet quite obscure in its details.

During the uptake of ammonia there was noted a very rapid and intensive synthesis of organic nitrogen compounds in plant sap. This organic nitrogen was composed chiefly of aminodicarboxylic acids and their amides. In addition to these alanine also seems to be of importance in this process.

The metabolic relations between aminodicarboxylic acids, their amides and alanine were investigated in detail by feeding the plants with these amino acids. Their easy interconvertibility was observed.

The question of the primary amino acids is discussed.

The formation of amides and their importance in the nitrogen metabolism of plants was also investigated and is discussed.

The relations between carbohydrate and amino acid metabolisms was investigated using malic acid as a representative of the carbohydrate derivatives.

REFERENCES

1. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S. *Ind. Eng. Chem., Anal. Ed.* **7** (1935) 152.
2. Schwab, G. *Planta* **25** (1936) 579.
3. Pucher, G. W., Vickery, H. B., and Wakeman, A. J. *Ind. Eng. Chem., Anal. Ed.* **6** (1934) 288.
4. Arhimo, A. A. *Suomen Kemistilehti* **B 12** (1939) 6.
5. Roine, P., and Rautanen, N. *Acta Chem. Scand.* **1** (1948) 854.
6. Virtanen, A. I., and Rautanen, N. *Biochem. J.* **41** (1947) 101.
7. Burström, H. *Svensk. Kem. Tid.* **54** (1942) 139.

8. Jendrassik, L., and Falcsik-Szabo, E. *Biochem. Z.* **261** (1933) 110.
9. Dittrich, W. *Planta* **12** (1931) 69.
10. Burström, H. *Lantbruks-Högskol. Ann.* **6** (1939) 1.
11. Meyer, V., and Schulze, E. *Ber.* **17** (1894) 1554.
12. Endres, G. *Naturwissenschaften* **22** (1934) 662.
13. Virtanen, A. I., and Laine, T. *Suomen Kemistilehti* **B 9** (1936) 12.
14. Endres, G. *Angew. Chem.* **49** (1936) 560.
15. Virtanen, A. I., and Arhimo, A. A. *Suomen Kemistilehti* **B 12** (1939) 24.
16. Virtanen, A. I., and Csáky, T. *Nature* 161 (1948) 814.
17. Blom, J. *Biochem. Z.* **194** (1928) 325.
18. Krebs, H. A. *Advances in Enzymol.* **3** (1943) 191.
19. Virtanen, A. I. *Biol. Revs. Cambridge Phil. Soc.* **22** (1947) 239.
20. Virtanen, A. I., and Linkola, H. *Nature* **158** (1946) 515.
21. Roine, P. *Ann. Acad. Sci. Fennicae* Ser. A. II. Chem. No. **26** (1947) 1.

Received January 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 140—146

Studies Related to Pristane

I. The Unsaponifiable Matter of the Liver Oil of the Basking Shark

NILS ANDREAS SÖRENSEN and JORUNN MEHLUM

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

Mitsumaru Tsujimoto [1] discovered in 1917 in the unsaponifiable matter of the liver oil of the basking shark (*Selache maxima* Gunnerus) a saturated hydrocarbon which later was named pristane [2]. Tsujimoto established the aliphatic nature of this hydrocarbon; as regards the molecular formula, Tsujimoto found it difficult to decide between $C_{17}H_{36}$, $C_{18}H_{38}$ and $C_{19}H_{40}$, we quote: »It is not safe to say that the substance was a single chemical compound, but judging from its boiling point and molecular weight, it appears to be very probable that it consisted mainly of $C_{18}H_{38}$ or an octadecane. But as the normal octadecane is a solid at the ordinary temperatures, the substance must be at least an isooctadecane.»

Later Tsujimoto's pupil Toyama [2] proved the occurence of pristane in some 5 further Elasmobranch-liver oils, but added very little to the chemistry of pristane. Unfortunately pristane in literature [2-4] is mostly quoted as iso-octadecane, regardless of Tsujimotos cautious statement. Probably the high m. p., — 11° C, stated by Toyama and Tsuchiya [5] has contributed to this disregarding of the statement of Tsujimoto [6], that purified pristane does not solidify at the temperature of solid carbon dioxide. This very low m. p. is quite incompatible with the structure of pristane as iso-octadecane = 2-methylheptadecane, the m. p. of which is + 5° [4].

Our interest was attracted to pristane as a result of biosynthetic discussions. As far as known, the fatty acids of the basking-shark liver oil are normal unbranched fatty acids. The unsaponifiable matter of the oil consists for the most part *cf.* [7-9] of the unsaturated triterpene hydrocarbon squalene $C_{30}H_{50}$. We would find it most remarkable if the basking-shark was capable of the normal fat synthesis, of the synthesis of terpenoid compounds as well as of the intermediate type of methylsubstituted chain compounds primarily discovered by Anderson and Spielman in the tubercelostearic acid [10]; in the latest years

increased through Weitkamp's excellent studies of the acidic constituents of degras[11]. So we found it worth while to prove the indicated constitution of pristane.

Chem. Eng. K. Toft-Eriksen[9] informed us that he once, on fractional destillation of one of his samples of basking-shark liver oil, observed crystals in the cooler at the end of the pristane fraction. As the temperature of the cooling water was greater than + 5°, the possibility, that the crystals were pure iso-octadecane, could at once be excluded. Some small samples of the oils investigated by Toft-Eriksen were at hand and we ascertained that two of them solidified at — 5°. So we decided to elucidate the nature of these crystals.

By carefully fractionating some kg of basking-shark oil in a 70 cm column we found that the oil regularly gives a broad fraction between pristane and squalene; the raw middle-fraction solidified by room temperature at once to a crystal jelly. By filtering and recrystallizing as described in the experimental part, this middle-fraction was divided into some pristane and squalene, and the three fatty alcohols cetyl-, stearyl- and oleyl alcohol.

The two saturated alcohols cetyl- and stearyl alcohol have a low solubility in pristane below room temperature and as they crystallize in great, but very thin leaflets which pile up on one another to a very voluminous framework even contaminations of 1 % of these saturated alcohols in pristane will bring the solution to solidify at 0°.

Besides the mentioned compounds, the basking shark liveroils as well as all liver oils of higher animals, contain cholesterol. The quantity was estimated, through precipitation with digitonin, to 0.4 % of the unsaponifiable matter. As regards the unsaponifiable matter of our great sample pure basking-shark oil, for which we are very much indepted to Chem. Eng. Alf Brautaset, Jahres Kjemiske Fabrikker A/S, Sandefjord, we may state the following approximate composition:

Cholesterol	0.4 %
Pristane	14 %
Squalene	84 %
Cetyl alcohol	0.6 %
Stearyl alcohol	0.4 %
Oleyl alcohol	0.3 %

The three fatty alcohols have before been found a few times in fish oils[12] each of them about 5 times, distributed in many classes of fish. Mostly they are found in small quantities and in all they are much more scarce than the glycerol-ether-alcohols, chimyl, batyl and selachyl alcohol. These alcohols very common among fishes are absent in basking-shark liver oil, as the oils give no isopropyliodide after Zeissel Fanto.

To eliminate the alcohols the pristane fractions were purified over metallic sodium. Redestilled once more, pristane is a colourless liquid, which does not solidify at — 80°. As shown by Tsujimoto [12], such refined pristane is not pure, but contains various small amounts of an unsaturated hydrocarbon, called zamene. Zamene can be quantitatively withdrawn through a few treatments with 100 % H_2SO_4. In this way refined pristane has physical constants in excellent agreement with the Japanese authors. With all constants referred to 20° and corrections of some evident misprints we have

	d_4^{20}	n_D^{20}
Tsujimoto 1917 [1]	0.78437	1.4398
Toyama 1923 [3]	0.7835	1.4390
Present investigation	0.78267	1.43848

From a biochemical point of view the simplest assumption would be that pristane is a saturated hydrocarbon of terpenoid structure. But as partially degraded terpenoids are much more rare than compounds with integral numbers of C_5, much importance is attached to the molecular weight of pristane. Professor dr. Endre Berner on our request most amiably carried out a precision determination of the molecular weight of our purest specimen (cryoscopic in benzene). As comparison compound we handed to Berner a sample of 2,6,11,15-tetramethyl-hexadecane = »Crocetane», synthesized for this purpose by Chem. Eng. Hugo Holtermann through hydrogenation of the previously unknown diterpene 2,6,11,15-tetramethyl-hexadeca-tetraene-2,6,10,14 = »Digeranyl». The details of this synthesis will follow in another publication of this series. For pristane Berner found mol. wt. 269, and 273, for »Digeranyl» 282, against calculated for $C_{20}H_{42} = 282.4$, $C_{19}H_{40} = 268.4$ and $C_{18}H_{38} = 254.4$.

So we must conclude that pristane is not an octadecane at all, but at least a nona-decane. It must be added that Tsujimoto gave two determinations *viz.* 265, 252; Toyama measurements on not less than nine samples; his values ranges from 259 to 270, mean 264.1 $\pm$ 1.2. The measurements of the Japanese authors themselves scarcely justify their own conclusion. As experimental errors systematically tend to lower the molecular weight determinations, already their original determinations suggest a molecular formula not lower than $C_{19}H_{40}$. However, we agree with Tsujimoto in his statement of the uncertainty of the homogeneity of the pristane hydrocarbon and so, in spite of the excellent agreement of professor Berners measurements for pristane with $C_{19}H_{40}$ we do not venture to exclude a molecular formula of $C_{20}H_{42}$.

Of saturated terpenoid hydrocarbons $C_{20}H_{42}$ Willstätter, Mayer and Hüni [14] has prepared »Phytane» (2,6,10,14-tetramethyl-hexadecane) from

phytol; Karrer and Golde [15] has transferred the carotinoid pigment Crocetin in the isomeric above mentioned hydrocarbon »Crocetane». From geraniol the same hydrocarbon has been synthesized by Fischer and Löwenberg [16]. As will be seen from Table 1, the physical constants of these two preparations differ appreciably. The data of Willstätter for »Phytane» and of Fischer and Löwenberg for »Crocetane» come very close to the physical constants of pristane and so augment the possibility that pristane is a saturated diterpene. As, however, there can be no doubt about the constitution of crocetin nor about the »Crocetane» of Karrer we found it necessary once again to synthesize this hydrocarbon.

Table 1. Physical constants of the saturated aliphatic diterpenes, $C_{20}\ H_{42}$.

Author	d	n
Phytane (Willstätter)	$(d_4^0 = 0.803)$	—
	$d_4^{20} = 0.78936$	—
Crocetane (Karrer)	$(d_4^{19.8} = 0.8027)$	$(n_D^{19.8} = 1.44937$
	$d_4^{20} = 0.8025$	$n_D^{20} = 1.4492$
Crocetane (Fischer)	$(d_4^{25} = 0.7853)$	$(n_D^{25} = 1.4382)$
	$d_4^{20} = 0.7887$	$n_D^{20} = 1.4402$
Crocetane (present investigation)	$d_4^{20} = 0.78584$	$n_D^{20} = 1.44055$

The synthesis was carried out from citral via tetrahydrogeraniol and tetrahydrogeranylbromide along much the same lines as by Fischer and Löwenberg. In Table 1, all data have been quoted from the originals and recalculated to 20°. As will be seen the data from our preparation of »Crocetane» is in accordance with those of Fischer. As to the deviating data of Karrers Crocetane, we shall return to them in a later communication.

EXPERIMENTAL

The crystalline alcohols

The unsaponifiable matter of basking-shark oil was destilled in batches of ca 550 g through a 70 cm column whose temperature could be regulated electrically from 75—350°. All destillations were carried out between 0.05—0.1 mm, the temperature of the column was regulated some 35° lower than the oil bath temperature. The fractions below 110° contain pristane contaminated with small amounts of some oxidation product of squalene with characteristic lemon odour, the fractions above 185° contain only squalene. There was no definite boiling fractions between pristane and squalene; arbitrarily, how-

ever, we separated the intermediary destillate in the fractions b. p. 110—145° and 145—185°. Fraction 110—145° solidified only when cooled, by the destillation of the fraction 145—185° the recipient and the connecting pipes had to be gently warmed to avoid tightening through crystallization. 550 g unsaponifiable matter gave approximately 55 g < 110°, 27 g between 110—145° and 38 g between 145—185°. The two last fractions were cooled down to — 17° and after some time each of them brought on porous porcelain at — 17°. In all 3,280 g unsaponifiable matter were in this way converted to 16 g raw crystals from the fraction 110—145° and 28 g from the fraction 145—185°. Both crystal fractions were recrystallized from acetone solution at 0° C, the second one then from alcohol at 0°. The m. p. showed the following displacement:

	Fraction I M. p.	Fraction II. M. p.
From acetone 1	8 g 44.5—47°	9 g 47 —49°
» » 2	6 » 48 —49°	6 » 54.5—56°
» » 3	4 » 48 —49°	4 » 55 —56.5°
From alcohol 4		1.5 » 56 —57°
» » 5		1 » 56 —57°

Through fractional destillation of spermaceti and repeated recrystallization there were prepared pure cetyl and stearyl alcohol. Cetyl alcohol from spermaceti m. p. 48—49°, mixed m. p. 48—49°.

Stearyl alcohol from spermaceti m. p. 56.5—57.5°, mixed m. p. 56—57°.

Fraction I, 3.

$C_{16}H_{34}O$ (242.3) Calc. C 79.25 H 14.14 Mol. wt. 242.3
Found » 78.9,79.2 » 14.1,14.35 » » 242.5,241

Fraction II, 5.

$C_{18}H_{38}O$ (270.3) Calc. C 79.95 H 14.13 Mol. wt. 270.3
Found » 79.1,79.7 » 13.85,14.05 » » 268,270

According to the procedure of Årtun [17] the cetyl alcohol as well as the stearyl alcohol from spermaceti and basking-shark liver oil was transformed into the 3,5-dinitro-benzoates.

3,5-dinitro-benzoate of cetyl alcohol from spermaceti m. p. 66—67°
» » » » » » » basking-shark m. p. 66—67°
» » » » stearyl alcohol from spermaceti m. p. 74—75°
» » » » » » » basking shark m. p. 74—75°
Mixed m. p. with each pair no depression.

Liquid alcohols

From all crystallizations and from the porous plates the mother liquors were collected and as much saturated alcohols as possible removed through crystallizations at low temperatures. The physical constants indicated that the liquid residues of Fraction I mainly consisted of pristane, that from II of squalene. Through »Entmischung» between petrol ether and methanol of 95, 92.5 and 90 % the remaining alcohols were separated

from the hydrocarbons, which constituted from 5/6 to 19/20 of the liquids. The hypophasic part showed

n_D^{20} = 1.4770, iodine number 102, and a m. p. about 0°.

Oleyl alcohol.

$C_{18}H_{36}O$ (268.3)	Calc. C 80.51	H 13.53	M. p. + 2—3°
	Found » 79.9,79.3 »	13.0,13.67 »	0°

The data are in accordance with a slightly contaminated oleyl alcohol. The most suitable manner to prove the occurence of oleyl alcohol is the hydrogenation to stearyl alcohol. This hydrogenation was carried out with Pt-silicagel at room temperature, the theoretical amount H_2 was taken up in 8 hours. The residue after hydrogenation was solid m. p. 56—57°, constant after recrystallization.

»Crocetane» from citral (stud. chem. Per Koch-Christensen)

Citral was carefully fractionated in a vacuum, and at once hydrogenated with Raney-nickel by 90° under 50—60 atm. H_2 (compare Palfray [18]). 3,7-dimethyloctanol-1 distilled at 114—116° at 21 mm and showed n_D^{20} = 1.4351; Palfray gives 106°/12 mm and n_D^{20} = 1.4367. 3,7-dimethyloctanol-1 were converted to the corresponding bromide according to [19] B. p. 108—111/24 mm; n_D^{19} = 1.4538.

Longinow and Margoliss [20] give n_D^{20} = 1.4534. 63 g 3,7-dimethyloctylbromide, 16 g metallic potassium and 250 g anhydrous benzene were boiled with reflux for 20 hours, and the reaction product fractionated. The fraction 144—155/2 mm was déstilled over Na, refined with repeated washings with 100 % H_2SO_4 and distilled over Na. Bath temperature 90°/0.001 mm d_4^{20} = 0.78584. Table 2 gives the observed dispersional data for »Crocetane» with the values calculated for

$$R_\lambda = \frac{92.648}{\lambda^2 \div 0.7837;} \quad R_{D\ calc.} = 94.56, \qquad \text{found } 94.79$$

$R_{\lambda=\infty}$, calc. = 92.389, found 92.648

λ calc. = 885.3 ÅU; standard value for saturated hydrocarbons λ_0 = 885.2 ÅU (compare Sørensen [21]).

Table 2. Dispersion of 2,6,11,15-tetramethyl-hexadecane (Crocetane).

λ	n_λ	$R_{\lambda\ obs.}$	$R_{\lambda\ calc.}$
6678.1 ÅU	1.43791	94.297	94.305
5895.8 »	1.44055	94.791	94.785
5875.7 »	1.44063	94.806	94.800
5460.7 »	1.44243	95.142	95.149
5015.6 »	1.44513	95.646	95.627
4471.48 »	1.44930	96.422	96.428
4358.3 »	1.45038	96.623	96.635

SUMMARY

The unsaponifiable matter of the liver oil of the basking-shark was reinvestigated and cetyl-, stearyl and oleyl alcohol were demonstrated as new compounds. Pristane has been shown to be at least a nonadecane, an eicosane formula can, however, not be excluded. The synthesis of »Crocetane» was repeated and its constants found in agreement with the data of Fischer and Löwenberg. »Crocetane» in its physical constants resembles purified pristane.

REFERENCES

1. Tsujimoto, M. *J. Ind. Eng. Chem.* **9** (1917) 1098.
2. Toyama, Y. *Chemische Umschau* **XXX** (1923) 181.
3. Ubbelohde, L. *Handbuch der Oele und Fette* Bd I, Leipzig (1929) 160.
4. Egloff, G. *Physical constants of Hydrocarbons* Vol. I, New York (1939) 103.
5. Toyama, Y., and Tsuchiya, T. *J. Soc. Chem. Ind. Japan* **32** (1929) suppl 374 B (quoted after 4).
6. Tsujimoto, M. *J. Soc. Chem. Ind. London* (**1932**) 317 T.
7. Tsujimoto, M. *J. Ind. Eng. Chem.* (**1916**) 889.
8. Andre, E., and Canal, H. *Ann. Chimie* [10] **7** (1927) 69.
9. Schmidt-Nielsen, S., and Toft-Eriksen, K. *Kgl. Norske Videnskab Selskabs-Forh.* **17** (1944) no. 35.
10. Spielman, M. A. *J. Biol. Chem.* **106** (1934) 87.
11. Weitkamp, A. W. *J. Am. Chem. Soc.* **67** (1945) 447.
12. In *Chlamydoselachus anguineus:* cetyl and oleyl alcohol (Toyama, Y. *Chem. Umschau* **29** (1922) 237 and 245. In *Ommastrephes sloani pacificus:* cetyl and probably oleyl alcohol (Tsujimoto, M. *Chem. Z.* **1932** I 1458). In *Mugil japonicus:* cetyl and stearyl alcohol (Tsujimoto, M. *Chem. Z.* **1934** I 1412). In *Ruvettus pretiosus:* cetyl, stearyl and oleyl alcohol (Warren, M. C. and Emmet Reid, E. *J. Am. Chem. Soc.* **54** (1932) 220). In *Gasterosteus aculeatus* and *Theragra chalcogramma:* oleyl alcohol (Ueno, S. and Komori, S. *Chem. Z.* **1936** II 1636). In *Pleurogrammus monopterygius* Pallas: oleyl alcohol (Ueno, S., and Iwai, M. *Chem. Z.* **1937** I 4174). In *Heptranchias Deani:* stearyl alcohol (Ono, T. *Chem. Z.* **1933** I 1218).
13. Tsujimoto, M. *Bull. chem. Soc. Japan* **10** (1935) 149.
14. Willstätter, R., Mann, E. W., and Hüni, E. *Ann.* **378** (1911) 107.
15. Karrer, P., and Golde, Th. *Helv. Chim. Acta* **13** (1930) 707.
16. Fischer, F. G., and Løwenberg, K. *Ann.* **475** (1929) 183.
17. Årtun, T. *Kgl. Norske Videnskab. Selskabs-Forh.* **14** (1941) no. 67.
18. Palfray, L. *Bull. Soc. Chim. France* [5] **7** (1940) 401.
19. *Organic Synthesis* Vol. XV p. 24.
20. Longinow, W., and Margoliss, E. *Bull. Soc. Chim. France* [4] **45** (1929) 166.
21. Sørensen, N. A. *Ann.* **546** (1940) 57.

Received January 17, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 147—165

A Contribution to the Physical Chemistry of Colloidal Metaphosphates *

HARRY MALMGREN

The Institute of Physical Chemistry, University of Upsala, Upsala, Sweden

In a previous paper [1] the results of dispersity measurements on high molecular potassium metaphosphates have been reported. This work is in some points a completion of that mentioned above and the investigations have also been extended to new fields. However, a brief summary of the older investigations will first be given.

I. These colloidal potassium metaphosphates are the salts of Kurol [2], insoluble in water but soluble in solutions of sodium- and ammonium salts. They are prepared by heating primary potassium phosphate:

$$n\ KH_2PO_4 \rightarrow (KPO_3)_n + n\ H_2O$$

If the heating is performed at constant temperature for a long time (150—250 hours) metaphosphates are obtained, the viscosity of which at a given concentration in a defined medium seems to approach a limiting value, corresponding to the temperature in question. $M/50$ basemolar solution in $M/10$ basemolar $Na_3(PO_3)_3$ were used to characterize these substances regarding viscosity.

II. These solutions are too viscous to be used for dispersity measurements. Sedimentation and diffusion were studied in 0.4 M NaCNS where the conditions are more normal.

The sedimentation in the ultracentrifuge [3] proved to be very dependent on concentration; the sedimentation velocity generally increases very much on dilution. The sedimentation constant used in calculations is generally the value at infinite dilution; it is given in Svedberg units, S.

The free diffusion measured by the scale method of Lamm [4] gave a normal picture within the concentration interval 0.1—0.5 %.

* Part of this investigation was presented at *Sjätte Nordiska Kemistmötet*, Lund, Aug. 1947.

III. If $(KPO_3)_n$ is dissolved in a solution containing an excess of Na^+-ions all the potassium goes into solution, and one can regard a polyanion surrounded by Na^+ ions as the sedimenting and diffusing substance. The partial spec. volume, V, calculated for $(NaPO_3)_n$, was found to be 0.39.

IV. A summary of the temperatures of preparation, viscosity, sedimentas tion and diffusion constants and molecular weights according to Svedberg'-wellknown formula has been drawn up in Table 1. In the table are also given these data for two later syntheses, namely K13 and K14. The molecular weights refer to the original $(KPO_3)_n$.

Table 1. Some molecular constants of the preparations.

Preparation	Temperature of synthesis	Viscosity 10^4 c. g. s.	s_0	D 10^7 c. g. s.	Molecular weight
K12	260°	153	10*	1.7	280,000
K10	290	193	14*	1.4	460,000
K13	305	170	26	1.5	790,000
K 9	382	347	27	1.0	1,100,000
K11	445	460	25	0.6	2,000,000
K14	495	260	34	1.2	1,300,000
K 8	665	422	30	0.6	2,300,000

In the temperature interval investigated the molecular weights seem to increase continuously with the temperature (exceptions, K11 and K14). The molecular weights have also been determined by means of the sedimentation equilibrium method; the results agree rather well with the values of Table 1.

V. In solutions these polymers are highly solvated particles, the shapes of which are probably not spherical.

As these polymetaphosphates are colloidal electrolytes, it is of course interesting to know how the viscosity, for instance, and the sedimentation are affected by electrolytes of different types, and how the charge of the colloidal ion varies in different media. Therefore the viscosity, sedimentation, diffusion and electrophoretic mobility have been studied under various conditions. Attention has also been paid to the question of the stability of the solutions, and the diffusion and sedimentation measurements have been calculated with regard to polydispersity. The problem of molecular shape and structure has been treated with the aid of the electron microscope and X-ray analysis.

* The preparations K10 and K12 seem to dissociate on dilution as shown by the fact that the sedimentation constant at first increases on dilution, but diminishes when the concentration is less than 0.5 %. The values of the table correspond to the maximal ones. An analogous phenomenon has not been observed with diffusion.

Viscosity

Pascal [2] tried to classify these high polymers by the viscosity of their $M/50$ basemolar solutions in $M/10$ basemolar sodium hexametaphosphate. For this purpose one could of course choose any salt solution in which the polymetaphosphate is soluble, however the result is very dependent on the medium chosen. Independently of the character of the electrolyte the viscosity decreases with increasing electrolyte concentration until salting out of the polymetaphosphate occurs. The effect of the salt may be due to a diminution of the charge of the colloidal anion and a decrease in the solvation.

When investigating the dependence of the viscosity on the ions present in solution one must not forget that the dissolved metaphosphate to some extend contributes to the ionic strength — especially at small concentrations of the low molecular electrolyte. Whence it is suitable to correlate the limiting value $(\eta_{sp}/c)_{c\to 0}$ against for example the normality of the electrolyte or ionic strength. As is known the specific viscocity η_{sp} is defined by: $\eta_{sp} = \eta_r - 1$; η_r = the relative viscosity. A KPO_3-polymer, preparation K14, has been investigated regarding its viscosity in NaCNS, Na_2SO_4, $Na_3(PO_3)_3$ and $Na_4P_2O_7$, *i. e.* an uni-univalent, an uni-bivalent, an uni-trivalent and an uni-tetravalent electrolyte with the same cation. The measurements have been made at 20° C in a capillary viscosimeter according to Ostwald; they are collected in Table 2. The limiting value $(\eta_{sp}/c)_{c\to 0}$ has been extrapolated from measurements at four or five concentrations; the curve η_{sp}/c falls to $c = 0$ without showing any minimum. c is expressed in no. of grams of substance per gram of solution.

Table 2. Dependence of the viscosity on the medium.

Medium	$(\eta_{sp}/c)_{c\to 0}$	Medium	$(\eta_{sp}/c)_{c\to 0}$
0.05 *N* NaCNS	645	0.30 *N* NaCNS	140
0.05 *N* Na_2SO_4	625	0.30 *N* Na_2SO_4	200
0.05 *N* $Na_3(PO_3)_3$	550	0.30 *N* $Na_3(PO_3)_3$	170
0.05 *N* $Na_4P_2O_7$	710	0.30 *N* $Na_4P_2O_7$	280
0.10 *N* NaCNS	350	0.40 *N* NaCNS	39
0.10 *N* Na_2SO_4	446	0.40 *N* Na_2SO_4	162
0.10 *N* $Na_3(PO_3)_3$	407	0.40 *N* $Na_3(PO_3)_3$	156
		0.40 *N* $Na_4P_2O_7$	245
0.20 *N* NaCNS	220		
0.20 *N* Na_2SO_4	261	0.70 *N* $Na_3(PO_3)_3$	115
0.20 *N* $Na_3(PO_3)_3$	250	0.80 *N* Na_2SO_4	118
0.20 *N* $Na_4P_2O_2$	344	0.80 *N* $Na_4P_2O_7$	126

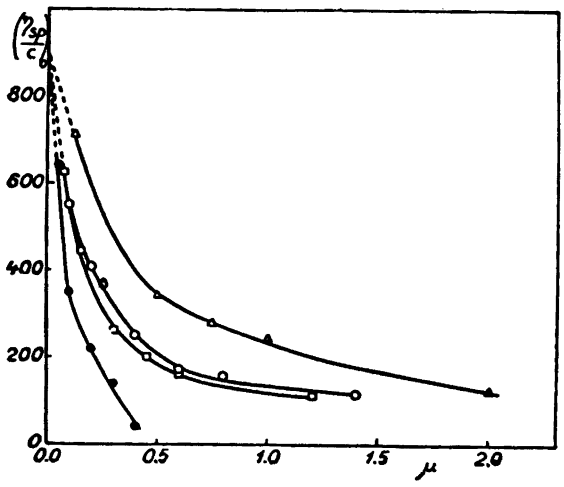


Fig. 1. $(\eta_{sp}/c)_{c\to 0}$ *as a function of the ionic strength.*
● *NaCNS;* □ Na_2SO_4
○ $Na_3(PO_3)_3$; Δ $Na_4P_2O_7$

The concentrations at which the polymetaphosphate is salted out by these electrolytes may also be mentioned in this connection. They are: for NaCNS 0.42 *N*, for Na_2SO_4 0.90 *N*, for $Na_3(PO_3)_3$ 0.72 *N*. A saturated solution of $Na_4P_2O_4$ (0.82 *N* at 20° C) does not salt out the metaphosphate.

As is seen from Table 2 the viscosity is not at all the same in, for example, 0.40 *N* NaCNS and 0.40 *N* $Na_4P_2O_7$. However as the great viscosity must be ascribed to the colloidal anion, the effect of electrolytes on the viscosity ought preferably, to depend on the cations present in the solutions if the viscosity were chiefly of an electro-character. Thus the electroviscous effect is only one of the factors acting on these conditions. Unfortunately it is impossible to perform a series of measurements analogous to that of Table 2 in the presence of 2-, 3- or 4-valent cations, as they precipitate the metaphosphate. A rather interesting result is obtained if the limiting value $(\eta_{sp}/c)_{c\to 0}$ is plotted against the ionic strength as abscissa. See Fig. 1.

The ionic strength μ is defined by the expression:

$$\mu = \tfrac{1}{2} \sum_i c_i z_i^2 \; ; \; c_i = \text{concentration of the ion of valency } z_i.$$

One might expect that the curves extrapolated to $\mu = 0$, would cross the ordinate at the same point which is approximately the case. As is seen from Fig. 1 an uni-univalent electrolyte has the most decreasing influence on the viscosity, followed by uni-bi-, uni-tri- and uni-tetravalent electrolytes respectively.

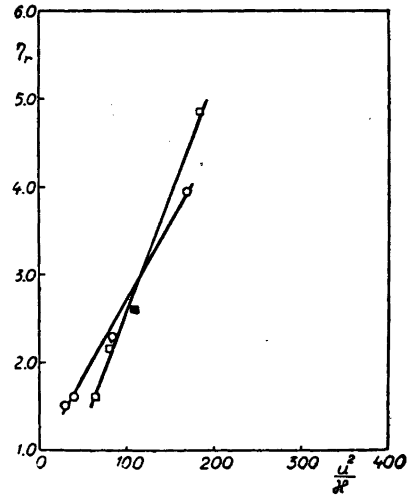


Fig. 2. The relative viscosity as a function of $\frac{\mu^2}{\varkappa}$.
□ *K 14 in* $Na_3(PO_3)_3$*-solutions*
○ *K 14 » NaCNS- »*

The addition of an electrolyte causes a denser ionic atmosphere around the particles thus making the approach of the water dipoles to the particles more difficult, which may explain a diminution in the solvation and viscosity. The electroviscous effect has been treated by Smoluchowski [5] and later by Krasny-Ergen [6]. In the formula

$$\eta_r - 1 = 2.5\,\varphi\left[1 + k\,\frac{1}{\varkappa\, a^2\,\eta_0}\left(\frac{\varepsilon\,\xi}{2\,\pi}\right)^2\right] \qquad (1)$$

φ is the volume of the particles relative to the volume of the solution, $\varkappa$ is the specific conductivity of the solution, a the radius of the particles, η_0 the viscosity, ε the dielectric constant of the solvent, ξ the electrokinetic potential of the particles and k a constant; its value being 3/2 according to Krasny-Ergen.

On addition of an electrolyte η_r dereases and this fact is mainly attributed to the increase in conductivity. Eq. (1) has been deduced for a suspended spherical particle and such a picture differs very much from that for a hydrophil colloid such as polymetaphosphate, for which a correcting factor, allowing for the shape, should be introduced in this equation.

If the electrokinetic potential is put proportional to the electrophoretic mobility u (see p. 154) a linear relation is to be expected between η_r and $\frac{u^2}{\varkappa}$, if the other quantities are regarded as constants. In solutions with high electrolyte concentrations such a relation seems to exist, see Fig. 2. Concentration of the polymetaphosphate 0.50 %.

For another colloidal electrolyte, *viz.* polyacrylic acid and its sodium salt Kern [7] has assumed that the viscosity is the product of two factors, the ionic factor, I, and the macromolecular factor, $(\eta_{sp}/c)_m$, allowing for the influence of the charge, and the degree of polymerization respectively.

$$\eta_{sp}/c = I \cdot (\eta_{sp}/c)_m \tag{2}$$

According to Kern an addition of salt only changes the ionic factor, the molecular weight and molecular shape being unchanged. Formally one could apply Eq. (2) to these metaphosphates, but Kern gives no detailed statement of the quantities involved in the ionic factor.

Stability

These polymers are not completely stable in solution [1, 8], as shown by the fact that the viscosity of a solution generally decreases with time. Above all the stability depends on the pH of the solution, but it is also greatly affected by microorganisms [9]; some metaphosphatases degrade the polymetaphosphate. On the assumption that the specific viscosity of the solution can be expressed as

$$\eta_{sp} = \sum_i c_i \eta_i \tag{3}$$

where c_i is the concentration and η_i the viscosity of the i the molecular class, η_{sp} is at least a qualitative measure of the concentration of the high molecular substance, as the contribution of the low molecular components is small. A preparation of $(KPO_3)_n$ K14, has been investigated with regard to the stability at some different pH values. The concentration of the polymetaphosphate was in each case 0.5 %. The ionic strength was 0.3, 0.1 of which was from the buffer substances and 0.2 from NaCl. The results are represented by Fig. 3.

As is easily seen, the metaphosphate solutions are most stable at the neutral point; on the acid side of the pH scale the break-down seems to occur more rapidly than on the alkaline. However as the break-down may occur in many ways, it is of course very difficult to determine a velocity constant of definite meaning, especially as the substance is polydisperse from the beginning. The curves of Fig. 3 can be expressed algebraically in the following manner:

Citrate	buffer	pH = 2.0	$\eta_{sp} = 1.366 - 1.34\ t + 0.612\ t^2$
Acetat	»	pH = 4.2	$\eta_{sp} = 0.765 - 2.6 \cdot 10^{-3}\ t + 1.3 \cdot 10^{-3}\ t^2$
Phosphate	»	pH = 6.9	$\eta_{sp} = 1.191 - 1.7 \cdot 10^{-3}\ t$
Borax-soda	»	pH = 9.4	$\eta_{sp} = 1.024 - 1.2 \cdot 10^{-3}\ t$
Borax-NaOH	»	pH = 11.5	$\eta_{sp} = 0.958 - 35 \cdot 10^{-3}\ t + 1.4 \cdot 10^{-3}\ t^2$

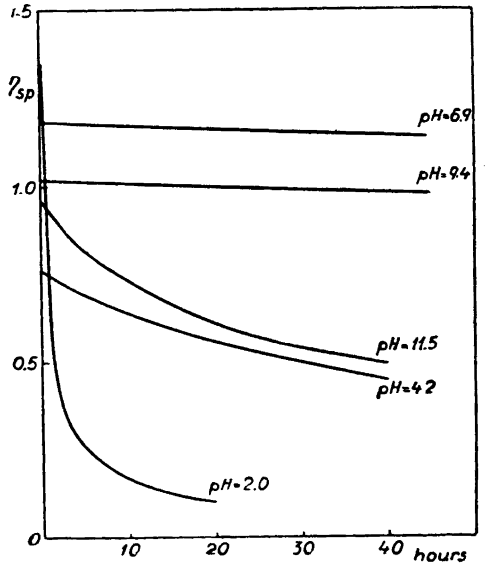


Fig. 3. The change of the specific viscosity with time at different pH-values.

In calculations of the coefficients special consideration has been given to low t-values (t is expressed in hours). The derivatives at $t = 0$, $\left(\frac{d\eta_{sp}}{dt}\right)_{t=0}$ are at these pH-values: -1.34, $-2.3 \cdot 10^{-3}$, $-1.7 \cdot 10^{-3}$, $-1.2 \cdot 10^{-3}$ and $35 \cdot 10^{-3}$ respectively; they may be considered at least as a qualitative measurement of the break-down at these pH values. The breakdown might occur at random. If for instance the PO_3-links were split off one after another, the amount of orthophosphate from hydrolysis would increase to a considerable concentration. By a method of analysis making possible the separation of meta- and orthophosphate is was proved that the orthophosphate formed by hydrolysis is scarcely present in measurable amounts [9, 10]. The orthophosphate can be determined in the colorimeter with ammonium molybdate and ascorbic acid at pH $\simeq$ 5 (acetate buffer) the presence of metaphosphate having no influence on the result.

ELECTROPHORESIS

As the colloidal properties of the polymetaphosphate are to be ascribed to the anion, it is of interest to know its mobility and charge, because these quantities are required for a theoretical treatment of the sedimentation and the diffusion. The electrophoretic measurements required for the calculation of the mobility, have been performed by observing the moving boundary

solution/solvent; the method has been worked out by Tiselius *et al.*[11, 12]. The mobility of the colloidal anion u, is expressed in cm^2/s volt (= the velocity in cm/s at the potentialgradient 1 volt/cm). The values of the descending and rising boundary usually agree. At low ionic strengths where differences may occur, u refers to the descending boundary which gives a more accurate value.

The dependence of the mobility on the electrolyte concentration

The measurements of viscosity have clearly shown hos dependent it is on the electrolyte concentration of the solution, and a quantity such as the electrophoretic mobility might be expected to be influenced in a similiar way which is in fact the case. The values of Table 3 refer to 0.50 % solutions of preparation K13.

Table 3. Dependence of the mobility on the medium.

Concentration of the low molecular electrolyte	$u \cdot 10^5$			
	NaCNS	NaCl	Na_2SO_4	$Na_3(PO_3)_3$
0.025 *N*	43	45		53
0.050 *N*	45	36	36	46
0.075 *N*	40			32
0.10 *N*	32	28	27	31
0.20 *N*	22	23	23	31
0.30 *N*	23	22		31
0.40 *N*	22	22	24	32
0.60 *N*			23	
0.80 *N*			23	
»limiting value»	22	22	23	31

In NaCNS, NaCl and Na_2SO_4 approximatively the same limiting value is obtained while that of $Na_3(PO_3)_3$ is considerably greater, which might be due to another state of solution in this medium for instance some kind of complex-formation.

As has been shown before, see *e. g.* Table 2, the viscosity of this polymetaphosphate decreases continuously until the salting-out point is reached, while on the other hand the mobility seems to approach a limiting value at a relatively low electrolyte concentration. A possible interpretation is that the ionic charge depends on the concentration of electrolyte in the solution only to a certain extent, whereas the solvation gradually decreases, until the substance is no longer soluble.

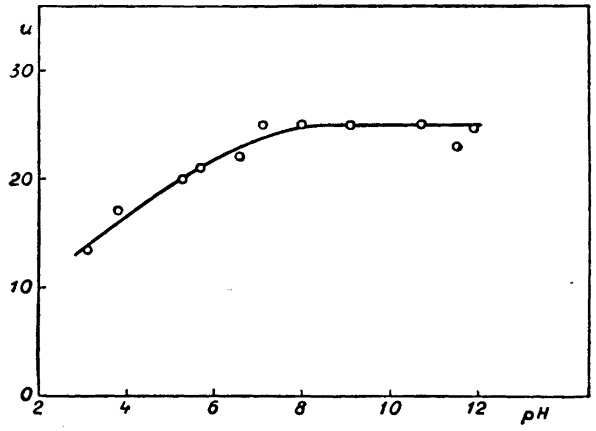


Fig. 4. The electrophoretic mobility as a function of pH.

The dependence of the mobility on the pH value

The dependence of the mobility on the pH of the solution is shown diagrammatically in Fig. 4. The preparation is K14; its concentration in all experiments 0.50 %. The ionic strength was 0.4, of which 0.1 is from the buffer substances and 0.3 from NaCl.

As is seen from Fig. 4 u is constant for basic and neutral reactions whereas the mobility decreases at low pH-values. In solutions more acid than pH $\simeq$ 3 it is difficult to make electrophoretic measurements because the break-down of the polymetaphosphate in acid medium appreciably affects the result. The polyacid, $(HPO_3)_n$ formally corresponding to these metaphosphates is rather strong*. Certainly the pH of a solution in neutral salts is about 6, but

* The strength of the acid has been estimated in the following manner: Suppose a metaphosphate $(KPO_3)_n$ is dissolved in a solution containing an excess of sodium ions. The process of solution is a permutit reaction resulting in practically all the potassium going into solution if $c_{Na} \gg c_K$. Assume furthermore that there is 1 »effective charge» in m PO_3-links. If we regard these n/m-valent ions as n/m molecules of the uni-univalent electrolyte Na $[Na_{m-1}(PO_3)_m]$ the following expression can be deduced for the dissociation equation of the corresponding acid, for the sake of brevity designed as HA.

$$\frac{C_{A^-}}{\frac{n}{m}C} = \frac{K_s}{C_{H^+} + K_s} \qquad (4)$$

K_s = the diss. constant of the acid; c = the conc. of the metaphosphate. Combining the equations at two different pH values (the quantities designed ′ and ″ resp.) yields:

$$\frac{m'\,c'_{A^-}}{m''\,C''_{A^-}} = \frac{C''_{H^+} + K_s}{C'_{H^+} + K_s} \qquad (5)$$

this slightly acid reaction is surely due to partial hydrolysis according to the brutto-formula

$$PO_3^- + H_2O \rightarrow H_2PO_4^-$$

Sedimentation

The sedimentation in the ultracentrifuge is also very dependent on the concentration of the low molecular electrolytes. The sedimentation constant s increases on increasing the electrolyte concentration without approaching a limiting value before the metaphopshate is salted out. The variations of the sedimentation constant in different media are shown in Fig. 5. The metaphosphate concentration was 0.50 % in all experiments; preparation K14.

As is seen in Fig. 5 the sedimentation constant at a given concentration of low molecular electrolyte is greatest in NaCl, the s-values diminish when the medium is changed to Na_2SO_4, $Na_3(PO_3)_3$ and $Na_4P_2O_7$ resp. At salt concentrations lower than 0.1 N the sedimentation curves show too great anomalies to be used for accurate calculations. However, it must be emphasized that on dialysing metaphosphate solutions of very low salt concentration against the buffer or water no dialysable metaphosphate has been found. It may therefore be assumed that the state of solution is colloidal even in a medium of low salt concentration.

This difference in the sedimentation constant between various media also remains at infinite dilution, see Fig. 6.

The effect of the low molecular salt on the sedimentation may be twofold. Partly that the presence of an electrolyte diminishes the solvation of the metaphosphate and therefor the frictional resistence on sedimentation, and partly that an excess of electrolyte contributes to decreasing the retarding influence of the charge effect on the sedimentation; the sedimentation constant s_1 of a colloidal ion being lower than would be the case if the particle carried no charge. A theoretical treatment of the subject has been given by Tiselius [13]. For a large excess of low molecular electrolyte the retarding influence of the charge effect is considerably diminished but not completely eliminated. For

---

On the assumption that the product $m \cdot c_{A^-}$ is proportional to the charge of the real polyanion and hence its mobility, Eq. (5) can be transformed to

$$\frac{u'}{u''} = \frac{C''_{H^+} + K_s}{C'_{H^+} + K_s} \tag{6}$$

From Eq. (6) the order of magnitude of K_s can be estimated to be 10^{-3}; the u-values put in Eq. (6) being chosen in the pH-range 3—5.

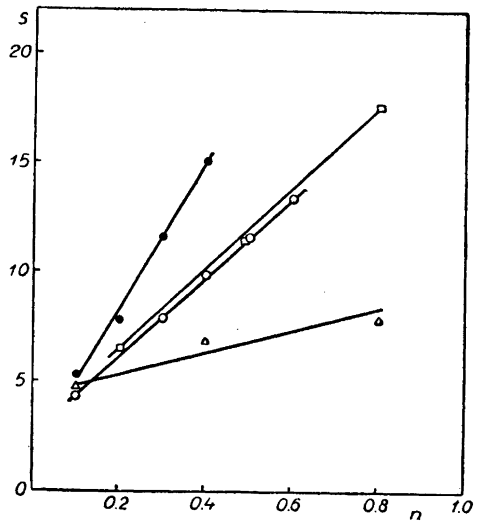


Fig. 5. The variation of s in electrolyte solution of different normalities.

● *NaCl;* □ Na_2SO_4
○ $Na_3(PO_3)_3$; △ $Na_4P_2O_7$

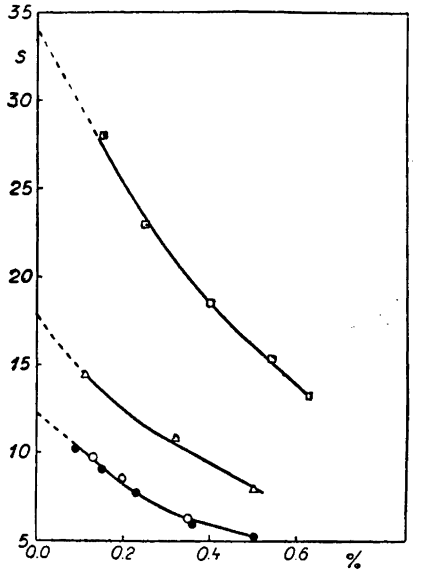


Fig. 6. The dependance of s on the polymetaphosphate concentration in some different electrolytes.

● *0,1-N NaCl;* ○ *0,2 N* $Na_4P_2O_7$
□ *0,4 N NaCl;* △ *0,8 N* $Na_4P_2O_7$

the sedimentation constant of a colloidal electrolyte A_nK in the presence of the salt AB ($C_{AB} \gg C_{A_nK}$) Lamm [14] has deduced the expression:

$$s_1 = \frac{M_1\,(1-V_1\varrho)\left[f_A + (1+\frac{nc_1}{c}\,f_B\right] - nM\,(1-V\varrho)\,f_A}{f_K\left[f_A + f_B\,(1+\frac{nc_1}{c})\right] + \frac{n^2c_1}{c}\cdot f_A\cdot f_B} \tag{7}$$

M_1 and M are the molecular weights of the colloid and the salt resp., V_1 and V the spec. volumes, c_1 and c the molar concentrations, a the density of the solution, f the frictional force on the ion indicated by the subscript and n the charge of the colloidal ion. If the sedimentation constant of the corresponding uncharged particle is s_K, we have:

$s_K = \frac{M_1\,(1-V_1\varrho)}{f_K}$. Assuming $f_A = f_B$ Eq. (7) can be written

$$\frac{s_1}{s_K} = \frac{1 - \frac{n}{2}\frac{M}{M_1}\cdot\frac{1-V\varrho}{1-V_1\varrho}}{1+\frac{n^2}{2}\cdot\frac{c_1}{c}\cdot f_A/f_K} \tag{8}$$

In 0.4 N NaCl solution, $(1 - V\varrho)$ is calculated to be 0.70; $(1 - V_1\varrho) = 0.61$. ($M = 58.5$.) Refering to preparation K 14 $c_1/c \simeq 10^{-5}$ in 0.5 % solutions and $M_1 = 1.3 \cdot 10^6$. On the assumption of free ionic mobility $nf_A/f_K = u/u_{Na}$, a ratio that might be of the order of 1/2. (u_{Na} is according to Landholt-Börnstein Tabellen $46 \cdot 10^{-5}$ cm²/s volt.) Putting these values in Eq. (8) we obtain

$$\frac{s_1}{s_k} = \frac{1 - 3n\ 10^{-5}}{1 + 3n\ 10^{-6}} \tag{9}$$

Assuming the molar frictional constant for the colloidal ion to have the same value in sedimentation as in electrophoretic motion the quantity n may be estimated. On this assumption the following expression for the number of effective charges, n_e, is obtained (Tiselius)

$$n_e = \frac{u\ M_1\ (1 - V_1\varrho)}{s_1\ F \cdot 10^7} \tag{10}$$

F = the Faraday const. The n-values calculated from this equation are collected in Table 4. The calculation is based on measurements in 0.4 N NaCl.

Table 4. The effective charge of some preparations in 0.4 N NaCl.

Preparation	Degree of polymerisation	$u \cdot 10^5$	n_e	Number of PO_3-groups per unit charge
K12	2400	17	26	90
K10	3900	16	28	140
K13	6600	22	38	175
K14	11000	25	53	210
K11	17000	15	64	260
K 8	19000	18	75	260

The mobility is, if not constant, at least rather independent of the degree of polymerization. This fact might be allowed for by assuming the charge per unit area of the molecules to be the same for all preparations in a given medium.

If the n_e-value of preparation K14 is put into Eq. (9) the charge effect appears to be less than 1 %. The errors of observation are certainly greater, especially as we deal with an unfractionated, polydisperse substance. With regard to the strong dependence of the sedimentation on the electrolyte concentration one might expect a considerably greater correction for the charge effect. Either Eq. (10) is not valid for these metaphosphates, *i. e.* the frictional

force on sedimentation and electrophoretic motion is not the same, or it must be assumed that the particle shape and eventually the size also vary with the salt concentration. One might also mention here the fact that s increases from 8 to 15 S (see Fig. 4) when the NaCl concentration increases from 0.2 to 0.4 N, the mobility being constant $\simeq$ 25 within this interval. However, if the charge effects can be omitted in 0.4 N NaCl, n must have very high values in solutions of lower electrolyte concentration. In 0.2 N NaCl n must amount to $\sim$ 15,000 to explain the decrease in the sedimentation constant from 15 to 8 S as a charge effect according to Eq. (9). However, this is impossible since the calculated number of PO_3-groups is less than 15,000.

Diffusion

The free diffusion is, like the sedimentation, very dependent on the electrolyte concentration. At very low salt concentrations the diffusion curves are skew and give a picture which is characteristic of swelling diffusion. At higher salt concentrations these anomalies disappear and the diffusion is more normal. Gralén [15] has worked out a method for the calculation of the diffusion coefficient at infinite dilution D_0, the diffusion being dependent on the concentration. In the formula

$$D_0 = D_m + \frac{A \cdot M_0}{4\ H \cdot t} \tag{11}$$

D_m is the diffusion coefficient calculated by the »moment» method, A the area bounded by the diffusion curve, H the height of the curve and M_0 the distance between the original boundary and the position of the peak after the time t.

However, the D_0-values determined for various media do not differ very much from each other after applying the correction term $\frac{A \cdot M_0}{4\ H \cdot t}$, see Table 5. Preparation K14, metaphosphate concentration 0.50 %. In comparison the diffusion coefficient calculated from the area of the curve D_A is also given; for a polydisperse substance $D_A < D_m$. D is given in 10^{-7} c. g. s.

Table 5. The diffusion coefficient in various media.

Medium	D_m	D_A	Correction	D_0
0.1 N NaCl	1.71	1.48	— 0.84	0.87
0.4 N NaCl	1.34	1.26	— 0.14	1.20
0.2 N $Na_4P_2O_7$	1.50	1.24	— 0.64	0.86
0.8 N $Na_4P_2O_7$	1.21	1.30	— 0.23	0.98

Fig. 7. Electron microscope picture of polymetaphosphate.

As illustrated in Table 5 the correction is considerable especially at low electrolyte concentrations. Of course the charge of the colloidal ion also influences the diffusion. The problem has been theoretically treated by Lamm [14], who for the diffusion coefficient D of the colloidal ion K^{n-} of the compound A_nK in the presence of the low molecular salt AB deduced the expression

$$D = \frac{RT}{f_k} \cdot \frac{1 + \frac{n^2 c_1}{c} \cdot \frac{f_B}{f_A + f_B}}{1 + \frac{n^2 c_1}{c} \cdot \frac{f_B}{f_A + f_B} \cdot \frac{f_A}{f_K}} \tag{12}$$

where the quantities have the meaning stated before. On the assumption that $f_A = f_B$ and $nf_A/f_K = u/u_{Na}$ the correction for diffusion in 0.4 N NaCl amounts to 1.5 %, which is much less than the value of Table 5. If the real n-value were greater than the value employed (53) the correction would increase rapidly with increasing n. However, the anomalies appearing on diffusion are probably due not only to charge effects but also to the fact that the substance is largely solvated; the swelling pressure might therefore be of importance.

Molecular weight

In Svedberg's formula for the molecular weight

$$M = \frac{s}{D} \cdot \frac{RT}{1 - V\varrho} \tag{13}$$

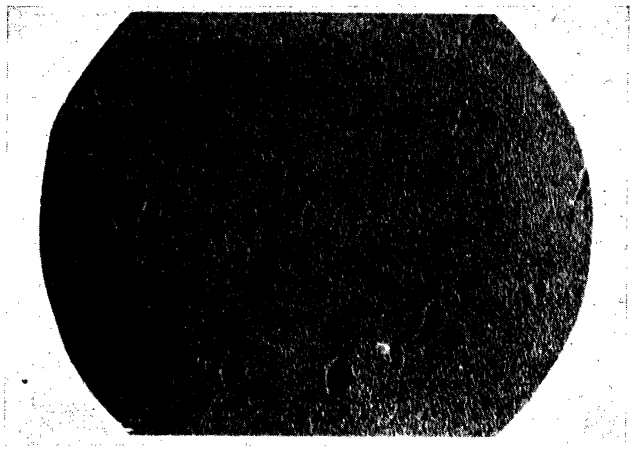


Fig. 8. Electron microscope picture of polymetaphosphate. The preparation is gold-shadowed to obtain a better contrast.

the ratio between the quantities s and D is involved. It appears that this ratio depends on the medium, see Table 6. The values refer to preparation K14, and the molecular weights to the original $(KPO_3)_n$.

Table 6. Dependence of molecular weight on the medium.

Medium	s_0	D_0	s_0/D_0	M
0.1 *N* NaCl	12	0.87	13.8	640,000
0.4 *N* NaCl	34	1.20	28.3	1,300,000
0.2 *N* $Na_4P_2O_7$	12	0.86	14.0	650,000
0.8 *N* $Na_4P_2O_7$	17	0.98	17.3	800,000

The calculated molecular weight is not constant but varies with the medium. Even if the true correction for the charge effect is considerably higher than the values estimated, this cannot account for the discrepancy between the molecular weights in different media, whence it might be inappropriate to speak of molecular weight in connection with these metaphosphates. Aggregation weight is probably a more correct expression, since all bonds cannot be of head valency character. However, even on this assumption the units held together by head valency bonds must be rather large. In spite of the breakdown of the polymetaphosphate by hydrolysis at weakly acid pH or by enzymes, the solutions still show colloidal properties and only very small amounts of orthophosphate are formed.

Polydispersity

A method of a calculating the polydispersity from the sedimentation curves has been worked out by Gralén [15]. By measuring the width of the curve, B, at successive distance from the centre of rotation, x, the quantity dB/dx is obtained, for which the value at infinite dilution is designated as $\left(\frac{dB}{dx}\right)_0$ By analogy with the assumption of Lansing and Kraemer [16] of a logarithmic distribution of the molecular weights a similar distribution might be assumed for the sedimentation constant

$$\frac{dc}{ds_0} = K_s \cdot e^{-\frac{1}{\gamma_s^2} \ln^2 \frac{s_1}{s_0}} \tag{14}$$

where K_s is the max. height of the distribution curve, γ_s the distribution coefficient of the sedimentation constant and s_1 the sedimentation constant at the max. point. Between $\left(\frac{dB}{dx}\right)_0$ and γ_s we have the relation

$$\left(\frac{dB}{dx}\right)_0 = \gamma_s \cdot \sqrt{\pi} \cdot e^{\gamma_s^2/4} \tag{15}$$

Assuming a logarithmic distribution of the diffusion coefficients

$$\frac{dc}{dD} = K_D \cdot e^{-\frac{1}{\gamma_D^2} \ln^2 \frac{D}{D_1}} \tag{16}$$

where K_D is the maximum height of the distribution curve and D_1 the diffusion coefficient at the maximum point of the curve, we can calculate the distribution coefficient γ_D from Eq. (17)

$$\frac{D_M}{D_A} = e^{3\gamma_D^2/8} \tag{17}$$

These calculations have been applied to part of the material. However, it must be emphasized that the accuracy is very dependent on the possibility of drawing correct base lines to the curves. The values collected in Table 7 are to be considered rather as a measure of the order of magnitude than as absolutely quantitative. The measurements refer to 0.4 N NaCNS as medium.

Table 7. γ_s- and γ_D-values of some preparations.

Preparation	γ_s	γ_D
K 8	0.12	0.36
K 13	0.10	0.44
K 14	0.17	0.40

The γ_s-values are all higher than the corresponding γ_D-values, probably partly depending on the fact that the diffusion measurements must be extended over several days and that the substance is not stable even at neutral pH. However, these distribution coefficients are still very low compared with those of other polydisperse systems. The following values are reported by Gralén [15]: for cellulose xanthate $\gamma_s = 0.3 - 0.6$ and $\gamma_D = 0.7 - 1.1$; for cellulose nitrate $\gamma_s = 0.1—0.7$ and $\gamma_D = 0.5—1.0$.

In fresh solutions these metaphosphates represent a system of rather low polydispersity in spite of their character of synthetic high polymers.

Molecular shape

Earlier attempts to determine the molecular shape by means of different methods have not given consistent results [2]. It might by expected that a direct measure of the molecular dimensions would be possible by means of the electron microscope. However, a solution of $(KPO_3)_n$ in NaCNS-solution, for instance, placed on to a thin film cannot simply be evaporated for insertion in the focal plane of the electron microscope. The excess of low molecular salt must first be dialyzed away or the metaphosphate would be covered with a salt crust. But when the salt has been dialyzed away the usual state of solution of the metaphosphate is greatly changed and, furthermore, the possibility of agglomeration at evaporation must be allowed for. Thus from the electron microscope pictures it is not possible to draw any valid conclusions concerning the shape and the dispersion of the polymetaphosphates in salt solutions. Fig. 7 shows a rather typical electron microscope picture. The form is irregular, round and rod-like particles are seen together with intermediate forms. Fig. 8 offers a more uncommon picture. Magnification 20,000 times. The pictures cannot represent exactly the molecules or aggregation of molecules as they are to be found in solution. The molecular weight of the preparation investigated is 1,300,000 in 0.4 *N* NaCNS. Thus a single molecule has the weight $1.3 \cdot 10^6 / 6.06 \cdot 10^{23} = 2.1 \cdot 10^{-18}$ g. Supposing the metaphosphate to be flattened on evaporation *in vacuo* the height h might be estimated by equating the weight

of a particle seen on the plate (area ~ 1—20 mm^2) and particle weight calculated above. (The density of the metaphosphate is 2.5). The h-values thus obtained are of the order of magnitude 0.1—1 Å, which values are evidently too low. However, if the electron microscope pictures represent aggregations of 10—100 molecules of the weight mentioned above, h might be estimated as several tens of Å's which is more probable. It is obvious, however, that the electron microscope pictures do not represent single molecules such as they are to be found in solution, where they may exist as »geknäuelte Molekeln», which should be treated statistically according to Kuhn.

Solvation

A method given by Sihtola [17] has been applied to the determination of the solvation. After a centrifuge run in a separation cell the buffer remaining in the upper part of the cell is more concentrated than the original buffer, because some water has followed the dissolved metaphosphate to the bottom of the cell. From these concentration differences determined by a refractometric method, the solvation is estimated. The values thus obtained, about 0.6 molecule of water per PO_3-group, are probably too low. (The values refer to 0.4 N NaCNS solution.) A co-sedimentation of low molecular electrolyte probably occurs, whence the measured concentration gradient is lower than if there were no co-sedimentation. The question of molecular shape and solvation must be left without a quantitative answer.

X-ray analysis

The synthesis of these polymetaphosphates is in principle the same for all preparations, *viz.* an intermolecular condensation, the degree of polymerisation being dependent on the temperature. Hence it might be assumed that the different preparations are rather alike with regard to structure, which is also confirmed by X-ray powder diagrams obtained by P. H. Lindgren in this institute. The measured spacings are in most cases the same within the errors of measurement although two preparations show interferences which are absent in the others. However, it must be remembered that the powder diagrams are also dependent on the granulation of the preparation, the time of exposure etc. The values of these measurements are collected in Table 8. The wave length of the radiation used, K_α-line of copper, is: $\lambda = 1.539$ A. Even if the preparations are, in the main, very alike these values do not exclude small differences.

Table 8. The spacings of some preparations.

Preparation	Spacing A					
K 12		3.58	3.29	3.00	2.61	2.19
K 9	6.27		3.38	3.07	2.70	2.26
K 14	6.63	4.02		3.14	2.58	2.35
K 8			3.31	3.03	2.63	2.22

SUMMARY

High molecular potassium metaphosphates, the so-called salts of Kurol, have been investigated with regard to their properties in solution.

It has appeared that viscosity, electrophoretic mobility, sedimentation and diffusion velocity are very dependent on the concentration of the low molecular electrolyte in the solutions.

The calculated molecular weights — or perhaps better aggregation weights — differ in various media and seem to increase with the concentration of the low molecular electrolyte in the solution.

The author desires to express his sincere thanks to Prof. The Svedberg for the privilege of carrying out this work in his laboratory.

REFERENCES

1. Malmgren, H., and Lamm, O. *Z. anorg. Chem.* **252** (1944) 255.
2. Pascal, P. *Bull. Soc. Chim. France* **33** (1923) 1611; **35** (1924) 1119, 1131.
3. Svedberg, T., and Pedersen, K. O. *The ultracentrifuge* Oxford (1940).
4. Lamm, O. *Nova Acta Regiae Soc. Sci. Upsaliensis IV* **10** (1937) no. 6.
5. Smoluchowski, M. v. *Koll.-Z.* **18** (1916) 190.
6. Krasny-Ergen, W. *Koll.-Z.* **74** (1936) 172.
7. Kern, W. *Z. physik. Chem.* **A181** (1938) 283.
8. Drucker, C. *Acta Chem. Scand.* **1** (1947) 221.
9. Ingelman, B., and Malmgren, H. *Acta Chem. Scand.* **1** (1947) 422.
10. Lowry, O., and Lopez, J. *J. Biol. Chem.* **162** (1946) 421.
11. Tiselius, A. *Nova Acta Regiae Soc. Sci. Upsaliensis IV* **7** (1930) no. 4.
12. Svensson, H. *Koll.-Z.* **87** (1939) 181.
13. Tiselius, A. *Koll.-Z.* **59** (1932) 306.
14. Lamm, O. *Arkiv Kemi, Mineral. Geol.* **17A** (1944) no. 6.
15. Gralén, N. *Sedimentation and diffusion measurement on cellulose and cellulose derivatives* Uppsala (1944).
16. Lansing, W. D., and Kraemer, E. O. *J. Am. Chem. Soc.* **57** (1935) 1369.
17. Sihtola, H. Unpublished.

Received January 22, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 166—176

Studies Related to Pristane

II. The Methyl-Octadecanes

JÖRGINE STENE SÖRENSEN and NILS ANDREAS SÖRENSEN

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

In the first communication in this series [1] it was shown that the saturated hydrocarbon pristane found in different fish liver oils is — in contrast to previous statements in literature — at least a nona-decane. As there were very few comparison substances in the nona-decane-series we decided to synthesize all the eight hitherto unknown mono methyl-octadecanes. All these syntheses, which will be found in the experimental part, are of a routine character, the only difficulty being to procure starting materials of great purity, the value of the entire work resting on the purity of the hydrocarbons obtained.

As the purity of a saturated hydrocarbon is difficult to ascertain by elementary analysis, we preferred to refine the final hydrocarbons with 100 % H_2SO_4 followed by redistillation over metallic sodium to constant physical properties (n_D^{20} and d_4^{20}), taking care that the refractive and dispersion constants were in accordance with the standard values of a saturated hydrocarbon. The dispersion was calculated by a method worked out by one of us [2]. We have been satisfied when $\lambda_{calc.}$ differs by less than 5 ÅU from the standard value for saturated hydrocarbons, *viz.* 885.2 ÅU.

Table 1. Physical constants of the x-methyl-octadecanols-x.

x	M. p.	d_4^{20}	n_D^{20}	$R_{D,\,obs.}$ ($R_{D,\,calc.} = 91.463$)	$R_{\lambda=\infty\,obs.}$ ($R_{\lambda=\infty\,calc.} = 89.386$)	λ_0 ÅU
3	+ 32—32.5°	—	—	—	—	—
4	+ 16°	0.8394	1.45192	91.36	89.32	880.1
5	+ 6.5—7.0°	0.83745	1.45080	91.38	89.335	881.3
6	— 23°	0.8385	1.45103	91.305	89.26	882.1
9	— 23°	0.8355	1.45085	91.60	89.54	883.5

All but one of the methyl-octadecanes — the 2-methyl-octadecane, which was synthesized according to Wurz — were prepared via the tertiary alcohols. Five of these tertiary alcohols were saturated, their constants are given in Table 1.

The two remaining carbinols were both prepared from Δ^{10}-undecenoic acid, and were used for the further synthesis as methyl-octadecene-17 ols. They were both liquids, m. p. below — 80°, their constants are given in Table 2.

Table 2. Physical constants of the x-methyl-octadecene-17-ols-x.

x	d_4^{20}	n_D^{20}	$R_{D,\ obs.}$ ($R_{D,\ calc.}$ = 90.996)
7	0.8470	1.4561	90.62
8	0.84296	1.4565	91.12

By splitting off water from the tertiary carbinols, three different ethylenes may arise; all, however, giving the same saturated hydrocarbon. Because of this uncertainty we have not designated any particular position to the ethylene bond. Table 3 gives the ethylenes from the carbinols in Table 1; Table 4 the dienes from the unsaturated alcohols in Table 2.

Table 3. Physical constants of the x-methyl-octadecenes.

x	d_4^{20}	n_D^{20}	$R_{D,\ obs.}$ ($R_{D,\ calc.}$ = 89,471)
3	0.8011	1.4490	89.16
4	0.7973	1.44975	89.72
5	0.7964	1.4489	89.67
6	0.7962	1.4494	89.78
9	0.7978	1.4487	89.48

Table 4. Physical constants of the x-methyl-octadeca-dienes (17,-).

x	d_4^{20}	n_D^{20}	$R_{D,\ obs.}$ ($R_{D,\ calc.}$ = 89,00)
7	0.8081	1.4553	88.79
8	0.8048	1.4557	89.21

Finally, Table 5 gives the eight theoretical mono-methyl-octadecanes. As these saturated hydrocarbons could be much more thoroughly purified than the intermediate compounds, the agreement between the constants of these 8 compounds is much better than between the different isomeric alcohols and olefines. The 2-methyl-octadecane, prepared by Wurz-synthesis, shows the greatest deviations, and although its m. p. + 12.5—13.2° fits in very well between the values + 5° for 2-methyl-heptadecane [3] and + 18.3° for 2-methyl-nonadecane [4], we are convinced that our preparation of 2-methyl-octadecane is not perfectly pure. The low values shown by the constants of the 2-methyl-compounds are, however, in accordance with the regularities which the petroleum chemists have worked out, chiefly on the basis of the physical constants of the lower parafines [5].

Table 5. Physical constants of the eight mono-methyl-octadecanes.

x	M. p.	d_4^{20}	n_D^{20}	$R_{D,\ obs.}$ ($R_{D,\ calc.}$ = 89,938)	$R_{\lambda=\infty,\ obs.}$ ($R_{\lambda=\infty,\ calc.}$ = 87,877)	λ_0 ÅU
2	+ 12.5—13.2°	0.77935	1.43707	90.212	88.146	892
3	+ 0.5°	0.7871	1.44110	90.039	88.005	885.4
4	— 1.0°	0.78607	1.44081	90.105	88.075	883.6
5	— 14— — 13°	0.78595	1.44067	90.094	88.071	884.25
6	— 4.0°	0.78589	1.44065	90.0975	88.067	884.25
7	— 16.5— — 16°	0.7871	1.44125	90.065	88.006	889.56
8	— 10°	0.78595	1.44098	90.149	88.109	887.07
9	— 16.5°	0.78555	1.44055	90.119	88.087	884.1

The general formula given by Calingaert *et. al.*[5] gives the following values for 2-, 3- and 4-methyl-octadecane:

	d_4^{20} calc. according to Calingaert [5]
2-Methyl-	0.78317
3- » -	0.78770
4- » -	0.78689

Our 2-methyl-compound shows far too great a depression, most likely due to lack of homogeneity in our preparation. The agreement is fairly good in the 3 and 4 series. Calingaert *et. al.* have given no formula for a-more central location of the methyl group. As will be seen from Table 5, the physical constants remain remarkably uniform from 4-methyl onwards to 9-methyl.

As to the problem of the constitution of pristane, it will be seen that of the physical constants of pure pristane (*cf.* Sörensen and Mehlum [1]),

$d_4^{20} = 0.7827—0.7843$; $n_D^{20} = 1.4385—1.4398$, $R_D = 90.08—90.12$,

the density and refractive indices of pristane are intermediate between the values for 2-methyl-octadecane and the remaining seven methyl-octadecanes. The molecular refraction is in complete accordance with the values found for the eight methyl octadecanes. As will be seen, however, all the methyl-octadecanes *have m. p. between — 16.5 and + 13°, whereas pristane does not solidify at — 80°*. We conclude that whether its molecular formula is $C_{19}H_{40}$ or $C_{20}H_{42}$, pristane will have to be more branched than a monomethylderivative. So, to some extent, the physical constants of the synthesized monomethyl-octadecanes increase the possibility that pristane is terpenoid in structure.

EXPERIMENTAL

2-Methyl-octadecane

From pure cetyl alcohol from spermaceti, m. p. 48—49°, cetyliodide was prepared by the method given in *Org. Syntheses 15* p. 29. From iso-propyliodide and cetyliodide the 2-methyl-octadecane was prepared by the standard Wurz-procedure.

Table 6. Dispersion of 2-methyl-octadecane.

$d_4^{20} = 0.7793_5$; $\lambda_0 = 892$ *ÅU*, $R_{\lambda=\infty} = 88.146$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.43447	89.744	89.748
5895.9	1.43707	90.212	90.214
5875.7	1.43717	90.230	90.226
5790.7	1.43761	90.309	90.289
5460.7	1.43897	90.553	90.563
5015.6	1.44173	91.048	91.027
4921.9	1.44240	91.168	91.141
4713.1	1.44396	91.447	91.422
4471.48	1.44597	91.806	91.801
4358.3	1.44704	91.997	92.001

3-Methyl-octadecane

Pure palmitic acid was converted into palmitoyl chloride with $SOCl_2$ (*cf.* Hann and Jamieson [6]). After destillation the chloride was poured under vigorous stirring into excess diluted ammonia. The palmitoyl amide was dried and crystallized from alcohol, m. p. 104—105°. Palmitoyl amide was converted into palmitonitrile with $SOCl_2$ according to Oldham and Ubbelohde [7]. Further, the palmitonitrile was transformed into methyl *n*-pentadecyl ketone with CH_3MgI according to the same authors; m. p. of this ketone 48—49°.

3-Methyl-octadecanol-3

To 2.4 g methyl *n*-pentadecyl ketone in abs. ether was slowly added the theoretical amount of C_2H_5MgBr in ether, the reaction mixture was left at room temperature for 15 hours and worked up in the usual way. B. p. in air bath 103—105°/0.001 mm.

$C_{19}H_{40}O$ (284.4)	Calc.	C 80.21	H 14.17
	Found	» 79.9	» 14.4

From 3-methyl-octadecanol-3 water was split off with $KHSO_4$-K_2SO_4, 1 hour at 150°. B. p. for this ethylene 87° in air bath/0.001 mm. It was redistilled over Na. The observed density agrees well with Eijkman's [8] $d^{13.4} = 0.8044$.

The ethylene was hydrogenated to 3-methyl-octadecane with PtO_2 (Adams) without solvent at 90—100° for 2 hours and refined as described above, b. p. 85—87° in air bath at 0.001 mm.

Table 7. Dispersion of 3-methyl-octadecane.

$d_4^{20} = 0.7871$, $\lambda_0 = 885.4$ ÅU, $R_{\lambda=\infty} = 88.005$.

λ	n_λ^{20}	R_λ obs.	R_λ calc.
6678.1	1.43849	89.575	89.579
5895.8	1.44110	90.039	90.035
5875.7	1.44119	90.055	90.050
5460.7	1.44300	90.376	90.381
5015.6	1.44571	90.855	90.836
4471.48	1.44989	91.593	91.596
4358.3	1.45096	91.781	91.793

4-Methyl-octadecane

From pure myristic acid *n*-tetradecanol-1 or myristyl alcohol was prepared as in *Org. Syntheses* **10** p. 62. The alcohol was converted into myristyl bromide by a procedure analogous to that given in *Org. Syntheses* **1** p. 7 for laurylbromide. 11.2 g myristyl bromide and 0.98 g Mg in abs. ether was heated until it had dissolved and slowly reacted at 10° with 3.2 g methyl *n*-propyl ketone (prepared according to Meerwein [9]), the carbinol 4-methyl-octadecanol-4, was worked up in the usual way.

$C_{19}H_{40}O$ (284.4)	Calc.	C 80.21	H 14.17
	Found	» 79.94	» 14.55

Table 8. Dispersion of 4-methyl-octadecanol-4.

$d_4^{20} = 0.8394$, $\lambda_0 = 880.11$ ÅU, $R_{\lambda=\infty} = 89.324$.

λ	n_λ^{20}	R_λ obs.	R_λ calc.
6678.1	1.44926	90.897	90.903
5895.8	1.45192	91.362	91.360
5875.7	1.45201	91.378	91.374
5460.7	1.45387	91.703	91.706
5015.6	1.45662	92.183	92.162
4471.48	1.46083	92.915	92.924
4358.3	1.46195	93.110	93.121

4-Methyl-octadecane was prepared from the carbinol in the same way as the 3-methyl-compound.

Table 9. Dispersion of 4-methyl-octadecane.

$d_4^{20} = 0.78607$; $\lambda_0 = 883.6$ ÅU; $R_{\lambda=\infty} = 88.078$.

λ	n_λ^{20}	$R_{\lambda,\ obs.}$	$R_{\lambda\ calc.}$
6678.1	1.43819	89.639	89.647
5895.8	1.44081	90.105	90.102
5875.7	1.44090	90.121	90.116
5460.7	1.44271	90.443	90.446
5015.6	1.44539	90.918	90.899
4471.48	1.44953	91.649	91.657
4358.3	1.45062_5	91.842_5	91.853

5-Methyl-octadecane

By the same procedure as that used in the synthesis of methyl *n*-pentadecyl ketone from palmitic acid, pure myristic acid was transformed into methyl *n*-tridecyl ketone m. p. 39°. This ketone in abs. ether (temperature below 10° C) was added slowly to the theoretical amount of $CH_3CH_2CH_2$-CH_2-Mg-Br, and 5-methyl-octadecanol-5 was worked up as usual.

$C_{19}H_{40}O$ (284.4) Calc. C 80.21 H 14.17
Found » 80.21 » 14.21

Table 10. Dispersion of 5-methyl-octadecanol-5.

$d_4^{20} = 0.83745$, $\lambda_0 = 881.31$ ÅU, $R_{\lambda=\infty} = 89.335$.

λ	n_λ^{20}	$R_{\lambda,\ obs.}$	$R_{\lambda,\ calc.}$
6678.1	1.44816	90.915	90.918
5895.8	1.45080	91.379	91.377
5875.7	1.45090	91.397	91.391
5460.7	1.45273	91.717	91.724
5015.6	1.45550	92.202	92.181
4471.48	1.45974	92.942	92.946
4358.3	1.46082	93.130	93.147

5-methyl-octadecane was prepared from the carbinol in the usual way.

Table 11. Dispersion of 5-methyl-octadecane.

$d_4^{20} = 0.78595$, $\lambda_0 = 884.25$ ÅU, $R_{\lambda=\infty} = 88.071$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.43802	89.623	89.639
5895.8	1.44067	90.094	90.092
5875.7	1.44076	90.110	90.107
5460.7	1.44256	90.430	90.436
5015.6	1.44525	90.906_5	90.889
4471.48	1.44940	91.640	91.645
4358.3	1.45048	91.831	91.842

6-Methyl-octadecane

Lauryl alcohol and lauryl bromide were prepared from pure lauric acid by the methods given in *Org. Syntheses* and used above for myristic acid. Ethyl *n*-butylacetoacetate was prepared as in *Org. Syntheses 7* p. 36, and decomposed to methyl *n*-amyl ketone according to Meerwein [9]. 18 g lauryl bromide in a few ml ether was Grignardized with 1.7 g magnesium. The methyl *n*-amyl ketone dissolved in ether was, as usual, added slowly at a temperature below 10°.
6-methyl octadecanol-6 was worked up in the usual way.

$C_{19}H_{40}O$ (284.4) Calc. C 80.21 H 14.17
Found » 80.30, 80.02 » 14.50, 14.23

Table 12. Dispersion of 6-methyl-octadecanol-6.

$d_4^{20} = 0.8385$, $\lambda_0 = 882.15$ ÅU $R_{\lambda=\infty} = 89.261$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.44838	90.840	90.846
5895.8	1.45103	91.305	91.305
5875.7	1.45118	91.331	91.319
5460.7	1.45297	91.644	91.653
5015.6	1.45582	92.142	92.110
4471.48	1.46000	92.871	92.876
4358.3	1.46105	93.053	93.074

The 6-methyl-octadecane was prepared in the usual way from the tertiary carbinol.

Table 13. Dispersion of 6-methyl-octadecane.

$d_4^{20} = 0.78589$, $\lambda_0 = 884.25$ ÅU, $R_{\lambda=\infty} = 88.067$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.43805	89.635	89.639
5895.8	1.44065	90.097_5	90.093_5
5875.7	1.44074	90.113	90.108
5460.7	1.44253	90.431	90.438
5015.6	1.44527	90.917	90.892
4471.48	1.44939	91.646	91.651
4358.3	1.45045	91.833	91.848

7-Methyl-octadecane

Methyl *n*-hexyl ketone was synthezised according to Newman and Booth jr.[10] by slowly adding an etheral solution of CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-Mg-Br to a mixture of acetic anhydride and abs. ether at — 75— — 80°, and stirring at this temperature for two hours. B. p. methyl *n*-hexylketone 172°.

From castor oil Δ^{10}-*n*-undecenoic acid was prepared according to Jones and Pyman[11]. The recrystallized acid was transformed into the ethyl ester by means of abs. C_2H_5OH + HCl at 70° C.

B. p. 134—138°/13 mm. $n_D^{20} = 1.4377$, $d_4^{20} = 0.8768$

$M_{D,\text{ obs.}} = 63.49$ $M_{D,\text{ calc.}} = 63.42$. After the abovementioned Bouveault-Blanc reduction the Δ^{10}-undecenoic acid ethyl ester was transformed into Δ^{10} undecenol-1. B. p. 133°/16 mm, $n_D^{20} = 1.4509$.

The 1-brom-undecene-10 was prepared from Δ^{10} undecenol-1 by the procedure given by Karrer and Helfenstein[12] for the preparation of farnesylbromide from farnesol. 1-Brom-undecene-10 distilled at 63° in air bath at 0.001 mm.

$$n_D^{20} = 1.4692, \quad d_4^{20} = 1.083 \quad M_{D,\text{ obs.}} = 59.96, \quad M_{D,\text{ calc.}} = 60.29$$

15 g 1-bromundecene-10 in 15 ml abs. ether was Grignardized with 1.55 g Mg, and methyl *n*-hexyl ketone in ether added slowly at below + 10°. 7-Methyl-octadecene-17-ol-7 was isolated by fractional distillation at 0.001, as — through some side reaction — it was originaly contaminated by some hydrocarbon with high boiling point.

$C_{19}H_{38}O$ (282.4)	Calc.	C 80.73	H 13.57
	Found	» 81.00	» 13.76

7-Methyl-octadecene-17-ol-7 was transformed into 7-methyl-octadecane in the usual way.

Table 14. Dispersion of 7-methyl-octadecane.

$d_4^{20} = 0.7871$, $\lambda_0 = 889.5$ ÅU, $R_{\lambda=\infty} = 88.006$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda\text{ calc.}}$
6678.1	1.43859	89.593	89.595
5895.8	1.44125	90.065	90.056
5875.7	1.44135	90.083	90.070
5460.7	1.44317	90.405_5	90.405
5015.6	1.44587	90.883	90.864
4471.48	1.45002	91.616	91.633
4358.3	1.45116	91.816_5	91.831

8-Methyl-octadecane

Δ^{10}-*n*-Undecenoic acid was transformed through the acid chloride and the amide into Δ^{10}-*n*-undeceno nitrile in the same way as described for the synthesis of 3-methyl-octadecane.

Δ^{10}-*n*-undeceno nitrile. B. p. 135/15 mm, $n_D^{20} = 1.4442$, $d_4^{20} = 0.8443$. By the usual Grignards synthesis Δ^{10}-*n*-undeceno nitrile was transformed into 2-keto-*n*-dodecene-11. B. p. 120°/15 mm. M. p. + 2.5. This ketone has been prepared by Polgar and Robinson [13], by the action of undecenoyl chloride on methylzinc iodide, they stated the b. p. as 114—115°/9 mm.

5.8 g 2-keto-*n*-dodecene-11 was slowly added to 6.7 g CH_3-$(CH_2)_5$-CH_2-Mg-Br in a little ether, and the tertiary carbinol worked up as above.

$C_{19}H_{38}O$ (282.4)	Calc.	C 80.735	H 13.57
	Found	» 80.16,80.36	» 13.52,13.49

The 8-methyl-octadecane was prepared from 8-methyl-octadecene-17-ol-8 as usual.

Table 15. Dispersion of 8-methyl-octadecane.

$d_4^{20} = 0.78595$, $\lambda_0 = 887.07$ ÅU, $R_{\lambda=\infty} = 88.109$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.43838	89.687	89.692
5895.8	1.44098	90.149	90.149
5875.7	1.44110	90.171	90.164
5460.7	1.44291	90.492	90.497
5015.6	1.44563	90.974	90.954
4471.48	1.44983	91.716	91.719
4358.3	1.45089	91.903	91.917

9-Methyl-octadecane

Methyl *n*-nonyl ketone was isolated from rue oil by distillation and recrystallization. *n*-Octanol was prepared from caprylic acid ethyl ester according to the above-mentioned method of Bouveault-Blanc. Further, 1-brom-*n*-octane according to Org. Syntheses 1 p. 7. 9-Methyl-octadecanol-9 was prepared after the standard Grignard procedure from 1-brom-*n*-octane and methyl-*n*-nonyl-ketone.

$C_{19}H_{40}O$ (284.4) Calc. C 80.21 H 14.17
Found » 79.93,79.76 » 14.16,14.16

Table 16. Dispersion of 9-methyl-octadecanol-9.

$d_4^{20} = 0.8355$, $\lambda_0 = 883.5$ ÅU, $R_{\lambda=\infty} = 89.542$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda\text{ calc.}}$
6678.1	1.44814	91.124	91.137
5895.8	1.45085	91.601	91.599
5875.7	1.45099	91.626	91.613
5460.7	1.45281	91.945	91.948
5015.6	1.45559	92.433	92.409
4471.48	1.45981	93.171	93.179
4358.3	1.46092	93.365	93.379

The 9-methyl-octadecane was prepared from 9-methyl-octadecanol-9 in the usual way.

Table 17. Dispersion of 9-methyl-octadecane.

$d_4^{20} = 0.78555$, $\lambda_0 = 884.1$ ÅU, $R_{\lambda=\infty} = 88.087$.

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.43791	89.649	89.658
5895.8	1.44055	90.119	90.113
5875.7	1.44062	90.131	90.128
5460.7	1.44245	90.456	90.458
5015.6	1.44513_5	90.932	90.912
4471.48	1.44932	91.673	91.671
4358.3	1.45033	91.851	91.868

SUMMARY

For comparison with pristane the eight monomethyloctadecanes were synthezised. The densities and refractive indices of the methyloctadecanes approached very closely to those of pristane, the m. p. however fell between

— 16.5° and + 13.2° for the methyloctadecanes as against $<-87°$ for pristane. As intermediate compounds in the synthesis of the methyloctadecanes the tertiary alcohols. 3-, 4-, 5-, 6- and 9-methyloctadecanols and the tertiary alcohols 7- and 8-methyloctadecene-17-ols were prepared. The physical constants including the dispersions of the new compounds are given.

REFERENCES

1. Sörensen, N. A., and Mehlum, J. *Acta Chem. Scand.* **2** (1948) 140.
2. Sörensen, N. A. *Ann.* **546** (1940) 57.
3. Landa, I., and Riedl, R. *Collection Czechoslov. Chem. Communs* **2** (1930) 520.
4. Mc. Kittrick, D. S. *J. Inst. Petroleum Technol.* **23** (1937) 630.
5. Calingaert, G. *Ind. Eng. Chem.* **33** (1941) 103.
6. Hann, R. M., and Jamieson, G. S. *J. Am. Chem. Soc.* **50** (1928) 1443.
7. Oldham, J. W. H., and Ubbelohde, A. R. *J. Chem. Soc.* (1939) 202.
8. Eijkman, C. *Recherches refractometriques* Harlem (1919).
9. Meerwein, H. *Ann.* **398** (1913) 249.
10. Newman, M. S., and Booth, jr., W. T. *J. Am. Chem. Soc.* **67** (1945) 154.
11. Jones, E. C. S., and Pyman, F. L. *J. Chem. Soc.* **127** (1925) 2596.
12. Karrer, P., and Helfenstein, A. *Helv. Chim. Acta* **14** (1931) 78.
13. Polgar, N., and Robinson, R. *J. Chem. Soc.* (1945) 394.

Received January 17, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 177—191

On the Crystal Structure of Silicon Carbide and its Content of Impurities

DICK LUNDQVIST

Elektrovärmeinstitutet, Stockholm, Sweden

The crystal structure of silicon carbide presents a pronounced peculiarity which has hitherto been observed in only a few other substances. It appears in a number of closely related crystal fomrs, which do not, however, constitute different modifications. Similar cases are met with in, for example, the tridymite [1], parisite and coquimbite [2]. We have in silicon carbide the formation of a special series of superstructures. The difference in lattice energy between these structures is definitely very small, and it has not been possible to detect any chemical or physical differences between them indicating that they belong to different modifications or phases.

The most common forms were investigated by Baumhauer [3] when he first studied the morphology of silicon carbide. He named the phenomenon polytypy. The old-established names of the common forms also date from him; he called them types I, II and III in turn, as he investigated them. The so-called amorphous silicon carbide, formerly named type IV, occupies a special position, and Thibault [2] has recently proposed the name β-SiC to distinguish it from all the other types which come under the name α-SiC.

This β-SiC is formed at a lower temperature than α-SiC, and has a structure characteristically different from the other types. I may, therefore, be justifiable to distinguish this form as a separate modification. However, as the use of the prefixes α and β may possibly cause some misunderstanding, since here they are applied in the reverse order from what is customary in phase diagrams, we shall call them here by more explicit names, the cubic (»amorphous») silicon carbide being called the *low temperature modification.* All the other types then belong to the *high temperature modification.*

The low temperature modification is normally formed at 1800—1950° C, but under special conditions, it may also be formed at temperatures down to

1200—1300° C [4]. At 1950—2200° C it is monotropically transformed into the high temperature modification, mainly via the gaseous phase.

The low temperature modification crystallizes in a diamond lattice. All the types of the high temperature modification constitute mean-stages between a diamond and a wurtzite lattice, but a pure wurtzite lattice is never formed.

The structures of types I, II and III were established by Ott [5], who also observed a new type called V, the structure of which has recently been calculated by Zhdanov and Minervina [6] and Ramsdell [7]. Ott, however, assumed that each type represented a separate modification and the long established denotions mod. I, mod. II, etc., are due to him. Later on Thibault [2], Ramsdell [7] and Zhdanov and Minervina [6] reported the appearance of further types, so that at present the number of closely investigated types is 7 or 8. It is, however, necessary to remember that only types I, II and III (and the low temperature modification) appear normally. The other types have only been observed in unique specimens. For a full account of these types, the reader must be referred to the literature. A complete survey of the range up to 1944 is to be found in Thibault [2].

In the following discussion we will avoid dividing the structures into cubic, hexagonal and rhombohedral systems as has often been done. Instead, we will describe all the types starting from their hexagonal cells. Then all the types have the same a-axis, whereas the length of the *c*-axis varies with the number of layers which characterizes the type in question. Each kind of atom is tetrahedrally coordinated by the other kind. The tetrahedrons may be stacked with successive layers, parallel or anti-parallel to each other.

Until now, very little has been known as to the reason for the development of certain characteristic sequences of alternating cubic and hexagonal closest packing in the silicon carbide structures. It is true that Baumhauer [3] could state that *green* crystals were characteristic of type II, *yellow* crystals of type I and that the *black* crystals were most frequently built up of intimately coalescent domains of types I, II and III. Some investigators have, on the whole, confirmed Baumhauer's observations, but Thibault [2], who has recently investigated a great number of silicon carbides, has not been able to find such a correlation for the American products. The other types are very rarely observed.

Zhdanov and Minervina [6] have assumed that the appearance of the different types is caused kinetically or by impurities, but no systematic comparisons between X-ray crystallographic investigations and chemical or spectro-chemical analyses are to be found anywhere in the literature. For a closer understandig of the connection and differences between the structure types it seemed, however, necessary to find if there exists some correlation between the types and the content of impurities in the silicon carbide.

A comprehensive investigation on the chemical and physical properties of silicon carbide has been in progress at the Electrothermal Institute since 1945. In these investigations we have considerad it especially important that *all* the samples of silicon carbide obtained should be studied by *all* the methods developed and the results systematically correlated. The results of the correlated series of spectrochemical analyses* and X-ray crystallographic investigations** are given below. The results of the remaining investigations will soon appear in a technical publication *(Acta Polytechnica)*.

EXPERIMENTS

The X-ray analyses were carried out by the powder method with focussing cameras of Bohlin-Phragmén type. Most of the photograms used for identifying the structural types were taken with cameras with dispersion angle $\Theta = 29°—57°$. For precision determination of the cell dimensions only cameras with dispersion angle 47°—82° were used. The wavelengths of the Cr-radiation used were taken as $K\alpha_1 = 2.289\ 62$ Å. U. and $K\alpha_2 = 2.293\ 52$ Å. U. The preparations had to be moved during the exposures, otherwise the lines became spotty.

The spectrochemical analyses have been made with a continuous d. c. arc between coal electrodes. The spectrograms have been taken in a large Hilger spectrograph with quartz optic. Standard No. 112 Silicon Carbide from the National Bureau of Standards has been used as the standard preparation. All the preparations have been crushed in an agate mortar to approximately the same grain size as the standard. The samples were taken from grains of 0.30—0.25 mm of the preparations, which had been carefully washed in hydrocloric acid.

The greater part of the spectrochemical analysis has comprised only the elements Al, Fe, Mn and Mg. In addition to these elements, the analysis of the standard also included Ti, Zr and Ca. Preparatory qualitative spectrochemical analyses have also shown the presence of V, Cr, Ni, Sn and Pb.

A preliminary investigation showed, however, that only the first-mentioned elements were present in such percentages that it was necessary to determine them in the preparations now studied.

Systematic determinations of the *specific weight* of the different products and *microscopical investigations* have also been carried out in connection with the analyses and X-ray investigations. The microscopical investigation comprised a study of the structure of natural crystals and cut specimens as well as a determination of the relative content of grains of different colours in crushed products of different grain-sizes.

A large number of silicon carbide products of Swedish, Norwegian and American origin have been examined. It has been considered of great importance to extend the investigation to comprise as many qualities and materials with as different an origin, prior

---

* The spectrochemical analyses have been performed at the Metallographic Institute by G. Carlsson and P. Spiegelberg to whom the author is much indebted.

** The X-ray photographs have been made at the Institute of Inorganic and Physical Chemistry at the University of Stockholm by S. Sahlquist and H. Larsson to whom many thanks are due.

treatment and content of impurities as possible. In several cases the normal commercial products have been supplemented by specially synthesized preparations. In all, about 200 products have been studied.

In most cases the preparations have been taken from crushed silicon carbide of the same origin and with the same grain-size (0.30—0.25 mm). The analyses obtained have been representative of a large quantity of silicon carbide — well mixed. This procedure has been necessary in view of the connection between these investigations and the others that have been carried on at the same time. By this procedure, however, it has not been possible to find the characteristic variations and their connections with the same accuracy as would have been the case if it had been possible to take only single homogeneous crystals for the X-ray investigations and the spectrochemical analyses. As large and homogeneous crystals, however, only constitute a minor part of the yield of a silicon carbide furnace, this inconvenience has been thought fully compensated by the advantage of a broader survey. In addition, the individual crystals are not usually homogeneous, but contain zones with different crystal types and probably with different content of impurities.

RESULTS

The common structure types, normally called types I, II and III, have appeared regularly in the X-ray photograms. No certain observations have been made of the types VI, V, IV, etc. which are reported in the literature in unique specimens. Usually the low-temperature modification does not appear in the commercial products. In most photographs, however, *extra reflexions* have appeared. The positions of these extra reflexions have varied from preparation to preparation. Only a few of them have reappeared in more than two different photograms. It has not been possible to identify the lines with any of the reflexions that might be expected to originate from the different conceivable impurities. A reasonable explanation is, therefore, that we are dealing here with effects of the same phenomenon that give rise to the appearance of the types I, II, III, etc. We have found many new types out of the great number of varieties that may be assumed to appear. In several cases it has been possible to calculate the probable axis lengths of these types. As the well-mixed material may always contain several new types at the same time, no special regard has been given to the calculations, which have been founded on only a few reflexions from each special photogram, and which are, therefore, quite uncertain.

Connection between the structure types and the colours of the crystals

Even at an early stage it was possible to confirm that a connection existed between the different colours of the grains and the structure types appearing in them. Preliminary indications in this direction were also in fact observed

during an investigation made in 1943—44 by N. Schrewelius*, who assumed the type II to be characteristic of white crystals. The relative content of the common types I, II and III in differently coloured preparations is listed in Table 1. The values given are means of three sets of preparations that have been separated from two different Swedish and one American product. The contents are estimated visually from the powder photographs.

Table 1. Relative content of structure types I, II and III in differently coloured fractions of silicon carbide products. Means of three sets of preparations.

Type	White light green	Green dark green	Yellow yellow-green	Blue-grey blue-green	Black half-opaque	Opaque
II	100	70	10	45	30	20
I	0	25	85	40	40	10
III	0	5	5	15	30	70

It was thus possible to state that type II seemed to be characteristic of pure white and green silicon carbide (free or nearly free from observable impurities). Type I seemed to appear most rich in yellow and yellow-green crystals, (in a moderately impure material) and type III seemed above all to characterize a more highly impure black product. This product has been called opaque-black, on account of its colour and appearance in the microscope, as compared with the half-opaque products which are more translucent in thin laminae and where the black colour seems largely to originate from a content of small particles of graphite. (See further below.)

The lattice dimensions

For a clear understanding of the causes of the appearance of the different types, it seemed of special importance to establish as accurately as possible the appearance of any characteristic variations in the lattice constants. No such variations are stated in the literature, but earlier comparisons have mainly dealt with possible variations in the *c*-axis in the same type, or have been carried out with cameras with a limited resolving power.

To get an elucidating comparison between the different types, it is necessary to investigate the relations between the smallest fundamental distance

* Private communication.

along the c-axis of the different types, *i. e.* the distance between the silicon layers.

To achieve the best possible precision in this respect, two preparations were exposed in the same camera and on the same film one after the other. The one preparation was of a very pure silicon carbide (Al and Fe less than 0.01 %) which contained exclusively type II, but for a few weak extra lines. The other preparation was of a very impure silicon carbide (Al 0.30 %, Fe 0.07 %) which contained only type III and a little of type I.

The lattice dimensions were:

Type II	a	$= 3.0800 \pm 0.0002$ Å. U.	
	c	$= 15.098 \pm 0.001$ Å. U.	
	$c/6$	$= 2.5164 \pm 0.0002$ Å. U.	
	Si-C	$= 1.8861$ Å. U.	
	$\sigma_{calc.}$	$= 3.213$	$\sigma_{obs.} = 3.209 + 0.001$
Type III	a	$= 3.0800 \pm 0.0002$ Å. U.	
	c	$= 10.081 \pm 0.001$ Å. U.	
	$c/4$	$= 2.5202 \pm 0.0002$ Å. U.	
	$\sigma_{calc.}$	$= 3.208$	$\sigma_{obs.} = 3.201 + 0.005$
Type I	a	$= 3.0800$ Å. U.	
	c	$= 37.817$ Å. U.	
	$c/15$	$= 2.521 (\pm 0.001)$ Å. U.	

At the transition from type II to type III (I), the silicon layers thus increase their distance from 2.5164 to 2.5202 Å. U. or 0.15 %. The observed specific weight of type II agrees well with the theoretical value, whereas the observed specific weights of the black opaque silicon carbides, rich in type III, show slightly greater deviations owing to their content of foreign matter. It is of importance to note that the reflexions in most cases have been very sharp. No general broadening of the lines is observed. Reflexions common to all types are, however, split up in a number of closely spaced fine lines when more than one type is present.

The spectrochemical analyses

For the spectrochemical analyses 60 preparations were selected from the total of about 200 preparations investigated by X-rays. The analyses showed great variations in the content of Al, smaller variations in the Fe content and very small in Mg and Mn. Systematic comparisons between the analyses and the structure types of the samples showed clearly a close connection between

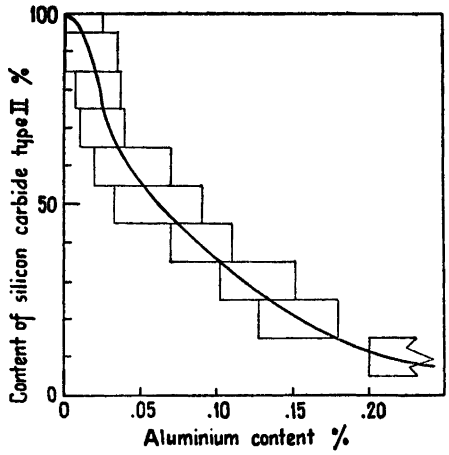


Figure 1. Correlation between the aluminium content of silicon carbide and its relative content of type II as estimated visually from the powder photograms. The relative uncertainty of the values is indicated by the rectangles.

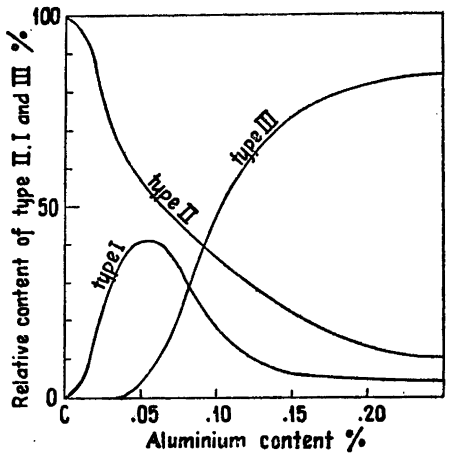


Figure 2. Correlation between the aluminium content and the relative content of the three main types of silicon carbide.

the Al content and the relative content of structure types III and I. This is illustrated in Fig. 1, where the Al content is plotted against the relative content of type II as estimated visually from the powder photograms. The relative uncertainty of the measurements is indicated by the size of the rectangles. In Fig. 2 the connection between the Al content and the relative content of all the three main types is drawn in an idealized form.

No such correlation could be observed between the structure types and the content of Fe, Mg or Mn. It must, however, be pointed out that the Al content in all the samples, with only a few exceptions, was larger than the content of any other impurity. In a few cases the Fe content was considerably higher than the Al content, but not even in these cases was it possible to find any transition of the structure types to type III with increasing Fe content.

Thus, silicon carbide when free from impurities, crystallizes in type II. With increasing content of impurities, above all of aluminium, there appears a transition to type I, the relative content of which has a maximum at about 0.05—0.06 % Al. At still increasing percentage of Al the transition proceeds to type III which begins to dominate at about 0.10 % Al. Samples with more than 0.20 % Al have 70—90 % of type III.

Even at very high contents of Al, however, the preparations still have appreciable percentages of type II (and I). If we want to explain the type

transition by the Al-atoms entering and causing the periodic re-arranging of the lattice, some difficulties arise at first as to how to explain why a lattice which is evidently saturated with Al does not crystallize exclusively in type III.

As mentioned above, however, the samples investigated were not homogeneous. They were prepared as representative samples from large quantities of crushed silicon carbide. Certainly in most cases the primary product came from the same silicon carbide furnace, and frequently from a very narrow range in this furnace. The average analysis should thus be highly characteristic of the silicon carbide in question. Accidental local variations in the concentration of the impurities in the raw material, as well as variations in the running of the furnace, will, however, cause small variations in the content of impurities from point to point in the product. Even large single crystals often show an irregular distribution of their impurities, resulting in differently coloured zones. Consequently, slightly impure material will always appear even in a mass of highly impure silicon carbide. At many points there will also be an excess of impurities and then other phases will appear. Analyses of differently coloured preparations, which have been separated from the same sample (under the microscope), have also shown that the impurities are unevenly distributed in the material.

Microscopic observations

The almost colourless or green crystals (which are of type II) when observed in the microscope show very little inclusions, and the very few inclusions observed are evidently carbon particles that have not reacted completely (Fig. 3). The yellow, yellow-green and black crystals, on the other hand, show considerable contents of inclusions. In the yellow (yellow-green) crystals, these inclusions are very finely dispersed (Fig. 4).

In black, half-opaque crystals, which have a higher but still relatively low content of impurities (Al = 0.10—0.15 %), the concentration of inclusions is very high, but the particles are small and the largest of them have the appearance of graphite laminae. When studying such a crystal with increasing amplification, an ever increasing number of smaller and smaller particles is observed (Fig. 5). Most suitable for this study is a thin rough plane crystal. At the same time the colour of the crystal becomes blue and increasingly lighter. Thus the black colour is evidently caused by the great number of more or less finely dispersed foreign particles in the crystal.

In black opaque silicon carbides with still higher Al contents (more than 0.20 %), and with high Fe contents, greater particles appear, the forms of

Figure 3. Green silicon carbide (× 150). Plane rough crystal. Only a few inclusions are observed. The limits of the crystal layers are observed as black streaks.

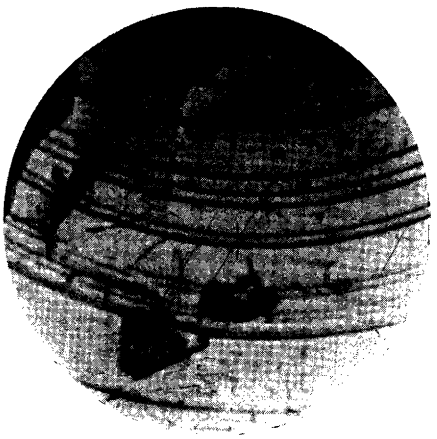


Figure 4. Yellow silicon carbide (× 150). Plane rough crystal. Slightly more opaque than green silicon carbide and containing more inclusions.

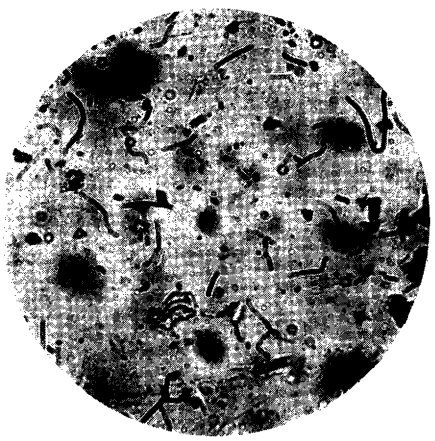


Figure 5. Black, half-opaque silicon carbide (× 150). Thin plane rough crystal. The number of small inclusions is large.

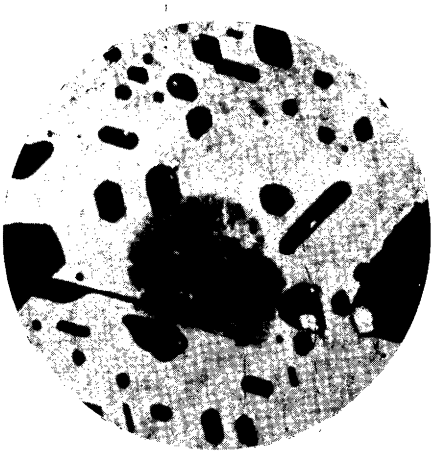


Figure 6. Black, opaque silicon carbide (× 150). Ground specimen. Two phases appear. The black, often pseudo-hexagonal, particles evidently are carbides, silicides or ternary compounds of iron and aluminium. Notice the cloud at the centre of the figure.

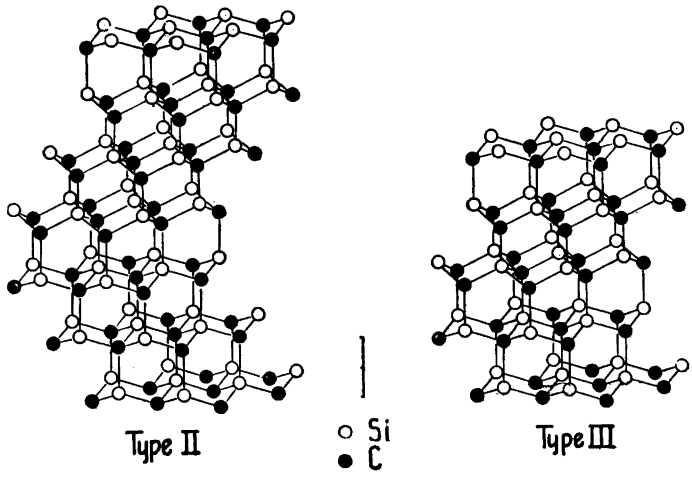


Figure 7. Structures of silicon carbide types II and III according to H. Ott.

which indicate that they were in a molten state when the crystals were formed, and that they were enclosed in cavities with hexagonal faces, thus obviously forming hexagonal crystals (Fig. 5). A direct comparison between a green and a black opaque rough crystal also indicates that the microscopical structure of the black crystal is strongly disturbed even at points where no foreign particles can be observed directly.

These observations, like the results from the X-ray investigation, indicate that the solubility of foreign atoms (*e. g.* Al) in the lattice of SiC is very small. It may be that the solubility is slightly greater at the elevated temperatures where the silicon carbide is formed, and that the Al-rich phase that appears on cooling causes strong strains in the crystal.

DISCUSSION OF THE RESULTS

We have found that type II is characteristic of pure silicon carbide and that type III is characteristic of silicon carbide saturated with aluminium, type I of the high-temperature modification evidently forming a mean-stage. For a clear understanding of the connection between all the types appearing, we must consider their crystal structures. Fig. 7 gives the structures for silicon carbide types II and III, according to Ott.

It is possible to describe the similarity, as well as the differences, between the types in a number of ways, all of which show more or less clearly the system according to which the variations appear. It seems most suitable to regard the

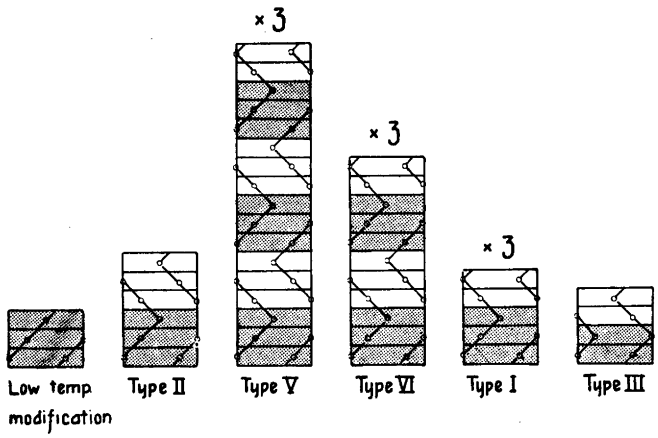


Figure 8. Schematic representation of silicon carbide types.

structure as a close-packed lattice of silicon atoms which is expanded a little by the carbon atoms, but we could also describe it as a close-packed lattice of carbon atoms expanded by the silicon atoms, or as a close-packed lattice of the Si-C-tetrahedrons.

In the low temperature modification the silicon atoms form a cubic closest packing. If we start from this as the fundamental type, we will come to all the realized and conceivable types by permitting a silicon layer to move with regular intervals on to a position above the two preceeding layers which belongs to a hexagonal closest packing. In a way we could, therefore, regard all the types of the high temperature modification as super-structures of the low temperature modification.

Now the low temperature modification crystallizes in a diamond lattice, whereas the layers of all the types of the high temperature modification may be grouped together in »diamond»-lamellae. These lamellae are stacked along the c-axis and each of them is rotated 180° about the c-axis relatively to the preceeding layer. It seems further to be a rule that the number of layers in each lamella is generally only 2 or 3. The only known exception is type IV where the numbers are 3 and 4. In Fig. 8 some of the silicon carbide types are shown schematically. The open rectangles represent the layers in a diamond lamella with all the Si-C-tetrahedrons orientated the same way, while the tetrahedrons in the layers represented by the dashed rectangles are antiparallel to the first set. The zigzag lines indicate the positions of the corresponding atoms in the different layers. (Compare Fig. 7.)

It is possible that the diamond lattice of the low temperature modification of silicon carbide is connected with the lowest free lattice energy. When the temperature rises and the thermal movements of the lattice increase, however, the growth of the diamond lattice is checked after every third layer, giving rise to type II. When the lattice is saturated with aluminium as in type III, the diamond lamellae will only be developed to 2 layers. At intermediat aluminium contents, there will now and then appear the regular alternation of 2 and 3 layer lamellae, which will give rise to the more rare types (IV, V, etc.). The probability of the formation of type I is evidently rather large under these circumstances.

Zhdanov and Minervina [6], who have discussed some of the silicon carbide structures in a similar manner, have chosen to represent the structures by numerical symbols only, in accordance with a system developed by Belov [8]. If we adopt their system, slightly modifying it by applying a dash above each other number to symbolise the different orientation of neighbouring lamealle, we obtain a very condensed representation of the types (Table 2). A similar system has also been developed by Ramsdell [7].

Table 2. Numerical symbols of the different types of silicon carbide.

Modification	Type	Hexagonal c-axis	Symbol
Low temp.		7.55 Å.U.	$\infty\,\bar{3}$
High temp.	II	15.098	$3.\bar{3}$
» »	V	128.3	$(3.\bar{3}.3.\bar{3}.3.\bar{2}) \times 3$
» »	VI	82.94	$(3.\bar{3}.3.\bar{2}) \times 3$
» »	IV	52.78	$(4.\bar{3}) \times 3$
» »	I	37.817	$(3.\bar{2}) \times 3$
» »	III	10.081	$2.\bar{2}$

Naturally a number of more complicated sequences will be possible, but the probability of their formation will be small. It is evident that type I, and the other more rare types of the same modification (IV, V, VI), may be looked upon as mean-stages between types II and III, or as coalescenses of them — or fractions of them — along the *c*-axis with a fortuitous, but in the special case, strictly regular sequence. In this connection we may remind the reader of the fact that the black crystals most often contain all three types intimately coalescing along the *c*-axis.

In a way, the phenomenon offers an analogy to the conditions of pure cobalt, which have been investigated by Lipson and Edvards [9] and Wilson [10].

In cobalt, however, there appears a *statistical* alternation of the cubic and hexagonal closest packed lattices of the two modifications of cobalt, while in silicon carbide the alternation is evidently not statistical, but strictly systematic over sufficiently long periods, as we have observed no broadening of the X-ray reflexions.

As has been shown above, Al is the only one of the impurities hitherto studied that has been observed to give a type transition and a perceivable expansion of the lattice. It is interesting to connect this fact with the great resemblance between the structures of SiC and that of the aluminium carbide Al_4C_3.

According to Stackelberg and Schnorrenberg [11], who established the latter structure, it is however more convenient to look upon it as a close-packed lattice of C^{4-} ions which is rather strongly deformed by Al^{3+} ions. The bonds in Al_4C_3 have clearly a more ionic character than the pure homopolar bond of SiC. It is thus highly conceivable that an Al-atom (ion) may be substituted for a Si-atom in SiC, but that it will at the same time introduce a strain and a tendency to deform the silicon carbide lattice. A local excess of Al must in turn result in the formation of another phase, *e. g.* Al_4C_3, possibly with some Si substituted for Al.

The pure SiC is a non-conductor of electricity. As Al has one valence electron less than Si, every Al-atom substituted for a Si-atom will give rise to a disturbing centre in the lattice, which will make the crystal a semi-conductor of the electron defect type. (A compensating subtraction of C-atoms would no doubt complicate the case.). Now the black silicon carbides (with their high aluminium contents) really *are* electron defect semi-conductors, and Busch and Labhart [12] have shown by measurements of the Halleffect that the concentration of disturbing centres is rather high. (Their highest values approximately correspond to an Al-concentration of 0.10 %.)

In many silicon carbide products the iron content seems to play an important rôle. Evidently the iron can cause no typetransitions, but in most cases it seems to be the Fe content that confers on thegreen silicon carbide its colour. The green crystals, which have an iron content equal to or even higher than the aluminium content, are semi-conductors of mainly excess electron type. Busch and Labhart have found the maximum concentration of disturbing centres in green SiC to be about a tenth of that of the black types. Probably the iron is still less soluble in the SiC lattice than aluminium, on account of the metallic character of the bonds in the iron carbide. Even with relatively low iron contents, the main part of the iron may be in colloidal dispersion. With increasing Fe content the main part is separated as discrete, molten particles

of iron carbides, silicides or ternary compounds, inside or on the surface of the crystal.

Naturally it is highly conceivable that a lot of other impurities could enter the silicon carbide lattice and disturb it more or less. In the first place this would be true of the other members of the fourth and third groups of the periodic system. It has been observed that boron is rather soluble in the lattice, but in this case it may be a question of an addition compound or of a substitution for the C-atoms.

Hitherto it has not been possible to investigate systematically the different possibilities in this respect on account of the difficulties of making in a laboratory reliable syntheses which would give products formed under almost the same conditions as in a large furnace, but with a better specified composition. Experiments in this direction are, however, planned.

Finally, we must remember that some silicon carbide products may also contain material with a deficit of Si or C in the lattice, which would also cause both lattice defects and semi-conducting power. It would, however, be very difficult to establish such defects.

SUMMARY

In connection with a general investigation on the properties of silicon carbide, extensive X-ray studies and spectrochemical analyses have been carried out. The material has also been studied by densitometric and microscopical methods.

It is suggested that, to avoid confusion, the cubic, »amorphous», silicon carbide (the β SiC of Thibault) be called the low temperature modification and that all the other types (the α-SiC of Thibault) be called the high temperature modification.

It is confirmed that different coloured silicon carbides are characterized by different structure types, only the common types I, II and III being considered.

A great number of extra reflexions with varying positions have been observed in the X-ray powder photographs, indicating the appearance of a great number of hitherto unknown types.

Lattice dimensions are given. The lattice is observed to expand along the c-axis by 0.15 % at the transition from type II to type III.

The spectrochemical analyses show that there is an obvious connection between the aluminium content and the structural types. Type II is characteristic of the pure SiC, type I of a SiC with moderate amounts of aluminium and type III of a SiC saturated with aluminium. No such connection is found

for other elements studied, *viz.* Mg, Mn, Fe. The percentages of other common impurities have been very low in the samples studied.

The results of the X-ray investigation and of the spectrochemical analyses are supported by the densitometric and microscopical investigations.

The bearing of the results is further discussed with reference to the close resemblance between the structure of SiC and Al_4C_3 and to the semi-conducting power of SiC.

This work has been made with financial support from the *Allmänna Svenska Elektriska AB.* and from the *Höganäs-Billesholms AB.* The latter concern has furnished most of the silicon carbide samples and kindly offered valuable information.

The author is much indebted to the director of this institute, Dr. B. Stålhane, who has discussed the work critically throughout and stimulated its publication.

Special thanks are due to Dr. N. Schrewelius, who has taken great interest in this investigation and done much to facilitate the work.

REFERENCES

1. Arnfelt, H. *Svensk Kem. Tid.* **53** (1941) 73.
2. Thibault, N. W. *Am. Mineral.* **29** (1944) 249, 327; **31** (1946) 512.
3. Baumhauer, H. *Z. Krist.* **50** (1912) 33; **55** (1915) 249.
4. Iley, R., and Riley, H. L. *Nature* **160** (1947) 468.
5. Ott, H. *Z. Krist.* **61** (1925) 515; **62** (1925) 201; **63** (1926) 1; *Sommerfeld-Festschrift* Leipzig (1928) 208.
6. Zhdanov, G. S., and Minervina, Z. V. *J. Phys. U.S.S.R.* **9** (1945) 244; *Acta Physicochim U.S.S.R.* **20** (1945) 3, 386; *Compt. rend. Acad. Sci. U.S.S.R.* **48** (1945) 182.
7. Ramsdell, L. S. *Am. Mineral.* **29** (1944) 431; **30** (1945) 519; **32** (1947) 64.
8. Belov, N. V. Thesis. Inst. of Gen. and Inorg. Ch. Moscow (1943) *see Zhdanov.*
9. Lipson, H., and Edwards, A. *Proc. Roy. Soc. London* **A 180** (1942) 268.
10. Wilson, A. J. C. *Proc. Roy. Soc. London* **A 180** (1942) 277.
11. v. Stackelberg, M., and Schnorrenberg, E. *Z. physik. Chem.* **B 27** (1934) 37.
    v. Stackelberg, M. *Z. physik. Chem.* **B 27** (1934) 53.
12. Busch, G., and Labhart, H. *Helv. Phys. Acta.* **19** (1946) 230.

Received January 21, 1948.

Short Communication

An Antibiotic Agent from *Marasmius graminum*

GERD BENDZ

Chemical Institute, University of Uppsala, Sveden

It has earlier been reported that species of the genus *Marasmius* produce substances inhibiting the growth of *Staphylococcus aureus* [1]. A chemical investigation of the culture medium from *Marasmius graminum*, obtained from the Institute of Physiological Botany, University of Uppsala, has shown that this substrate contains at least two different agents active against *Staphylococcus aureus;* one of these is volatile with steam *in vacuo* and has been obtained in a crystalline form. By distilling 8 litres of culture medium *in vacuo*, with a bath temperature of 25° C, a yellow aqueous distillate showing activity was collected in the receiver. The distillation was continued while maintaining the volume in the distillation flask constant by the addition of water, until the distillate was colourless and did not show any activity. The active principle was then extracted from this distillate with small portions of ether or chloroform; the solvent was dried over anhydrous sodium sulphate and removed under reduced pressure, leaving a red residue of 0.1 g of crude material melting at 70—74° C. This substance, which can be recrystallized from water or ligroin giving red crystals with a sharp melting point of 84.5° C, is sparingly soluble in water yielding a yellow solution, which turns violet if alkali is added. The solution inhibits the growth of *Staphylococcus aureus* and certain other pathogenic bacteria *in vitro*.

A detailed report will appear later.

1. Melin, E., Wikén, T., and Öblom, K. *Nature* **159** (1947) 840.

Received July 6, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 193—203

Utilisation des Cellules Multiplicateurs d'Electrons en Spectrographie Raman. I

PER-OLOF KINELL et PHILIPPE TRAYNARD

Institut de Chimie-Physique, Université d'Upsal, Suède

L'apparition des cellules multiplicateurs d'électrons est récente, et, dès le début, elle a suscité un grand nombre de travaux. Les spectroscopistes ont été les premiers à s'en emparer, heureux de pouvoir remplacer la plaque photographique par un nouveau récepteur qui convenait particulièrement bien pour les mesures des faibles intensités (grossièrement, celles qui peuvent donner une image photographique en moins de deux heures).

En analyse spectrochimique minérale, les travaux de Boettner [1], Diecke [2] et leurs collaborateurs ont conduit Saunderson [3] et coll. à donner la description d'un spectrographe à réseau donnant directement la teneur en un constituant donné d'un alliage déterminé. En spectrographie Raman, les travaux que Rank et son école [4, 5, 6] mènent depuis 1942 sont très importants et ont abouti dernièrement à la publication [7] de 172 enregistrements de spectres Raman d'hydrocarbures. En dehors de ces deux grandes applications on a essayé aussi d'appliquer les cellules multiplicateurs d'électrons en radioactivité [8], en astronomie [9,10], et en roentgenographie [11].

Comme on le voit, le domaine d'utilisation est très grand et, bien que la sensibilité des cellules soit rapidement limitée vers le rouge, on peut penser que ce domaine ira encore en s'aggrandissant, tellement, ces cellules offrent d'avantages en comparaison de la plaque photographique.

Dans les travaux spectrographiques on peut distinguer trois façons principales d'examiner un spectre avec une cellule:

1) emploi d'un spectrographe à réseau. C'est la solution de Rank [4–7]. Elle est la plus simple. En effet, la cellule peut être fixe, l'examen se faisant simplement, par suite de la focalisation automatique, en tournant le réseau. On évite ainsi, au maximum, les complications mécaniques;

2) emploi d'un spectrographe à prisme. On peut envisager deux solutions: a) le travail que l'on a à faire est toujours le même (par exemple, analyse d'un alliage déterminé). Il semble alors que le plus simple soit une solution analogue à celle de Saunderson [3] qui emploie autant de cellules qu'il y a de raies à examiner. Ces cellules sont fixées une fois pour toutes, mais, évidement, cela exige que l'alliage ne soit pas trop riche en constituants sinon on se heurte à un sérieux problème d'encombrement. b) On ne veut examiner qu'une petite portion du spectre et l'on peut se ramener à la solution de Diecke [12] qui emploie des cellules semi-fixes.

Le but de cet article est de combler une lacune et de décrire un spectrographe à prisme dans lequel toute l'étendue du spectre est examinée avec une seule cellule. (Une telle solution a, peut être, été réalisée par Chien et Bender [13] mais ils ne donnent aucun détail.) Nous avons appliqué ce spectrographe à l'étude de l'effet Raman, et, pour cela, nous avons été amené à construire un organe d'illumination, dérivé de celui de Rank [5], mais beaucoup plus puissant.

CARACTERISTIQUES GÉNÉRALES DES CELLULES MULTIPLICATEURS D'ÉLECTRONS

Par suite de la guerre, puis des difficultés d'importation, les cellules multiplicateurs d'électrons sont peu connues, aussi croyons-nous utile de donner ici les caractéristiques principales de ces nouvelles lampes, cependant que pour plus de détails on pourra se rapporter à l'article d'Engstrom [14].

Une cellule multiplicateur d'électrons se compose comme une cellule ordinaire d'une couche sensible à base de Césium d'où le flux lumineux arrache des électrons. Alors que dans les cellules ordinaires les électrons sont immédiatement collectés par une plaque, ici, ils sont accélérés et dirigés sur une électrode auxiliaire appelée dynode, portée à un potentiel positif par rapport à la couche sensible. Sur cette première dynode les électrons rebondissent en arrachant d'autres électrons (phénomène de l'émission secondaire) et l'ensemble est à nouveau accéléré et dirigé sur une seconde dynode où le processus se renouvelle.

A chaque passage sur une dynode le nombre des électrons croit donc en progression géométrique. Le résultat final est une amplification d'environ 10^6 lorsque, comme c'est le cas pour les tubes RCA, on utilise 9 dynodes et une différence de potentiel de 100 volts par dynode. Le facteur d'amplification croit d'ailleurs extrêmement rapidement avec le voltage et, au voisinage de 100 volts, une variation de 10 volts par étage permet approximativement de le doubler.

Il s'en suit que si nous voulons avoir un courant total stable à 1 % il faut que le voltage soit stable à environ 1 ‰. On a donc besoin d'une source de voltage continu extrêmement stable. C'est là un inconvénient de ces nouvelles cellules.

SENSIBILITÉ SPECTRALE

On peut trouver dans le commerce quatre sortes de tubes qui se rattachent à trois régions spectrales principales, qui s'étendent de 3000 à 6500 et 7500 Å pour les types 931 A, 1P21 et 1P22 RCA et de 2000 (enveloppe en verre spécial) à 6500 Å pour le type 1P28 RCA. Pour les trois types le maximum de sensibilité se trouve vers 3500—4000 Å, mais l'extension vers le rouge du domaine de sensibilité n'a pu être réalisé qu'au dépend de la sensibilité générale, si bien que le tube 1P22 ne peut être employé que pour des flux lumineux relativement intenses. Au contraire, les tubes 931 A et 1P21 sont très sensibles et surtout sont particulièrement stables, c'est à dire que la probabilité pour en trouver un de stable dans un lot donné est relativement assez élevée (20 %), car dans l'état actuel de la fabrication on ne peut être certain de la parfaite identité de tous les tubes.

FATIGUE

Bien que dans notre travail nous n'ayons pas eu, par suite de l'extrême faiblesse des flux lumineux, à nous inquiéter de la fatigue des cellules, c'est à dire de la variation avec le temps du courant photoélectrique lorsque, à la fois, l'excitation lumineuse et le voltage sont maintenus constants, nous croyons utile de donner ici quelques indications à ce sujet.

Chaque cellule possède à cet égard une conduite spéciale et, seules, quelques considérations générales qualitatives peuvent être données.

Toutes les cellules se fatiguent assez rapidement lorsque l'intensité du courant débité dépasse dix micro-ampères, et la baisse de courant constatée peut atteindre 30 % pour des intensités plus fortes après une heure de fonctionnement. Un repos prolongé, à l'obscurité, permet à la cellule de retrouver sa sensibilité première, mais, si le courant qui a provoqué la fatigue a dépassé un milliampère la sensibilité initiale ne peut être retrouvée qu'après plusieurs jours de repos. Ainsi que l'ont montré Marshall et coll. [11], la fatigue ne provient pas d'une baisse des propriétés de la couche sensible, mais d'une diminution de l'émission secondaire sur les dernières dynodes.

Pour remédier à cet inconvénient, il est recommandé de n'utiliser les cellules qu'avec des intensités de l'ordre du micro-ampère et même moins si cela est possible. Dans l'appareil que nous avons conçu les courants sont de l'ordre de 0,1 micro-ampère et, par suite, la fatigue est extrêmement faible; mais il peut

se trouver en analyse spectrochimique des courants assez forts et alors la solution de Saunderson, utilisant une lampe pour chaque raie, semble être la meilleure car elle conduit aux temps d'examen les moins longs.

COURANT DE FOND

En l'absence de toute lumière les cellules multiplicateurs d'électrons fournissent tout de même un courant dont l'existence est extrêmement gênante pour la mesure des petites intensités lumineuses. Ce courant est généralement de l'ordre de 10^{-8} à 10^{-7} ampère et provient de deux sources: 1) fuites ohmiques et 2) émission thermoionique de la couche sensible, amplifiée par le tube comme un courant photoélectrique ordinaire.

Les fuites ohmiques peuvent être internes à la cellule et sont dues, alors, à des traces de métal déposées sur le verre ou à toute autre cause qui réduirait l'isolement, mais, surtout elles sont dues à la résistance trop faible du diélectrique qui forme le culot de la lampe. Nous avons constaté que cette résistance est d'environ 4000 megohms, ceci permet d'expliquer un courant de fuite de l'ordre de 10^{-8} ampère.

Nons avons essayé de supprimer le culot de la lampe et nous avons réussi, de cette facon, à annuler presque complèment le courant de fond jusque vers un voltage d'alimention de 50 volts par étage, valeur pour laquelle apparait le courant d'origine thermoionique qui croit, ensuite, très rapidement. Cependant, ce qui est le plus gênant ce n'est pas tant l'existence de ce courant que les fluctuations qui l'accompagnent et qui limitent pratiquement la sensibilité.

Si l'on essaie de pousser le tube au delà de 120 volts par étage, il apparait rapidement un courant de fond intense et tout à fait instable qui est du aux phénomènes d'ionisation accompagnés peut être de fluorescence de la paroi de verre.

Toutes ces raisons jumelées font que le pouvoir d'amplification de la cellule dans des conditions normales de travail se tient généralement aux environs de 10^6 avec 100 à 120 volts de différence de potentiel par étage.

BRUIT DE FOND

Dans les conditions normales d'utilisation, nous venons de le voir, le courant de fond est surtout d'origine thermoionique. Ce courant n'est donc pas stable, mais fluctue autour d'une valeur moyenne avec une »pseudopériode» d'environ $^1/_2$ seconde. Dans le procédé de mesure que nous avons utilisé c'est la valeur de ces fluctuations qui limite la sensibilité. Il est donc de la plus

grande importance de se baser pour le choix d'un tube non sur la valeur du pouvoir d'amplification, mais sur la valeur des fluctuations du courant de fond. Plusieurs auteurs et en particulier Engstrom [14], ont montré que le rapport signal-bruit de fond diminuait légèrement lorsque la différence de potentiel augmentait et que l'éclairage restait constant. Il y a donc avantage à choisir le tube qui possède le meilleur rapport signal-bruit de fond et à l'utiliser avec le voltage maximum autorisé par l'apparition du courant désordonné provenant de l'ionisation intense de la cellule.

On peut utiliser deux méthodes pour réduire les fluctuations à l'enregistrement:

1) Augmenter la constante de temps du système détecteur. C'est la solution de Diecke, par exemple. Les mesures rapides sont exclues et l'examen du spectre doit se faire assez lentement: Remarquons qu'en spectrographie Raman cela n'est pas très gênant, mais cela peut la devenir en analyse spectrochimique, par exemple.

2) Refroidir la cellule pour diminuer l'émission thermoionique. C'est un moyen extrêmement efficace, mais qui complique beaucoup le dispositif mécanique. Nous avons constaté qu'à la température de la glace sèche le courant de fond était environ 100 fois plus faible qu'à température ordinaire. Les fluctuations ne sont plus décelables et le pouvoir d'amplification n'est pratiquement pas touché. C'est donc une solution de choix, cependant, les complications qu'elle entraine, spécialement pour préserver la cellule de tout dépot de buée qui l'opacifie, ont fait que nous lui avons préféré la première solution. Engstrom [15] a donné la description d'un réfrigérateur très bien conçu mais qui s'applique surtout bien aux installations semi-fixes.

APPLICATIONS AUX MESURES D'INTENSITÉS LUMINEUSES

Après toutes les réserves que nous avons faites sur les modalités d'utilisation des cellules multiplicateurs d'électrons il reste à s'assurer que cet instrument permet non seulement de détecter mais aussi de mesurer un rayonnement lumineux.

C'est un fait maintenant bien établi que le courant photoélectrique de ces nouvelles cellules est fonction linéaire, dans la limite des erreurs expérimentales, de l'intensité de la lumière monochromatique reçue. Engstrom [14] a fait des mesures depuis un flux lumineux de 10^{-13} lumen, où l'on peut compter les photons, jusqu'à 1 lumen. Le courant débité varie depuis 10^{-12} ampère jusqu'à 45 milliamperes qui se présente comme une valeur de saturation atteinte dès 10^{-3} lumens. En excluant la région de saturation, on peut tenir comme certaine la linéarité de la réponse entre 10^{-13} lumen (10^{-12} ampère) et 10^{-3} lumen (10^{-2}

ampère). Pratiquement la région de travail se situe entre 10^{-10} et 10^{-4} lumen correspondant à des courants de l'ordre de 10^{-9} à 10^{-3} ampère. Dans cette région la déviation à la loi linéaire ne dépasse pas 3 %.

DESCRIPTION D'UN SPECTROGRAPHE RAMAN À PRISME ET À ENREGISTREMENT DIRECT

L'ensemble comprend trois parties:

1) Organe d'illumination.
2) Spectrographe.
3) Organes récepteurs.

Organe d'illumination

C'est un cylindre vertical à parois internes réflechissantes de 20 cm de haut et de 60 cm de diamètre. Il est muni d'un waterjacket. Sur la périphérie interne sont montées 6 lampes Philips à vapeur de mercure (H 2000) émettant chacunes 2000 lumens spécialement dans le bleu et le vert. Le spectre compris entre le triplet 4358 et la raie vert-chou 4960 Å est relativement assez pur, mais au delà de 4960 Å il présente des raies étrangères au mercure qui rendent difficile l'examen des raies Raman dont le nombre d'onde dépasse 2000, sauf si on utilise, comme nous le faisons des filtres lumineux qui nettoient cette région.

La lumière de chacune des lampes est focalisée au centre du cylindre par 6 lentilles cylindriques (6 cm de diamètre) parcourues par un courant d'eau.

Au centre du cylindre se trouve le tube Raman. C'est un tube de pyrex, de 1 cm de diamètre et de 22 cm de haut, muni d'une chemise qui peut être rempli par un filtre lumineux ou parcourue par un courant d'eau à température constante. Autour du tube se trouvent encore 2 autres cylindres concentriques destinés à recevoir éventuellement, l'un un filtre lumineux et l'autre un courant d'eau froide pour maintenir la température du filtre dans des limites convenables. Les filtres utilisés sont des filtres à éosine et nitrite de soude indiqués par Taboury [16].

Enfin, l'ensemble est parcouru par un violent courant d'air provoqué par un ventilateur. Toutes les précautions prises pour assurer la réfrigération sont nécessaires. L'eau du water-jacket évacue environ 20 grandes calories par minute et le ventilateur à peu près autant moyennant quoi la température, au centre du cylindre, se maintient aux environ de 25° C.

Le tube Raman occupe une position verticale, la lumière diffusée est donc reprise par un miroir à 45° et, par l'intermédiaire d'une lentille, projetée sur la fente du spectrographe.

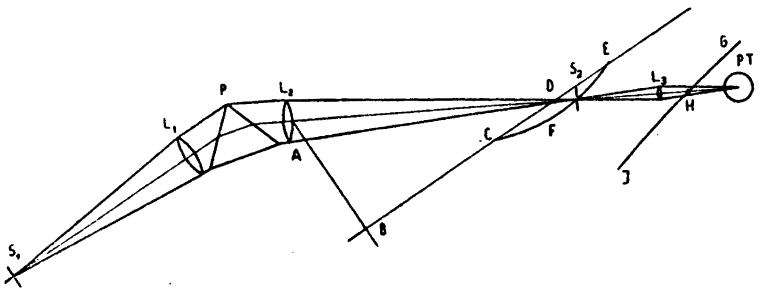


Fig. 1. Schéma de principe du montage d'une cellule dans un spectrographe à prisme.

Spectrographe

C'est un spectrographe Hilger à un seul prisme de verre et d'ouverture assez faible. Sa dispersion dans la région bleue- bleu-vert est d'environ 30 Å par millimètre, mais, concurremment avec cette faible dispersion il possède une bonne luminosité, ce qui permet d'utiliser des fentes d'entrée relativement étroites (1/10 mm).

Il est conçu pour être utilisé avec des plaques photographiques en verre mince, car la courbe focale CFE (fig. 1) n'est pas rectiligne mais présente une nette courbure. Le chassis peut, bien entendu, être facilement enlevé et remis.

Organes récepteurs

Les organes récepteurs sont constitués par tout un ensemble massé autour de la cellule photoélectrique.

Pour des raisons d'encombrement, il n'est pas possible de placer la couche sensible de la cellule sur la courbe focale CFE. On est donc obligé de reprendre le spectre par une fente S_2 suivie d'une lentille L_3 et de la cellule PT. Au fur et à mesure que le point F décrit la courbe focale, il faut, bien entendu, que l'axe optique soit constament aligné avec le rayon lumineux incident, il faut donc que l'ensemble S_2L_3PT, lorsque le point S_2 se déplace, tourne autour du point A centre de la lentille L_2 d'ou l'on peut, en première approximation supposer issus tous les rayons lumineux.

Ceci représente un problème de mécanique difficile à résoudre. Notre solution présente un intérêt général qui nous pousse à en donner une description plus complète.

L'axe optique du système récepteur est matérialisé par deux points D et H. Le point D est le point d'attache d'un écrou qui est assujetti à se déplacer

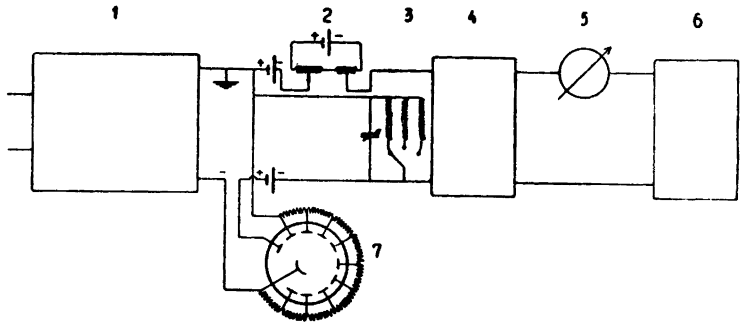


Fig. 2. Schéma de principe du montage électrique d'une cellule multiplicateur d'électrons: (1) stabilisateur de voltage, (2) voltage de compensation, (3) resistances et condensateur d'amortissement, (4) amplificateur, (5) milliampèremetre, (6) enregistreur et (7) cellule.

sur la droite CE matérialisée par une vis. La distance DH étant constante, si D décrit une droite, H décrit la conchoïde de cette droite par rapport au point A, soit la courbe JHG. L'axe optique étant de cette façon toujours dirigé vers le point A il reste à faire décrire à la fente S_2 la courbe focale CFE. Dans ce but, un premier chariot C_1 solidaire de D et de H supporte un deuxième chariot C_2 qui porte la fente S_2, la lentille L_3 et la cellule PT. C_2 peut glisser sur C_1 dans la direction ADH grâce à un roulement à bille. Un ressort appuie constament S_2 sur la courbe focale.

L'ensemble doit être réalisé avec grande précision, en particulier, la vis BCE et l'écrou D doivent permettre d'atteindre un mouvement dont la précision soit de l'ordre du 1/100 de millimètre. Les jeux doivent être réduits au minimum car, pour certaines cellules, la surface sensible n'est pas uniforme et un très léger déplacement peut donner de grandes variations dans l'intensité du courant débité.

Alimentation de la cellule. Amplification du courant photoélectrique

Ainsi que nous l'avons déjà dit, la cellule (7) (fig. 2) est alimentée en courant continu stabilisé au $1\,{}^0/_{00}$. Le stabilisateur (1) permet de faire varier le voltage depuis 300 volts jusqu' à 2000 volts couvrant largement le domaine d'utilisation de la lampe.

Les courants sont extrêmement faibles, 10^{-9} à 10^{-7} ampère et nécessitent une amplification de 10^6 pour être enregistrés avec facilité.

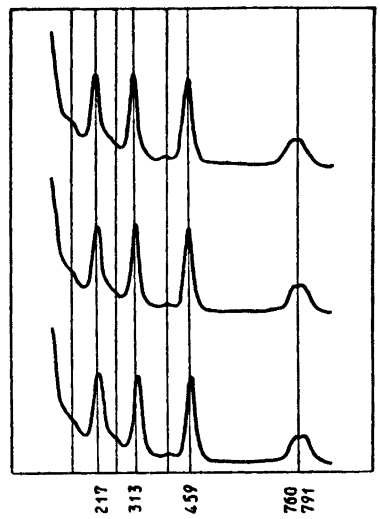


Fig. 3. Spectres Raman du tétrachlorure de carbonne.

L'amplificateur (4) que nous utilisons est en réalité un amplificateur de voltage. Le courant à mesurer circule à travers une grande résistance de 2 à 100 mégohms (3) et c'est le voltage résultant qui est repris par un montage dont nous donnerons ultérieurement la description. Ce montage permet de mesurer avec précision un voltage compris entre 0 et 0.9 volt, limites entre lesquelles la réponse est linéaire. Or, si le courant de fond est de l'ordre de 10^{-8} ampère et la résistance utilisée de 10^8 ohms, le voltage du au courant de fond est déjà de 1 volt. Il faut donc, avant tout, compenser ce voltage et ne mesurer que l'excédent.

Cette compensation est produite très simplement (2). Le courant à mesurer circule à travers une grande résistance (3) dont une extrémité est à la terre et l'autre est reliée à l'une des bornes d'entrée de l'amplificateur. L'autre borne est portée à un potentiel voisin grâce à une pile dont l'extrémité négative est à la terre. Un accumulateur (2) permet d'avoir entre les deux bornes un voltage aussi proche que possible de zéro.

Ce dispositif permet entre autre, de traiter la fluorescence, qui est un sérieux inconvénient de la spectrographie Raman, comme un courant de fond et d'obtenir un spectre »pur» avec des substances qui ne donneraient rien sur une plaque photographique.

L'emploi d'une grande résistance rend toute simple l'augmentation de la constante de temps du système grace à un condensateur placé en parallèle. Comme nous l'avons expliqué, on est alors obligé de réduire la vitesse d'examen du spectre (1 mm par minute).

Resultats

Nous nous sommes surtout intéressés aux mesures d'intensités lumineuses et, à titre d'exemple, nous donnons trois enregistrements successifs du spectre Raman du tétrachlorure de carbonne (fig. 3), qui montrent que la précision obtenue dans la mesure des intensités atteint facilement 2 %. La présence des raies satellites (excitation par le triplet 4358) est aussi une preuve de la sensibilité de l'appareil.

Notre travail s'oriente maintenant vers l'étude de certains problèmes pour la résolution desquels la mesure des intensités peut présenter un grand intérêt, par exemple, étude des mélanges anormaux, mesures de certaines vitesses de réactions, étude des processus de polymérisation.

RÉSUMÉ

Apres avoir donné les principales caractéristiques des cellules multiplicateurs d'électrons, telles qu'elles résultent de la littérature et de notre expérience personelle, nous avons décrit un spectrographe Raman à prisme utilisant ces cellules comme organe récepteur. L'organe d'illumination est particulièrement puissant, mais nous avons surtout insisté sur le dispositif utilisé pour permettre à la cellule de parcourir toute l'étendue du spectre. Après amplification convenable, les courants photoélectriques, enregistrés sur un voltmètre enregistreur à plume, permettent d'obtenir des spectrogrammes sur lesquels les mesures d'intensités sont possibles à environ 2 % près.

Nous tenons tous deux à remercier le Professeur The Svedberg pour l'intêret et les facilités de toutes sortes qu'il a mises à notre disposition, l'un de nous désire surtout souligner la généreuse hospitalité qu'il a reçue dans ses laboratoires et l'en remercier ici vivement.

Les conseils de E. Blomgren dans toutes les questions qui touchent l'électronique, nous ont été particulièrement précieux.

BIBLIOGRAPHIE

1. Boettner, E. A., et Brewington, G. R. *J. Optical Soc. Am.* **34** (1944) 330.
2. Diecke, G. H., et Crosswhite, H. M. *J. Optical Soc. Am.* **35** (1945) 471.
3. Saunderson, J. L., Caldecourt, V. J., et Peterson, E. W. *J. Optical Soc. Am.* **35** (1945) 681.
4. Rank, D. H., Pfister, R. J., et Coleman, P. D. *J. Optical Soc. Am.* **32** (1942) 390.
5. Rank, D. H., Pfister, R. J., et Grimm, P. *J. Optical Soc. Am.* **33** (1943) 31.
6. Rank, D. H., et Wiegand, R. V. *J. Optical Soc. Am.* **36** (1946) 325.
7. Rank, D. H., Fenske, N. R., Braun, W. G., Wiegand, R. V., Quiggle, D., et McCormick, R. H. *Ind. Eng. Chem. Anal. Ed.* **19** (1947) 700.

8. Blau, M., et Dreyfus, B. *Rev. Sci. Instruments* **16** (1945) 24.
9. Kron, G. E. *Astrophys. J.* **103** (1946) 324.
10. Whitford, A. E., et Kron, G. E. *Rev. Sci. Instruments* **8** (1937) 78.
11. Marshall, F. H., Coltman, J. W., et Hunter, L. P. *Rev. Sci. Instruments* **18** (1947) 504.
12. Diecke, G. H. *War Production Board, Washington, Research Report No. W-193-PB-32156* May 1 (1945).
13. Chien, J.-Y., et Bender, P. *J. Chem. Phys.* **15** (1947) 376.
14. Engstrom, R. W. *J. Optical Soc. Am.* **37** (1947) 420.
15. Engstrom, R. W. *Rev. Sci. Instruments* **18** (1947) 587.
16. Taboury, F. J. *Bull. soc. chim.* **10** [5] (1943) 205.

Manuscript reçu le 9 Fevrier 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 204—208

Spectrophotometric Determination of Acetaldehyde in Mono-vinyl Acetate

INGVAR JULLANDER and KURT BRUNE

Research Laboratory, Mo och Domsjö AB, Örnsköldsvik, Sweden

It is well known that in the polymerisation of mono-vinylacetate the presence of acetaldehyde decreases the molecular weight of the poly-vinyl-acetate obtained [1, 2]. Addition of acetaldehyde has even been used as a regulator for the viscosity of the end product [3]. It is accordingly of interest to have available reliable and sensitive methods for determination of acetaldehyde in vinyl acetate, if possible down to 0.1 g/l.

Physical methods of analysis are preferred to chemical methods because whenever vinyl acetate is brought into contact with water there is always the danger of saponification of the ester and subsequent increase in the aldehyde content.

Absorption in the ultraviolet region has been used by a German company for determination of acetaldehyde [4]. No details as to procedure, accuracy etc. were given, and it was also reported that another company found the method unreliable.

We have tested the method with the aid of a Beckman Spectrophotometer Model DU [5] and obtained an entirely satisfactory result. All measurements were made at room temperature with a cell thickness of 1.004 cm.

Vinyl acetate of commercial origin was distilled three times in a Podbielniak column modified by Skärblom and Linder [6]. The height of the column is 200 cm, and the fractionating efficiency corresponds to 30—35 theoretical plates. Fig. 1 shows the absorption curve for the original product and the different distillates. The optical density D is calculated from

$$D = -^{10} \log T/T_0$$

(T and T_0 transmission of sample and reference substance resp.)

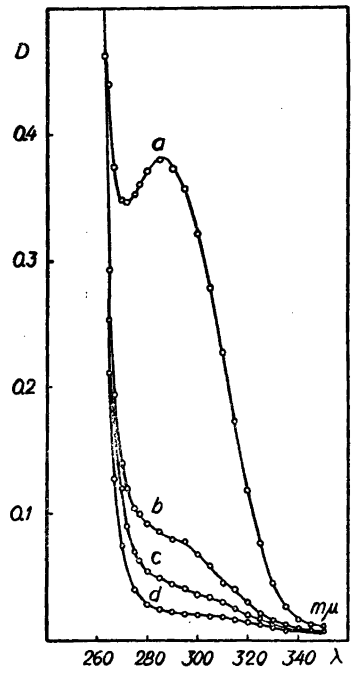


Fig. 1. Optical density D of mono-vinylacetate as function of wave-length. Distilled water was used as reference substance. Curve a obtained with the commercial product, curves b, c and d after single, double and triple distillations respectively.

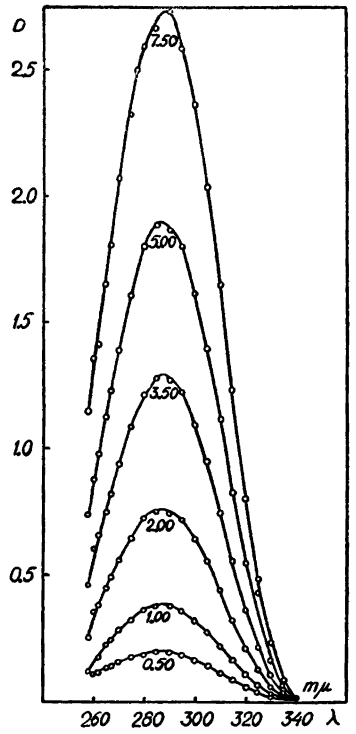


Fig. 2. Optical density D of acetaldehyde (conc. in g/l denoted for each curve) in mono-vinylacetate as function of wave-length. Triple distilled vinyl acetate (curve d in Fig. 1) used as reference substance.

Addition of different amounts of acetaldehyde gave the absorption curves in Fig. 2. From a similar series of curves the extinction at $\lambda = 287$ mμ (= position of the peak) plotted against concentration gave the curve in Fig. 3, which shows that the Lambert-Beer law is well fulfilled within the concentration interval 0—6 g/l. The fact that the point corresponding to the highest concentration falls below the straight line can easily be explained as experimental error, the transmission in this case being less than 1 %.

From the values of D the extinction coefficient ε (concentration in moles per liter, thickness of absorbing layer in cm) has been calculated in Table 1. The average is $\varepsilon = 17.3$.

Fig. 1 shows that at $\lambda = 287$ mμ even the triple distilled vinyl acetate has a certain absorption compared with distilled water. It is uncertain whether

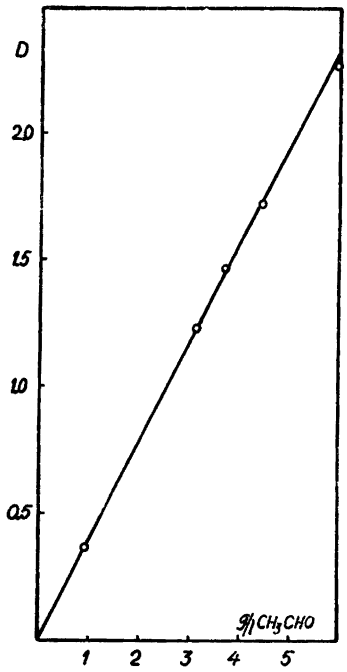


Fig. 3. Optical density D at $\lambda = 287$ mμ for acetaldehyde in vinyl acetate.

Table 1. Extinction coefficient ε calculated from experiments at different concentrations.

Conc. g/l	ε
0.94	17.3
3.13	17.3
3.72	17.4
4.46	17.0
5.95	(16.8)

this residual optical density should be ascribed to the absorption of vinyl acetate itself or to acetaldehyde still present in the liquid, but the latter alternative seems more probable. Previous investigations of the ultraviolet absorption spectrum of vinyl acetate [7, 8] were not continued beyond $\lambda = 280$ mμ. The maximum amount of acetaldehyde that can be present in the triple distilled product is 0.06 g/l. In another experiment double distillation resulted in a maximum amount of acetaldehyde = 0.09 g/l.

In order to test the reproducibility of the analysis different amounts of acetaldehyde were added to the distilled vinyl acetate. The result is seen in Table 2. The acid number, *i. e.*, mg KOH required for neutralisation per g of sample, was also determined. From the outset it was expected that the presence of acetic acid in the vinyl acetate should not interfere with the aldehyde analysis. Addition of acetic acid in two of the tests confirmed this. From the table we may draw the conclusion that the accuracy of the method is about 0.1 g/l. The minimum detectable amount is estimated to be a few thousands of a percent. It might be mentioned that some of the discrepancies in Table 2 can undoubtedly be ascribed to the difficulty of accurately preparing dilute solutions of an easily volatile solute like acetaldehyde.

Table 2. Analytical tests.

Conc. CH_3CHO in g/l		Acid number
Added	Found	
0.09	0.11	< 0.1
0.68	0.73	< 0.1
2.16	2.25	9.9
2.18	2.39	1.2
2.33	2.40	< 0.1
4.67	4.61	< 0.1

SUMMARY

A method for determining acetaldehyde in mono-vinylacetate down to a few thousands of a per cent by means of ultraviolet absorption is described. From the law of Lambert-Beer the extinction coefficient at $\lambda = 287$ mμ is calculated to be $\varepsilon = 17.3$ (conc. in moles per liter).

The authors wish to express their sincere thanks to Mr S. Johanson for his helplful advice.

REFERENCES

1. Blaikie, K. G., and Crozier, R. N. *Ind. Eng. Chem., Ind. Ed.* **28** (1936) 1155.
2. Skirrow, F. W. *Brit. Plastics* **10** (1939) 416, 507.
3. DeBell, J. M., Goggin, W. C., and Gloor, W. E. *German plastics practice.* Springfield, Mass. (1946) p. 106.

4. Rosenquist, E. N. *The production of mono-vinylacetate. F. I. A. T. Report* no. 860 (1946).
5. Cary, H. H., and Beckman, A. O. *J. Optical Soc. Am.* **31** (1941) 682.
6. Skärblom, K. I., and Linder, Å. *Tek. Tid.* **67** no. 7, *Kemi* (1937) 12.
7. Paist, W. D., Blout, E. R., Uhle, F. C., and Elderfield, R. C. *J. Org. Chem.* **6** (1941) 273.
8. Calvin, M., Magel, Th. T., and Hurd, C. D. *J. Am. Chem. Soc.* **63** (1941) 2174.

Received February 10, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 209—219

Corticrocin, a Pigment from the Mycelium of a Mycorrhiza Fungus. II *

HOLGER ERDTMAN

Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden

Mycorrhiza, »fungus root», is a product of symbiosis between the roots of most higher plants and mycelia of certain fungi. Although, on a suitable substrate, each participant usually grows quite normally it now appears to be assumed by most botanists that under certain conditions frequently occurring in Nature mycorrhiza is essential to the plants involved in this symbiosis, a mutual exchange of metabolic products probably taking place.

Although the mycorrhiza symbiosis possesses great economical importance in agriculture and forestry, and therefore has been extensively studied by physiologists [1], very little is known about its chemistry.

Some of the mycorrhiza-forming fungi have been subjected to chemical investigation *e. g. Amanita muscaria*, the fly agaric, but only the fruit bodies, not the mycelium.

On the roots of *e. g.* Norway spruce, Scots pine and whorthleberry (cowberry, *Vaccinium vitis idaea*) growing on poor, acid soil very frequently a mycorrhiza is found which is characterised by its bright yellow colour. Melin assumes that the mycelium belongs to the fungus *Corticium croceum* Bres. (= *Corticium sulphureum* (Fr.) Fr.). This question, however, is not yet settled (Melin, private communication).

Since this yellow mycorrhiza is very important in Swedish forests attempts were made to isolate the pigment responsible for its colour. Colouring matters are frequently physiologically active and this pigment may perhaps play an essential *rôle* in the metabolism of the fungus, therefore being of importance

* Part I, preliminary note, *Nature* **160** (1947) 331. Through an editorial change of the nomenclature from the »diacid» to the »dicarboxylic acid» basis in this report, $(CH_2)_{12}(COOH)_2$ and corticrocin were given erroneous names (»tetradeca» instead of »dodeca»). The paper was unfortunately published before the author had an opportunity to read the proofs.

for this particular mycorrhiza. According to Melin [1] also the isolated mycelium is characterised by a yellow colour. It is not known with certainty, but appears to be very probable, however, that the pigment of the isolated mycelium and that of the mycorrhiza are identical. Several species of the genus *Corticium* (natural order *Thelephoraceae*) produce pigments *e. g. Corticium salicinum*, and an investigation of the pigment of the fruit body of the true *Corticium croceum* and other *Corticium* species may contribute to the elucidation of the systematic position of the fungus of the yellow mycorrhiza. Such investigations are at present in progress.

Most mycorrhiza fungi grow very slowly on a synthetic medium and the mycelium of the yellow mycorrhiza is not exceptional [1]. Hence it was necessary to gather the starting material directly from the forest soil. I take this opportunity of expressing my gratitude to all those collaborators who assisted me in this very tedious work. This resulted in the recovery of about thirty rucksacks (450 liters) of soil containing roots with mycorrhiza heavily contaminated by the normal constituents of the raw humus *e. g.* decomposing branches, needles, bark, cone fragments, sand and mud.

It was of course impossible to isolate the mycorrhiza from this material manually, except for micro tests performed in order to gain experience regarding the best method for the isolation of the pigment. This could be extracted from the mycorrhiza with hot pyridine or long extraction with acetone. The pigment was found to be extremely sparingly soluble in most organic solvents. It readily formed crystals and could be purified by carefully conducted sublimations in a high vacuum. When the mycorrhiza was ground with water a turbid yellow solution was obtained, perhaps containing the pigment in combination with proteins. Sublimed crystals were completely soluble in very dilute alkali but not in more concentrated, when insoluble salts were formed. Consequently, its acidic nature was at first overlooked.

The isolated mycorrhiza appears to contain about 4 % of this pigment.

After previous concentration of the mycorrhiza, mainly involving washing with large amounts of water, procedures which are described in the experimental part, the dried soil was exhaustively extracted with acetone. The isolation of the pigment from the accompanying large amount of bituminous substances is facilitated by its extremely low solubility. From hot pyridine long yellow needles containing pyridine easily removable in a vacuum over concentrated sulphuric acid were obtained.

Elementary analysis of different preparations gave very inconsistent results. The percentage of carbon varied from 64 to 68 and that of hydrogen from 5 to 6. The preparations were free from sulphur and nitrogen. They were almost ashfree but sometimes contained a little »methoxyl» (0—1.3 %).

The melting points were high and of very little use as indications of degrees of purity.

Repeated recrystallisations, involving great losses of pigment, appeared to yield purer products, the percentage of carbon ultimately rising to about 68.

It appeared to be possible that this inconstancy in the composition of the preparations, which all »looked very pure», might be due to an admixture of compounds deriving from the soil and possessing the same low solubility as the pigment. Therefore raw humus from the same source but without yellow mycorrhizas was pretreated and extracted in exactly the same manner as soil containing the fungus. The yellow pigment was now replaced by an almost colourless material, which according to its elementary composition and chemical behaviour, *e. g.* hydrolysis with strong alkali was obviously a »wax». This material was soluble in hot ethyl benzoate in which, fortunately, the pigment is almost insoluble. After extractions with boiling ethyl benzoate and subsequent recrystallisations from pyridine different preparations, previously yielding varying analytical results, furnished materials which all had a composition agreeing with the formula $C_{14}H_{14}O_4$. Six consecutive recrystallisations from pyridine followed by sublimation in a high vacuum and a final recrystallisation from pyridine yielded a material of unchanged composition.

The yield of pure pigment from one rucksack of raw humus generally amounted to 0.2—0.3 grams.

The pigment, which has been termed *corticrocin*, dissolves in concentrated sulphuric acid to a red solution, which soon turns yellow, green and finally colourless. Corticrocin reacts with diazomethane with brisk evolution of nitrogen and formation of a yellow, sparingly soluble methyl derivative, which may be recrystallised from chloroform yielding orange-red needles or from acetic acid when golden yellow, glistening plates are obtained. Both materials melt at 230—232° (uncorr.). The molecular weight of this substance, corticrocin dimethyl ester $C_{12}H_{12}(COOCH_3)_2$ could be estimated by the Rast method, but it was necessary to work very quickly since the ester is liable to decompose in hot camphor giving too high values.

Corticrocin dimethyl ester is sufficiently soluble in alcohol to permit the investigation of the U. V. absorption, the complete curve being given in Fig. 1.

The curve shows three characteristic maxima of high absorption ($\lambda = 374$ mμ, $\log \varepsilon = 4.79$; $\lambda = 393$ mμ, $\log \varepsilon = 4.95$; $\lambda = 416$ mμ, $\log \varepsilon = 4.92$) and is very similar to those of several carotenoids. The absorption is especially reminiscent of dihydrobixin methyl ester[2], which contains eight conjugated double bonds. On the assumption, however, that corticrocin is a dicarboxylic acid of true carotenoid type (derived from isoprene) it is impossible to accommodate eight conjugated double bonds in the molecule.

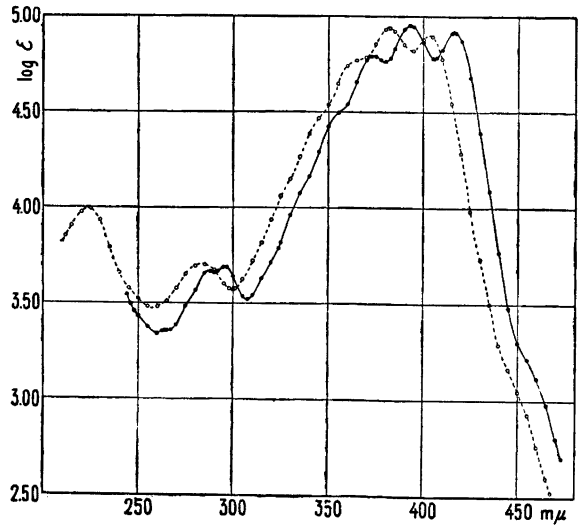


Fig. 1. Ultraviolet absorption of corticrocin dimethyl ester in chloroform (●——●) and of corticrocin in dilute alkali (○– – –○).

Moreover, chromic acid oxidation indicated that corticrocin contains no C—CH_3 groups. This led to the conclusion that corticrocin is not a normal carotenoid dicarboxylic acid but a polyene carboxylic acid of the structure

$$HOCO \cdot (CH = CH)_6 \cdot COOH$$

This was confirmed by the result of the catalytic hydrogenation of corticrocin dimethyl ester. Six molecules of hydrogen were absorbed and the dimethyl ester of *n*-dodecane dicarboxylic acid-1,12 was formed and identified by mixed melting point determination.

In a series of brillant studies of the polyene dicarboxylic acids Kuhn and his collaborators [3] prepared the acids of the general structure $HOCO \cdot (CH = CH)_n \cdot COOH$ with $n = 3$, 4, 5 and 7. The missing link in Kuhns series is corticrocin, the first polyene dicarboxylic acid of this type to be found in Nature.

The melting points of the dimethyl esters $CH_3OCO \cdot (CH = CH)_n \cdot COOCH_3$ are placed together in Table 1.

The melting point of corticrocin dimethyl ester (232°) fits well in this series.

In their papers on polyene dicarboxylic acids Kuhn and his co-workers only give wavelength data for the position of the maxima. Quantitative

Table 1. Melting points of the dimethyl esters $CH_3OCO(CH = CH)_nCOOCH_3$.

n	M. p. n uneven	M. p. n even	Difference
0		53°	49
1	102		54
2		156	16
3	172		40
4		212	11
5	223		9
6		232	4
7	236		

measurements, however, had been carried out and this enabled Kuhn to predict the maxima for corticrocin with great accuracy. »Für Corticrocindimethylester in Chloroform würde ich etwa erwarten λ^1_{max} 415 mμ, λ^2_{max} 393 mμ.« (Found: $\lambda^1_{max} = 416$ mμ, $\lambda^2_{max} = 393$ mμ).

I am very much indebted to Professor Kuhn for the following figures regarding wavelengths and molar extinction coefficients of the absorption maxima of the dimethyl esters of the acids $HOCO(CH = CH)_nCOOH$ with $n = 5$ and 7 in chloroform. (Table 2.)

Table 2. Absorption maxima of the methyl esters $CH_3OCO(CH = CH)_nCOOCH_3$ in chloroform.

n	λ^1_{max}	$\varkappa^1_{max}$	log ε^1_{max}	λ^2_{max}	$\varkappa^2_{max}$	log ε^2_{max}
5	384 mμ	$153 \cdot 10^3$	4.82	365 mμ	$174 \cdot 10^3$	4.88
6	416 »	$191 \cdot 10^3$	4.92	393 »	$205 \cdot 10^3$	4.95
7	440 »	$216 \cdot 10^3$	4.97	414 »	$220 \cdot 10^3$	4.98

Corticrocin is too insoluble in alcohol to permit a quantitative study of its U. V. spectrum. The solubility of its sodium salt in weak sodium hydroxide solution, however, is sufficient for this purpose and Fig. 1 gives the absorption of the salt.

Small specimens of corticrocin dimethyl ester can be sublimed unchanged in a high vacuum. The absorption spectrum is not altered appreciably if a trace of iodine (1 % of the weight of the ester) is added to the chloroform solution and the absorption examined after 30 minutes in diffuse light [4]. A

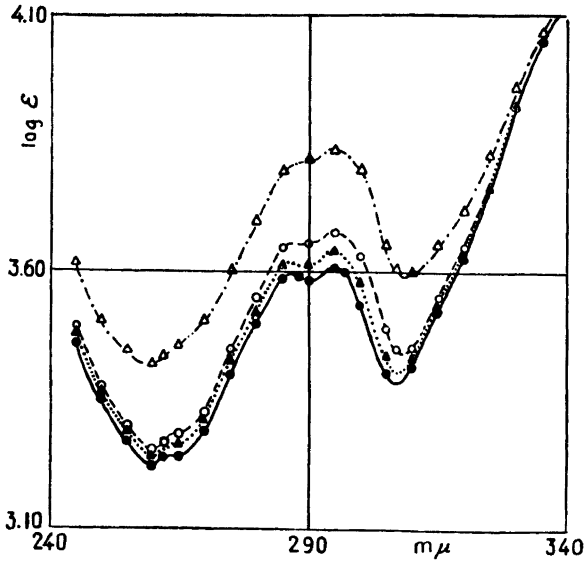
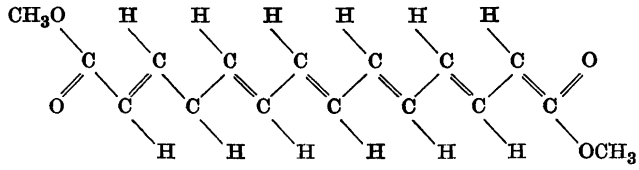


Fig. 2. Effect of traces of iodine on the absorption spectrum of corticrocin dimethyl ester in chloroform.

●——● *Without addition of iodine. No illumination.*
▲·····▲ *Without addition of iodine. Illumination 45 minutes.*
○— — —○ *1 % of iodine added. 30 minutes in diffuse day light. (No change after 43 hours in the dark.)*
△— · —△ *1 % of iodine added. 30 minutes in diffuse light. Then illumination 45 minutes.*

definite change of the absorption in the region of short waves takes place if the solution is illuminated. (45 min, 100 Watt lamp, distance 15 cm). The maxima of high absorption were practically unchanged. (Fig. 2.)
This appears to show that the corticrocin dimethyl ester preparation employed had the stable *trans* configuration throughout the chain:



Illumination in the presence of iodine probably causes an isomerisation.

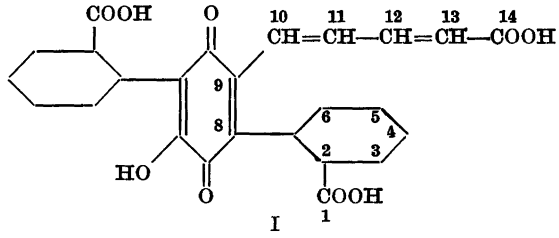
It is not yet known whether corticrocin possesses any physiological activity or plays any *rôle* in the metabolism of the fungus. If this ultimately proves to

be the case it is probable that it functions as a hydrogen carrier. Like the polyene dicarboxylic acids studied by Kuhn corticrocin and its dimethyl ester are readily hydrogenated by zinc and pyridine to the corresponding dihydro-compounds, but a description of these experiments will be the subject of a forthcoming publication.

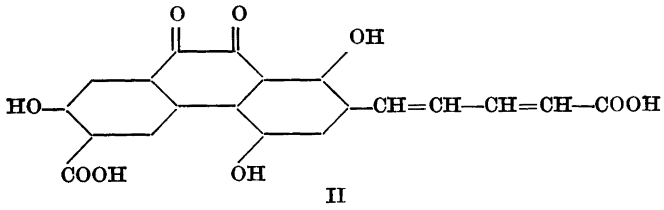
In view of the remarkable oxidation of benzene to muconic acid and especially the ω-oxidation of polyene monocarboxylic acids to the corresponding dicarboxylic acids discovered by Kuhn [5], reactions which take place in the animal body, it appears probable that corticrocin is formed in nature by ω-oxidation of the acid $CH_3(CH = CH)_6COOH$. Serious attempts were made to isolate this acid from the mother liquors but since the latter are heavily contaminated with bituminous products and waxes this task was similar to the search for a needle in a hay-stack.

Corticrocin accommodates in its molecule all the carbon atoms of phenanthrene and anthracene, derivatives of which are common products of fungus metabolism. It is interesting to note that some other components of fungi show close similarities to corticrocin.

The structure of muscarufin (I), the red colouring matter of the fly agaric *(Amanita muscaria)*, according to Kögl [6] possesses a side chain which resembles corticrocin.



I



II

Moreover, the right-hand part of the molecule indicated by the numbers 1—14 accommodates the whole molecule of corticrocin, part of which has been cyclicised to a benzene ring. If one assumes that the OH-group in the quinone nucleus of muscarufin is formed by an oxidation process during which a side chain similar to C_{10}—C_{14} is split off, the whole molecule of muscarufin appears

to be built up from two molecules of corticrocin, joined at C_8 and C_9 by two carbonyl groups.

The structure of thelephoric acid from *Thelephora* species (II), also proposed by Kögl [7], invites similar speculations but the relation to *corticrocin* is, apart from the presence of the —CH = CH—CH = CH—COOH side chain, less obvious. *Thelephora* and *Corticium* are closely related (natural order *Thelephoraceae*).

It appears not to be out of the way to assume that sometimes benzene nuclei may be formed in Nature by cyclisation of highly unsaturated fatty acids. Among the true »carotenoids» bixin and crocetin bear very close structural relationship to corticrocin. According to Kuhn and Moewus [8] crocetin possesses great biological importance *e. g.* as a sex determining factor of certain algae.

EXPERIMENTAL PART

Isolation of corticrocin

The material was collected in the neighbourhood of Stockholm. The best places to search for this subterranean fungus mycelium are on stones or rocks losely covered with mossy raw humus and inhabited by *Vaccinium vitis idaea*. It is sometimes possible to roll off a carpet of such raw humus interwoven with yellow mycorrhiza. The layer next to the rock in particular is sometimes bright yellow.

Some concentration of the mycorrhiza from the raw humus was necessary and several methods were tried, including washing with saponine solutions and different synthetic detergents. In this manner a large amount of soily material could be removed but the washings were frequently coloured faintly greenish yellow, indicating loss of pigment. Extraction of this material invariably yielded less pigment than the untreated raw humus.

The method finally adopted was to stir the raw humus for several hours with large volumes of water, then filter the mixture through a wire gauze (mesh 4 × 4 mm). When still on the screen a rapid stream of water was directed on to the raw humus to wash away impurities. The material was then kneaded manually with water containing a little fat alcohol sulphonate and washed again with large amounts of water. The residue was pressed mechanically and dried in a stream of hot air. The coarser material, *e. g.* bigger roots, stones, lumps of bark and cones were removed by hand. The material was then packed in an apparatus for continuous extraction with acetone. The black extract obtained after 0.5—1 hours extraction was collected separately and the extraction continued for about two days or until no more yellow mycorrhiza could be detected in the material.

From the first extract a sediment slowly deposited which was isolated by filtration, boiled with much acetone and filtered through a hot filter leaving undissolved a crude, yellow pigment. From the second extract the precipitate was recovered and the crude material boiled with acetone and filtered. The two acetone filtrates from the second extract were combined and concentrated. After a few days a precipitate had appeared which was treated in the same way as the sediment from the first extract, yielding a

second crop of pigment. The combined crude pigment fractions were boiled with ethyl acetate and finally with ethyl benzoate, filtered hot and washed with acetone. The dried corticrocin was dissolved in large amounts of boiling pyridine, filtered through a hot filter and left to crystallise. Sometimes supersaturated solutions were formed which crystallised on seeding. The corticrocin should not be left in contact with pyridine and air longer than necessary. Three to five recrystallisations generally yielded an analytically pure product. The mother liquors were poured into water and acidified with concentrated hydrochloric acid. The fine suspension was centrifuged and the precipitate triturated with boiling methanol and recrystallised from pyridine. The pyridine-containing yellow needles or flat, elongated prisms, sometimes attaining a length of several centimeters, were spread in a thin layer on a watch glass and dried in an evacuated desiccator over concentrated sulphuric acid. In this way the pyridine was quickly removed and the crystal form retained. The pyridine can also be removed by washing with ether, acetone or methanol but in this way the crystal form is spoiled and an orange red powder obtained.

$C_{14}H_{14}O_4$	Calc.	C 68.3	H 5.7
	Found	» 68.1, 68.4, 68.3	» 5.9, 5.7, 5.8

Less carefully purified samples which had been boiled with ethyl benzoate usually gave somewhat low values for carbon and hydrogen, (67.8—68.0 *viz.* 5.4—5.7 %).

When heated in evacuated capillary tubes pure corticrocin starts to sublime at about 270°, a red »gas» filling the tubes. The sublimate usually melts at about 317° leaving a network of a faintly coloured decomposition product. Similar results, sublimation at 260—280° and decomposition between 300 and 317° with formation of a product not melting below 340°, were obtained in tubes filled with nitrogen or in open capillary tubes. (All the melting points were observed on the »Mikroheiztisch»).

With bromine corticrocin suspended in chloroform yields a colourless product which, unlike the pigment, is easily soluble in ether, acetone and benzene, less readily soluble in chloroform and insoluble in petroleum ether and ligroin.

Percentage of corticrocin in the isolated mycorrhiza

In order to get an idea of the percentage of corticrocin in the mycorrhiza 0.4736 g were isolated manually from the raw humus and extracted for one week with acetone in a Soxhlet apparatus, by which time the material had become white. (It is much more difficult to remove the pigment from the isolated mycorrhiza than from the raw humus.)

The acetone extract was evaporated and the residue, 42.3 mg, washed with chloroform. Yield 15.0 mg of crude corticrocin (3.7 % calculated on ash free, dry mycorrhiza).

Corticrocin dimethyl ester

Pure corticrocin was treated with an excess of diazomethane in ether. A vigorous evolution of nitrogen took place and the crystals were transformed into a yellow powder. After some hours the ether was evaporated and the methyl ester recrystallised from acetic acid or chloroform. The ester is more soluble than the acid. From acetic acid golden yellow, glistening plates are obtained. From chloroform orange red, glistening needles separate. Both types of crystals melt at the same temperature. M.p. after one recrystal-

lisation generally 228—230°, after two or more recrystallisations 230—232° (open tubes, uncorrected). The ester is more easily sublimed in a high vacuum than the acid and may be purified in this way. Pure specimens of the ester could easily be obtained from crude corticrocin, which, however, had been boiled with ethyl benzoate. It is soluble in concentrated sulphuric acid with a red colour, which soon turns brownish.

$C_{14}H_{12}O_2(OCH_3)_2$	Calc.	C 70.0	H 6.6	OCH_3 22.6
	Found	» 69.9	» 6.6	» 22.9

Molecular weight (Rast, camphor, depression: 13.8° 10.1°) 285, 305 (calc. 274). It was found advisable to introduce the capillary tubes into a preheated bath. If this precaution was not observed too low depressions were obtained corresponding to molecular weights varying between 300 and 400.

Catalytic hydrogenation of corticrocin dimethyl ester

Corticrocin dimethyl ester (0.5 g) was suspended in acetic acid (35 ml), platinum oxide (0.2 g) added and the mixture shaken with hydrogen. The methylester gradually passed into solution. After four hours the calculated amount of hydrogen had been consumed. The solution was now colourless and no further absorption of hydrogen took place. The acetic acid was removed in a vacuum, the high-boiling oily residue dissolved in ether and the solution shaken with sodium hydrogen carbonate. The ether was evaporated and the oil distilled in a high vacuum. The distillate crystallised on cooling and was recrystallised from methanol. M. p. 43—45°, alone or mixed with an authentic specimen of dodecane dicarboxylic acid-(1,12) dimethyl ester.

$C_{12}H_{24}(COOCH_3)_2$	Calc.	C 67.1	H 10.5	OCH_3 21.6
	Found	» 67.0	» 10.5	» 21.5

The ester was hydrolysed with alkali to the corresponding acid, which was recrystallised from water or ethanol. M. p. 125—126° alone or in admixture with an authentic specimen of dodecane dicarboxylic acid - (1, 12).

$C_{12}H_{24}(COOH)_2$	Calc.	C 65.1	H 10.1
	Found	» 65.1	» 10.2

SUMMARY

From the yellow mycorrhiza frequently found in Sweden on the roots of pine, spruce and *Vaccinium vitis idaea* (fungus probably *Corticium croceum* Bres.) an orange yellow pigment, corticrocin, has been isolated. It has been shown to be dodecahexaene-(1,3,5,7,9,11)-dicarboxylic acid-(1, 12). The U. V. absorption curve shows three characteristic maxima: $\lambda = 374$ mμ, log $\varepsilon = 4{,}79$; $\lambda = 393$ mμ, log $\varepsilon = 4.95$; $\lambda = 416$ mμ, log $\varepsilon = 4.92$.

Corticrocin is the first unbranched polyene dicarboxylic acid to be found in Nature.

The author wishes to express his sincere gratitude to *Statens Tekniska Forskningsråd* for generous financial support of this work. My cordial thanks are also due to Mrs. A. Timell for skilful assistance in the laborious preparations, to Fil.lic. G. Aulin-Erdtman for spectrographic assistance and to Mr. W. Kirsten for the microanalysis.

LITERATURE

1. Melin, E. In Abderhalden *Handbuch der biologischen Arbeitsmethoden* Abt. XI. Teil 4 : 2 (1936) 1025.
2. v. Euler, H., Karrer, P., Klussmann, E., and Morf, R. *Helv. Chim. Acta* **15** (1932) 504.
3. Kuhn, R., and Grundmann, Ch. *Ber.* **69** (1936) 1557, 1979; **70** (1937) 1318; *Angew. Chem.* **49** (1936) 928; *Chem.-Ztg* **61** (1937) 17.
4. LeRosen, A. L., and Zechmeister, L. *J. Am. Chem. Soc.* **64** (1942) 1075; Zechmeister, L., and Pinckard, J. H. *J. Am. Chem. Soc.* **69** (1937) 1930, 1934.
5. Kuhn, R., Köhler, F., and Köhler, L. *Z. Physiol. Chem.* **247** (1937) 197.
6. Kögl, F., and Erxleben, H. *Ann.* **479** (1930) 1.
7. Kögl, F., Erxleben, H., and Jänecke, L. *Ann.* **482** (1930) 170.
8. Literature: Kuhn, R. *Angew. Chem.* **53** (1940) 1.

Received February 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 220—224

Tuberculostatic Derivatives of *p*-Aminobenzoic Acid

I. Esters and Amides of 4-Aminosalicylic Acid

KAI ARNE JENSEN, KARL-GUSTAF ROSDAHL and HELMUTH INGVORSEN

Research Laboratory of AB Ferrosan, Malmö, Sweden

Lehmann [1], in 1946, reported that 4-aminosalicylic acid possessed bacteriostatic properties against the tubercle bacillus. The compound is effective in the suppression of tuberculous infections of laboratory animals and has under the name PAS (*para*-amino-salicylic) been applied in the treatment of tuberculosis in man, which it seems to influence favourably.

On the basis of the observation by Bernheim [2], that salicylic acid and benzoic acid stimulate the respiration of the tubercle bacillus, Lehmann investigated several derivatives of salicylic acid and *p*-amino-benzoic acid in the hope of finding a compound acting as antagonist to salicylic acid. The most bacteriostatic active of these compounds was 4-aminosalicylic acid. It soon turned out, however, that its effect could not be considered as inhibitory towards salicylic acid. Considering the findings of Johnson *et al.*[3] and Wyss *et al.*[4], *viz.* that certain 2-substituted *p*-aminobenzoic acids act as competitors to *p*-aminobenzoic acid, it also seems more likely, that the effect of 4-aminobenzoic acid should be due to competition with the metabolite *p*-aminobenzoic acid.

As a matter of fact it could be shown that the inhibitory effect of *p*-aminosalicylic acid could be neutralized by addition of *p*-aminobenzoic acid. The bacteriostatic effect of *p*-aminosalicylic acid against bacteria, other than *Mycobacterium tuberculosis*, is very slight (*cf.* Sievers [5]); on pneumococci there was no inhibition until a concentration between 0.012 and 0.025 *M*; by addition of *p*-aminobenzoic acid making the solutions 0.0002 *M* as to this compound, there was no inhibition in the 0.025 *M* PAS-solution and only slight inhibition in a 0.05 *M* PAS-solution.*

* We thank Kai Schmith, M. D., for the performance of these experiments.

Although the competitive effect cannot be demonstrated so clearly as with the sulfonamides, due to the slight bacteriostatic effect of PAS against pneumococci, we think that these experiments prove that the effect of PAS is in fact inhibitory to *p*-aminobenzoic acid. Lehmann has obtained similar results from experiments with *M. tuberculosis* (private communication).

In order to investigate whether the bacteriostatic effect of 4-aminosalicylic acid could be enhanced by substitutions, we have prepared some of its esters and amides, derived from aliphatic amines. Amides with heterocylic substituents, which cannot be prepared in the same way as the aliphatic derivatives, will be described in a subsequent paper.

The bacteriostatic effect of these new compounds against *M. tuberculosis* has been determined by Lehmann, who will publish his results elsewhere. It may, however, be mentioned here that all the compounds inhibit the growth of tubercle bacilli, most of them having about the same bacteriostatic effect as the free acid.

EXPERIMENTAL

The esters of 4-aminosalicylic acid were prepared by catalytic hydrogenation of the corresponding nitroesters. 4-Nitrosalicylic acid was prepared from 4-nitrophthalimide [6], and the acid was esterified by refluxing with the appropriate alcohol in the presence of sulphuric acid:

20 g of *p*-nitrosalicylic acid and 8 ml of conc. sulphuric acid were dissolved in 160 ml of the alcohol and refluxed for 16—24 hours. The alcohol was removed *in vacuo*, water was added and the precipitate filtered, dried and crystallized from methanol. The nitroesters were recrystallized from the corresponding alcohol, decolourizing the solutions with a little charcoal (methanol or ethanol may also be used without any ester interchange). Yields varying from 40—60 %.

The esters form light yellow crystals, which are easily soluble in alcohol, benzene etc. and slightly soluble in water. Melting points and analyses are presented in Table 1. (The ethylester has previously been synthezised by Borsche [7], who describes it as white, it has, however, about the same colour as sulphur.)

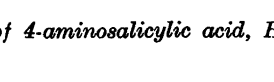
Table 1. Esters of 4-nitrosalicylic acid, O_2N *COOR.*

R	Formula	M.p. °C	N analyses, % calc.	found
Ethyl	$C_9H_9O_5N$	90	6.63	6.61
n-Propyl	$C_{10}H_{11}O_5N$	33	6.23	6.25
iso-Propyl	$C_{10}H_{11}O_5N$	73	6.23	6.28
n-Butyl	$C_{11}H_{13}O_5N$	35	5.86	5.90
iso-Butyl	$C_{11}H_{13}O_5N$	62	5.86	5.79

The nitro-esters were hydrogenated in ethanolic solution (the use of higher alcohols is not convenient):

10 g of ethyl-4-nitrosalicylate were suspended in 50 ml of ethanol and 0,5 g PtO_2 was added. The suspension was shaken in an atmosphere of hydrogen at 4 atm.; the ester dissolves gradually under noticeable heat evolution. After two hours the calculated amount of hydrogen was absorbed, the solution was cold and a white crystalline product formed. The crystals were dissolved by heating, the solution filtered from platinum and concentrated *in vacuo*. By addition of water a white precipitate separated; it was filtered off and recrystallized from dilute alcohol or benzene. The hydrogenation of the other esters was carried out in smaller amounts (*e. g.* 1 g of ester, 20 ml of alcohol and 0.1 g of PtO_2).

The esters of 4-aminosalicylic acid form white crystals, which are very soluble in alcohol. They may conveniently be recrystallized from dilute alcohol or benzene. The yields amount to 70—80 %. Melting points and analyses are presented in Table 2.

Table 2. Esters of 4-aminosalicylic acid, 

R	Formula	M. p. °C	N analyses, % calc.	found
Ethyl	$C_9H_{11}O_3N$	115	7.73	7.51
n-Propyl	$C_{10}H_{13}O_3N$	102—03	7.17	7.11
iso-Propyl	$C_{10}H_{13}O_3N$	75—76	7.17	7.20
n-Butyl	$C_{11}H_{15}O_3N$	93—94	6.70	6.78
iso-Butyl	$C_{11}H_{15}O_3N$	84—85	6.70	6.73

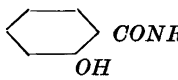
4-Nitrosalicylamide has been prepared by Borsche by reaction of ethyl 4-nitrosalicylate with alcoholic ammonia. It is, however, more convenient to use aqueous ammonia:

10 g of ethyl 4-nitrosalicylate and 50 ml of concentrated aqueous ammonia were heated at 100° for two hours in a closed vessel. The solution was turned to dryness in vacuo and the residue recrystallized from water, charcoal and a few drops of acetic acid being added.

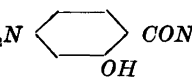
The methylamide was made in the same way. The other amides were prepared from the anhydrous amines:

Ethyl 4-nitrosalicylate (1—5 g) was placed in a thick walled glasstube and the same weight of the appropriate amine added under cooling. The tube was sealed and heated at 100° in water bath. At first a yellow crystalline solid (alkylammonium salt of the phenol) is formed, but gradually it deliquesces and is transformed into a syrup. The time

of heating varies from 1 hour (ethyl- and propylamides) to about 14 hours (diethyl- and dipropylamide). The reaction products were dissolved in 50 ml of ethanol; the solution was diluted to 250—300 ml with water, decolorized with carbon, filtered and the amides precipitated by addition of hydrochloric acid. The amides were recrystallized from dilute alcohol (1 : 1) containing about ½ % acetic acid. The amides of 4-nitrosalicylic acid (Table 3) form beautiful white or yellowish crystals, slightly soluble in water, very soluble in alcohol.

Table 3. Amides of 4-nitrosalicylic acid,  *CONRR'.*

R	R'	Formula	M. p. °C	N analyses, % calc.	found
H	H	$C_7H_6O_4N_2$	194	15.40	15.61
CH_3	H	$C_8H_8O_4N_2$	218	14.29	14.35
C_2H_5	H	$C_9H_{10}O_4N_2$	157	13.33	13.52
C_3H_5	H	$C_{10}H_{10}O_4N_2$	134	12.66	12.57
n-C_3H_7	H	$C_{10}H_{12}O_4N_2$	128	12.50	12.35
i-C_3H_7	H	$C_{10}H_{12}O_4N_2$	187	12.50	12.63
C_2H_5	C_2H_5	$C_{11}H_{14}O_4N_2$	183	11.76	11.75
n-C_4H_9	H	$C_{11}H_{14}O_4N_2$	122	11.76	11.82
i-C_4H_9	H	$C_{11}H_{14}O_4N_2$	167	11.76	11.86
n-C_3H_7	*n*-C_3H_7	$C_{13}H_{18}O_4N_2$	131	10.52	10.42
$C_6H_5CH_2$	H	$C_{14}H_{12}O_4N_2$	154	10.28	10.07

Table 4. Amides of 4-aminosalicylic acid,  *CONRR'.*

R	R'	Formula	M. p. °C	N analyses, % calc.	found
H	H	$C_7H_8O_2N_2$	162	18.42	18.40
CH_3	H	$C_8H_{10}O_2N_2$	158	16.86	16.93
C_2H_5	H	$C_9H_{12}O_2N_2$	138	15.55	15.73
C_3H_5	H	$C_{10}H_{12}O_2N_2$	134	14.58	14.54
n-C_3H_7	H	$C_{10}H_{14}O_2N_2$	142	14.43	14.27
i-C_3H_7	H	$C_{10}H_{14}O_2N_2$	161	14.43	14.58
C_2H_5	C_2H_5	$C_{11}H_{16}O_2N_2$	141	13.46	13.50
n-C_4H_9	H	$C_{11}H_{16}O_2N_2$	125	13.46	13.51
i-C_4H_9	H	$C_{11}H_{16}O_2N_2$	127	13.46	13.51
n-C_3H_7	*n*-C_3H_7	$C_{13}H_{20}O_2N_2$	121	11.86	11.98
$C_6H_5CH_2$	H	$C_{14}H_{14}O_2N_2$	129	11.57	11.48

The nitroamides were converted into the corresponding aminoderivatives by catalytic hydrogenation, 25 ml of ethanol and 0.1 g of PtO_2 being used for 1 g of the nitro-amide. After absorption of the calculated amount of hydrogen the solutions were filtered and evaporated almost to dryness *in vacuo;* the

residues were dissolved in boiling water, the solutions decolourized with carbon and filtered. By cooling the amides precipitate in good yields (70—80 %) in form of colourless crystals, which are more soluble in water than the corresponding nitro compounds.

By catalytic hydrogenation the allylamide of 4-nitrosalicylic acid is transformed into the *n*-propylamide of *p*-aminosalicylic acid. The allylamide of *p*-aminosalicylic acid was prepared by reduction of the corresponding nitro compound with zinc and hydrochloric acid in alcoholic solution.

4-Aminosalicylic amide has been synthesized also from methyl 4-aminosalicylate [8] and ammonia: A mixture of 8 g of methyl 4-aminosalicylate and 8 ml of conc. aqueous ammonia was heated in a closed vessel for two hours at 100°. The solution was evaporated almost to dryness *in vacuo*, and the crystals, which separated, were recrystallized from water. The compound was identified as the amide of 4-aminosalicylic acid. With *n*-butylamine and methyl 4-aminosalicylate the corresponding amide was isolated too. The higher esters on the contrary react very slowly with ammonia and amines, even at 140°, and only unchanged ester or oily products could be obtained.

SUMMARY

The bacteriostatic effect of 4-aminosalicylic acid is antagonized by *p*-aminobenzoic acid.

Some esters and amides of 4-aminosalicylic acid were prepared by catalytic hydrogenation of the corresponding nitro derivatives.

REFERENCES

1. Lehmann, J. *Lancet* **250** (1946) 15; *Svenska Läkartidn.* **43** (1946) 2029.
2. Bernheim, F. *Science* **92** (1940) 204; *J. Bact.* **41** (1941) 387.
3. Johnson, O. H., Green, D. E., and Pauli, R. *J. Biol. Chem.* **153** (1944) 37.
4. Wyss, O., Rubin, M., and Strandskov, F. B. *Proc. Soc. Exp. Biol. Med.* **52** (1943) 106.
5. Sievers, O. *Svenska Läkartidn.* **43** (1946) 2041.
6. Seidel, H., and Bittner, J. C. *Monatsh.* **23** (1902) 431;
   Seidel, H. *Ber.* **34** (1901) 4352.
7. Borsche, W. *Ann.* **390** (1912) 18.
8. Rosdahl, K.-G. *Svensk Kem. Tid.* **60** (1948) 12.

Received February 21, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 225—252

Beitrag zur Kenntnis der Natriummetaphosphate. I

W. TEICHERT und K. RINMAN

Ångvärmeinstitutet, Ingeniörsvetenskapsakademien, Stockholm, Schweden

Natriumpolymetaphosphat hat während der letzten Jahre bei der Behandlung von Rohwässern für verschiedenartige Zwecke technische Bedeutung gewonnen. Gewöhnlich wird diese polymere, anorganische Verbindung als Grahamsches Salz bezeichnet nach ihrem Entdecker Thomas Graham [1], und hat früher lediglich in der qualitativen, anorganischen Analyse eine gewisse Rolle gespielt auf Grund seiner Fähigkeit, in geschmolzenem Zustand verschiedene Metalloxyde zu lösen unter Entwicklung einer für das betreffende Metalloxyd charakteristischen Färbung, die sog. Perlreaktion.

Grahamsches Salz zeichnet sich jedoch ausserdem durch eine Reihe anderer für phosphorsaure Salze recht ungewöhnliche Eigenschaften aus. Natriumpolymetaphosphat bildet z. B. mit einer Anzahl Schwermetall-Ionen in Wasser leicht lösliche, stabile Komplexverbindungen, besonders mit Erdalkali-Ionen, also denjenigen Kationen, welche die Härte dess Wassers bedingen, und ist auf Grund dessen erstmalig von Hall [2] in die Wasseraufbereitung eingeführt worden. Ausserdem ist dieses Salz im Stande, die Kristallbildung und Ausfällung von unter gewöhnlichen Umständen in Wasser schwer löslichen Salzen teilweise oder ganz zu verhindern, und dies bei so niedrigen Metaphosphat-Koncentrationen, dass eine Komplexbildung praktisch nicht mehr zur Wirkung kommen kann. Grahamsches Salz ist aus diesem Grunde besonders in U. S. A. und Deutschland zur Anwendung gekommen bei der Behandlung von stark Bikarbonat-harten Rohwässern, welche schon weit unterhalb der Siedetemperatur zur Kalkausfällung und Steinbildung z. B. in Kondensatoren u. ä. neigen. Welche Verbesserungen man bei solchen, oft sehr schwierigen betriebstechnischen Problemen durch eine Metaphosphatdosierung erreichen kann, ist u. a. von Döring [3] eingehend dargelegt worden.

Die grosse Anzahl der in der letzten Zeit auf dem Gebiet der Alkalimetaphosphate veröffentlichten Arbeiten, welche oft einander widersprechende Aussagen enthalten, hat gezeigt, dass die Chemie dieser in vieler Hinsicht recht

eigenartigen Verbindungen Probleme enthält, welche bisher noch keine zufriedenstellende Lösung gefunden haben. Dies gilt besonders für die Frage der chemischen Konstitution der Metaphosphate, welche dem von den übrigen Alkaliphosphaten abweichenden Verhalten zu Grunde liegen muss.

Karbe und Jander[4] haben vor einigen Jahren in einem umfassenden Artikel eine Zusammenstellung der bis zu diesem Zeitpunkt in der Fachlitteratur über Metaphosphate veröffentlichten Arbeiten gegeben und dabei gleichzeitig eigene Forschungsergebnisse mitgeteilt. Seither sind von verschiedenen Seiten weitere Beiträge zur Lösung der hier interessierenden Fragen geliefert worden.

Die vorliegende Arbeit begrenzt sich auf Untersuchungen an Natriumtrimetaphosphat und Natriumpolymetaphosphat, Grahamsches Salz, einmal deshalb weil Natriumpolymetaphosphat, wie eingangs erwähnt, in der Technologie des Wassers praktische Bedeutung gewonnen hat und gerade betreffs dieses Salzes bei den in der letzten Zeit publizierten Arbeiten widersprechende Angaben zu finden sind, zum anderen weil sich während dieser Untersuchung herausgestellthat, dass Trimetafosfat und Polymetaphosphat in naher Beziehung zu einander zu stehen scheinen besonders bezüglich der räumlichen Konfiguration der beiden Verbindungen.

Natriumtrimetaphosphat und Natriumpolymetaphosphat sind reversibel in einander umwandelbar gemäss Gleichung 1

$$\frac{n}{3}\,Na_3P_3O_9 \underset{t<600°C}{\overset{t\approx 640°C}{\rightleftharpoons}} \left[NaPO_3\right]_n \qquad (1)$$

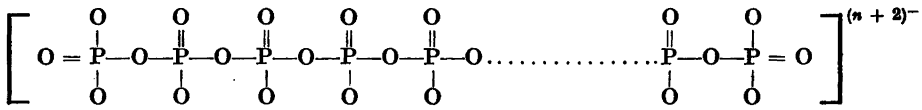
Erhitzt man $Na_3P_3O_9$ über die Schmelztemperatur, bildet sich unter Polymerisation Grahamsches Salz, welches seinerseits beim Tempern nahe unterhalb des Schmelzpunktes allmählich in Trimetaphosphat übergeht.

Karbe und Jander wiesen als erste darauf hin, dass Grahamsches Salz nicht als eine definierte salzartige Verbindung anzusprechen ist entsprechend der bis dahin geltenden Auffassung, dass diese Verbindung eine Hexametaphosphat sei. Mit Hilfe der von Brinzinger[5] entwickelten Dialysen-Methode zur Bestimmung des Ionen- bezw. Molekulargewichts fanden Jander und Mitarbeiter für Präparate, welche auf Temperaturen zwischen 650 und 1200° C erhitzt worden waren, Ionengewichte, welche auf eine Aggregation von 34 bis 88 (PO_3)-Gruppen im Makroion hinzudeuten schienen.

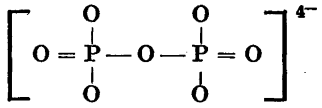
Die absolute Genauigkeit dieser Ionengewichtsbestimmungen ist später von anderen Autoren angezweifelt worden mit der Begründung, dass Trimetaphosphat als Eich-Ion benutzt worden war und Unterschiede der Grösse

der Hydrathülle des Bezugsions und des zu messenden Ions bedeutende Messfehler mit sich führen können.

So hat z. B. Samuelsson [6] bei der Titration von Polymetaphosphorsäuren welche aus verschieden-hoch erhitzten Grahamschen Salzen durch Ionenaustausch dargestellt worden waren, mit Lauge gegen Phenolphtalein und Methylorange eine geringe und mit der Erhitzungs Temperatur des Ausgangsproduktes variierende Differenz des Laugenverbrauchs festgestellt. Diese Differenz im Laugenverbrauch schreibt Samuelsson schwächer-sauren Endgruppen zu bei einem kettenförmigen Anion von folgendem Typ:



Eine Endgruppierung also, welche dem Pyrophosphat-Anion entsprechen würde.



Samuelsson zieht dabei den Schluss, dass der Unterschied im Laugenverbrauch bei der Titration gegen Phenolphtalein und Methylorange von der Länge der Kette abhängig ist und somit ein Kriterium für die Grösse des Polymetaphosphat-Anions darstellen würde. Wäre diese Annahme richtig, so müsste der Verlauf der pH-Kurve bei einer sorgfältigen potentiometrischen Titration von frisch bereiteter Polymetaphosphorsäure mit Lauge einen Potentialsprung zwischen pH = 10 und pH = 11 andeuten, Phenolphtalein dagegen hat seinen Farbumschlag bei einem pH-Wert von ca 9. Eine solche kettenförmige Formulierung des Polymetaphosphat-Anions gibt keine Erklärung für die bei dem Grahamschen Salz sehr ausgeprägte Fähigkeit, mit Erdalkalimetall-Ionen stabile, in Wasser leicht lösliche Komplexverbindungen zu bilden, und dass bei der Hydratation und Spaltung der Polymetaphosphorsäuren Spaltungsprodukte entstehen, die in einem definierten Mengenverhältnis zu einander stehen. Auch mit dem reversiblen Uebergang von Trimetaphosphat in Polymetaphosphat ist eine solche Formulierung schwer in Einklang zu bringen.

Es hat sich jedoch bei einer über die hier mitgeteilten Versuchsresultate hinaus weitergeführten Untersuchung [7] erwiesen, dass Trimetaphosphat und Grahamsches Salz in stuktureller Hinsicht grosse Ähnlichkeit aufweisen, und es erscheint durchaus wahrscheinlich, dass die mit Hilfe der Dialyse bestimm-

ten Ionengewichte ein richtiges Bild des Polymerisationsgrades der auf verschiedenhohe Temperaturen erhitzten Grahamschen Salz ergeben.

Die für diese Untersuchung verwendeten Präparate wurden erhalten durch allmähliches Erhitzen eines äquimolaren Gemisches von Mononatriumorthophosphat und Ammoniumnitrat auf Temperaturen zwischen 240 und 630° C. Die Erhitzungsdauer erstreckte sich über 18 bis 20 h. Darüberhinaus wurden aus den hierbei erhaltenen kristallinen Produkten verschiedene Schmelzen (Grahamsches Salz) dargestellt durch Erhitzen dieser kristallinen Produkte über die Schmelztemperatur. Die Natriummetaphosphat-Schmelzen wurden 1—2 h auf einer bestimmten Temperatur über dem Schmelzpunkt gehalten und dann rasch abgekühlt durch einsetzen des Schmelzgefässes, eine flache Nickel- oder Platinschale, in eine Kältemischung von — 20° C. Die Präparate mit einer Darstellungstemperatur unter 630° C bestanden aus einer porösen, kristallinen Masse, welche sich nach dem Zerkleinern meist ohne Rückstand von sogen. Madrell-Salz in Wasser lösten. Durch Umfällung aus der wässrigen Lösung mit Äthylalcohol wurde das Salz gereinigt. Es entstanden hierbei anfänglich immer sehr voluminöse Fällungen, die jedoch bei kräftigen Umrühren und Erhöhung des Alcohol-haltes auf ca 70 % in leicht-filtrierbare gut sedimentierende Niederschläge übergingen. Lediglich bei Präparaten mit einer Erhitzungstemperatur zwischen 240 und 260° C erhielt man beim Versetzen der wässrigen Lösung mit Alcohol zuerst eine Emulsion, aus welcher sich nach einigen Minuten eine schwere, wasserklare, ölartige Flüssigkeit abschied, die bei wiederholter Behandlung mit absoluten Alcohol in ein wasserfreies Kristallpulver überging. Die Schmelzen wurden direkt ohne vorherige Reinigung untersucht. Für sämtliche Präparate wurde ein Atomverhältnis Na : P : O = 1 : 1 : 3 gefunden. Der pH-Wert einer 0,1-*N* Lösung lag bei den niedrigpolymeren Natriumsalzen bei 6,2, während für die Lösungen von Grahamschen Salz pH-Werte von 5,7—5,8 gefunden wurden.

I. IONENGEWICHTSBESTIMMUNGEN

Die Messgenauigkeit von *Brinzingers* Dialysenmethode zur Bestimmung von Ionengewichten beruht in erster Linie auf der Wahl eines geeigneten Bezugsions. Dieses muss betreffs des Verhältnisses zwischen wahrem Ionengewicht und der das Ion in wässriger Lösung umgebenden Hydrathülle ungefähr dem des zu messenden Ions entsprechen. Im Verlaufe dieser Untersuchung erwies es sich, dass eine Eichung der verwendeten Kollodiummembranen im Falle der Metaphosphat-Anionen, auch für die hochpolymeren Verbindungen, das Chlor-Ion am meisten geeignet erschien. Die gefundenen Mess resultate beziehen sich daher sämtlich auf Eichwerte, welche durch Dialyse

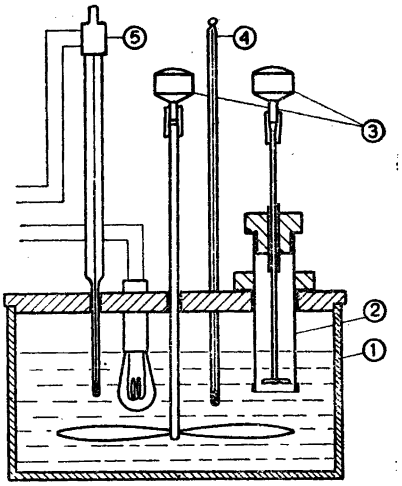


Fig. 1. Apparat zur Ionengewichtsbestimmung.

von 0,1-normaler NaCl-Lösung in 1,0-normaler Ammoniumnitratlösung als Fremdelektrolyt erhalten worden waren.

Die Versuchsanordnung ist in Fig. 1 wiedergegeben. Sie besteht aus einem cylindrischem Glasgefäss (1) mit ca 10 l Fassungsvermögen, welches den Aussenelektrolyten, Ammoniumnitrat, enthält. Die eigentliche Dialysierzelle besteht aus einem plangeschliffenem, ziemlich weitem Glasrohr (2), welches am unteren Ende durch eine Kollodiummembran verschlossen ist. Die Dialysierzelle kann leicht gehoben oder gesenkt werden zur Einstellung von gleichem Flüssigkeitsniveau zwischen Aussen- und Innenlösung. Die Aussenlösung wurde jeweils nach 5 Dialysen erneuert. Durch 2 Glaspropeller mit Synchronmotoren (3) wurden die Lösungen in gleichmässiger Bewegung gehalten. Das Aussengefäss arbeitete gleichzeitig als Thermostat, dessen Temperatur mit einem geeichten Normalthermometer (4) kontrolliert, und durch ein Kontaktthermometer (5) auf 25 $\pm$ 0,5° C konstant gehalten wurde. Der Koncentrationsunterschied im Innengefäss vor und nach der Dialyse wurde gravimetrisch bestimmt.

Der Dialysenmethode liegt bekanntlich die von Riecke [8] gefundene Beziehung zwischen Diffusionskoefficient und Molekulargewicht zu Grunde. Innerhalb gleichartiger Systeme besteht ein enger Zusammenhang zwischen Diffusions- bezw. Dialysenkoefficient und Molekular- bezw. Ionengewicht gemäss Gleichung (2) und (2) a.

$$D_1 \cdot \sqrt{M_1} = D_2 \cdot \sqrt{M_2} \qquad (2)$$

$$\lambda_1 \cdot \sqrt{M_1} = \lambda_2 \cdot \sqrt{M_2} \qquad (2a)$$

D = Diffusionskoefficient.
λ = Dialysenkoefficient.
M = Molekular- bezw. Ionengewicht.

Dialysenkoefficient λ wird nach Gleichung (3) berechnet.

$$\lambda = \frac{1}{t} \cdot \ln \frac{c_2}{c_1} \tag{3}$$

c_1 = Koncentration am Anfang des Versuches.
c_2 = » » Ende » »
t = Dialysendauer, min.

Die hierbei erhaltenen Dialysenkoefficienten können nicht ohne weiteres mit den von Karbe und Jander gefundenen Werten verglichen werden, da man bei diesem Messverfahren lediglich relative Werte erhält, die von der verwendeten Apparatur und den Dialysier-Membranen abhängig sind.

In Tabelle 1 sind die nach dieser Methode bestimmten Ionengewichte derjenigen Präparate zusammengefasst, welche bei Temperaturen von 240 bis 630° C dargestellt worden waren. Aus den Messresultaten geht hervor, dass Natriummetaphosphat im Gegensatz zu bisher geltenden Auffassungen innerhalb eines Temperaturbereichs zwischen 260 und 625° C nur in der *trimeren* Form, $Na_3P_3O_9$, vorzuliegen scheint. Bei Temperaturen unter 260° C entspricht das gefundene Ionengewicht der *dimeren* Form, $Na_2P_2O_6$. Dies steht in Uebereinstimmung mit dem von Travers und Yu Kwong Chu [9] kryoskopisch gefundenem Wert. Das für Präparat 12 mit einer Erhitzungstemperatur von 630° C gefundene Ionengewicht deutet zwar auf eine 4-fache Aggregation hin, jedoch wäre das Existenzgebiet für dieses Metaphosphat ausserordentlich begrenzt, und der höhere Wert kann ebenso in einer beginnenden Polymerisation zu hochpolymeren Anionen seine Ursache haben.

Die in Tabelle 1 in Klammern aufgeführten Ionengewichte wurden für die entsprechenden Salze vor der Reinigung durch Umfällung erhalten und lassen darauf schliessen, das eine Depolymerisation hierbei nicht stattgefunden hat. Präparat 20 wurde durch Tempern einer erstarrten Schmelze von Grahamschen Salz bei ca 600° C während 18 h erhalten. Hierbei geht die glasartige Substanz allmählich in eine weisse, undurchsichtige, kristalline Masse über, ohne dass dabei die äussere Form der Bruchstücke verändert wird, wie dies z. B. bei dem sogen. Natriumtripolyphosphat der Fall ist.

Ausser den in Tabelle 1 wiedergegebenen Versuchsresultaten an niedrigpolymerisierten Metaphosphaten wurde das Ionengewicht für eine Anzahl bei

Tabelle 1. Ionengewichte der niedrig-polymeren Natriummetaphosphate.

Präparat Nr	Erhitzungs-temperatur °C	Ionengewicht $(PO_3)^-$	Molekulargewicht $NaPO_3$
17	245	161	205
9	250	169 (154)	216
8	265	232	296
7	275	256	327
6	305	232	296
5	320	236 (233)	301
13	325	236	301
4	350	247 (249)	315
10	405	248 (241)	316
11	530	221	282
20	600	257 (236)	328
18	625	260	332
12	630	314	397

verschiedenen Temperaturen dargestellten Grahamschen Salzen auf die gleiche Weise bestimmt. Die aus den Dialysenkoefficienten berechneten Ionengewichte sind in Tabelle 2 wiedergegeben.

Tabelle 2. Ionengewichte der hoch-polymeren Natriummetaphosphate.

Präparat Nr	Erhitzungs-temperatur °C	Ionengewicht $(PO_3)^-_n$	Molekulargewicht $[NaPO_3]_n$
14	650	1250	1592
15	710	1460	1860
16	750	1809	2304
19	790	2592	3306
techn. sogen. Hexametaphosphat		2941	3750

Die für Grahamsches Salz in dem in Tabelle 2 wiedergegebenen Temperaturbereich gefundenen Ionengewichte liegen durchweg niedriger als die von Karbe und Jander seinerzeit mitgeteilten Werte. Der Verlauf der Molekulargewichts-Temperatur-Kurve in Fig. 2 deutet darauf hin, dass der Polymerisationgrad nicht eine nahezu lineare Funktion der Erhitzungstemperatur ist, wie aus den experimentellen Befunden sowohl bei Karbe und Jander als auch aus dem in Samuelssons Artikel wiedergegebenen Diagramm zu schliessen war. Tabelle 3 enthält eine Uebersicht über die Anzahl (PO_3)-bezw. $(P_3O_9)^{3-}$ Gruppen, welche in Abhängigkeit von der Darstellungstemperatur am Aufbau des Makro-Ions beteiligt zu sein scheinen.

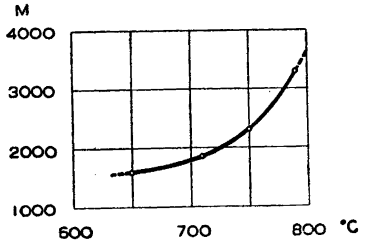


Fig. 2. Molekulargewicht des Grahamschen Salzes in Abhängigkeit von der Darstellungstemperatur.

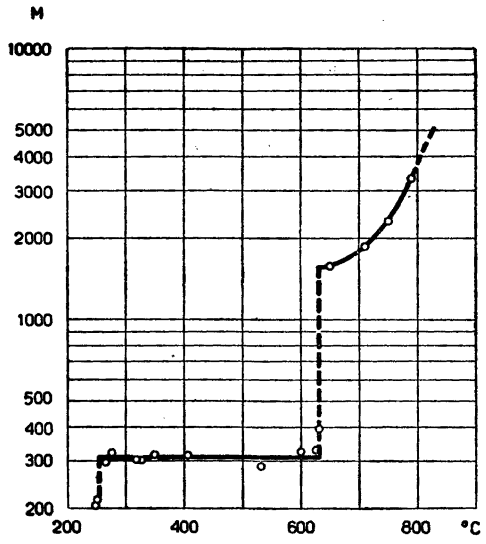


Fig. 3. Molekulargewichte der Natriummetaphosphate bei Darstellungstemperaturen zwischen 200° C und 800° C.

Tabelle 3. Polymerisation der Natriummetaphosphate in Abhängigkeit von der Darstellungstemperatur.

Darstellungs-Temperatur °C	Anzahl im Makro-Ion $(PO_3)^-$	Anzahl im Makro-Ion $(P_3O_9)^{3-}$
230—260	2	—
265—625	3	1
630	(4)	—
650	15—16	5
710	18	6
750	23	8
790	32—33	11

Fig. 3 gibt einen Ueberblick über die durch Bestimmung des Ionengewichts für Natriumdimeta- und Natriumtrimetaphosphat gefundenen Stabilitätsbereiche und den Uebergang in Natriumpolymetaphosphat mit einer von der Erhitzungstemperatur in starkem Masse abhängigen Polymerisation, welche als Molekulargewicht in logarithmischer Skala wiedergegeben ist. Der Kurvenzug von 650° C aufwärts zeigt deutlich, dass die Polymerisation keine lineare Funktion der Temperatur ist. Es ist anzunehmen, dass der Verlauf der Polymerisation in der Hauptsache durch zwei sich überlagernden und einan-

der entgegengesetzt wirkende Faktoren beeinflusst wird. In positiver Richtung wirkt die sicherlich exotherme Polymerisationswärme bei der Zusammenlagerung der Elementargruppen, während in entgegengesetzter Richtung die mit zunehmender Temperatur steigende Schwingungsenergie zwischen den Elementargruppen und die intramolekularen Schwingungen wirken und bei genügend hoher Temperatur die Verknüpfungen zwischen den einzelnen Gruppen zum Zerreissen bringen. Es erscheint unwahrscheinlich, dass sich hieraus eine lineare Temperaturfunktion ergibt.

Der Uebergang von Natriumtrimetaphosphat in die hochpolymere Form verläuft möglicherweise über Zwischenstufen, eine Existenz eines Hexametaphosphats hat bei diesen Versuchen jedoch nicht festgestellt werden können.

Auf die gleiche Weise wurden die Anionengewichte einer Anzahl freier Metaphosphorsäuren bestimmt. Die Säuren wurden durch Umsetzung von in Wasser suspendierten Bleimetaphosphat mit Schwefelwasserstoff erhalten. Der Ueberschuss an H_2S wurde durch einen lebhaften Stickstoff-Strom entfernt, und das gebildete Bleisulfid von der Säure abfiltriert. Aus Tabelle 4 ergibt sich, dass die für die Säuren erhaltenen Anionengewichte denen der Natriumsalze, welche als Ausgangsstoffe verwendet worden waren, grössenordnungsmässig entsprechen. Der für ein hoch-polymeres Anion gefundene Wert liegt zwar etwas niedriger als das Anionengewicht des entsprechenden Grahamschen Salzes, jedoch ist der Unterschied grundsätzlich unbedeutend und erklärlich, da die Metaphosphorsäuren äusserst instabile Gebilde sind, wie weitere Untersuchungen erwiesen haben, und bei der oben beschriebenen langwierigen Darstellungsmethode kann eine teilweise Spaltung sehr wohl statt gefunden haben. Auch ein geringer Anteil niedrig-molekularer Spaltungsprodukte hat bei dieser hoch-polymeren Säure eine stärkere Einwirkung auf den durch Dialyse bestimmten Wert als beispielsweise bei Trimetaphosphorsäure wegen des grösseren Unterschiedes der Anionengewichte zwischen Makro-Ion und den Anionen der Spaltungsprodukte.

Tabelle 4. Ionengewichte der Metaphosphorsäuren und der den Säuren entsprechenden Natriumsalze.

Präparat Nr	Darstellungstemperatur C°	Ionengewicht	
		Natriumsalz	Säure
7	275	256	259
6	305	232	247
11	530	221	237
16	750	1808	1546

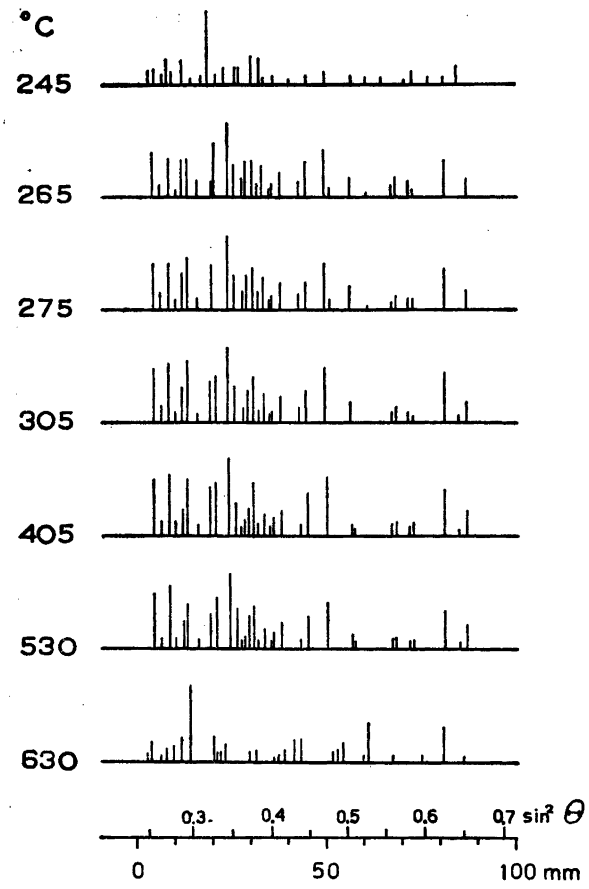


Fig. 4. Pulverdiagramme der niedrig-polymeren Natriummetaphosphate.

II. PULVERDIAGRAMME

Parallel mit den Dialysenversuchen wurden von einer Anzahl Präparate mit verschiedenen Darstellungstemperaturen Röntgendiagramme aufgenommen. Auch hierbei zeigte es sich, dass die erhaltenen Diagramme für Natriummetaphosphate, welche bei Temperaturen zwischen 265 und 530° C dargestellt worden waren, mit einander identisch sind, während das bei 245° C dargestellte Präparat in einem anderen Gittertyp kristallisiert. Die Pulverdiagramme sind in Fig. 4 und Fig. 5 schematisch wiedergegeben. Für rekristallisiertes Grahamsches Salz (Präparat Nr. 20) wurde ein Diagramm erhalten, welches sich von den Diagrammen der Präparate mit Darstellungstemperaturen zwischen 265 und 530° C deutlich unterscheidet und möglicherweise einer Hochtemperaturform des Natriumtrimetaphosphates zuzuschreiben ist.

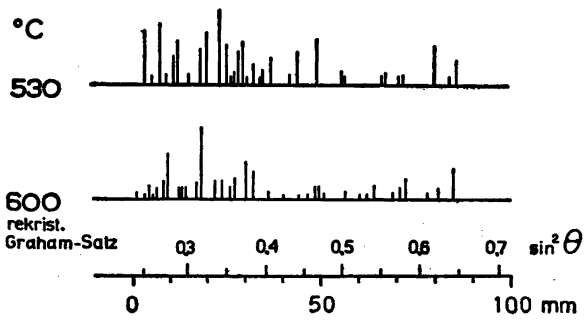


Fig. 5. Pulverdiagramm von rekristallisiertem Grahamschen Salz und Natriumtrimetaphosphat (530° C).

III. TEMPERATURVERLAUF BEI ERHITZUNG VON NATRIUMMETAPHOSPHAT

Der Temperaturverlauf bei der allmählichen Erhitzung von Natriummetaphosphat, welches durch Dehydratation eines äquimolaren Salzgemisches von $NaH_2PO_4 \cdot H_2O$ und NH_4NO_3 bei 235° C während 15 h dargestellt worden war, zeigt gemäss dem in Fig. 6 wiedergegebenen Temperatur-Zeit-Diagramm drei Haltepunkte. Bei 260° C scheint das primär gebildete Dimetaphosphat in Trimetaphosphat überzugehen, dies steht in Uebereinstimmung mit den durch Dialyse bestimmten Anionengewichten und den erhaltenen Röntgendiagrammen. Bei ca 550° C findet anscheinend eine Umwandlung statt in eine bei niederer Temperatur instabile Hochtemperaturform des Trimetaphosphats. Eine Aufnahme von Hochtemperaturdiagrammen und Bestimmung der Lösungswärmen in einem Temperaturbereich zwischen 400 und 600° C könnte hierüber Klarheit bringen. Der dritte Haltepunkt bei ca 645° C entspricht der Schmelztemperatur, bei welcher gleichzeitig der Uebergang in die hochpolymere Form stattfindet. Nach der verhältnismässig geringen Haltezeit zu urteilen, ist die Polymerisation ein exothermer Vorgang, durch welchen die Schmelzvärme herabgesetzt wird.

Auf Grund von Ionengewichtsbestimmungen, Pulverdiagrammen und einer einfacheren thermischen Analyse dürfte folgendes Umwandlungsschema aufgestellt werden können für die bei Zimmertemperatur stabilen bezw. metastabilen Modifikationen des Natriummetaphosphats, welches durch Dehydratation von Mononatriumorthophosphat dargestellt wird.

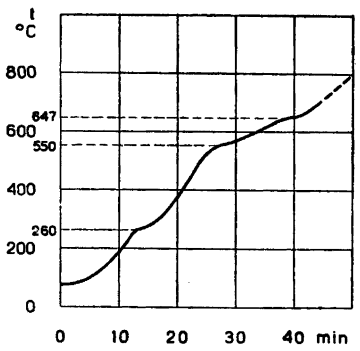


Fig. 6. Zeit-Temperatur-Diagramm bei der Erhitzung von Natriummetaphosphat.

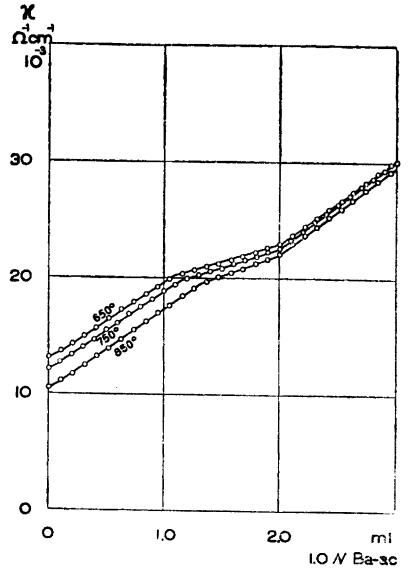


Fig. 7. Konduktometrische Titration von Grahamschen Salzen mit Bariumacetat. 20 ml 0,1 N $[NaPO_3]_n$ titriert mit 1,0 N Ba-Ac.

$$2\ NaH_2PO_4 \cdot H_2O \xrightarrow[-3\ H_2O]{t \approx 230^\circ C} Na_2P_2O_6 \qquad (4)$$

$$3\ Na_2P_2O_6 \xrightarrow{t \approx 260^\circ C} 2\ Na_3P_3O_9 \qquad (5)$$

$$\frac{n}{3}\ Na_3P_3O_9 \xrightarrow{t \approx 645^\circ C} [NaPO_3]_n \qquad (6)$$

IV. KOMPLEXBILDUNG DES GRAHAMSCHEN SALZES MIT ERDALKALI-IONEN

Wässrige Lösungen von Grahamschen Salzen besitzen bekanntlich die Fähigkeit, Erdalkali- und andere Schwermetall-Ionen in Form von leicht löslichen, stabilen komplexen Ionen zu binden. Die Aussagen darüber, wie viele Äquivalente Natrium hierbei beispielsweise durch Erdalkali-Ionen ersetzt werden können, ohne dass eine Fällung von schwerlöslichen Erdalkalipolymetaphosphat entsteht, stimmen nicht mit einander überein. Verschiedenartige Bruttoformeln sind für den löslichen Komplex vorgeschlagen

worden, und die Angaben variieren zwischen 1/3, 1/2 und 2/3 der Natrium-Äquivalente, welche z. B. durch Ca^{2+} oder Ba^{2+} ersetzt werden könnten gemäss Formel I, II und III

$$[Na_4\ (Ca\ (PO_3)_6)]_n \qquad \text{I}$$

$$[Na_2\ (Ca\ (PO_3)_4)]_n \qquad \text{II}$$

$$[Na_2\ (Ca_2(PO_3)_6)]_n \qquad \text{III}$$

Rudy, Schloesser und Watzel [10] haben gefunden, dass I der richtigen Zusammensetzung entsprechen müsste, während Jahr und Wendland [11] auf Grund von konduktometrischen Titrationen der Auffassung sind, dass die Hälfte der Na^+-Äquivalente durch Erdalkali ersetzt werden kann, ohne dass eine Fällung entsteht. Andress und Wüst [12] schliesslich bestimmten das Calcium-Bindevermögen von hochpolymeren Natriummetaphosphat durch nephelometrische Titration mit $CaCl_2$-Lösung zu maximal 2/3 des ionogen gebundenen Natriums gemäss Formel III.

Konduktometrische Titrationen von 0,1 *N* Lösungen bei verschiedenen Temperaturen hergestellter Grahamscher Salze mit 1,0 *N* Bariumacetat-Lösung haben dagegen gezeigt, dass keine der Formeln I—III allein ein richtiges Bild der chemischen Bruttozusammensetzung von derartigen Komplexverbindungen zu geben im Stande ist. Auch die Fähigkeit des Grahamschen Salzes zur Bildung leichtlöslicher Komplexverbindungen mit Erdalkali-Ionen scheint sich mit der Darstellungstemperatur zu ändern, d. h. das Komplexbindungsvermögen ist abhängig von der Grösse des Polymetaphosphat-Anions. Die bei der Titration für verschiedene Grahamsche Salze erhaltenen Konduktivitätskurven sind in Fig. 7 wiedergegeben und zeigen, wie dies früher schon von Jahr und Wendland gefunden worden war, zwei deutlich hervortretende Knickpunkte, welche durch nahezu gerade Linien verbunden werden. Der erste geradlinige Anstieg der Kurven gibt das Komplexbindungsvermögen des betreffenden Grahamschen Salzes wieder. Während dieser Phase entsteht beim Zufliessenlassen von Bariumacetat-Lösung keine beständige Fällung, sondern sämtliches zugeführte Erdalkali wird zur Bildung des löslichen Komplexes verbraucht. Der erste Knickpunkt bei allen Titrationskurven liegt jenseits des halben Äquivalenzpunktes. Bei Ueberschreiten des ersten Knickpunktes entsteht eine bleibende Trübung, welche allmählich in eine voluminöse Fällung von Erdalkalipolymetaphosphat übergeht. Während dieser Fällungsperiode zeigt die Leitfähigkeitskurve ebenfalls einen geradlinigen Verlauf jedoch mit bedeutend geringeren Neigungswinkel. Im Äquivalenzpunkt schliesslich ändert sich die Richtung wiederum gemeinsam für alle untersuchten Salzlösungen, und nimmt einem steileren Verlauf.

Der erste Knickpunkt der Konduktivitetskurven verschiebt sich mit steigender Darstellungstemperatur des Natriumpolymetaphosphats immer weiter nach rechts, und es scheint hieraus hervorzugehen, dass das Komplexbindungsvermögen in einem bestimmten Zusammenhang mit der Grösse des Anions steht. Das Äquivalenzverhältnis Na : Ba in dem löslichen Komplex kann demnach nicht durch eine Formel gemäss I—III wiedergegeben werden. Der Quotient $\frac{Ba^{2+}/2}{Na^{+}}$ war bei allen untersuchten Grahamschen Salzen grösser als 1,0 und steigt mit zunehmender Darstellungstemperatur des Salzes. Eine Formulierung nach I erscheint demnach nicht richtig, wenn es sich darum handelt, durch eine Bruttoformel das maximale Komplexbindungsvermögen des Grahamschen Salzes zum Ausdruck zu bringen. Die Formeln II und III stellen augenscheinlich zwei Grenzfälle dar, zwischen welchen das Komplexbindungsvermögen variieren kann. Es ergibt sich hieraus weiterhin, dass diese Titrationsmethode für eine analytische Bestimmung des Grahamschen Salzes umgeeignet ist. Treadwell und Leutwyler [13] haben die Calcium-Titration als Bestimmungsmethode angewandt bei einer Untersuchung an sogen. Hexametaphosphat.

V. ÄQUIVALENTLEITFÄHIGKEIT VON NATRIUMTRIMETAPHOSPHAT UND NATRIUMPOLYMETAPHOSPHAT

Bei Versuchen, die Anionengrösse, d. h. den Polymerisationsgrad verschiedener Metaphosphate zu bestimmen, ist bei früheren Arbeiten öfters die sogen. *Ostwald-Waldensche* Regel herangezogen worden. Diese Regel sagt in etwas vereinfachter Form aus, dass der Quotient

$$\frac{\Lambda_{1024} - \Lambda_{32}}{10} \approx \text{der Valenz des Anions.}$$

Für eine grosse Anzahl in wässriger Lösung stark dissociierter Elektrolyte dürfte diese Regel Gültigkeit haben, eine Bedingung hierfür scheint jedoch die von Kohlrausch gefundene lineare Beziehung zwischen Λ und $\sqrt{c}$ bezw. $\sqrt[3]{c}$ zu sein.

v. Knorre und Wielser [14] zogen betreffs der Ionengrösse eines Natriummetaphosphats den Schluss, dass es sich bei dem betreffenden Salz um ein Trimetaphosphat handelte, da die Differenz $\Lambda_{1024} - \Lambda_{32}$ einen Wert von 32,9 ergab, und es sich somit um ein dreibasisches Anion handeln müsse. Die Messresultate v. Knorre's zeigen jedoch, dass der Verlauf der Kurve des Äquivalentleitvermögens dieses Salzes in wässriger Lösung bei grösseren Ver-

dünnungen gegen Λ_∞ keinen linearen Verlauf zeigt. In einer Anzahl anderer Veröffentlichungen über Metaphosphate ist diese Messmethode in weitem Ausmasse zur Anwendung gekommen, und die erhaltenen Resultate werden als Argumente für die vertretenen Anschauungen bezüglich der Polymerisation der untersuchten Metaphosphate herangezogen.

Da es nunmehr doch als gesichert angesehen werden kann, dass Grahamsches Salz nicht ein chemisches Individuum ist, welches einer Formulierung als Hexametaphosphat, $Na_6P_6O_{18}$, entspricht, sondern ein Polymetaphosphat ist, dessen Anion eine bedeutend grössere Anzahl (PO_3)-Gruppen in sich einschliesst, erschien es daher in Anschluss an die Ionengewichtsbestimmungen dieser Verbindungen von Interesse, die Äquivalentleitfähigkeit von auf verschieden hohe Temperatur erhitzten Natriumpolymetaphosphaten und von Natriumtrimetaphosphat in wässriger Lösung zu studieren, zumal schon Wiesler [15] an verschiedenen Präparaten deutliche Unterschiede in der Äquivalentleitfähigkeit gefunden hatte, dies aber auf eine beginnende Hydratation zurückführte.

An wässrigen Lösungen von Grahamschen Salzen und Natriumtrimetaphosphat wurde Λ bei verschiedenen Verdünnungen bestimmt. Gleichzeitig wurden Parallelversuche an NaCl-Lösungen durchgeführt, um eine Auffassung über die Genauigkeit der Messresultate zu erhalten. Die bei diesen Messungen erhaltenen Werte für $[NaPO_3]_n$, $Na_3P_3O_9$ und für NaCl sind in Tabelle 5 und 6 zusammengefasst. Tabelle 5 enthält darüber hinaus die für 20° C umgerechneten Werte für NaCl nach Lorenz und Michael [16]. Aus den Kurvenzügen in Figur 8 geht hervor, dass der Gang der Äquivalentleitfähigkeit gegen $\sqrt{c}$ für NaCl und $Na_3P_3O_9$ zwischen den Verdünnungen 8 und 256 einen nahezu linearen Verlauf hat, bei grösseren Verdünnungen dagegen zeigen sämtliche Metaphosphate plötzlich einen steilen Anstieg, ähnlich den Salzen schwacher Säuren, trotzdem es sich bei diesen Verbindungen um Salze stark dissociierter Säuren handelt. Λ_∞ für $Na_3P_3O_9$ scheint sogar den entsprechenden Wert für NaCl zu übersteigen. Die Äquivalentleitfähigkeitskurven der verschiedenen Grahamschen Salze sind einander ähnlich. Λ sinkt mit steigender Darstellungstemperatur und von einer Proportionalität zu $\sqrt{c}$ kann nicht mehr gesprochen werden. Aus dem Verlauf der Kurven gegen grosse Verdünnungen hin zu schliessen liegen die Werte für Λ_∞ bei sämtlichen untersuchten Metaphosphaten ziemlich nahe bei einander. Die von v. Knorre für Natriumtrimetaphosphat gefundenen Werte sind etwas höher als die bei diesen Versuchen ermittelten Leitfähigkeiten, jedoch ist der Verlauf der Kurve von Λ gegen $\sqrt{c}$ ein ähnlicher. v. Knorre's Werte gelten wahrscheinlich für 25° C.

Aus den Versuchsresultaten geht hervor, dass man mit Hilfe der *Ostwald-Waldenschen* Regel im Falle der Metaphosphate nicht im Stande ist, irgend-

welche Schlüsse betreffs des Polymerisationsgrades dieser Verbindungen zu ziehen. Dass man für Trimetaphosphat einen annehmbaren Wert von 32,7, also den Wert für ein dreibasisches Anion, erhält, dürfte hier mehr ein Zufall sein.

Tabelle 5. Äquivalentleitfähigkeit von $Na_3P_3O_9$ und NaCl.

Verdünnung $\frac{1}{c}$	Λ 20° $Na_3P_3O_9$	Λ $Na_3P_3O_9$ nach v. Knorre	Λ 20° NaCl	Λ ber. f. 20° NaCl nach Lorenz-Michael
8	67,8	73,9	90,3	94,8
16	76,2	83,6	96,4	98,9
32	84,5	89,5	100,9	100,5
64	93,5	97,9	105,6	105,5
128	96,8	105,9	106,9	107,8
256	100,5	109,5	109,4	109,6
512	109,4	118,7	110,6	111,1
1024	117,2	123,4	112,4	112,1
2048	123,6	—	113,2	112,9
4096	128,8	—	—	—

Tabelle 6. Äquivalentleitfähigkeit von bei verschiedenen Temperaturen dargestellten Grahamschen Salzen.

Verdünnung $\frac{1}{c}$	Λ 20° Darstellungstemperatur° C 650	710	750	790	1000
10	46,5	37,0	31,6	26,6	—
20	49,2	39,2	34,3	29,4	—
40	54,2	43,5	36,2	31,6	—
80	60,0	49,1	41,4	35,3	—
160	69,5	55,4	47,5	39,4	—
320	81,0	64,0	53,9	45,5	—
640	89,1	73,2	62,9	52,8	—
1280	98,8	84,2	71,9	62,7	—
64	—	—	—	—	32,6
2048	—	—	—	—	67,4

Es dürfte schwierig sein, zu entscheiden, was diesem von den Eigenschaften der Salze stark-dissociierter Säuren abweichenden Verhalten der Metaphosphate zu Grunde liegt. Es wäre jedoch denkbar, dass die Beweglichkeit der Ionen trotz weitestgehender Dissociation durch den sterischen Bau der Makro-Ionen beeinflusst wird, und dass dieser Effekt bei grösseren Verdünnungen aufgehoben wird. pH-Bestimmungen an einer wässrigen Lösung eines Gra-

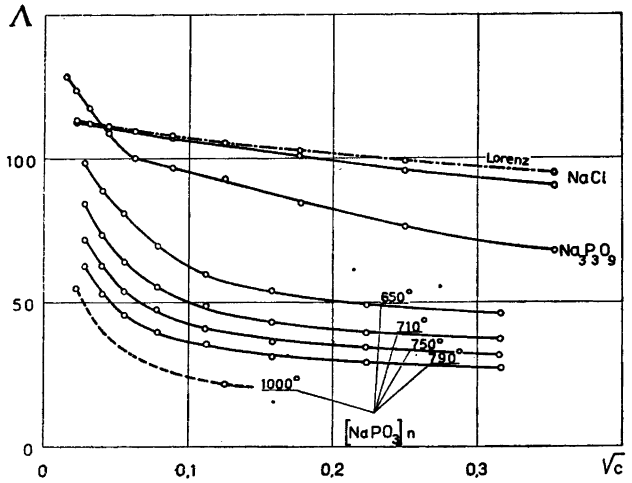


Fig. 8. Äquivalentleitfähigkeit von Natriumtrimetaphosphat und Grahamschen Salzen in Abhängigkeit von $\sqrt{c}$.

hamschen Salzes (800° C) bei verschiedenen Koncentrationen zeigen eine ähnliche Erscheinung. Tabelle 7 gibt eine Zusammenstellung von pH-Werten wieder, welche bei 32—2084 facher Verdünnung bestimmt wurden. Es zeigte sich hierbei, dass der pH-Wert der Lösungen eine auffällige Erhöhung um 0,4 Einheiten innerhalb des gleichen Koncentrationsgebietes erfährt, bei welchem die Kurven für die Äquivalentleitfähigkeit der verschiedenen Polymetaphosphate einen steileren nach oben gerichteten Verlauf nehmen. Eine andere Erscheinung, welche möglicherweise hiermit in Zusammenhang gebracht werden kann, ist die Trägheit der Austauschreaktion $Na^+ - - - - H^+$ in einem mit Wasserstoff-Ionen gesättigten Ionenaustauscher, welche bei wässrigen Lösungen von Grahamschen Salzen verglichen mit Natriumtrimetaphosphat besonders ausgeprägt ist.

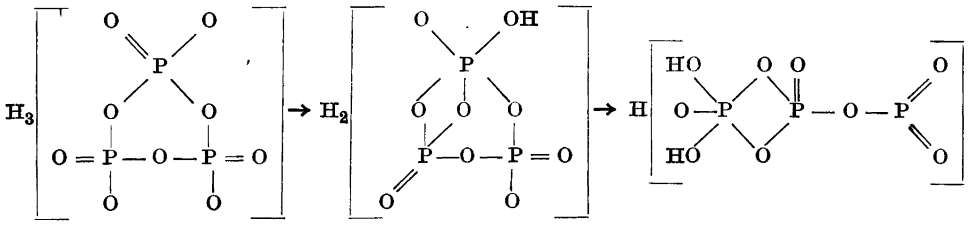
Tabelle 7. pH-Wert einer wässrigen Lösung von Grahamschen Salz (800° C) bei verschiedenen Verdünnungen.

$\frac{1}{c}$	$\sqrt{c}$	pH bei 20° C
32	0,177	5,7
64	0,125	5,7
128	0,0878	5,7
256	0,0626	5,7
512	0,0442	5,9
1024	0,0313	6,0
2048	0,0221	6,1

VI. TRIMETAPHOSPHORSÄURE UND POLYMETAPHOSPHORSÄUREN

Der Unterschied in dem gefundenem Anionengewicht für ein Grahamsches Salz und der daraus über das Bleisalz dargestellten freien Polymetaphosphorsäure (Tabelle 4) deutet darauf hin, dass die Metaphosphorsäuren äusserst instabile Gebilde sind. Es ist anzunehmen, dass die fortschreitende Hydratation parallel mit einer Spaltung in niedrig-molekulare, beständige Hydratationsprodukte geht, und die sich teilweise widersprechenden Anschauungen über der Eigenschaften dieser Säuren dürften ihre Erklärung gerade in der Instabilitet der Metaphosphorsäuren haben.

Ueber das Verhalten der Trimetaphosphorsäure stimmen die während der letzten Zeit vertretenen Anschauungen darin überein, dass diese Säure sich wie eine starke Mineralsäure verhalten muss, bei welcher sämtliche 3 H^+-Ionen die gleiche Dissociationskonstante besitzen. Diese Säure hat keine Pufferwirkung wie beispielsweise Ortho- und Pyrophosphorsäure. Dies ist von Rudy und Schloesser [17] und von Treadwell und Leutwyler [13] durch Verdrängungstitrationen an Natriumtrimetaphosphat mit Säure bezw. Lauge eindeutig erwiesen worden. Es wurden hierbei Titrationskurven erhalten, welche charakteristisch für die Salze einbasischer, stark-dissociierter Säuren sind. Jander und Mitarbeiter sind bei der Titration einer über das Bleisalz hergestellten Trimetaphosphorsäure mit Lauge zu dem gleichen Ergebnis gekommen, haben jedoch bei dem Altern einer solchen Säure eine Verschiebung des für *alle* Phosphorsäuren gemeinsamen Potentialsprunges bei einem Verhältnis Na : P = 1 : 1 festgestellt. Dieses Verhalten würde nicht auf eine Spaltung des Anions hindeuten, sondern müsste seine Ursache in einer Art Disproportionierung des ursprünglich auf sämtliche 3 P-Atome gleichmässig verteilten, ionogen gebundenen Wasserstoffs haben etwa gemäss folgendem Umwandlungsschema.



Es ist jedoch wenig wahrscheinlich, dass eine solche Verschiebung von der elektrostatisch neutralen Ausgangsform zu einer stark assymmetrischen Konfiguration stattfinden könnte ohne gleichzeitige Spaltung in die stabileren Ortho- oder Pyrophosphatanionen. Dieser von Jander beobachtete

Effekt konnte bei dieser Untersuchung in keinem Falle reproduciert werden, obgleich eine grössere Anzahl Metaphosphorsäuren untersucht wurden.

Ueber das Verhalten der Polymetaphosphorsäuren, also jener Säuren, welche den Grahamschen Salzen entsprechen, sind die in der Litteratur mitgeteilten experimentellen Ergebnisse weniger mit einander übereinstimmend. Allerdings haben Rudy und Schloesser und auch Partidge [18] durch Verdrängungstitrationen gefunden, dass es sich auch bei diesen Salzen um Salze starker Säuren handeln muss, während dagegen Treadwell und Leutwyler [19] auf Grund ähnlichen Titrationen annehmen, dass eine sogen. Hexametaphosphorsäure zwei deutlich hervortretende Potentialsprünge aufweisen muss. Salih [20] kommt auf Grund konduktometrischer Titrationen an einer über das Bleisalz aus Grahamschen Salz hergestellten Metaphosphorsäure zu dem Schluss, dass diese Säure 4 stark-dissociierte und 2 schwächere H^+-Ionen auf 6 P-Atome haben muss. Für diese Auffassung spricht z. B. die Fähigkeit der Grahamschen Salze zur Bildung von Komplexverbindungen mit Erdalkali-Ionen.

Es ergibt sich also, dass betreffs dieser Metaphosphorsäuren noch keine völlige Klarheit besteht, was jedoch, wie schon eingangs erwähnt, damit zu erklären ist, dass diese Säuren äusserst instabil sind, zu ihrer Darstellung ungeeignete Methoden angewandt worden sind, und dass es sich bei dem Versuchsmaterial teilweise nicht um reine Metaphosphate mit einem Atomverhältnis $Na : P : O = 1 : 1 : 3$ gehandelt haben kann.

Um irgendwelche exakten Aussagen über das Verhalten dieser Säuren machen zu können, ist es einmal unerlässlich, dass das Ausgangsmaterialen reines Metaphosphat ist, d. h. frei ist von Salzen mehr hydratisierter Phosphorsäuren, und dass die Herstellung schnell genug erfolgt, damit eine Hydratation und Spaltung nach Möglichkeit ausgeschlossen werden kann.

Samuelsson [21] hat während der letzten Jahre eine Anzahl neue Arbeitsmethoden besonders auf dem Gebiet der analytischen Chemie entwickelt, welche sich auf Austauschreaktionen mit Hilfe von organischen Ionenaustauschern gründen und welche sich vor allem durch eine operative Schnelligkeit und Einfachheit auszeichnen. Es war deshalb zu erwarten, dass eine solche Methode mit grösserer Aussicht auf Erfolg bei der Darstellung dieser instabilen Säuren angewendet werden konnte. Die für die im Folgenden beschriebenen titrimetrischen Untersuchungen verwendeten Metaphosphorsäuren wurden ausschliesslich durch eine solche Austauschreaktion gemäss Gleichung (7) dargestellt.

$$\text{Na-Salz} + HX \longrightarrow \text{Säure} + NaX \qquad (7)$$

(X = Kationenaustauscher *Wofatit K*)

Die Austauschreaktion wurde in einer Bürette, gemäss Figur 9, vorgenommen. Der Ionenaustauscher wurde vor jeder Austauschreaktion mit H^+-Ionen gesättigt dadurch, dass ca 250 ml 6 *N* HCl die Austauscherschicht passierten, darauf wurde mit dest. Wasser chlorfrei gewaschen. Die Darstellung der Metaphosphorsäure geschah auf folgende Weise: 300 ml 0,1 *N* Natriummetaphosphat-Lösung wurden durch die Austauscherschicht geschickt. Die ersten 50—75 ml Säure wurden verworfen, da die Säure im Anfang etwas schwächer war, weil der Ionenaustauscher in der Bürette unter Wasser aufbewart wurde. Von der danach abrinnenden Lösung wurden 200 ml aufgefangen und zur Untersuchung verwendet. 20 ml der so erhaltenen Säure wurden unmittelbar darauf und bei verschiedenen Zeitpunkten nach der Darstellung potentiometrisch und konduktometrisch mit 1,0 *N* KOH titriert. Hierbei wurden die pH-Werte mittels einer Glaselektrode, gekoppelt mit einem Röhrenpotentiometer, bestimmt. Die verwendete Glaselektrode wurde innerhalb eines Messbereichs zwischen pH = 1,0 und pH = 13.0 durch Vergleich mit Pufferlösungen nach Clark-Sörensen geeicht. Für die konduktometrischen Bestimmungen wurde die sogen. Ausschlagsmethode angewendet. Die zweite Decimale der pH-Werte ist visuell geschätzt.

POTENTIOMETRISCHE UND KONDUKTOMETRISCHE TITRATION VON FRISCH BEREITETER TRIMETAPHOSPHORSÄURE UND POLYMETAPHOSPHORSÄURE

Bei der potentiometrischen Titration beider Säuren wurden Kurven erhalten, welche zweifelsfrei erweisen, dass sich beide Säuren wie starke Mineralsäuren verhalten. Es wurde nur *ein* Potentialsprung im Äquivalenzpunkt bei Na : P = 1 : 1 gefunden. Sämtliche 3 oder *n* H^+-Ionen haben die gleiche Dissociationskonstante, wie aus Tabelle 8 resp. 9 und den pH-Kurven in Figur 10 und 11 eindeutig hervorgeht. Der pH-Wert von 0,1 *N* $H_3P_3O_9$ liegt bei 1,10—1,15, während die Polymetaphosphorsäuren etwas schwächer sind mit einem pH-Wert = 1,23—1,28 für eine 0,1 *N* Säure. Der Potentialverlauf sowohl bei Trimetaphosphorsäure als auch bei Polymetaphosphorsäuren ist identisch mit den von Rudy und Schloesser durch Verdrängungstitrationen an den Natriumsalzen dieser Säuren mit Säure bezw. Lauge mitgeteilten Titrationskurven.

Eine sprungweise Potentialänderung ausser im Neutralpunkt bei einem Verhältnis Na : P = 1 : 1 hat hierbei nicht beobachtet werden können. Dasselbe gilt für den Gang der Leitfähigkeit bei der konduktometrischen Titration. Trimetaphosphorsäure ergibt hierbei einen scharfen Wendepunkt, wie

aus Figur 12 hervorgeht, während sich das Konduktivitätsminimum bei den Polymetaphosphorsäuren über ein etwas grösseres Intervall ausdehnt, doch mit ausgeprägtem Minimum bei Na : P = 1 : 1.

Tabelle 8. Potentiometrische Titration von $H_3P_3O_9$, 20 ml 0,1 N.

ml 1,0 *N* KOH	pH	ml 1,0 *N* KOH	pH
0	1,10	1,95	2,85
0,20	1,12	1,97	3,30
0,40	1,17	2,00	8,60
0,60	1,22	2,05	11,35
0,80	1,30	2,10	11,73
1,00	1,38	2,20	12,20
1,20	1,49	2,30	12,50
1,40	1,60	2,40	12,70
1,60	1,80	2,60	13,00
1,80	2,10	2,80	13,10
1,90	2,48	3,00	13,16

Tabelle 9. Potentiometrische Titration von $[HPO_3]_n$, 20 ml 0,1 N.

ml 1,0 *N* KOH	pH	ml 1,0 *N* KOH	pH
0	1,28	1,80	2,78
0,20	1,32	1,90	3,28
0,40	1,38	1,95	3,91
0,60	1,47	2,00	7,18
0,80	1,55	2,05	11,00
1,00	1,68	2,10	11,78
1,20	1,85	2,20	12,31
1,40	2,03	2,40	12,90
1,60	2,31	2,60	13,25
1,70	2,50	2,80	13,41

Der Verlauf der beiden pH-Kurven für Trimeta- und Polymetaphosphorsäure weist keine principiellen Unterschiede auf. Die beiden Kurven unterscheiden sich lediglich dadurch, dass die Neigung der pH-Kurve der Polymetaphosphorsäuren in saueren und alkalischen Gebiet etwas stärker ist als bei Trimetaphosphorsäure.

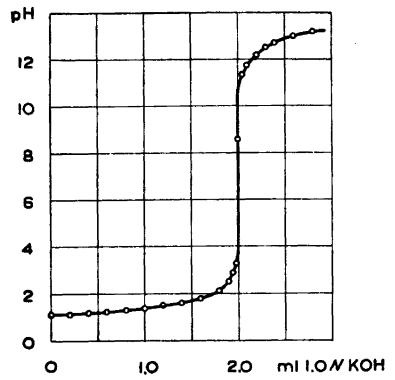


Fig. 10. Potentiometrische Titration von 20 ml 0,1 N Trimetaphosphorsäure mit 1,0 N KOH.

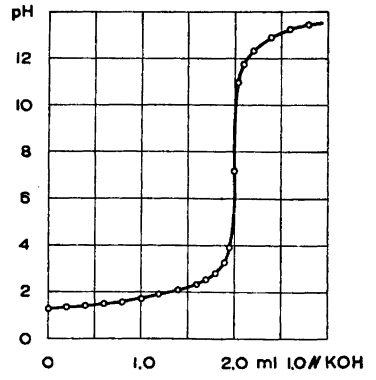


Fig. 11. Potentiometrische Titration von 20 ml 0,1 N Polymetaphosphorsäure mit 1,0 N KOH.

HYDRATATION DER METAPHOSPHORSÄUREN

Kürzlich hat Bell [22] Untersuchungsergebnisse veröffentlicht über den Verlauf und die Bildung von Spaltungsprodukten bei der Hydratation einiger phosphorsaurer Natriumsalze, welche für die technische Wasseraufbereitung Bedeutung gewonnen haben. Es interessieren hier in diesem Zusammenhang besonders die dabei an Grahamschen Salz und Natriumtrimetaphosphat beschriebenen Versuchsergebnisse, welche zeigen, dass der Verlauf der Spaltungsreaktion in starkem Masse von den Versuchsbedingungen abhängig ist.

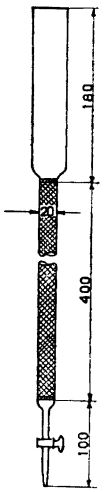


Fig. 9. Realktionsbürette zur Darstellung der Metaphosphorsäuren durch Ionenaustausch.

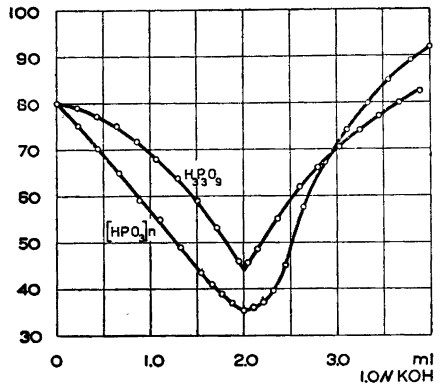


Fig. 12. Konduktometrische Titration von 20 ml 0,1 N Trimetaphosphorsäure und 20 ml 0,1 N Polymetaphosphorsäure mit 1,0 N KOH.

Die Stabilität sowohl der Metaphosphat-Anionen als auch der bei der Spaltung eventuell auftretenden Zwischenstufen, z. B. das sogen. Tripolyphosphat, $Na_5P_3O_{10}$, wechselt ausserordentlich stark mit der Wasserstoff-Ionenkonzentration des Systems. Im Verlauf der vorliegenden Untersuchung hat es sich gezeigt, dass 0,1 *N* Metaphosphorsäure schon nach ca 240 h bei 18° C vollständig in beständigere Phosphorsäuren gespalten ist, während die Natriummetaphosphate in wässriger Lösung sich unter den gleichen Bedingungen wochenlang unverändert halten.

Um den Verlauf der Hydratation dieser Säuren verfolgen zu können, wurden die durch Ionenaustausch dargestellten Metaphosphorsäuren bei verschiedenen Zeitpunkten nach der Darstellung potentiometrisch und konduktometrisch titriert. Hierbei wurden schon nach wenigen Stunden Kurven erhalten, welche sich deutlich von den für frisch-bereitete Säuren erhaltenen Titrationskurven unterschieden. Gleichzeitig mit einem langsamen Ansteigen des pH-Wertes entwickelte sich ein zweiter Potentialsprung bei pH $\approx$ 10, welcher sich anfänglich rasch verschiebt hinsichtlich des Laugenverbrauchs, wie aus Figur 13 hervorgeht. Allmählich nähert sich dieser Potentialsprung jedoch einem konstanten Wert bei einem Laugenverbrauch, welcher einem Atomverhältnis Na : P = 2 : 1 entspricht, was auf eine vollständige Hydratation in Ortho- resp. Pyrophosphorsäure hindeutet.

Bei den hierbei erhaltenen konduktometrischen Titrationskurven kommt die oben beschriebene Veränderung dadurch zum Ausdruck, dass sich das Konduktivitätsminimum bei Na : P = 1 : 1 immer mehr verbreitert und dass im alkalischen Gebiet zwei Knickpunkte auftreten von welchen derjenige, welcher am weitesten nach rechts liegt, dem zweiten Potentialsprung bei der potentiometrischen Titration entspricht gemäss Fig. 14.

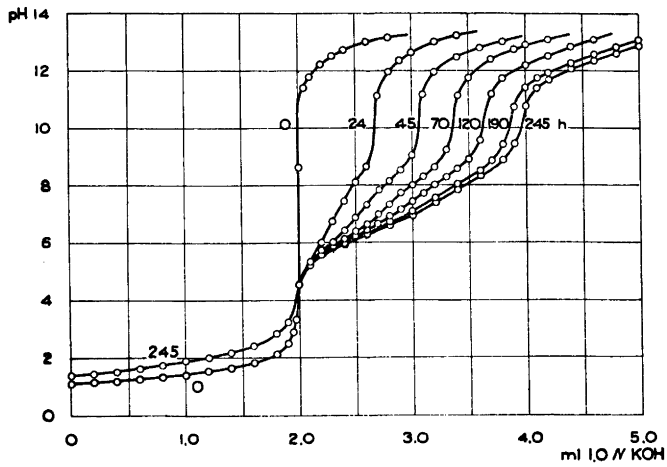


Fig. 13. Potentiometrische Titration von 20 ml 0,1 N Trimetaphosphorsäure mit 1,0 N KOH bei verschiedenen Zeitpunkten nach der Darstellung.

Vergleicht man die von Treadwell durch Verdrängungstitration an einem Grahamschen Salz gefundene Kurve und den Verlauf der von Salih beschriebenen konduktometrischen Titration einer sogen. Hexametaphosphorsäure mit den in Fig. 13 und Fig. 14 wiedergegebenen Kurven für verschiedene Zwischenstufen zwischen reiner Metaphosphorsäure und den Hydratationsprodukten, so liegt die Annahme nahe, dass es sich in beiden Fällen nicht um reine Metaphosphate bezw. Metaphosphorsäure gehandelt haben kann sondern um Präparate, welche auf Grund ungeeigneter Darstellungsmethoden schon mit Hydratationsprodukten vermischt waren.

Auch an Hand von Ionengewichtsbestimmungen konnte festgestellt werden, dass sowohl Trimetaphosphorsäure als auch die den Grahamschen Salzen entsprechenden Polymetaphosphorsäuren nach ca 10 Tagen Lagerung bei Zimmertemperatur vollständig in niedrig-molekulare Hydratationsprodukte gespalten waren.

Um Aufschluss darüber zu gewinnen, ob die oben beschriebene Veränderung der Titrationskurven allein eine Folge der Bildung von Orthophosphorsäure ist, und um durch Identificierung der Spaltungsprodukte eventuelle Rückschlüsse auf die Konstitution der Metaphosphat-Anionen ziehen zu können, wurde die gebildete Orthophosphorsäure bei verschiedenen Zeitpunkten nach der Darstellung der Säuren analytisch bestimmt nach einem an anderer Stelle beschriebenem kolorimetrischem Verfahren [23]. Die hierbei erhaltenen Analysenwerte sind in Tabelle 10 zusammengefasst und als % 0,1 *M* H_3PO_4 auf den totalen Phosphorhalt der Lösungen berechnet.

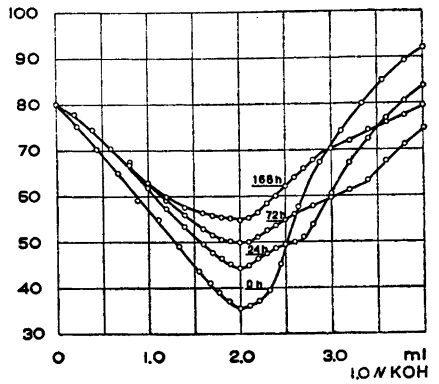


Fig. 14. Konduktometrische Titration von 20 ml 0,1 N Polymetaphosphorsäure mit 1,0 N KOH bei verschiedenen Zeitpunkten nach der Darstellung.

Tabelle 10. Bildung von Orthophosphorsäure bei der Hydratation der Metaphosphorsäuren.

0,1 *N* $H_3P_3O_9$ Alter der Säure h	% 0,1 *M* H_3PO_4	0,1 *N* $[HPO_3]_n$ Alter der Säure h	% 0,1 *M* H_3PO_4
0	0	0	0
24	11,1	20	10,8
45	17,8	74	29,2
65	21,7	99	32,3
72	22,7	190	38,8
96	26,2	264	41,1
100	27,3	289	41,5
120	29,0	336	42,0
147	30,1	458	42,2
192	31,0		
243	32,1		
295	33,3		
312	33.7		

Aus diesen Analysenergebnissen geht hervor, dass sich bei der Hydratation und Spaltung der Metaphosphorsäuren nicht ausschliesslich Orthophosphorsäure bildet, sondern dass, wie über diese Untersuchung hinaus weitergeführte Versuche ergeben haben, der grössere Anteil des in der Lösung enthaltenen Phosphors in Pyrophosphorsäure umgewandelt wird.

Dies konnte durch potentiometrische Modellversuche bestätigt werden und wird Gegenstand einer weiteren Mitteilung sein.

Eine Erklärung für die Bildung von Spaltprodukten, welche in einem definierten Mengenverhältnis zu einander stehen, ist mit einer einfachen kettenförmigen Konstitutionsformel nach I oder II schwer in Einklang zu bringen,

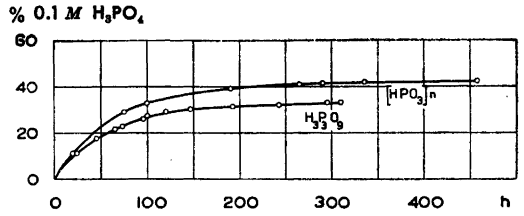
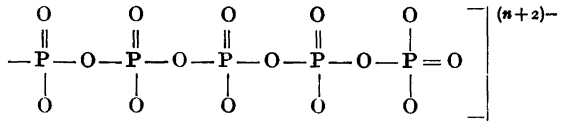
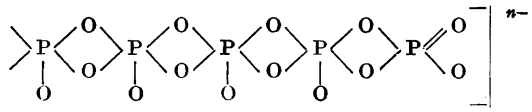


Fig. 15. Bildungsgeschwindigkeit von Orthophosphorsäure bei der Hydratation von Trimetaphosphorsäure und Polymetaphosphorsäure.

und es muss hiernach als wahrscheinlich angesehen werden, dass die Konstitution der Polymetaphosphat-Anionen wesentlich von diesen Formeln abweicht.



I



II

ZUSAMMENFASSUNG

Die Anionengewichte von Natriummetaphosphaten wurden bestimmt, welche durch thermische Dehydratation von Mononatriumorthophosphat bei verschiedenen Temperaturen dargestellt worden waren. Aus den hierbei erhaltenen Versuchsergebnissen ist zu schliessen, dass sich nach diesem Darstellungsverfahren nur *drei* verschiedene Modifikationen bilden, nämlich Dimetaphosphat, Trimetaphosphat und die unter der Bezeichnung »Grahamsches Salz» bekannten Natriumpolymetaphosphate mit einem von der Darstellungstemperatur in starkem Masse abhängigen Polymerisationsgrad. Dieses Ergebnis wurde durch Aufnahme von Pulverdiagrammen und durch eine einfachere thermische Analyse bestätigt. Weiterhin hat diese Untersuchung gezeigt, dass die sogen. *Ostwald-Waldensche* Regel zur Bestimmung der Valenz der verschiedenen Metaphosphat-Anionen nicht herangezogen werden kann. Die Fähigkeit des Grahamschen Salzes zur Bildung von Komplexverbindungen mit Erdalkali-Ionen wurde untersucht, und dabei festgestellt, dass das Komplexbindungsvermögen der Polymetaphosphate abhängig ist von der Darstellungstemperatur und damit von der Grösse der Polymetaphosphat-Anionen.

Aus Natriumtrimetaphosphat und aus Grahamschen Salz wurden durch Ionenaustausch die entsprechenden Metaphosphorsäuren dargestellt. Aus an diesen Säuren durchgeführten potentiometrischen und konduktometrischen Titrationen hat sich ergeben, dass sowohl Trimetaphosphorsäure als auch Polymetaphosphorsäuren sich wie starkdissociierte Mineralsäuren verhalten, bei welchen sämtliche Wasserstoff-Ionen die gleiche Dissociationskonstante haben. Die Metaphosphorsäuren sind äusserst instabile Gebilde und werden schon bei Zimmertemperatur unter Spaltung hydratisiert.

LITTERATUR

1. Graham, Th. *Pogg. Ann.* **32** (1834) 33.
2. U. S. patent 1956515 (1934).
3. Döring, R. *Die Wärme* **67** (1944) 91.
4. Karbe, K., und Jander, G. *Kolloid-Beihefte* **54** (1944) 1.
5. Brinzinger, H. *Naturwissenschaften* **18** (1930) 354.
6. Samuelsson, O. *Svensk Kem. Tid.* **56** (1944) 343.
7. In Kürze werden weitere Versuchsresultate mitgeteilt werden, aus welchen hervorzugehen scheint, dass der Trimetaphosphat-Ring die Elementargruppe darstellt, aus welcher die Anionen des Grahamschen Salzes aufgebaut sind.
8. Riecke, E. *Z. physik. Chem.* **6** (1890) 564.
9. Travers, A., und Yu Kwong Chu. *Compt. rend.* **198** (1934) 2100.
10. Rudy, H., Schloesser, H., und Watzel, R. *Z. angew. Chem.* **53** (1940) 531.
11. Jahr, F., und Wendland. Zitiert bei Karbe und Jander (*l. c.*).
12. Andress, K. R., und Wüst, K. *Z. anorg. u. allg. Chem.* **237** (1938) 121.
13. Treadwell, W. D., und Leutwyler, F. *Helv. Chim. Acta* **21** (1938) 1450.
14. v. Knorre, G. *Z. anorg. Chem.* **24** (1900) 369.
15. Wiesler, A. *Z. anorg. Chem.* **28** (1901) 207.
16. Lorenz, R., und Michael, W. *Z. anorg. u. allgem. Chem.* **116** (1921) 161.
17. Rudy, H., und Schloesser, H. *Ber.* **73** (1940) 484.
18. Partridge, E. P. *Dual Service News, Hall Lab.* Pittsburgh (1937).
19. Treadwell, W. D., und Leutwyler, F. *Helv. Chim. Acta* **20** (1937) 931.
20. Salih, R. *Bull. soc. Chim. France* [5] **3** (1936) 1391.
21. Samuelsson, O. *Svensk Kem. Tid.* **54** (1942) 124; **57** (1945) 91, 158, 250.
22. Bell, R. N. *Ind. Eng. Chem.* **39** (1947) 136.
23. Teichert, W. *Iva* **17** (1946) 135.

Eingegangen am 7. Februar 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 252—263

The Cupric-Ion Catalysis in the Bromination of Ethyl Acetoacetate

KAI JULIUS PEDERSEN

The Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, Denmark

When bromine water is added to an aqueous solution of ethyl acetoacetate the following consecutive reactions take place

$$CH_3COCH_2COOC_2H_5 + Br_2 \rightarrow CH_3COCHBrCOOC_2H_5 + H^+ + Br^- \quad (1)$$

$$CH_3COCHBrCOOC_2H_5 + Br_2 \rightarrow CH_3COCBr_2COOC_2H_5 + H^+ + Br^- \quad (2)$$

They are of the first order with respect to the esters, and the rate is independent of the concentration of bromine. Both reactions are catalysed by bases in general, *i. e.* by molecules that can take up a proton, and the catalytic power of a base increases with increasing basic strength. The rate-determining step is, therefore, a bimolecular proton transfer from the ester to the base. Also water molecules act as proton acceptors. When no basic catalyst is added, the rate measured is exclusively that of the proton transfer to the water molecules of the solvent. The bromination of ethyl acetoacetate was studied experimentally, and the theory of the basic catalysis given in earlier papers [1, 2, 3]. In this paper it is shown that, if also cupric ions are added, an additional acceleration of reaction 1 is found. The effect of cupric ions appears as a catalysis of the bimolecular proton transfer from the ethyl acetoacetate to the base. When the medium contains both cupric ions and a base B, the first order velocity constant of reaction 1 may therefore be written

$$k = k_0 + k'_0\, c_{Cu^{++}} + k_B\, c_B + k'_B\, c_{Cu^{++}}\, c_B \quad (3)$$

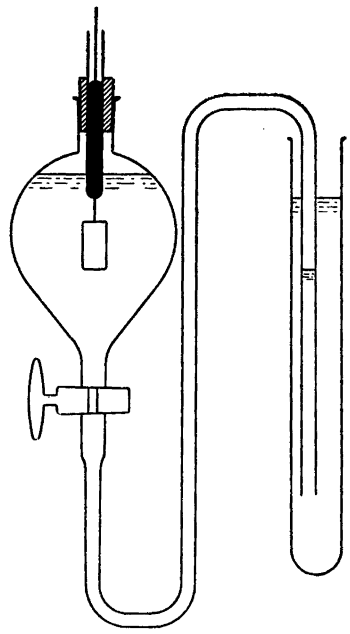


Fig. 1. Reaction vessel.

PROCEDURE AND MATERIALS

As reaction vessel served a separatory funnel with glass stopper and a long stem bent as shown in Fig. 1. The stem and the bore of the stopcock were first filled with a solution of the same composition as the reacting mixture but without ester and bromine. The funnel was then charged with solution of the ester and the other substances for the experiment except bromine. It was placed in a water thermostat at 18.0° C. The end of the stem was dipping into a test tube with 3.5 molar potassium chloride forming the connexion to a calomel electrode. A weight burette with bromine water [4] was also placed in the thermostat. When temperature equilibrium was attained the weight burette and the reaction vessel were taken out, and bromine water, insufficient for completing the reaction, was quickly added to the solution in the funnel which was again placed in the thermostat. The total volume of the reacting mixture was always 125 ml. When most of the bromine had reacted, the glass stopper was replaced by a cork carrying a bright platinum electrode, and the stopcock was opened (see Fig. 1). The solution always contained 0.02 molar potassium bromide which secured a well defined potential of the cell. A valve potentiometer (maker »Radiometer», Copenhagen) had, beforehand, been so adjusted that its galvanometer would give no deflection when about 10^{-6} molar bromine was left in the reaction vessel. With an initial ester concentration of 0.004 molar, this corresponds to only 0.0125 per cent of the bromine necessary for complete bromination. The moment when all the free bromine had disappeared was therefore indicated by a quick change of sign of the deflection of the galvanometer. By repeating the experiment with different initial concentrations of bromine, it was possible to follow the reaction kinetically.

The ethyl acetoacetate used for the measurements was an old preparation which had 15 years before been purified through the sodium hydrogen sulphite compound [1, 2]. The bromine water was prepared from Kahlbaum's bromine »for analysis». The other substances used were the purest commercial preparations. The cupric nitrate had further been recrystallized from water.

THE CUPRIC-ION CATALYSIS OF THE WATER REACTION

The bromination was studied in 0.002 molar hydrochloric acid and in the same solution containing, in addition, barium or cupric nitrate. When c is the initial molar concentration of the ester, x the equivalent concentration of bromine used by the reaction at the time t minutes after the start, k and h the first order velocity constants of, respectively, reaction 1 and 2, the following formula holds [1, 2]

$$c - \frac{x}{4} = c\left[A\ 10^{-k^*t} - (A - 1 + \frac{\varepsilon}{2})\ 10^{-h^*t}\right] \qquad (4)$$

$$\text{where} \qquad A = (1 - \varepsilon)\left(1 + \frac{k^*}{2\ (h^* - k^*)}\right)$$

and ε (= 0.0037) is the degree of enolisation [1,2] of ethyl acetoacetate at equilibrium in the solution. An asterisk denotes that the factor log e has been omitted, thus $k^* = 0.4343\ k$. The method [1, 2] used for computing the velocity constants is very time-consuming. An easier and very ingenious method for the analysis of irreversible consecutive first order reactions has been worked out by Swain [4]. His paper was, however, not yet available here when the computations were carried out.

The results of the measurements and computations are given in Tables 1 and 2. Table 1 shows that a good agreement with formula 4 is obtained when the values of k^* and h^* given in Table 2 are chosen. The accuracy with which the two constants may be determined from a given set of measurements depends upon the ratio h^*/k^*. The smaller of the two constants may always be estimated most accurately. When no cupric nitrate is present, h^* is about 11 times as great as k^* which ratio allows a good determination of k^* but only a moderate estimate of h^*. When cupric nitrate is added, the ratio decreases and, consequently, the degree of uncertainty in k^* increases.

It is seen from Table 2 that k^* rises considerably when cupric nitrate is added while h^* does not change appreciably (considering the smaller accuracy of its estimation). The increase of k^* is proportional to the concentration C of cupric nitrate. The measurements with barium nitrate show that this salt has no, or only a slight, effect on the two constants. The effect of cupric

Table 1. Bromination of ethyl acetoacetate (0.003896 molar) at 18.0° C in solutions containing 0.0020 M HCl, 0.020 M KBr, and the salts given below. The table shows x (gram-equivalents of bromine per liter which had reacted t minutes after the start) found by experiment and calculated from formula 4 when the values of k and h* given in Table 2, and ε = 0.0037 are used.*

t	$x10^3$ found	$x10^3$ calc.	t	$x10^3$ found	$x10^3$ calc.	t	$x10^3$ found	$x10^3$ calc.
No other salt			13.75	8.06	8.09	5.26	5.44	5.46
3.27	1.58	1.58	17.10	9.42	9.42	5.49	5.68	5.68
6.11	3.08	3.08	23.00	11.21	11.22	6.13	6.29	6.28
7.69	3.91	3.88	40.08	13.90	13.98	8.38	8.12	8.16
11.27	5.48	5.53				9.60	9.03	9.04
19.55	8.55	8.52	0.00500 M $Cu(NO_3)_2$			13.44	11.19	11.19
36.32	12.13	12.13	2.21	1.62	1.62	4.06[a]	8.43	8.48
			3.54	2.70	2.72	11.42[a]	20.37	20.32
0.0500 M $Ba(NO_3)_2$			5.21	4.09	4.06			
3.12	1.53	1.52	7.88	6.04	6.04	0.0200 M $Cu(NO_3)_2$		
7.58	3.87	3.89	11.44	8.22	8.20	1.73	2.52	2.58
13.53	6.58	6.60	15.05	9.92	9.92	2.72	4.04	4.09
21.55	9.22	9.22	19.41	11.47	11.48	3.48	5.28	5.21
33.00	11.72	11.72				4.51	6.62	6.62
			0.0100 M $Cu(NO_3)_2$			4.82	7.04	7.02
0.00250 M $Cu(NO_3)_2$			1.19	1.10	1.16	6.33	8.70	8.77
1.84	1.10	1.09	1.44	1.28	1.42	8.30	10.55	10.56
3.78	2.36	2.39	2.79	2.84	2.87	11.77	12.76	12.70
6.47	4.21	4.18	3.50	3.67	3.64	3.30[b]	4.99	5.26
9.47	5.97	5.98	4.06	4.18	4.24	5.14[b]	7.38	7.41

[a]0.007792 molar ester [b]0.0100 molar HCl

nitrate may therefore be considered as a specific cupric-ion catalysis of reaction 1. As a mean of the values in Table 2, the catalytic constant of cupric ions k_0'* (*cf.* equation 3) is found to be 2.69.

Table 2. Bromination of ethyl acetoacetate at 18.0° C in solutions containing 0.002 M HCl, 0.02 M KBr, and C molar barium or cupric nitrate. k and h* are the velocity constants (per minute) of, respectively, reaction 1 and 2.*

	C	k^*	h^*	$\frac{k^*-k_0^*}{C}$
	0	0.01855	0.208	
Barium nitrate	0.0500	0.01897	0.205	0.01
Cupric nitrate	0.00250	0.02538	0.187	2.73
» »	0.00500	0.03200	0.183	2.69
» »	0.0100	0.04563	0.185	2.71
» »	0.0200	0.07136	0.192	2.64

The value of k^* determined here for the solution without added barium or cupric salt is 3 per cent higher than that found in the earlier paper [1, 2]. The object of the present examination was more to demonstrate the catalytic effect of the cupric ions than to obtain accurate absolute values of the velocity constants. No effort was therefore made to find the reason for the discrepancy. It is possible that it is connected with the different methods of following the reaction used in the two papers.

THE COMBINED CUPRIC- AND ACETATE-ION CATALYSIS

In order to show that cupric ions catalyse also the proton transfer from ethyl acetoacetate to acetate ions (*cf.* equation 3), measurements were carried out in an acetate buffer solution containing in addition cupric nitrate. Owing to the production of hydrogen ions by reactions 1 and 2 and to the complex formation between cupric and acetate ions, both the cupric and acetate ion concentration change during the reaction. The mathematical analysis of the reaction is therefore very complicated, and it has been necessary to introduce different simplifying approximations.

If we denote the concentrations of ethyl acetoacetate, ethyl monobromoacetoacetate, and ethyl dibromoacetoacetate by, respectively, α, β, and γ, where

$$\alpha + \beta + \gamma = c \tag{5}$$

we have

$$-\frac{d\alpha}{dt} = k\ \alpha \tag{6}$$

and

$$\frac{d\beta}{dt} = k\ \alpha - h\ \beta \tag{7}$$

where k and h are variables given by expression 3 and a similar expression for h. We introduce the approximation that $r = h/k$ is constant. From equation 6 and 7 we obtain

$$\frac{d\beta}{d\alpha} = r\frac{\beta}{\alpha} - 1$$

Integrating and using $\alpha = c$ when $\beta = 0$, we get

$$\frac{\beta}{\alpha} = \frac{1}{r-1} - \frac{1}{r-1}\left(\frac{\alpha}{c}\right)^{r-1} \tag{8}$$

It follows from this equation that, if $r > 2$, $\frac{\beta}{\alpha}$ approaches $\frac{1}{r-1}$ when $\frac{\alpha}{c}$ decreases toward nought, that is, when t increases without limit; and the greater r the more rapid the approach. When r and t are sufficiently great we may use the approximation

$$\frac{\beta}{\alpha} = \frac{1}{r-1} \tag{9}$$

The equivalent concentration of bromine used by the reaction is

$$x = 2\beta + 4\gamma \tag{10}$$

From equations 5, 9, and 10 we obtain

$$c - \frac{x}{4} = \alpha\left[1 + \frac{1}{2\ (r-1)}\right]$$

Equation 6 may therefore be written as follows

$$-\frac{d\left(c - \frac{x}{4}\right)}{dt} = k\left(c - \frac{x}{4}\right) \tag{11}$$

where k is given by equation 3.

We now introduce the approximation that both the acetate and the cupric ion concentration vary linearly with the concentration of hydrogen bromide formed by the reaction $(= \frac{x}{2})$. We therefore may write

$$c_{Ac^-} = c_{Ac^-(\infty)} + p(c - \frac{x}{4}) \tag{12}$$

and

$$c_{Cu^{++}} = c_{Cu^{++}(\infty)} - q(c - \frac{x}{4}) \tag{13}$$

where

$$p = (c_{Ac^-(o)} - c_{Ac^-(\infty)})/c \tag{14}$$

and

$$q = (c_{Cu^{++}(\infty)} - c_{Cu^{++}(o)})/c \tag{15}$$

The subscripts (o) and (∞) denote that the concentration corresponds to, respectively, $t = 0$ and $t = \infty$.

It is assumed that the complexes formed between cupric and acetate ions have no catalytic effect (see later). We introduce equations 3, 12, and 13 into equation 11 and use the abbreviations

$$k_\infty = k_0 + k_0' \, c_{Cu^{++}(\infty)} + k_{Ac^-} \, c_{Ac^-(\infty)} + k_{Ac^-}' \, c_{Cu^{++}(\infty)} \, c_{Ac^-(\infty)} \tag{16}$$

$$l = p[k_{Ac^-} + k_{Ac^-}' \, c_{Cu^{++}(\infty)}] - q \, [k_0' + k_{Ac^-}' \, c_{Ac^-(\infty)}] \tag{17}$$

$$m = pqk_{Ac^-}' \tag{18}$$

$$n = \sqrt{l^2 + 4 \, k_\infty m} \tag{19}$$

We obtain

$$-\frac{d\left(c - \frac{x}{4}\right)}{dt} = \left[k_\infty + l\left(c - \frac{x}{4}\right) - m\left(c - \frac{x}{4}\right)^2\right]\left(c - \frac{x}{4}\right) \tag{20}$$

and, by integration,

$$k_\infty{}^* t = F(x) + I \tag{21}$$

where

$$F(x) = \frac{n+l}{2n} \log \frac{\frac{n-l}{2m} + \left(c - \frac{x}{4}\right)}{\frac{n-l}{2m} + c} + \frac{n-l}{2n} \log \frac{\frac{n+l}{2m} - \left(c - \frac{x}{4}\right)}{\frac{n+l}{2m} - c} - \log \frac{c - \frac{x}{4}}{c} \tag{22}$$

and I is an integration constant.

In the special case when no cupric nitrate is present in the acetate buffer, we have $q = 0$, $l = pk_{Ac^-}$, and $m = 0$. We therefore obtain by integration of equation 20

$$k_\infty{}^* t = f(x) + I \tag{23}$$

where

$$f(x) = \log \frac{\frac{k_\infty}{pk_{Ac^-}} + \left(c - \frac{x}{4}\right)}{\frac{k_\infty}{pk_{Ac^-}} + c} - \log \frac{c - \frac{x}{4}}{c} \tag{24}$$

Owing to the neglect of the last term of eq. 8, formulae 21 and 23 will hold only when t is sufficiently great. If we plot $F(x)$ or $f(x)$ against t we therefore obtain a curve which, when t increases, more or less rapidly approaches a straight line having the slope $k_\infty{}^*$. In computing $k_\infty{}^*$ from a set of experi-

mental data by means of equations 21 and 23, it is necessary to use a method of successive approximations since $k_\infty{}^*$ enters into both sides of the equations.

Measurements were first carried out in an acetate buffer without cupric salt. The initial composition of the solution and the experimental data are given in Table 3. The dissociation constant of acetic acid was estimated [6]

Table 3. Acetate-ion catalysis in the bromination of ethyl acetoacetate at 18.0° C. The solution contained initially 0.003873 m ethyl acetoacetate, 0.00974 M sodium acetate, 0.100 M acetic acid, 0.090 M sodium nitrate, and 0.020 M potassium bromide. The table shows $f(x)$ computed from the experimental data and, in the last column, $f(x)$ calculated from the linear equation: $f(x) = 0.04022\ t - 0.0129$.

t	$\left(c - \frac{x}{4}\right)10^3$	$f(x)$	$f(x)$ calc.
0	3.873	0.0000	
1.36	3.068	0.0432	0.0418
1.88	2.777	0.0636	0.0627
3.08	2.262	0.1087	0.1110
4.42	1.794	0.1651	0.1649
7.98	1.108	0.3077	0.3081

to be 2.84×10^{-5}. Hence, $c_{Ac^-(o)} = 0.01002$, and $c_{Ac^-(\infty)} = 0.00264$. From equation 14 we obtain $p = 1.905$. *Suppose* that $k_{Ac^-}{}^* = 8.21$. From equation 16 and $k_0{}^* = 0.01855$ we then find $k_\infty{}^* = 0.04022$. $f(x)$ is now computed from the experimental data by means of equation 24. The values obtained are given in the third column of Table 3. When they are plotted against t, all the points fall close to a straight line of the equation given in the text to the table. When $f(x)$ is calculated from this equation the values presented in the last column are found. The slope of the straight line is $k_\infty{}^* = 0.04022$. Hence, we find, in agreement with the supposition, $k_{Ac^-}{}^* = 8.21$.

Experimental data for the same acetate buffer solution, containing, in addition, 0.00998 molar cupric nitrate, are given in Table 4. From an earlier study [6] of the complex formation between cupric and acetate ions, it may be estimated that, for the solution examined,

$$\frac{c_{CuAc^+}}{c_{Cu^{++}}\, c_{Ac^-}} = 52 \quad \text{and} \quad \frac{c_{CuAc_2}}{c_{Cu^{++}}\, c_{Ac^-}{}^2} = 360$$

and that the dissociation constant of acetic acid is 2.84×10^{-5}. Hence, we find $c_{Ac^-(o)} = 0.00720$, $c_{Cu^{++}(o)} = 0.00717$, $c_{Ac^-(\infty)} = 0.00256$, and $c_{Cu^{++}(\infty)} = 0.00880$. From equations 14 and 15 we find $p = 1.198$ and $q = 0.421$. *Suppose* that $k_{Ac^-}{}'^* = 1143$. Using $k_0{}^* = 0.01855$, $k_0{}'^* = 2.69$,

and $k_{Ac^-}{}^* = 8.21$, we then find from equation 16 that $k_\infty{}^* = 0.0890$, and, from equations 17, 18, and 19, that $l^* = 19.52$, $m^* = 576$, and $n^* = 24.2$. $F(x)$ is now calculated from the experimental data by means of equation 22. The values obtained are given in the third column of Table 4. When they are plotted against t, all the points fall close to a straight line of the equation given

Table 4. Combined acetate- and cupric-ion catalysis in the bromination of ethyl acetoacetate at 18.0° C. The solution contained initially 0.003873 M ethyl acetoacetate, 0.00974 M sodium acetate, 0.100 M acetic acid, 0.00998 M cupric nitrate, 0.0900 M sodium nitrate, and 0.020 M potassium bromide. The table shows $F(x)$ computed from the experimental data and, in the last column, $F(x)$ calculated from the linear equation: $F(x) = 0.0890\ t - 0.0220$.

t	$\left(c - \frac{x}{4}\right)10^3$	$F(x)$	$F(x)$ calc.
0	3.873	0.0000	
1.08	2.908	0.0749	0.0741
1.33	2.690	0.0964	0.0964
1.99	2.189	0.1563	0.1551
2.25	2.046	0.1768	0.1782
3.27	1.538	0.2673	0.2690
4.54	1.095	0.3831	0.3821
7.11	0.591	0.6111	0.6108

in the text to the table. When $F(x)$ is calculated from this equation the values presented in the last column are found. The slope of the straight line is $k_\infty{}^* = 0.0890$. Hence, we find from equation 16, in agreement with the supposition, $k_{Ac^-}{}'^* = 1143$.

DISCUSSION

The results may be summarized in the formula

$$k^* = 0.01855 + 2.69\ c_{Cu^{++}} + 8.21\ c_{Ac^-} + 1143\ c_{Cu^{++}}\ c_{Ac^-}$$

which may also be written in the following way

$$k^* = 0.01855\ (1 + 145\ c_{Cu^{++}}) + 8.21\ (1 + 139\ c_{Cu^{++}})\ c_{Ac^-}$$

It is seen from the latter expression that cupric ions increase the velocities of both the water reaction and the acetate-ion catalysis by nearly the same factor. The rate-determining step in the base-catalysed bromination of ethyl acetoacetate is considered to be the bimolecular reaction by which a proton is transferred from the ester to the base (*e. g.* water or acetate ion). The cupric ion has no perceptible tendency to take up a proton. It is therefore excluded

that it may act simply as a basic catalyst. We may also exclude the possibility that the effect of cupric nitrate is due to the extremely small concentration of hydroxy-cupric ions [7] present in the solution, because it has been shown (see Table 1) that the rate in 0.02 molar cupric nitrate does not change essentially when the concentration of hydrochloric acid is increased from 0.002 to 0.01 molar. The effect of cupric nitrate is too great to be interpreted as a case of a general kinetic salt effect, and its specific character has been demonstrated by the fact that barium nitrate, which is of the same valency type as cupric nitrate, has almost no influence on the velocity (see Table 2). The effect may therefore be classified as a specific cupric-ion catalysis of the proton transfer from the ester to the base. A termolecular mechanism of this reaction is so unlikely that it need not be considered. The rate-determining step is therefore a bimolecular proton transfer, either from the ester molecule to a complex between the base and the cupric ion, or from a complex between the ester and the cupric ion to the base. The former possibility is strongly opposed and the latter supported by the well known experience that, when a base or an acid forms a complex with a cupric ion (or another cation), the strength of the base will highly decrease while that of the acid will greatly increase. The following examples will illustrate this rule. In a study of the complex formation between cupric and acetate ions [6], no tendency of the acetato-cupric ion to combine with protons was detectable. For that reason it was also assumed in the present paper, when the rate in solutions containing both acetate and cupric salt was analysed, that acetato-cupric complexes do not catalyse. On the other hand, in a study of the acidity of the hydrated cupric ion [7], it was found that the acid strength of the water molecules in this ion is some 10^7 times as great as that of the free water.

On the basis of these considerations, the following explanation of the cupric-ion catalysis is suggested. It is assumed that the cupric ion and ethyl acetoacetate (HR) in aqueous solution form a complex, and that the equilibrium

$$Cu^{++} + HR \rightleftharpoons Cu(HR)^{++}$$

is attained practically instantaneously, but only a small fraction of the cupric ions and the ester molecules is transformed into complex ions. When K is the complexity constant we have

$$c_{Cu(HR)++} = K c_{Cu++}\, c_{HR}$$

The proton is bound much more loosely, and the ease with which it is given off to a base is much greater, in $Cu(HR)^{++}$ than in HR. If $\varkappa_0$ denotes

the pseudo-unimolecular, and $\varkappa_B$ the bimolecular velocity constant for the proton transfer from $Cu(HR)^{++}$ to, respectively, water and a base B, we, therefore, have $\varkappa_0 \gg k_0$, and $\varkappa_B \gg k_B$. The first order velocity constant for the bromination of ethyl acetoacetate in an aqueous solution containing both cupric ions and a base B may now be written

$$k = k_0 + \varkappa_0 K c_{Cu^{++}} + k_B c_B + \varkappa_B K c_{Cu^{++}} c_B$$

When we, in this equation, set $\varkappa_0 K = k_0'$ and $\varkappa_B K = k_B'$ we obtain equation 3.

While no other experimental facts indicate the existence of cupric complexes of ethyl acetoacetate, it is known that its (enolic) anion combines with cupric ions in aqueous solution. The stability of the complex formed, and of the cupric compounds of a series of anions of β-diketones, has recently been studied by Calvin and co-workers [8]. Mellor and Maley [9] have examined similar compounds with other metal ions. There is, however, nothing in these papers that indicates a complex formation between cupric ions and the keto-form of ethyl acetoacetate.

The experimental work reported here was planned only as an introduction to a more careful investigation of the problem including the examination of other metal ions. Owing to the difficulty in obtaining sufficiently accurate velocity constants when consecutive reactions take place, it was decided to continue the investigation with, instead of ethyl acetoacetate, a β-keto-ester having only one α-hydrogen atom, namely 2-carbethoxycyclopentanone, $(CH_2)_3COCHCOOC_2H_5$. A paper dealing with the metal-ion catalysis in the bromination of this ester will soon appear.

SUMMARY

When ethyl acetoacetate reacts with bromine in aqueous solution the two α-hydrogen atoms are replaced by bromine atoms by two consecutive first order reactions which are both catalysed by bases in general. The rate-determining step is, in both changes, a proton transfer from the ester to the base.

In this paper, the effect of cupric nitrate on the rate of bromination in very dilute hydrochloric acid and in acetate buffer has been examined. It was found that cupric ions catalyse the first of the two consecutive reactions (velocity constant k *) while they have no detectable influence on the second reaction. The results at 18.0° C may be summarized in the formula

$$k^* = 0.01855 + 2.69\ c_{Cu^{++}} + 8.21\ c_{Ac^-} + 1143\ c_{Cu^{++}}\ c_{Ac^-}$$

It was also shown that barium nitrate has only an insignificant influence on the rate of bromination in dilute hydrochloric acid.

The effect of cupric ions appears as a catalysis of the proton transfer from the ester to the base (water or acetate ion). In order to explain this catalysis, it is assumed that a small amount of a complex between cupric ions and ethyl acetoacetate is formed. In such a complex, the proton is bound much more loosely than in the free ester, and the rate with which it is transferred to a base is, therefore, much greater.

I wish to express my thanks to the head of the laboratory, Professor Niels Bjerrum, for his kind interest in my work.

REFERENCES

1. Pedersen, K. J. *Den almindelige Syre- og Basekatalyse* Copenhagen (1932).
2. Pedersen, K. J. *J. Phys. Chem.* **37** (1933) 751, **38** (1934) 581, 601.
3. Pedersen, K. J. *Trans. Faraday Soc.* **34** (1938) 237.
4. Pedersen, K. J. *Acta Chem. Scand.* **1** (1947) 433.
5. Swain, C. G. *J. Am. Chem. Soc.* **66** (1944) 1696.
6. Pedersen, K. J. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **22** (1945) no. 12.
7. Pedersen, K. J. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **20** (1943) no. 7.
8. Calvin, M., and co-workers *J. Am. Chem. Soc.* **67** (1945) 2003, **68** (1946) 557, 949.
9. Mellor, D. P., and Maley, L. *Nature* **159** (1947) 370.

Received February 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 264—289

A Spectrophotometric Method for the Simultaneous Determination of Myoglobin and Hemoglobin in Extracts of Human Muscle

CHRISTIAN de DUVE*

Biochemical Department, Medical Nobel Institute, Stockholm, Sweden

Except for the detailed study made by Whipple and his collaborators [1-3] on dogs, practically no data are available on the myoglobin (Mgb) content of muscle tissue and on its variations. As far as we have been able to ascertain, there are none on that of human muscle. It is true that, in his review, Millikan [4] alludes to a personal communication by Jonxis referring to the Mgb content of the heart and diaphragm of new born infants and, in an article on myodistrophy, Meldolesi [5] quotes the extensive investigations carried out by his assistant De Orchi. Unfortunately, no trace of these two works has been found in the literature.

Myoglobin may not deserve this lack of attention. Its essential physiological importance with respect to muscular function has been clearly demonstrated by Millikan [4] and the possibility of a myoglobin insufficiency underlying unexplained heart- or skeletal muscle-dysfunctions must not be overlooked. A study of the ratio: blood hemoglobin (Hb)/muscle Mgb may also yield useful information as to the relation existing between these closely similar pigments, especially in view of the increasing attention which has been given in recent years to the pathological variations of other hemoproteins. One can almost safely predict that a careful investigation of the Mgb content of heart and peripheral muscle in various pathological cases must bring to light some sort of Mgb pathology and, if it should not, the finding would in itself be of considerable importance, singling out myoglobin from hemoglobin and other essential iron-porphyrin pigments.

* Present address: Department of Physiological Chemistry, University of Louvain, Belgium.

The present work was initiated at the suggestion of Prof. H. Theorell as a preliminary to the above mentioned investigations. Its aim was to provide an analytical tool which, while requiring a minimum amount of tissue and furnishing sufficiently dependable results, would at the same time be simple enough for large scale routine determinations. As will appear from the details given below, this aim has only been reached so far by compromising between these somewhat conflicting exigencies. However, the method as it has been worked out so far, is not less accurate than most other methods applying to tissue extracts and can also be of utility in the determination of *blood-hemoglobin* in muscle extracts. It is hoped that further investigations, to be carried out by Dr. G. Biörck who has assumed the task of making a detailed survey of the possible pathological variations of the Mgb content, may lead to further improvements of the method described. Several suggestions arising from the experience already gained will be made at the end of this paper.

The main difficulty in the estimation of human myoglobin is that hemoglobin-free samples cannot be obtained, as is the case with animals, and that quantitative separation of both pigments cannot be effected without loss (Theorell and de Duve [6]). Determination of their relative concentrations in a mixture can only be made by taking advantage of the small spectral differences existing between the two pigments. By introducing additional precautions into the classical spectrophotometric technique and carefully determining the constants of the pure components, a satisfactory degree of accuracy has been attained. A further complication, which has been recognized by all the authors who have worked in this field, arises from the fact that extracts which are both truly representative of the Mgb content of the tissue and suitable for spectrophotometric determination, are very difficult to obtain. So far these two qualities have not proved entirely compatible. It will be more convenient to deal first with the spectrophotometric technique; the extraction procedure will be described in a second section and the third section will be devoted to an analysis of the results obtained on a variety of samples of both heart and peripheral muscle.

SPECTROPHOTOMETRIC TECHNIQUE

Principle of the method

Two differential methods have been used for the estimation of Mgb in animals. Shenk, Hall and King [7] measured the absorption at 542, 577, and 582 mμ on untreated muscle extracts, which were assumed to contain mixtures of oxy-hemoglobin and oxy-myoglobin. The spectral differences between these two compounds were used as a basis for the calculation of their respective concentrations. Owing to the ease with which myoglobin is auto-oxidized, it is doubted whether all the myoglobin still was present in

oxy-form in their extracts. Watson's [8] method is also based on the difference between the oxy-compounds, but his extracts are freshly reduced and reoxygenated before the reading, which is done with the Hartridge reversion spectroscope. The position of the maximum of the α-band which varies between 577.4 and 580.8 mμ is used for determining the ratio Hb/Mgb in the mixture. The total concentration of pigment is then measured by the somewhat involved process of reading the absorption of the CO-compounds at 560 and 540 mμ. As the extinction-coefficient of CO-Mgb and CO-Hb are not the same at those wave-lengths, a different value, depending on the distribution determined by the first method, has to be used in each case for this calculation. It is not clear why the CO-compounds were not used as well for the determination of the ratio Hb/Mgb as the difference between the absorption maxima of the CO-compounds is much larger than in the case of the O_2-compounds (10 mμ instead of 3.4). Moreover, Watson uses the coefficient of horse-Hb and Mgb for measurements on other animal species, which is probably incorrect.

The present method is based on the difference in light-absorption of the carbon-monoxide compounds of Mgb and Hb, readings being taken at wave-length values particularly suited to the problem at hand. As an independent control, the total concentration of pigments is measured as reduced pyridine-hemochromogen. It will be clear that much of the accuracy of the measurement depends on the perfect reproducibility of the wave-lengths used. In order to ensure this, it was found necessary to check each wave-length before a reading was taken, using a calibrated glass filter. The constants were determined on solutions of known concentration made up from pure Mgb and Hb. The validity of the method was checked on various mixtures of these solutions and the applicability of Beer's law was checked by widely varying the concentrations of the two compounds, both in pure solutions and in mixtures.

Determination of the constants

Material. — *Human myoglobin* was crystallized according to a slight modification of the method described before [6].

Instead of adding solid $(NH_4)_2SO_4$ to the concentrated extract to separate hemoglobin, the extract was dialyzed first against a 50 % and then against a 75 % saturated solution of $(NH_4)_2SO_4$ brought to pH 8 with ammonia. This procedure had the double advantage of further concentrating the extract and removing the acetate and phosphate present. A known concentration of neutral salt was reached in this way. After centrifuging away the bulk of the Hb, the concentration of $(NH_4)_2SO_4$ was brought to 79 % saturation. The small precipitate formed contained a mixture of Hb and Mgb. Above this point the solution was essentially free of Hb and further treated in the way already described.

After recrystallization, the sample proved homogenous both in the ultra-centrifuge and in the Tiselius apparatus. The iron content was 0.34 %, the nitrogen content 16.8 % of the dry weight.

Human Hemoglobin was prepared from fresh washed erythrocytes, hemolyzed with distilled water saturated with CO_2, the cell-debris being removed by centrifugation. The sample was electrophoretically homogenous and contained 0.34 % iron, and 16.6 % nitrogen.

The spectrophotometric constants for the pyridine-hemochromogens and for the CO-compounds of Hb and Mgb were determined on solutions prepared from these samples and standardized by simultaneous iron, nitrogen, and sometimes dry-weight-determinations.

Apparatus and reproducibility of wave-lengths. — The Beckmann spectrophotometer was used. This apparatus was found extremely reliable as far as the absolute value of the readings was concerned. However, it was observed in the course of this work, that even when the apparatus is perfectly calibrated, the wave-length scale is not entirely reproducible at the higher wave-lengths. In the region between 550 and 600 mμ, shifts of 1 to 2 mμ may occur. These shifts do not affect the entire scale in the same way and are probably due to a very slight unavoidable looseness in the mechanical parts of the monochromator. Whereas no change can be detected in the ultra-violet region, where the dispersion of the prism is greatest, the corresponding discrepancy between the position of the scale and that of the prism will become more and more perceptible, the smaller the dispersion of the prism.

In most cases, a slight error as to the wave-length will not considerably affect the accuracy of a measurement, but, in the present instance, perfect reproducibility of the wave-length was an essential condition. In order to insure this, the following device was used:

A glass light-filter showing a suitable absorption-spectrum (see p. 271) was mounted vertically on a stand fitting into the movable plate normally supporting the cell-holder in the Beckmann apparatus. The extinction-value of the filter was then determined against air at every required wave-length, measurements being carried out simultaneously on the standard solutions without altering the position of the monochromator. The spectra of CO-Mgb, and CO-Hb and of the filter were thus determined, every single set of three points being perfectly matched as to the wave-length. Any desired wave-length corresponding to one of the standard readings could then be obtained independently of the scale itself by first inserting the filter and moving the monochromator in the neighborhood of the required wave-length until the extinction-value previously obtained was reproduced.

The reliability of this simple procedure has been repeatedly checked and it has proved very useful in other spectrophotometric determinations as well. It requires three conditions: 1. The light-absorption of the filter must vary sufficiently with the wave-length in the range where readings have to be made; the steeper the spectral curve of the filter, the more accurate the wave-length adjustment will be. 2. The position of the filter in respect to the light-beam must be kept constant. 3. The spectrum of the filter must remain constant.

The filter used in this work was Seitz VG 20, 1 mm. Its spectrum is given in Fig. 2, along with the standard curves of CO-Hb and CO-Mgb. It is not ideally adapted to the present case as its spectrum shows only a small slope in the region of 568 mμ, where a reading has to be taken. A consideration of the shape of the standard curves will show, however, that a small error in wave-length at that point will not affect the accuracy of the determination in a significant way. On the other hand, at 583.8 mμ, where high accuracy in the wave-length is essential, the slope of the filter's spectrum is such that the wave-length can be reproduced within 1 Å.

The stability of the filter's spectrum can be checked from time to time against a solution of CO-Hb. The concentration of this solution need not be known accurately as the shape of the curve of CO-Hb with respect to the absolute values of the filter will show whether these values have suffered any significant change.

Nomenclature. — In expressing the extinction-coefficient, the classical formula: $\varepsilon = \frac{1}{cd} \log \frac{I_0}{I}$ was used.

d was 1 cm in all cases.
c was expressed in milligrams of dry substance/ml.

The dry equivalent weight of both compounds being 16.200/atom of iron, the equivalent absorption coefficient β can be calculated from the values of ε listed below by the equation: $\beta = 2.3 \times 16.200 \times \varepsilon \times 10^3$ and the equivalent extinction-coefficient by multiplying ε by 16.200.

Furthermore, the values of ε for reduced pyridine-hemochromogen are expressed in mg of dry Hb or Mgb/l ml of the solution used. As this solution is diluted four times for the reading, the true values of molar ε and β of the pyridine-hemochromogen itself will be obtained by multiplying those values, calculated as above, by 4.

Pyridine-hemochromogen standard. — The samples were prepared by adding 3 ml of a solution containing: pyridine 100 ml, *N* NaOH 30 ml, *aq. dist. ad* 300 ml., to 1 ml of the standard Mgb or Hb solution directly in a 1 cm cuvette,

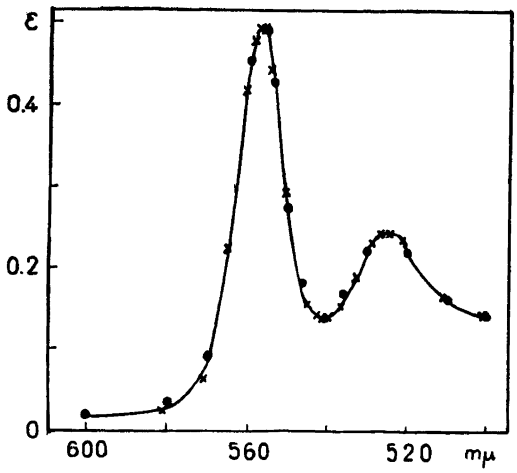


Fig. 1. Spectrum of reduced pyridine-hemochromogen.

● *From hemoglobin*
× *From myoglobin*

and mixing thoroughly with a thin glass rod. Oxidized pyridine-hemochromogen is formed, which is a relatively stable compound. Immediately before taking the spectrophotometric reading, an excess of solid $Na_2S_2O_4$ is added and quickly dissolved by stirring. The reduced hemochromogen is not stable for more than 5 to 10 min and readings have to be taken quickly.

Fig. 1 reproduces graphically the standard values for reduced pyridine-hemochromogen from hemoglobin and from myoglobin between 500 and 600 mμ. It is clear that they are undistinguishable.

As a means of determining protohemin quantitatively, the pyridine-hemochromogen method is not very trustworthy. Even with pure solutions, the error easily attains 2 %. In order to obtain reproducible results, it is essential that the solutions should always be prepared in the same way. We have found the method described above much more reliable than the usual one which consists in splitting off the hemin first with alkali and adding pyridine afterwards. A sufficient excess of hydrosulphite should also be used.

For quantitative measurements readings are usually taken at 557.5 mμ (max.). A reading may eventually also be taken at 540 mμ (min.), the difference between the absorption at both wave-lengths being used in the latter case. The constants for these two wave-lengths are given in Table 1.

Standard extinction values of the carbon-monoxide compounds. — The carbon-monoxide compounds of Hb and Mgb were prepared immediately before the readings, using the apparatus described previously [6] and pure CO. The gas was first allowed to bubble through the solution during 5 minutes, then an

Table 1. Constants of reduced pyridine-hemochromogen. (All values expressed for 1 cm cell-depth.)

Wave-length	Max. 557.5 mμ	Min. 540 mμ	Difference 557.5 mμ—540 mμ
$\varepsilon = \log I_0/I$ for 1 mg Hb or Mgb dissolved in 4 ml pyr-NaOH-hydrosulph.	0.494	0.139*	0.355*
Molar extinction coeffic. $\varepsilon = \log I_0/I$ for $c = 1$ mM red. pyr.-hemochr. p. l.	32.0	9.0	23.0
Molar absorption coeffic. $\beta = \ln I_0/I$ for $c = 1$ mole red. pyr.-hemochr. p. ml. $\times 10^{-7}$	7.36	2.07	5.29

excess of $Na_2S_2O_4$ was added under CO atmosphere and carbon-monoxide led again during 5 more minutes. The solution was quickly transferred into the cuvette, care being taken to fill the cuvette as completely as possible, and to cover it immediately.

At each wave-length, readings were taken simultaneously on CO-Mgb, CO-Hb and on the filter, as described above. The solutions had been brought to identical concentrations in order to enable an accurate determination of the isobestic wave-lengths. The spectra of both compounds and of the filter are given in Fig. 2 and the numerical values of the extinction coefficients have been listed in Table 2 in order to facilitate reproduction of the method. A glass filter of the same type or any other suitable and stable standard can be used for this purpose and it will be sufficient to standardize it against a solution of CO-Hb, as the corresponding values for CO-Mgb can then be obtained by interpolation from the chart below.

In our previous communication on human myoglobin [6] a spectrum of CO-Mgb significantly different from the one published in this paper has been recorded. The values put forward previously rested on a single experiment which, for technical reasons, could not be repeated, and were not given as definitive. Three peculiarities had been observed: 1) The extinction values were uncommonly low compared to those of horse myoglobin. These values had been calculated on the basis of the iron content of the solutions, deter-

* In crude muscle extracts, the absorption at 540 mμ is somewhat raised, probably owing to the impurities present. Better agreement is obtained with $\varepsilon_{(540)} = 0.156$ and $\varepsilon_{(557.5)} - \varepsilon_{(540)} = 0.338$. This empirical value was used for calculation of results on extracts with the aid of formula (7): see further.

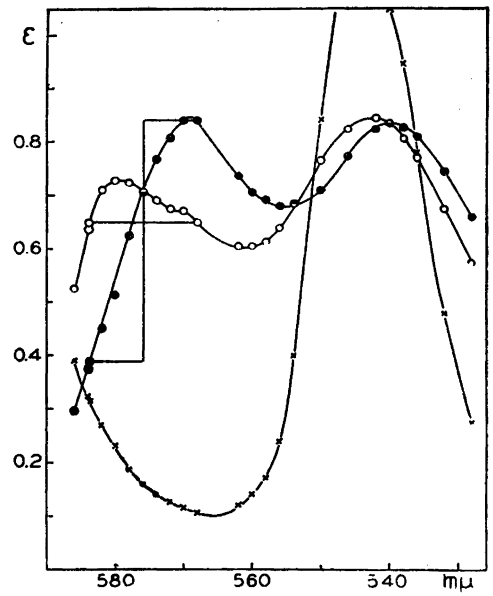


Fig. 2. Spectra of the carbon-monoxide compounds.

●——● *Hemoglobin*
○——○ *Myoglobin*
×——× *Calibration filter*

Table 2. Constants of CO-Hb and CO-Mgb: log I_0/I for d = 1 cm and c = 1 g dry pigment (1/16.200 equivalent)/liter.

mμ	CO-Hb	CO-Mgb	mμ	CO-Hb	CO-Mgb
586	0.296	0.525	560	0.706	0.605
584	0.375	0.636	558	0.693	0.613
583.8	0.388	0.649	556	0.680	0.640
582	0.451	0.710	553.8	0.685	0.685
580	0.513	0.727	550	0.710	0.766
578	0.625	0.723	546	0.773	0.824
575.7	0.706	0.706	542	0.824	0.844
574	0.767	0.690	540	0.833	0.833
572	0.806	0.675	538	0.826	0.806
570	0.839	0.671	536	0.809	0.769
568	0.840	0.649	532	0.744	0.675
562	0.736	0.605	528	0.659	0.574

mined *after the readings*. It has now been ascertained that the added sodium hydrosulphite contained traces of iron, sufficient to decrease the calculated coefficients by about 15 %. The true values approach closely those of horse myoglobin. 2) The peak of the alpha-band was found to be at 577 mμ whereas the corresponding value for myoglobins of other species ranges between 579 and 580 mμ. It will be clear from the dis-

cussion given above that differences of 2 mμ cannot be considered as significant with the type of apparatus used. In the course of a great number of observations on human Mgb, using the Beckmann spectrophotometer, the peak of the alpha-band has been found at every point between 577 and 580 mμ. The mean value of 578.5 mμ has been adopted here. 3) The dissymetry of the two bands has been confirmed. Moreover a faint band has been regularly observed, but with somewhat varying intensity, at 570 mμ. As denatured CO-Mgb has a band at 570 mμ, it is possible that a slight denaturing of the product may be responsible for it.

Stability of the compounds. — CO-Mgb is not as stable as CO-Hb and is relatively rapidly oxidized to met-Mgb in the presence of air. However, if the cuvette is completely filled and quickly covered, this process is sufficiently slow not to interfere with the measurement. After 3 hours, the decrease in extinction at 578 mμ was only 2 %, and the increase at 635 mμ negligible.

Influence of pH. — Between 6.5 and 8, the pH has no measurable influence on the spectral curves. An acid reaction must be avoided, as it tends to accelerate both auto-oxidation and denaturation. In view of the strong acidifying effect of $Na_2S_2O_4$, the solutions must be sufficiently buffered, preferably towards the alkaline range. M/15 Na_2HPO_4 has been used throughout the present work.

Influence of concentration. — Both CO-Hb and CO-Mgb obey Beer's law perfectly within a range of concentration of 0.05 to 1 g/l at the various wave-lengths used for their determination.

Choice of wave-lengths and calculations

Theoretically, the concentrations of Hb and Mgb can be determined by two independent readings taken at two wave-length values where the extinction coefficients of Hb and Mgb are sufficiently different, according to the general formula

$$C_{Mgb} = \frac{A_{(1)}\,\varepsilon_{Hb(2)} - A_{(2)}\,\varepsilon_{Hb(1)}}{\varepsilon_{Mgb(1)}\varepsilon_{Hb(2)} - \varepsilon_{Mgb(2)}\varepsilon_{Hb(1)}} \qquad (1)$$

$$C_{Hb} = \frac{A_{(1)}\,\varepsilon_{Mgb(2)} - A_{(2)}\,\varepsilon_{Mgb(1)}}{\varepsilon_{Hb(1)}\,\varepsilon_{Mgb(2)} - \varepsilon_{Hb(2)}\,\varepsilon_{Mgb(1)}} \qquad (2)$$

where

C_{Mgb} and C_{Hb} are the concentrations of Hb and Mgb
$A_{(1)}$ and $A_{(2)}$ the density values read at wave-length (1) and (2)
ε_{Hb} and ε_{Mgb} the extinction coefficients of Hb and Mgb at both wave-lengths.

The choice of the wave-lengths to be used, which is arbitrary, will be dictated, for obvious reasons, by the shape of the two spectral curves, the points where a variation of the ratio $\frac{C_{Mgb}}{C_{Hb}}$ produces the greatest change in light absorption being the most suitable for the determination of this ratio. In the present case, three points are particularly interesting because, besides satisfying the above requirements, they have the advantage of greatly simplifying the calculations involved and of immediately providing a figure averaging three independent determinations. The first is the isobestic point at 575.7 mμ (see Fig. 2). At this wave-length CO-Mgb and CO-Hb show the same extinction coefficient so that the total concentration in pigment can be determined, irrespective of their relative concentrations, from one single reading at that wave-length, and compared with the value obtained by the reduced pyridine-hemochromogen method. The other two points are 568 and 583.8 mμ (see Fig. 2). At these wave-lengths, CO-Mgb shows identical extinction values and will therefore contribute the same value to the densities read at both wave-lengths. CO-Hb, on the contrary, has widely differing extinction-coefficients and is solely responsible for the difference between the two density values. The concentration of Hb can therefore be calculated directly from this difference irrespective of the amount of Mgb present, and, for that matter, of any other impurity showing approximately the same extinction value at both wave-lengths. The concentration of Mgb will then be obtained as the difference between the value read at the isobestic point and the value obtained for Hb.

In the present case, the following set of equations will be valid

$$C = C_{Mgb} + C_{Hb} = \frac{A_{(575.7)}}{0.706} \tag{3}$$

$$C_{Hb} = \frac{A_{(568)} - A_{(583.8)}}{0.452} \tag{4}$$

$$C_{Mgb} = C - C_{Hb} \tag{5}$$

The value of C can be checked independently by the reduced pyridine-hemochromogen method. If the method described above is applied (1 ml of the solution to be analysed plus 3 ml NaOH-pyridine), a value of C directly comparable with that calculated from equation (3) can be obtained from either of the following expressions

$$C = \frac{A_{(557.5)}}{0.494} \tag{6}$$

$$C = \frac{A_{(557.5)} - A_{(540)}}{0.338} \qquad (7)^*$$

Equation (7) has the advantage of eliminating the error arising from impurities which absorb light equally at 557.5 and at 540 mμ.

Remarks. — It is interesting to note that the values computed from equations (3), (4), and (5) actually average three sets of calculations. With the three readings made, three different combinations of 2 wave-lengths can be used to calculate C_{Hb} and C_{Mgb} according to the general formulae (1) and (2): $A_{(568)} + A_{(575.7)}$; $A_{(583.8)} + A_{(575.7)}$; $A_{(568)} + A_{(583.8)}$. Averaging of the three expressions of C_{Mgb} obtained in this way leads to the following equation

$$C_{Mgb} = 1.3746 \times A_{(575.7)} - 2.1859 \times A_{(568)} + 2.2314 \times A_{(583.8)} \qquad (8)$$

A comparable expression can be obtained from equations (3) and (4)

$$C_{Mgb} = 1.4160 \times A_{(575.7)} - 2.2123 \times A_{(568)} + 2.2123 \times A_{(583.8)} \qquad (9)$$

It is seen that equations (8) and (9) are closely similar and the value of C_{Mgb}, as calculated by the simple expressions (3) and (4) will correspond almost identically to the true mean of the values which could be calculated by combining in the classical way, the values read at 3 different wave-lengths, the only difference being that equation (9) averages the three readings in a slightly different way as compared to equation (8). Both methods of calculation were used on more than 100 sets of determinations on muscle extracts and in no case was the difference between the values obtained more than 1 %. The use of equation (3) and (4) is thereby justified as it leads to greater simplicity without loss of accuracy.

2) Fig. 2 shows the existence of two more isobestic points (553.8 and 540 mμ). Readings at those points might provide a valuable check of the reading at 575.7 mμ, especially as the properties of the filter render these wave-length values very accurately reproducible. Their use is however prohibited in muscle extracts as the disturbing effect of impurities increases at shorter wave-lengths (especially that of cytochrome-c in this particular case). For a similar reason, no attempt was made to take advantage of the differences in the Soret-region.

3) The determination of C_{Hb} is less exposed to error than that of C_{Mgb}, as interference of impurities which like Mgb show the same absorption-value at 568 and at 583.8 mμ is eliminated in equation (3). Such impurities will, on the other hand, appear as Mgb. Unfortunately, the spectral curves do not favor a reverse combination, whereby C_{Mgb} could be calculated directly from the difference in densities at two points at which Hb contributes equally. The most favorable combination of that sort would be 575.7 and 560 mμ, but even there the difference brought about by Mgb is very small and moreover, even small amounts of cytochrome-c will cause a substantial error, as was shown by a few trial tests made on muscle extracts.

---

* For pure solutions $C = \frac{A_{(557.5)} - A_{(540)}}{0.355}$: see previous footnote, p. 270.

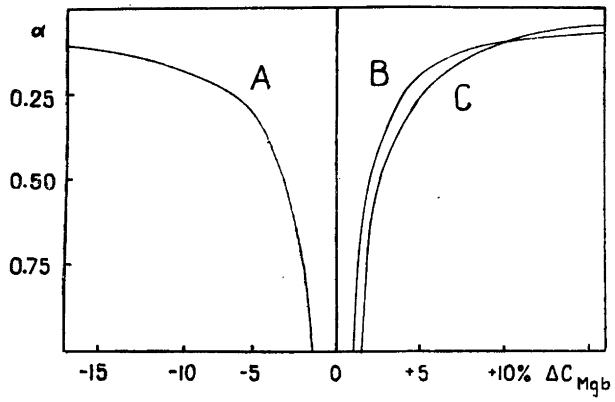


Fig. 3. Percentage of error of C_{Mgb} ($\triangle C_{Mgb}$) *as a function of* $\alpha = \frac{C_{Mgb}}{C}$.

A = If an error of + 1 % is made in $A_{(568)}$
B = » » » » » » » » » $A_{(575.7)}$
C = » » » » » » » » » $A_{(583.8)}$

Evaluation of the error

The present discussion will be limited to a theoretical evaluation of the error inherent to the method itself, applied under the best possible conditions. The close similarity of the two components between which simple spectrophotometry is asked to differentiate, can be expected to assign unavoidable limitations to the accuracy of the determination. Therefore, a range of error larger than the one affecting usual photometric measurements may be predicted. Errors in the extinction value obtained, arising either from personal errors or technical defects of the apparatus, will generally be magnified in the value calculated for C_{Mgb} and it is interesting, in order to define the degree of accuracy of the method, to estimate the extent of this magnification. In this respect, two factors are of importance in the present case: 1) the ratio $\frac{C_{Mgb}}{C}$; 2) the accuracy of the wave-length setting. Furthermore, in order to make it possible to evaluate the applicability of the method to crude muscle extract, where unknown factors may influence the measurements in an uncontrollable way, an independent check of the reliability of each determination will be found necessary. Such a check will be provided by a comparison between the value of C obtained at the isobestic wave-length and that given by the pyridine-hemochromogen method.

Influence of the ratio $\frac{C_{Mgb}}{C}$. — It is obvious that the error affecting the value of C_{Mgb} will be greater, the smaller the ratio $\alpha = \frac{C_{Mgb}}{C}$. Fig. 3 shows the variation of the percentage of error of C_{Mgb}, ΔC_{Mgb} as a function of the relative concentration α of myoglobin in the mixture, in three simple theoretical cases. Curves A, B, and C apply to an error of + 1 % in the estimation of respectively $A_{(568)}$ $A_{(575.7)}$, and $A_{(583.8)}$, the other two values being in each case assumed to be the correct ones. It is seen that the error in the readings are magnified by a factor which is roughly equal to $\pm \frac{1.5}{\alpha}$. If sufficient care is taken, reading errors should not exceed $\pm$ 1 %. Therefore, the value $\pm \frac{1.5}{\alpha}$ % can be taken as the margin of error of the determination of C_{Mbg}, at least if no error is made in the setting of the wave-lengths.

Influence of the wave-length setting. — Assuming the extinction-coefficient of the filter to remain constant and to be measurable within $\pm$ 1 %, the wave-length can be reproduced within 0.2 mμ at 568 mμ, 0.15 mμ at 575.7 mμ, and 0.1 mμ at 583.8 mμ. The maximal error arising from such wave-length shifts will be 0.3 % at 568 mμ, 0.75 % at 575.7 mμ and 1 % at 583.8 mμ, giving a mean maximal error of 0.7 %. It follows that the error in the extinction-values can reach 1.7 %, and the total maximal error in the determination of C_{Mgb} will be $\pm \frac{1.7 \times 1.5}{\alpha} = \pm \frac{2.5}{\alpha}$ %. This value characterizes the range of deviation of a given value of C_{Mgb}.

Test of reliability. — Application of this method to crude muscle extract introduces new possible errors in the evaluation of C_{Mgb}. Impurities may influence the spectrophotometric data either by being themselves light-absorbing in the wave-length region used, or by increasing the natural instability of the hemoglobins and catalysing their transformation into compounds of different spectral characteristics. The extraction procedure itself might also be responsible for similar changes. Unless the resulting modifications of the absorption-values are very important, a plausible value for C_{Mgb} may nevertheless be obtained from the spectrophotometric data. In most cases, however, errors in the data obtained on the CO-compounds will be reflected in a discrepancy between the value of C obtained at the isobestic point and that furnished by the pyridine-hemochromogen determination.* Agreement between these two

* It has been ascertained, for instance, that CO-myoglobin subjected to mild denaturing agents (increasing acidity-concentrated urea) shows a variety of spectra closely resembling those of mixtures of undenatured CO-Mgb and CO-Hb. But, for the same amount of hemin, the extinction-coefficients of the denatured Mgb are significantly lower than those of undenatured mixtures.

values will generally mean that the amount of unmodified protoheme which can be extracted, is present in the solution of CO-compounds as native CO-Mgb and CO-Hb and that interference by impurities is negligible.

Practically, the following test is suggested for the reliability of a given determination. If the pyridine-hemochromogen value agrees within 3 % with the corresponding value obtained at 575.7 mμ on the CO-compounds, it is felt that the whole set of determinations carried out on the CO-compounds can be considered as reliable and the resulting value of C_{Mgb} obtained will be labeled R. A discrepancy of 3—6 % between the pyridine-hemochromogen and the isobestic values should not yet be viewed too seriously, in view of the sensitivity of the test and of the somewhat wide range of error to be admitted in any case. Values of C_{Mgb} falling in this group have been labeled A for acceptable. If the discrepancy is greater than 6 %, the value of C_{Mgb} must be considered as unreliable (U).

A similar classification can also be made, using the difference between the two pyridine-hemochromogen values calculated from equations (6) and (7), on the principle that a significant disagreement between both values tends to cast a doubt on their validity, and, consequently, on the validity of the whole determination.

Judging from the numerous determinations which have been carried out on various mixtures of pure Hb and Mgb, it is felt that this estimation of the error inherent to the method is a generous one. In fact, the practical error in these determinations was usually much smaller than the one allowed theoretically, probably because, in most cases, the errors made tend to cancel each other to a great extent. However, the rule which has been set will be found useful in analyzing the results obtained on extracts where greater discrepancies occur.

EXTRACTION TECHNIQUE

To be suitable for spectrophotometric analysis according to the technique outlined in the previous section, 6 ml of a clear, well buffered, and slightly alkaline (M/15 Na_2HPO_4) solution containing between 0.3 and 0.8 mg of total pigment per ml are required.

Several difficulties were encountered in the attempt to prepare muscle extracts conforming to this rule. When water is used as extracting medium on ground muscle, the extract is invariably turbid and cannot be clarified by centrifuging. None of the reagents which were tried in order to remove this turbidity proved satisfactory. Whenever clarification was achieved, some pigment was carried down and could not be recovered. It was found however

that if a slightly acid medium is used for extraction, clear solutions can be obtained. In order to retain the plasmolytic effect of H_2O, very dilute solutions of varying acidity were tried and $N/100$ acetate buffer pH 4.5 was found to give satisfactory results, the buffering capacity of the tissue being sufficient to bring the pH up to about 6.

Various grinding techniques were tried, with the aim of minimizing the amount of muscle tissue necessary for the determination, enabling an accurate correlation of the results to a dry-weight basis and making the extraction as quantitative as possible. Homogenizing was found more applicable to small quantities of tissue than grinding with sand, but true homogenates could only be obtained by first subjecting the tough muscle structure to a preliminary disruption by freezing and grinding with dry ice. The advantage of this procedure was that the grinding medium was automatically eliminated and that quantitative recovery of the minced tissue was not necessary, as the dry-weight determination could be made on an aliquot of the homogenate.

Practically, the following method was followed:

Approximately 0.5—1 g of muscle tissue was dissected free of fat and connective tissue and washed free of part of the blood by gently squeezing under tap-water. A mortar with some powdered dry ice was prepared and the piece of muscle cut in small strips which were embedded in the dry ice. After allowing a little while for freezing, the mixture was vigorously ground under addition of dry ice when necessary, until a pink homogenous powder was obtained. Before thawing had set in, this powder was transferred to a pyrex-glass homogenizer and further ground in the tube by means of the glass pestle until thawing was completed. 3 ml of $N/100$ acetate buffer pH 4.5 was added and the mixture was homogenized. This part of the procedure was best carried out by hand, under visual control. About 0.6 ml of the homogenate was then pipetted into a tared weighing-vessel with a ground glass stopper (»pig» type) and the remaining quantity into a tared stoppered centrifuge tube of about 6 ml capacity. Both vessels were carefully weighed and the weighing-vessel was reweighed after standing 12 hours at 110°. The ratio: dry weight/wet weight of this aliquot gave the amount of dry matter/g of homogenate and, by multiplying by this factor the weight of homogenate used for the determination, the amount of dry matter analyzed was obtained.

To the aliquot of the homogenate transferred into the centrifuge-tube 3 ml of acetate buffer was added. After thorough mixing with a glass rod, the sample was centrifuged and the clear supernatant decanted into a 10 ml graduated cylinder. The precipitate was washed twice with acetate buffer and the washings added to the first extract. This part of the procedure had to be carried out carefully, as quantitative recovery of the extract and wash-

ings was essential. The total volume of fluid was then read as accurately as possible, after which the extract and washings were mixed in order to equilibrate the concentration. From now on, losses of fluid became of secondary importance, as only the concentrations needed be determined.

The extract was then carefully examined for the presence of any turbidity or solid particles and eventually recentrifuged until a crystal-clear solution was obtained. In this case, a medicine dropper was used to decant the supernatant, of which only 6 ml were necessary for the subsequent determination. To the clear extract a total of 25 mgm solid $Na_2HPO_4 \cdot 12\ H_2O$ p. ml of fluid was added and dissolved by stirring, bringing the pH to about 8 and the phosphate molarity to about $M/15$. This resulted in a volume increase of 1.2 %. It was essential that a perfectly clear extract be obtained *before the addition of phosphate* as solid particles usually disperse in the more alkaline medium, creating unremovable turbidity.

By carefully following the above procedure, extracts suitable for spectrophotometric determination were regularly obtained. 1 ml of the extract was then pipetted into a 1 cm cuvette for pyridine-hemochromogen determination and the remainder used for conversion into the CO-compounds and analysis, according to the technique described in the previous section. The values of C_{Mgb}, C_{Hb}, and C were converted into mg/100 mg of dry weight by the following formula:

$$\% \text{ Mgb} = C_{Mgb} \frac{1.012 \times V \times 100}{d.\ w.}$$

where V is the volume of the extract.
1.012 the correction factor for the addition of phosphate.
$d.\ w.$ the dry weight of the sample analysed.

RESULTS AND DISCUSSION

A total of 113 single determinations have been performed according to the technique which has been described. In a number of cases, determinations were made in duplicate on aliquots of the *same homogenate* as well as on different pieces of the *same muscle sample*. Detailed results will be only presented in as much as they help to evaluate the degree of accuracy and the reliability of the method used. No attempt will be made to correlate the results obtained with clinical data.

Single determinations

Examination of each single set of results separately can only furnish information as to their reliability, according to the reliability-test described in

the first section. On the basis of the deviation between the pyridine-hemochromogen values obtained from equation (6) and (7), 59 out of 90 measurements were found reliable, 18 acceptable and 13 unreliable. From the deviation between the mean pyridine-hemochromogen value and the isobestic value, it was found that 58 out of 113 determinations could be labeled reliable, 28 acceptable and 27 unreliable. The results of the combined tests are summarized in Table 3.

Table 3. Reliability-test.

Deviation between the 2 pyridine-hemochromogen values.	Deviation between pyridine-hemochromogen value and isobestic value.		
	0—3 % Reliable	3—6 % Acceptable	over 6 % Unreliable
0—3 % Reliable	35	15	9
3—6 % Acceptable	9	6	3
over 6 % Unreliable	2	5	6
Not determined	12	2	9

According to this survey, the number of unreliable determinations is abnormally high (30 %), which seems to suggest that the procedure followed is not as harmless when applied to extracts as it is when pure solutions are used. Excluding reading errors, which could hardly account for such a high percentage, significant discrepancies between the 3 values considered will be caused mainly by the presence of light absorbing impurities or of cloudiness or by partial denaturation of the pigments. There is no doubt that interfering impurities may be present in some extracts, but their influence should show equally in duplicate determinations. As this was not always the case, it is obvious that the experimental procedure itself must in some way be responsible for the observed discrepancies.

The most harmful step may well be the bubbling procedure used for saturating the extracts with CO. Watson [8] reports that this technique invariably provoked turbidity in his extracts. This was only exceptionally the case in the present experiments but the possibility of partial denaturation unaccompanied by cloudiness cannot be excluded. In this respect, it must be emphasized that both human hemoglobin and myoglobin are very unstable

in dilute solutions and this instability may well be enhanced in a crude muscle extract.

It is therefore suggested, as a possible improvement, to substitute a more gentle saturation technique to the one described in this paper, for instance the one advocated by Watson [8] which consists in bringing the reduced solution under pure CO atmosphere and equilibrating by simply rotating the tube (without shaking).

Finally, it may be noted that the preceeding argument partly rests on the assumption that the pyridine-hemochromogen determinations are generally more reliable than the measurements at the isobestic point of the CO-compounds. In view of the delicacy of the pyridine-hemochromogen test, which has been commented upon in the first section, a reverse grading might have been hypothecated. However, it will be seen from the figures given in Table 3 that better agreement is obtained between the 2 pyridine-hemochromogen values than between their mean and the isobestic value. Moreover, consideration of the results obtained in duplicate determinations which will be discussed presently, seems to show that the former values are more trustworthy than the latter. The validity of the pyridine-hemochromogen value has been further checked in a number of cases by a reading at 650 mμ, where pyridine-verdohemochromogen shows a strong absorption-band. The absorption was very low at that wave-length, which seems to indicate that no oxidation of the hemin-group had taken place in the extract.

Duplicate determinations

29 homogenates were analysed in duplicate. These determinations show several interesting features and have for that reason, been recorded fully in Table 4.

It will be seen first that better agreement is generally obtained between the pyridine-hemochromogen values than between the isobestic values. Especially when one or both of the determinations of a set are labeled unreliable, the discrepancy is seen to be generally due to erroneous isobestic values, whereas the pyridine-hemochromogen values show satisfactory agreement.

Surprisingly close agreement is obtained between the two myoglobin values of a duplicate set. Only in 4 cases (*) does the observed deviation exceed the allowed deviation calculated by the formula $\frac{2.5}{\alpha}$ and only in one case (**) is the observed deviation more than twice the allowed deviation. In other words the error involved by the complete succession of steps starting with the weighing of an aliquot of a given homogenate is usually smaller than that

Table 4. Results of duplicate determinations. — All values in mg/100 mg of dry tissue.

No.	Reliability	Total pigment (as pyr.-hem.)		Total pigment (as CO-comp.)		Hemoglobin		Myoglobin		
		Mean	Deviation	Mean	Deviation	Mean	Deviation	Mean	Deviation	Allowed deviation
41/42	R R	4.60	±0.015	4.64	±0.09	2.58	±0.06	2.06	±0.03	±0.11
47/48	R R	3.84	±0.05	3.845	±0.165	1.67	±0.09	2.175	±0.075	±0.095
57/58	R R	6.11	±0.11	6.205	±0.045	3.005	±0.005	3.20	±0.04	±0.16
66/67	R R	1.65	±0.045	1.64	±0.06	0.69	±0.04	0.95	±0.02	±0.04
71/72	R R	2.875	±0.005	2.91	±0.08	1.18	±0.11	1.73	±0.03	±0.075
77/78	R R	3.315	±0.09	3.355	±0.065	1.925	±0.005	1.43	±0.06	±0.08
79/80	R R	2.585	±0	2.59	±0.07	1.345	±0.025	1.245	±0.045	±0.06
105/106	R R	3.00	±0.04	2.985	±0.005	0.055	±0.055	2.93	±0.06	±0.075
107/109	R R	3.03	±0.025	3.025	±0.035	0.13	±0.04	2.895	±0.075	±0.075
53/54	R A	3.25	±0.01	3.21	±0.07	1.56	±0.01	1.65	±0.06	±0.085
61/62	R A	4.55	±0.035	4.63	±0.08	2.455	±0.085	2.175	±0.005	±0.105
63/64	R A **	1.975	±0.045	2.05	±0.02	0.97	±0.09	1.08	±0.11	±0.05
73/74	R A*	2.33	±0.005	2.275	±0.035	1.045	±0.045	1.23	±0.08	±0.06
75/76	R A	3.01	±0.095	2.91	±0.03	1.54	±0	1.37	±0.03	±0.07
81/82	R A*	2.83	±0.025	2.795	±0.075	1.305	±0.155	1.49	±0.08	±0.07
97/99	R A	2.98	±0.06	3.045	±0.015	0.36	±0.03	2.685	±0.045	±0.08
98/100	R A	2.98	±0.09	3.015	±0.025	0.655	±0.035	2.36	±0.01	±0.075
110/111	R A	2.81	±0.01	2.915	±0.045	0.03	±0.01	2.885	±0.035	±0.07
25/26	R U	3.95	±0.34	3.66	±0.16	2.16	±0.07	1.50	±0.09	±0.09
49/50	R U	2.685	±0.035	2.535	±0.045	0.995	±0.105	1.54	±0.06	±0.065
51/52	R U	2.03	±0.03	2.10	±0.11	0.71	±0.12	1.39	±0.01	±0.055
101/102	R U	4.60	±0.025	4.365	±0.195	3.005	±0.245	1.36	±0.05	±0.11
103/104	R U	3.58	±0.015	3.51	±0.07	0.26	±0.02	3.25	±0.05	±0.08
45/46	A A	3.02	±0.04	3.14	±0.06	1.11	±0	2.03	±0.06	±0.08
59/60	A A	6.85	±0.045	7.195	±0.105	4.02	±0.03	3.175	±0.075	±0.175
55/56	A U	2.56	±0.01	2.535	±0.095	0.895	±0.125	1.69	±0.02	±0.065
43/44	U U	1.50	±0.02	1.60	±0	0.68	±0.04	0.92	±0.04	±0.04
69/70	U U*	3.725	±0.015	3.365	±0.065	1.725	±0.205	1.64	±0.14	±0.08
112/113	U U	4.39	±0.075	5.05	±0.15	0.335	±0.065	4.715	±0.085	±0.12

allowed for the spectrophotometric determination only. It is clear that the Mgb-content of a given homogenate can be estimated with good accuracy and that the homogenates made according to the technique described are true homogenates.

* Experiments in which the observed deviation is higher than the allowed deviation.

** Experiments in which the observed deviation is more than twice the allowed deviation.

The fact that the agreement is equally good when one or two of the determinations in a set have to be considered unreliable seems to be a very favorable circumstance. Apparently, when the isobestic value turns out to be higher (turbidity?) or lower (denaturation ?) than the pyridine-hemochromogen value, there seems to be a compensation between the errors made so that a correct Mgb-value is obtained. As will be seen in Table 4, the error mainly reflects in such cases on the Hb-value.

Serial determinations on the same muscle sample

In 20 cases, more than one independent analysis, either single or duplicate, was made on the same muscle sample, the number of analyses ranging from 2 to 4. In these cases where the entire procedure was duplicated, less satisfactory agreement was obtained, as is shown by the figures given in Table 5. Only the values for Mgb-content have been given, as the Hb-content is by definition a variable. The number of determinations refers to the number of *independent* determinations made (S = single, D = duplicates on one homogenate). The observed deviation has been calculated as the sum of the deviations of each result from the mean (in absolute value), divided by the number of determinations. The corresponding allowed deviation is the mean of the allowed deviations $\left(\frac{2.5}{a}\right)$ of each determination.

Table 5 shows that a perfect check (within the allowed deviation) was obtained in 8 out of 20 sets of determinations; 7 showed a satisfactory check (up to twice the allowed deviation); 5 displayed significant discrepancies. Considering the numerous factors involved in an analysis of this kind, these discrepancies should not be viewed too seriously. In most cases the agreement is more satisfactory than that generally obtained in tissue-analyses, and only in extreme cases does the range of error actually approach the 30 % limit usually admitted in this type of determination.

However, in view of the good agreement which can be obtained from the homogenizing step onwards, it was felt that better constancy could be expected in serial analyses and considerable time was devoted to a study of the factors which might be responsible for the observed discrepancies. Obviously, they can only be due to one of the three following reasons: 1) Unequal distribution of Mgb in muscle tissue; 2) Failure to extract Mgb completely; 3) Errors in the determination of dry-weight.

Weighing errors could not account for the observed discrepancies: The dry samples were weighed carefully on a sensitive microbalance and duplicate weighings showed an accuracy of 1—2 %. The possibility of unequal distri-

Table 5. Results of serial determinations. — All values in mg/100 mg of dry tissue. S = Single determinations. D = Duplicate determinations on the same homogenate.

No.	Sample	Source	Number of determinations	Reliability	Myoglobin-content in % of dry matter		
					Mean value	Deviation	Allowed deviation
1	Heart-muscle	New-born	2 S	R/R	0.15	±0.05	±0.365
2	»	»	2 S	R/A	0.655	±0.085	±0.245
3	»*	Adult	2 S	R/R	1.37	±0.07	±0.05
4	»*	»	4 D	RR/RR/RA/RA	1.385	±0.076	±0.07
5	»	»	2 D + 1 S	RR/R/RA	1.02	±0.05	±0.05
6	»	»	2 D	RA/RU	1.595	±0.045	±0.075
7	»**	»	3 D + 1 S	RR/RA/UU/U	1.59	±0.18	±0.07
8	»*	»	4 S	R/A/A/U	1.31	±0.115	±0.09
9	»*	»	2 S	R/U	0.905	±0.045	±0.04
10	»*	»	2 S	R/U	1.265	±0.135	±0.085
11	Abdom.muscle	Biopsy	2 S	R/A	3.05	±0.03	±0.18
12	»**	»	3 D	RR/RA/AA	2.85	±0.675	±0.15
13	»**	»	2 S	A/U	2.80	±0.34	±0.155
14	»	Post-mortem	2 D	RR/AA	2.10	±0.075	±0.09
15	»*	»	2 D	RA/RA	2.52	±0.16	±0.08
16	»	»	2 D	RR/RA	2.89	±0.005	±0.07
17	»*	»	2 D	RR/RU	3.09	±0.16	±0.08
18	»**	»	2 S	R/U	1.64	±0.28	±0.05
19	»**	»	2 D	RU/AU	1.54	±0.15	±0.06
20	»	»	2 S	A/U	1.605	±0.005	±0.055

bution of Mgb in muscle tissue cannot be ruled out a priori but appears improbable: Muscle shows an essentially homogenous color and the samples taken for analysis were taken as free of fat and connective tissue as possible. This possibility should therefore only be accepted by excluding the other two with certainty.

There remains the possibility of incomplete extraction. This is by far the most probable explanation for the observed discrepancies. Moreover, direct evidence was obtained in a number of cases that detectable quantities of hemin remained in the tissue after extraction:

Treatment of the residue on a water-bath at 95° with glacial acetic acid saturated with NaCl gave a slightly colored extract, showing the presence of chlorhemin. In a few cases, it was possible to extract the hemin by neutralizing with concentrated NaOH under cooling and treating the moderately warm

super-saturated solution of sodium acetate with a small amount of pyridine. Most of the hemin went over in the pyridine and could be determined spectrophotometrically. This was not always the case and this procedure often brought about partial destruction of the hemin. Treatment of the tissue directly with pyridine was not found satisfactory as the hemochromogen is extremely rapidly oxidized under those conditions, but, by carrying out this procedure in an evacuated Thunberg-tube, a strong pyridine-hemochromogen band could be observed spectroscopically. Yellowish extracts were also obtained by treating the tissue residue with dilute ammonia, but the strong turbidity of the solution rendered spectrophotometric determination impossible.

In 10 cases, somewhat reliable values for unextracted hemin could be obtained and they were found to be quite high, ranging between 5 and 30 % of the extracted material. That such large quantities of hemin should belong to other hemoproteins different from Mgb or Hb appears very improbable, and the observed discrepancies seem to show that at least part of the unextracted hemin is Mgb-hemin.

Muscle-tissue is notoriously resistant to homogenizing devices and incomplete disruption of the cells could therefore easily be responsible for the observed differences. There is however evidence that such was not the case in the present experiments. Parallel experiments made on tissue extracted according to the method described, and on tissue either thoroughly ground with sand or finely divided with a freezing-microtome and subsequently homogenized furnished similar results and hemin could be detected in the residue in each case. Overnight extraction in the cold did not increase the amount of Mgb extracted. Histological examination performed by Dr. Biorck on a number of homogenates, showed the absence of intact cells. It is therefore felt that the technique used allowed complete disruption of the cells.

The possibility that part of the dissolved pigments may be retained by adsorption to the cell structures is suggested by an observation by Dirken [9] who actually describes the adsorption of Hb by washed minced muscle tissue and further states that the adsorbed pigment cannot be washed away with water but can easily be removed by an isotonic salt solution. In the present experiments, washing of the residue with M/10 phosphate buffer pH 6 did not liberate any of the retained pigment. Moreover, there is no correlation between the loss sustained and the amount of tissue extracted, whereas in Dirken's experiments the amount of Hb adsorbed depended solely on the amount of tissue present.

It seems therefore that the unextracted Mgb (and perhaps also Hb) was truly insoluble and the question then arises whether this insolubility is a natural or an artifical phenomenon.

Native Mgb is obviously an intracellular component and it is further known that both mechanical injuries of the muscles (crush-syndrome myoglobinuria) and an unexplained pathological condition (paralytic myoglobinuria) may release myoglobin from the cell-interior, in which case it appears in the blood-stream and is excreted in the urine. Whether these conditions are essentially associated with alterations of the cell-membrane or with the rupture of a linkage between Mgb and the cell-structure is not known. The possible existence of an insoluble structure-bound form of Mgb cannot therefore be ruled out with certainty and it is not impossible that complete release of Mgb from the muscle structure may necessitate disruption not only of the muscle cells but even of the smaller granules which form the homogenate. It may be pointed out in this connection that the heavier particles in the homogenate generally appeared slightly colored, whereas the smaller fragments seemed to be colorless.

On the other hand it is also quite possible that the solubility of Mgb and Hb may be affected by secondary changes, either occuring spontaneously or produced by some step in the extracting procedure.

The fact that the »insoluble» form can be extracted with dilute ammonia is consistant with either explanation, as ammonia causes complete disruption of the granules but will also dissolve denatured proteins. So is the following observation: An ammonia extract of tissue-residue was clarified by ultracentrifuging. Spectroscopical examinations showed bands typical of a mixture of oxy-Mgb and oxy-Hb. After reduction with $Na_2S_2O_4$ and treatment with CO, the spectrophotometric analysis gave a distribution of 50 % Mgb and 50 % Hb, but the isobestic value was much lower than the pyridine-hemochromogen value. A solution of partly denatured Mgb would furnish a similar result. Neutralization or dialysis of the ammonia extract against dist. H_2O caused precipitation of the pigment, which could not be redissolved in a slightly acid or neutral salt solution but dissolved readily in ammonia or an alkaline buffer of pH 9. These facts seem to support the denaturation theory, but it must be remembered that ammonia itself is a strong denaturing agent.

If the solubility of Mgb were affected by spontaneous post-mortal changes, one would expect these alterations to be reflected in a difference between the values obtained on biopsy-samples and those furnished by post-mortem samples. The limited number of determinations made so far does not make a statistical comparison possible but it will be seen from the values listed in Table 6 that there is no indication of a significant difference of the kind surmised. It seems at least probable that Mgb suffers no rapid post-mortal alterations, as long as the muscle remains *in situ*. This is confirmed by the fact that the time elapsing between the time of death and the excision of the sample does not seem to affect the results in a significant way.

Table 6. Myoglobin content of abdominal muscle.

Post-mortem	Biopsy
3.09	3.05
2.89	2.85
2.52	2.80
2.10	2.64
1.64	2.53
1.605	2.16
1.54	2.06
	1.85
	1.36
	1.36

The influence of prolonged storage in the frozen state was also investigated. After 10 days storage, one sample gave a smaller value, one a slightly higher and one a significantly higher value than the one determined previously. A marked increase was also observed in one case after 4 days' storage. Successive determinations performed one or two days running showed variations in both directions. There is however some indication that too long standing at room temperature of a muscle fragment which has been previously kept frozen may cause denaturation *in situ*. This seems to have been the case in experiments no. 7 and 12 of Table 5, in which respectively 3 and 2 of the analyses first performed gave results in perfect agreement and the last one a significantly lower value.

The evidence presented above does not furnish conclusive proof of either of the two hypotheses propounded but seems to agree best with a combination of both and to suggest the following tentative conclusions: Myoglobin can be taken to be intimately linked to the muscle structure and not to be readily separable from this structure. Preliminary freezing seems to facilitate extraction but also to render Mgb more susceptible to subsequent denaturing *in situ*. Even after freezing, complete extraction can only be effected by very careful grinding of the cell particles.

From these characteristics, the optimal conditions for extraction can be deduced.

CONCLUSION

Careful analysis of the results obtained with the method described in this paper applied under a variety of experimental conditions leads to the following conclusions:

The differential determination of human Hb and Mgb in a mixture of both pigments can be made with good accuracy, using the Beckmann spectrophotometer, provided reproducibility of the wave-lengths can be ensured. The device described in this paper has been found satisfactory.

Spectrophotometric errors are larger in extracts than in pure solutions but affect the value for Hb more than that for Mgb, which shows surprising constancy. The greater cause of error is probably the bubbling procedure used for saturating with CO and the use of a gentler technique has been suggested.

Errors due to incomplete extraction seem to be due to a combination of two factors: incomplete release of Mgb from the cell-structure and denaturation *in situ*. Optimal extraction conditions have only been worked out on a compromise basis. Agents like ammonia, which will release Mgb completely cannot be used because they furnish turbid solutions and moreover exert a denaturing effect. The best procedure appears to be freezing followed by thorough grinding with dry ice and homogenizing, using a slightly acid extracting fluid. Under those conditions, a clear extract is obtained but special care has to be taken because Mgb appears to be more labile *in situ* after the tissue has been frozen and subsequently thawed.

In more than half the determinations performed, the variation between two independent analyses was smaller than 10 %.

In view of the fact that the conditions were purposely varied in the experiments described, it is felt that this figure characterizes the range of error of the method applied under optimal conditions.

SUMMARY

1. Accurate spectrophotometric constants have been determined for the carbonmonoxide compounds of human hemoglobin and myoglobin between 600 and 520 mμ, and for the reduced pyridine hemochromogens formed from these substances in $N/10$ NaOH.

2. A differential spectrophotometric method for the determination of human hemoglobin and myoglobin in mixtures of these pigments has been worked out on the basis of the determined constants.

3. The reliability and degree of accuracy of the method have been discussed theoretically and shown to be satisfactory, using a variety of mixtures of both pigments.

4. A method is described for obtaining clear extracts from human heart- and skeletal muscle-tissue, suitable for spectrophotometric analysis according to the method described.

5. Application of the spectrophotometric method to these extracts was found to furnish results reproducible within the limit of error which has to be admitted on theoretical grounds. Extracts have been found, however, to require more careful treatment than pure solutions, and suggestions as to possible improvements have been made accordingly.

6. Serial determinations performed independently on the same samples of muscle tissue gave satisfactory agreement in more than half the cases. Significant discrepancies occured in one fourth of the measurements and were found to be due to incomplete extraction of the pigment which either remained bound to the tissue-granules or had suffered denaturation *in situ*.

7. The factors responsible for incomplete extraction have been investigated and the optimal extracting conditions deduced from this analysis.

8. On the basis of the results obtained so far it is believed that the method, applied under optimal conditions, will allow to determine the Mgb-content of muscle tissue with less than 10 % error.

The author wishes to express his very sincere thanks to Professor Hugo Theorell for his constant help and invaluable advice in the planning and the performance of this work, as well as for the generous hospitality which was extended to him at the Medical Nobel Institute, Stockholm. The kind support of Professor G. Nylin is also gratefully acknowledged.

Special thanks are due to Dr. R. K. Bonnichsen and Ing. Å. Åkeson for their friendly help and particularly to Dr. Gunnar Biörck, who not only provided the necessary specimens but also actively collaborated in the final adjustment of the method described. The technical assistance of Miss L. Rudberg has been invaluable in the performance of a great number of the analyses.

The cost of this work was partly supported by a grant from *Stiftelsen Therese och Johan Andersons minne.*

REFERENCES

1. Whipple, G. H. *Am. J. Physiol.* **76** (1926) 693.
2. Whipple, G. H. *Am. J. Physiol.* **76** (1926) 708.
3. Whipple, G. H., and Robscheit-Robbins, F. S. *Am. J. Physiol.* **78** (1926) 675.
4. Millikan, G. A. *Physiol. Rev.* **19** (1939) 501.
5. Meldolesi, G. *Deut. med. Wochschr.* **63 II** (1937) 1654.
6. Theorell, H., and de Duve, Chr. *Arch. Biochem.* **12** (1947) 113.
7. Shenk, J. H., Hall, J. L., and King, H. H. *J. Biol. Chem.* **105** (1934) 741.
8. Watson, R. H. *Biochem. J.* **29** (1935) 2114.
9. Dirken, M. N. J. *J. Physiol. London* **67** (1929) 282.

Received February 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 290—295

Short Communications

Szilard-Chalmers Reaction on Antimony

LARS MELANDER

Nobel Institute for Physics, Stockholm, Sweden

In order to obtain a Szilard-Chalmers concentration of the β-active isotopes formed by the action of slow neutrons on antimony, some experiments were carried out with the compounds phenylstibinic acid, $C_6H_5SbO(OH)_2$, and triphenylstibine, $(C_6H_5)_3Sb$. With the first compound the results were negative, presumably owing to the difficulties in the separation of the liberated activity from the bulk. The second compound proved to be suited to the purpose, as could be anticipated from the analogous experiments by Maurer and Ramm [1] on bismuth and lead by means of their tri- and tetraphenyl compounds respectively.

Experiments with phenylstibinic acid. An attempt was made to separate the activity by hydrogen sulphide precipitation and also by zinc reduction. The results were negative, and in view of the success with the triphenyl compound the failure appears to have been due to bad separation. In the case of the sulphide, for instance, an organic antimony sulphide is formed under about the same conditions as Sb_2S_3.

A very slight concentration was obtained when the solid acid was irradiated, dissolved in chloroform, and the solution extracted with an aqueous solution containing antimony trichloride and tartaric acid. The antimony was finally precipitated as Sb_2S_3. The specific activity of the antimony in this compound was 1.5 times that of the original antimony of the acid and contained 1.5 % of the total activity.

It is generally assumed that the phenylstibinic acid dissolves as a colloid in solvents like chloroform. The greater part of the activity may then be retained in the interior of the particles, which seems to be a reasonable explanation of the poor yield.

Experiments with triphenylstibine. Triphenylstibine is readily soluble in benzene. When such a solution has been irradiated, it is possible to extract about 50 % of the activity by means of water containing small amounts of antimony and tartaric acid. Some activity may also be obtained in a second extraction. The trisulphide is finally precipitated. Two typical experiments are given in Table 1 (γ-radiation measured).

Table 1. Two typical concentrations of active antimony.

	$(C_6H_5)_3Sb$ irradiated	Sb_2S_3 obtained	Spec.act. of Sb increased	Yield %
I (first. extr.)	10 g	24 mg	102 t.	51
(sec. extr.)		—	—	9
II (first extr.)	24 g	35 mg	135 t.	41
(sec. extr.)		—	—	6

The product contains both activities formed (Sb^{122} and Sb^{124}), but it has not been established whether the ratio is the

same as that in which they are formed by the nuclear reactions, a matter which may be of some interest.

By the use of less carrier it should be possible to attain higher concentrations.

*Preparation of antimony compounds.** The phenylstibinic acid was prepared according to Schmidt [2], and the triphenylstibine by the method given in *Org. Syntheses* [3].

Szilard-Chalmers reaction by means of $(C_6H_5)_3Sb$. The compound is dissolved in benzene (1 g in 5 ml), and the solution clarified if necessary by means of charcoal, which is filtered off. The solution is irradiated with slow neutrons in the cyclotron.

After the irradiation a sample of the solution is saved for comparative activity measurements. The main part of it is mixed with the same volume of an aqueous solution containing 2 % tartaric acid and 0.05 % potassium antimonyl tartrate, and the solutions are shaken together for 15 minutes. (It seems to be of importance for the yield to use an adequate amount of aqueous solution and to shake long enough.) After separation the solutions are filtered clear, and the organic solution is ready for a new irradiation. From the aqueous solution Sb_2S_3 is precipitated with H_2S, washed and dried. The sulphide prepared in this simple manner may not have the correct composition, but that is of little importance in the present experiments.

1. Maurer, W., and Ramm, W. *Z. Physik* **119** (1942) 602.
2. Schmidt, H. *Ann.* **421** (1920) 188.
3. *Org. Syntheses*, Coll. vol. I, 2nd ed., New York (1946).

Received May 24, 1948.

* Much of the experimental work has been carried out by Mr. K. Halvarson, Ing., to whom my gratitude is due.

A New „Daylight-device" for Titrations

K. RICHTER AND BENT BARFOD

Philips Ltd., the Neon-factory, Copenhagen,
and
Medicinalco Ltd., the Chemical-Biological Laboratories, Copenhagen, Denmark

The work in the analytical laboratory would be greatly facilitated if the micro titrations which have to be carried out in broad daylight in order to obtain accurate readings (*e. g.* iodometric titrations) could be performed with the same precision by artificial light.

The so-called »daylight-bulbs» which so far have been on the market, do not produce light of such a quality that very slight colour changes near the end-point of the titration can be read with the precision necessary in micro titrations.

We have succeeded in constructing an artificial »daylight device» which satisfies all our demands.

The light-source is a helical, high-voltage fluorescent lighting tube which with a current of 25 mA will produce light having a luminous flux of about 100 lumen. The nature of the light justifies the term: daylight of a colour temperature of about 5000° K. The tube will stand about 8000 lighthours.

A leak transformer having a primary voltage of 220 V and a secondary no load voltage of 1500 V has been used for the operation of the above fluorescent lighting tube. The drop in voltage between the electrodes of the tube will amount to about 300 V at 25 mA. It is therefore sufficient to use a transformer having a no load voltage of 6—700 V if such a transformer is available. The consumption will in the latter case be 6—8 W.

The helical light-source has a total diameter of 6.5 cm. The transformer and the electrodes of the tube are insulated mounted in a lattice case (height 14.5 cm, length

same as that in which they are formed by the nuclear reactions, a matter which may be of some interest.

By the use of less carrier it should be possible to attain higher concentrations.

*Preparation of antimony compounds.** The phenylstibinic acid was prepared according to Schmidt [2], and the triphenylstibine by the method given in *Org. Syntheses* [3].

Szilard-Chalmers reaction by means of $(C_6H_5)_3Sb$. The compound is dissolved in benzene (1 g in 5 ml), and the solution clarified if necessary by means of charcoal, which is filtered off. The solution is irradiated with slow neutrons in the cyclotron.

After the irradiation a sample of the solution is saved for comparative activity measurements. The main part of it is mixed with the same volume of an aqueous solution containing 2 % tartaric acid and 0.05 % potassium antimonyl tartrate, and the solutions are shaken together for 15 minutes. (It seems to be of importance for the yield to use an adequate amount of aqueous solution and to shake long enough.) After separation the solutions are filtered clear, and the organic solution is ready for a new irradiation. From the aqueous solution Sb_2S_3 is precipitated with H_2S, washed and dried. The sulphide prepared in this simple manner may not have the correct composition, but that is of little importance in the present experiments.

1. Maurer, W., and Ramm, W. *Z. Physik* **119** (1942) 602.
2. Schmidt, H. *Ann.* **421** (1920) 188.
3. *Org. Syntheses*, Coll. vol. I, 2nd ed., New York (1946).

Received May 24, 1948.

* Much of the experimental work has been carried out by Mr. K. Halvarson, Ing., to whom my gratitude is due.

A New „Daylight-device" for Titrations

K. RICHTER AND BENT BARFOD

Philips Ltd., the Neon-factory, Copenhagen,
and
Medicinalco Ltd., the Chemical-Biological Laboratories, Copenhagen, Denmark

The work in the analytical laboratory would be greatly facilitated if the micro titrations which have to be carried out in broad daylight in order to obtain accurate readings (*e. g.* iodometric titrations) could be performed with the same precision by artificial light.

The so-called »daylight-bulbs» which so far have been on the market, do not produce light of such a quality that very slight colour changes near the end-point of the titration can be read with the precision necessary in micro titrations.

We have succeeded in constructing an artificial »daylight device» which satisfies all our demands.

The light-source is a helical, high-voltage fluorescent lighting tube which with a current of 25 mA will produce light having a luminous flux of about 100 lumen. The nature of the light justifies the term: daylight of a colour temperature of about 5000° K. The tube will stand about 8000 lighthours.

A leak transformer having a primary voltage of 220 V and a secondary no load voltage of 1500 V has been used for the operation of the above fluorescent lighting tube. The drop in voltage between the electrodes of the tube will amount to about 300 V at 25 mA. It is therefore sufficient to use a transformer having a no load voltage of 6—700 V if such a transformer is available. The consumption will in the latter case be 6—8 W.

The helical light-source has a total diameter of 6.5 cm. The transformer and the electrodes of the tube are insulated mounted in a lattice case (height 14.5 cm, length

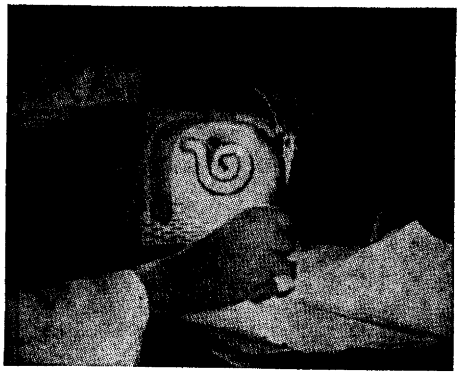


Fig. 1. The daylight-device in use.

29 cm, depth 13.5 cm). The case has four supporting legs which elevate the center of the lamp to 14 cm above the table. This is the distance found suitable for our purposes.

The light is shielded on two sides by a 6 cm wide cover.

The lamp is placed as illustrated by the photograph. The light falls obliquely from the left hand side down on the titration vessel. The colour of the liquid is thus observed against a background of non-reflecting white (a sheet of filter paper). If the light is mounted below the titration vessel, the end-point cannot be determined with the same accuracy as with the arrangement shown on the photograph. The light cover prevents direct light from reaching the eyes of the analyst. The case serves very conveniently as a support for the left hand which operates the cock of the micro burette.

Using this lighting device the colour at the end-point of the titration is clearly observed and at the same values as for titrations carried out in daylight, even when liquids of very low normalities are used (0.002 *N*).*

* The daylight-device can be obtained from PHILIPS Ltd. the Neon-factory, Copenhagen, Denmark. The complete apparatus is not yet put into serial production, but the tube and transformer, without mounting, will cost about 100 Danish crowns.

Received June 16, 1948.

The Synthesis of 1,2-Diiodoethene

ALEXIS C. PAPPAS

Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

This paper deals with a method for the synthesis of 1,2-diiodoethene (acetylene-diiodide), which was invented in studying analytical methods for determining impurities in acetylene generated from calcium carbide and acetylene from storage cylinders and studying methods for purification of acetylene for laboratory use.

Among the analytical methods that may be used for the quantitative determination of gaseous phosphorus compounds in acetylene, the oxidation of these impurities by the action of iodine in an aqueous potassium iodide solution proposed by Maurichéau — Beaupré [1] is recommended. From the amount of iodine used for the oxidation the content of phosphine is calculated by using an empirical factor. This method was tried. It proved, however, impossible to obtain conformable results.

To purify acetylene from gaseous phosphorus, arsenic and sulphur compounds the method of Conn, Kistiakowskey and Smith [2] was used. The crude acetylene was passed through wash bottles containing iodine in aqueous potassium iodide solution. The acetylene leaving this solution had a characteristic odour. If the solution in the wash bottles was allowed to stand only for a few days with acetylene, the solution became pale and long needle-shaped crystals were formed. At the same time the purifying capacity of the solution diminished and became very low.

As will be shown in this paper, the cause of these two phenomena is the formation of 1,2-diiodoethene.

According to Beilstein's Handbuch der Organischen Chemie [3] diiodoethene may be prepared by the method of Berthelot, by the action of acetylene on iodine at an

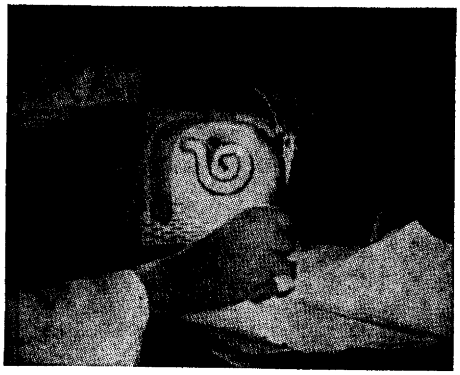


Fig. 1. The daylight-device in use.

29 cm, depth 13.5 cm). The case has four supporting legs which elevate the center of the lamp to 14 cm above the table. This is the distance found suitable for our purposes.

The light is shielded on two sides by a 6 cm wide cover.

The lamp is placed as illustrated by the photograph. The light falls obliquely from the left hand side down on the titration vessel. The colour of the liquid is thus observed against a background of non-reflecting white (a sheet of filter paper). If the light is mounted below the titration vessel, the end-point cannot be determined with the same accuracy as with the arrangement shown on the photograph. The light cover prevents direct light from reaching the eyes of the analyst. The case serves very conveniently as a support for the left hand which operates the cock of the micro burette.

Using this lighting device the colour at the end-point of the titration is clearly observed and at the same values as for titrations carried out in daylight, even when liquids of very low normalities are used (0.002 *N*).*

* The daylight-device can be obtained from PHILIPS Ltd. the Neon-factory, Copenhagen, Denmark. The complete apparatus is not yet put into serial production, but the tube and transformer, without mounting, will cost about 100 Danish crowns.

Received June 16, 1948.

The Synthesis of 1,2-Diiodoethene

ALEXIS C. PAPPAS

Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

This paper deals with a method for the synthesis of 1,2-diiodoethene (acetylene-diiodide), which was invented in studying analytical methods for determining impurities in acetylene generated from calcium carbide and acetylene from storage cylinders and studying methods for purification of acetylene for laboratory use.

Among the analytical methods that may be used for the quantitative determination of gaseous phosphorus compounds in acetylene, the oxidation of these impurities by the action of iodine in an aqueous potassium iodide solution proposed by Maurichéau — Beaupré [1] is recommended. From the amount of iodine used for the oxidation the content of phosphine is calculated by using an empirical factor. This method was tried. It proved, however, impossible to obtain conformable results.

To purify acetylene from gaseous phosphorus, arsenic and sulphur compounds the method of Conn, Kistiakowskey and Smith [2] was used. The crude acetylene was passed through wash bottles containing iodine in aqueous potassium iodide solution. The acetylene leaving this solution had a characteristic odour. If the solution in the wash bottles was allowed to stand only for a few days with acetylene, the solution became pale and long needle-shaped crystals were formed. At the same time the purifying capacity of the solution diminished and became very low.

As will be shown in this paper, the cause of these two phenomena is the formation of 1,2-diiodoethene.

According to Beilstein's Handbuch der Organischen Chemie [3] diiodoethene may be prepared by the method of Berthelot, by the action of acetylene on iodine at an

elevated temperature, or by Ssabanejeff's method by passing acetylene through a solution of iodine in absolute alcohol. The best method, however, is that of Biltz: Imbibing iodine with absolute alcohol and passing acetylene in the mixture. After three or four days the mixture is poured into water and 1,2-diiodoethene separates. Beside the solid *trans*-isomer varying quantities of the liquid *cis*-isomer are formed.

Experimental. Acetylene was passed through a 0.2 *N* iodine in aqueous potassium iodide solution in Erlenmeyer flasks. These were then closed and the solutions were left under a slight pressure of acetylene. One sample was placed in complete darkness and another in the sunlight. After a short time, one day or more, long, white needle-shaped crystals were found to have been formed in both samples. The crystals were much entangled and looked like serpentine asbestos. The solutions had lost colour and a characteristic odour was noticed. A sumblimate of short, pink-coloured (due to some disengaged iodine) crystals was found to have deposited in the upper part of the flasks that had been exposed to sunlight, whereas there was no such deposit in the flasks placed in darkness.

The mass of crystals was filtered off, washed with potassium iodide solution, sodium hydroxyde solution and water, and dried between filter-papers. The mass was then recrystallized from alcohol. In the filtrate, *i. e.* in the iodine potassium iodide solution, the iodine content was determined by titration with sodium-thiosulphate.

In one experiment when the samples had been left for two days, 96 per cent of the iodine was found to have taken part in the reaction. As the addition of iodine to acetylene occurs slowly and the reaction stops when one molecule of iodine has been added [4], the probability is that only 1,2-diiodoethene is formed in the above mentioned reactions:

$$C_2H_2 + I_2 \rightarrow C_2H_2I_2$$

By comparing the weight of the mass of crystals with the calculated amounts of 1,2-diiodoethene corresponding to the iodine consumption, the yield was found to be 91 per cent.

The identity reactions that were made are given in the table and the data referring to the *trans*-isomer of 1,2-diiodoethene [5] are given for comparison.

	M.p. °C	B. p. °C	Mol. wt.	% I
$C_2H_2I_2$-*trans*-isomer	73	192	279.86	90.7
Product prepared by us	73.4	196—197	285.2	91.0

The molecular weight was determined by Pirsch' micromethod [6] by measuring the lowering of the freezing point in camphor. Tetrabromomethane could not be used because of its reaction with the compound *.

The data found for the unknown compound give evidence of the fact that the *trans*-isomer of 1,2-diiodoethene had been formed.

The *trans*-1,2-diiodoethene obtained has a characteristic not bad odour and crystallizes from alcohol in long monoclinic prisms, which could be pulverized only with great difficulties.

The crystals have a slight double refraction. They are insoluble in water, soluble in alcohol, ether and other organic solvents, volatile with alcohol and water vapour.

The compound is slightly volatile at room temperature, not sensitive to diffused light, but decomposes slightly under the direct influence of sunlight and by distillation.

* The author is indebted to Reidar Lie, stud.mag.scient., for his kind assistance in carrying out the determination of molecular weights.

There might also be a possibility that some of the *cis*-isomer of 1,2-diiodoethene were formed during the reaction. This is an oily liquid and has a melting point of 13.8° C below zero [5]. Attempts were made to isolate this compound by a fractional crystallization of the mother liquor when the *trans*-isomer had crystallized. It was, however, impossible to prove the presence of a *cis*-isomer, apparently it is not formed by this method. The reason may be that the *cis*-isomer decomposes slowly in the dark and faster in the light and that it is transferred to the *trans*-isomer in the presence of iodine.

After concluding this work the author came across a paper written by Keiser [7], in which he briefly mentions that acetylene-diiodide may be obtained by passing acetylene through a solution of iodine in potassium iodide. Keiser gives no reference and no details of the process and the method is not mentioned in Beilstein's Handbuch der Organischen Chemie.

The author is indebted to Professor Dr. H. Haraldsen for permission to work in his laboratory and to *Nansenfondet* for a grant.

1. Maurícheau-Beaupré, Z. *Calciumcarbid und Acetylen* (1907) 369; *cf.* Vogel, J. H. *Das Acetylen* Leipzig (1923) p. 96.
2. Conn, J. B., Kistiakowskey, G. B., and Smith, E. A. *J. Am. Chem. Soc.* **61** (1939) 1868.
3. Beilstein, F. K. *Handbuch der Organischen Chemie* 4th edition, Berlin, Vol. 1, syst. 11 (1918) p. 194.
4. Nieuwland, J. A., and Vogt, R. R. *The chemistry of acetylene* New York (1945) p. 106.
5. Lange, N. A. *Handbook of chemistry* Cleveland (1944) p. 322—23.
6. Pirsch, J. Z. *Z. Angew. Chem.* **51** (1938) 73.
7. Keiser, E. H. *Am. Chem. J.* **21** (1899) 261.

Received August 13, 1948.

Methylpyrazolonethiocarbamide and Aromatic Halogene Ketones

TORSTEN EKSTRAND

The Central Laboratories, Astra, Södertälje, Sweden

In attempts to prepare thiazolesubstituted pyrazolones we have used 3-methylpyrazolone(5)-1-thiocarbamide (I) [1]. It seemed probable that this compound in a normal way should react with halogene ketones to form thiazole derivatives. However, an unexpected reaction took place with some aromatic halogene ketones. *m*-Nitrophenacyl bromide reacted in the expected way with formation of II, R = $m\text{-}NO_2 \cdot C_6H_4$.

If the thiocarbamide was treated with phenacyl chloride no thiazole compound was obtained but phenacyl rhodanide (III, R = H; R′ = C_6H_5) in a yield of more than 70 %. Desyl chloride reacted in the same way, furnishing the corresponding rhodanide in a yield of nearly 90 % (III, R = R′ = C_6H_5).

In these cases the thiocarbamide obviously was splitt. Probably the pyrazolone nucleus was desintegrated too, because it was impossible to isolate the simple methylpyrazolone from the mother liquor.

Methylpyrazolonthioamide and m-nitrophenacyl bromide. Finely ground thioamide (5 g) and *m*-nitrophenacyl bromide (8 g) were suspended in ethanol (100 ml) and heated on a water bath for 30 minutes with reflux. After cooling to 0° over night the precipitate (8 g) was sucked off. Recrystallised from ethanol (500 ml), m. p. 235—236°. The analytical data agree with those of compound II, R = $m\text{-}NO_2 \cdot C_6H_4$

$C_{13}H_{10}O_3N_4S$ (302.3)

Calc.	N 18.5	S 10.6
Found	» 18.4	» 10.5

There might also be a possibility that some of the *cis*-isomer of 1,2-diiodoethene were formed during the reaction. This is an oily liquid and has a melting point of 13.8° C below zero [5]. Attempts were made to isolate this compound by a fractional crystallization of the mother liquor when the *trans*-isomer had crystallized. It was, however, impossible to prove the presence of a *cis*-isomer, apparently it is not formed by this method. The reason may be that the *cis*-isomer decomposes slowly in the dark and faster in the light and that it is transferred to the *trans*-isomer in the presence of iodine.

After concluding this work the author came across a paper written by Keiser [7], in which he briefly mentions that acetylene-diiodide may be obtained by passing acetylene through a solution of iodine in potassium iodide. Keiser gives no reference and no details of the process and the method is not mentioned in Beilstein's Handbuch der Organischen Chemie.

The author is indebted to Professor Dr. H. Haraldsen for permission to work in his laboratory and to *Nansenfondet* for a grant.

1. Maurícheau-Beaupré, Z. *Calciumcarbid und Acetylen* (1907) 369; *cf.* Vogel, J. H. *Das Acetylen* Leipzig (1923) p. 96.
2. Conn, J. B., Kistiakowskey, G. B., and Smith, E. A. *J. Am. Chem. Soc.* **61** (1939) 1868.
3. Beilstein, F. K. *Handbuch der Organischen Chemie* 4th edition, Berlin, Vol. 1, syst. 11 (1918) p. 194.
4. Nieuwland, J. A., and Vogt, R. R. *The chemistry of acetylene* New York (1945) p. 106.
5. Lange, N. A. *Handbook of chemistry* Cleveland (1944) p. 322—23.
6. Pirsch, J. Z. *Z. Angew. Chem.* **51** (1938) 73.
7. Keiser, E. H. *Am. Chem. J.* **21** (1899) 261.

Received August 13, 1948.

Methylpyrazolonethiocarbamide and Aromatic Halogene Ketones

TORSTEN EKSTRAND

The Central Laboratories, Astra, Södertälje, Sweden

In attempts to prepare thiazolesubstituted pyrazolones we have used 3-methylpyrazolone(5)-1-thiocarbamide (I) [1]. It seemed probable that this compound in a normal way should react with halogene ketones to form thiazole derivatives. However, an unexpected reaction took place with some aromatic halogene ketones. *m*-Nitrophenacyl bromide reacted in the expected way with formation of II, R = $m\text{-}NO_2 \cdot C_6H_4$.

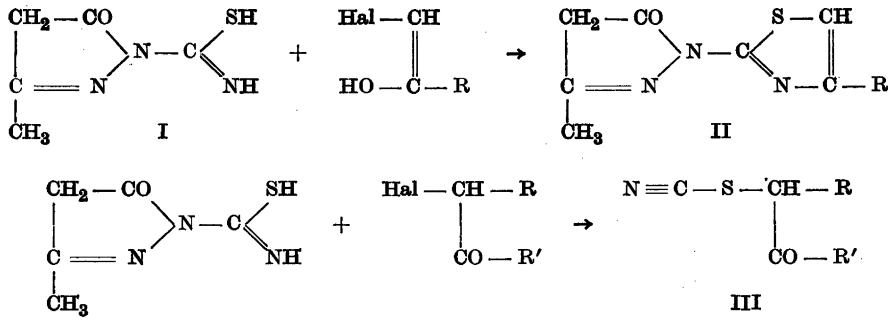
If the thiocarbamide was treated with phenacyl chloride no thiazole compound was obtained but phenacyl rhodanide (III, R = H; R′ = C_6H_5) in a yield of more than 70 %. Desyl chloride reacted in the same way, furnishing the corresponding rhodanide in a yield of nearly 90 % (III, R = R′ = C_6H_5).

In these cases the thiocarbamide obviously was splitt. Probably the pyrazolone nucleus was desintegrated too, because it was impossible to isolate the simple methylpyrazolone from the mother liquor.

Methylpyrazolonthioamide and m-nitrophenacyl bromide. Finely ground thioamide (5 g) and *m*-nitrophenacyl bromide (8 g) were suspended in ethanol (100 ml) and heated on a water bath for 30 minutes with reflux. After cooling to 0° over night the precipitate (8 g) was sucked off. Recrystallised from ethanol (500 ml), m. p. 235—236°. The analytical data agree with those of compound II, R = $m\text{-}NO_2 \cdot C_6H_4$

$C_{13}H_{10}O_3N_4S$ (302.3)

Calc.	N 18.5	S 10.6
Found	» 18.4	» 10.5



Methylpyrazolonthioamide and phenacyl chloride. The thioamide (5 g) and phenacyl chloride (5 g) were suspended in ethanol (100 ml) and heated on water bath. After 4 hours a clear solution was obtained. It was evaporated to a volume of 40 ml and cooled in ice water. The crystals (5 g) were sucked off and recrystallised from petrol ether. M. p. 72—73°.

C_9H_7ONS (177.2)

Calc.	C 61.0	H 3.96	N 7.92	S 18.0
Found	» 60.9	» 3.99	» 8.05	» 17.9

Phenacyl rhodanide was prepared according to the method of Arapides [2]. M. p. 73—74°, mixed m. p. with the substance above 72.5—73.5°.

Methylpyrazolonthioamide and desyl chloride. Desyl rhodanide was prepared in the same way from thioamide (5 g), desyl chloride (7.5 g) and ethanol (80 ml). Yield 5.7 g. After three recrystallisations from ethanol-water 3 : 1 m. p. 110—110°.

$C_{15}H_{11}ONS$ (253.3)

Calc.	C 71.2	H 4.35	N 5.53	S 12.6
Found	» 71.0	» 4.33	» 5.62	» 12.3

Desyl rhodanide, prepared according to the method of Wheeler and Johnson [3], m. p. 108—109°. Mixed m. p. with the substance above 108—110°.

1. Chandra De, S. *Q. J. Indian Chem. Soc.* **3** (1926) 30.
2. Arapides, L. *Ann.* **249** (1888) 10.
3. Wheeler, H. L., and Johnson, T. B. *Am. Chem. J.* **26** (1901) 202.

Received August 18, 1948.

New Book

Advances in Enzymology and Related Subjects of Biochemistry. Volume 7. Edited by F. F. Nord. Interscience Publishers Inc, New York, 1947. 665 pp. $8.75.

It is a real pleasure to announce Volume 7 of the important series Advances in Enzymology, which first appeared in 1941. Editors of the first five volumes were F. F. Nord and C. H. Werkman; since Volume 6 F. F. Nord has acted as sole editor. The names of the editors are sufficient assurance for the quality of the series, and all expectations in this respect have also been wholly fulfilled. It is hardly necessary to point out how valuable a series of this type is for the active student and research worker in the field of biochemistry. He finds the actual problems carefully treated in comprehensive papers by the most competent authors, papers which are not more or less mechanical compilations of the existing literature (such compilations are, of course, necessary, but are already in existence) but critical surveys by authors who are really able to separate the wheat from the chaff.

Volume 7 contains 12 papers and in addition to the ordinary indexes also a cumulative index of Volumes 1—7. The following short survey of the content shows that the many branches of enzymology and its related fields are duly considered. S. C. Brooks (Berkeley) writes on permeability and enzyme reactions, W. Seifriz (Philadelphia) on the properties of protoplasm with reference to the influence of enzymic reactions. P. D. Ditchie (Birmingham) gives a survey of recent views on asymmetric synthesis and related processes, and G. de Hevesy (Stockholm) of applications of radioactive indicators in turnover studies. There are also chapters on bacterial luminiscence by F. H. Johnson (Princeton), heme-linked groups and mode of action of some hemoproteids by H. Theorell (Stockholm), distribution, structure and properties of the tetrapyrroles by S. Granick (New York), oxidation of organic sulfur in animals by C. Fromageot (Paris), interrelations in microorganisms between growth and the metabolism of vitaminelike substances by H. McIlwain (Sheffield), antibacterial substances from fungi and green plants, by F. Kavanagh (New York), kidney enzymes and essential hypertension by O. Schales (New Orleans) and recent progress in industrial fermentation by F. M. Hildebrandt (Baltimore).

K. Myrbäck

ACTA CHEMICA SCANDINAVICA 2 (1948) 297—318

Metal Ammine Formation in Aqueous Solution

VI. Stability and Light Absorption of Copper Ethylenediamine Ions

JANNIK BJERRUM and ERLING JUHL NIELSEN

Chemical Laboratory of the University, Copenhagen, Denmark

In continuation of earlier investigations in this field[1] the present paper reports a study of the complex formation between ethylenediamine and copper. The cupric complexes have recently been investigated by Carlson, McReynolds and Verhoek[2], and Table 1 furnishes a survey of their data and the results obtained in the present paper. Carlson *et al.* carry out their measurements by means of the glass electrode and make their calculations in exactly the same way as J. Bjerrum in his work on »Metal Ammine Formation»[3]. In the present investigation the two first consecutive formation constants are likewise determined by means of glass electrode, but, besides, together with the gross complexity constant $K_2 = k_1 k_2$ and its temperature coefficient, these constants are also determined by measurement with copper amalgam electrode. All determinations seem to be in satisfactory agreement, and only the results of Job[4] with respect to K_2 are, as previously pointed out (ref. 3, p. 75), completely misleading. The 3rd consecutive constant has previously, using Werner and Spruck's old freezing point measurements[5] (ref. 3, p. 110) been estimated with considerable uncertainty to have the value $k_3 \sim 3$. It is now, by accurate spectrophotometric measurements, found to be much smaller ($k_3 = 0.10$).

THE CONSTITUTION OF THE COPPER ETHYLENEDIAMINE COMPLEXES

As chelate ligand, ethylenediamine occupies two co-ordinate positions, and if N in general denotes the number of the first more firmly and uniformly bound ligands, J. Bjerrum's (ref. 3, p. 80) characteristic co-ordination number in systems with chelate-bound ethylenediamine is given by the expression $Z = 2N$. In the cupric-ethylenediamine system the two first consecutive constants k_1 and k_2 are both very large and of the same order of magnitude,

Table 1. Concentration complexity constants for the copper ethylenediamine complexes in salt solutions.

$$k_n = \frac{[\text{Me en}_n^{+\nu}]}{[\text{Me en}_{n-1}^{+\nu}]\,[\text{en}]}\,,\quad K_n = k_1 k_2 \ldots . k_n$$

$Me^{+\nu}$	Complexity constant	Temp. coef.	$t°$	Medium	Method	References
Cu^{++}	log k_1					
	10.72		25°	1.3 N (1 N KNO_3)	glass.electr.	This paper
	10.75		25°	1.3 N (1 N KNO_3)	Cu,Hg-electr.	» »
	10.55		30°	~ 1 N (KNO_3)	glass electr.	Carlson *et al.*[2]
Cu^{++}	log k_2					
	9.31		25°	1.3 N (1 N KNO_3)	glass electr.	This paper
	9.28		25°	1.3 N (1 N KNO_3)	Cu,Hg-electr.	» »
	9.05		30°	~ 1 N (KNO_3)	glass electr.	Carlson *et al.*[2]
		$\frac{d \log K_2}{d t°}$				
Cu^{++}	log K_2					
	20.03		25°	1.3 N (1 N KNO_3)	glass electr.	This paper
	20.07	— 0.064	25°	1.07 N (1 N KNO_3)	Cu,Hg-electr.	» »
	19.60		30°	~ 1 N (KNO_3)	glass electr.	Carlson *et al.*[2]
	5.8 ?*		16°		spectroph.	Job[4]
Cu^{++}	log k_3					
	— 1.0		25°	1.07 N (1 N KNO_3)	spectroph.	This paper
Cu^{+}	log K_2					
	~ 10.8		25°		estimation	» »

and the smallness of the 3rd consecutive constant therefore shows that the cupric ion quite distinctly has the characteristic co-ordination number 4.

For statistical reasons the ratio between k_1 and k_2 must be as $\frac{4}{1} : \frac{1}{2}$ when assuming that the cupric ion has planar square configuration (ref. 3, pp. 40, 101), and it will be seen that the corresponding statistical effect $S_{1,2} = 0{,}9$ explains the main part of the difference between the logarithms of the two constants.

The chelate-fortifying effect of ethylenediamine relative to the simple binding of ammonia molecules is relatively greater for the cupric- than for the zinc- and cadmium ion, and, as far as one can judge from the present experimental material, the other metal ions of the 1st transition-group fall

* The basis for the calculation of this value is not correct (*cf.* ref. 3, p. 75).

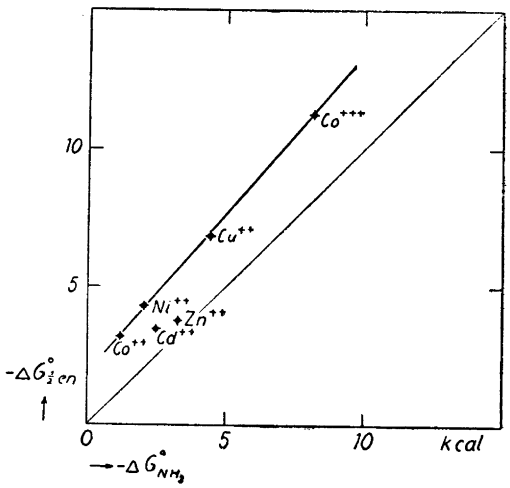


Fig. 1. The chelate-fortifying action of ethylenediamine relative to ammonia. The affinity of the metal ions to ethylenediamine is plotted as ordinate and their affinity to ammonia at the same temperatur (25° or 30°) is plotted as abscissa.

in the same class as the cupric ion. This is directly apparent in Fig. 1 where the affinity between metal ions and ethylenediamine per amino group

$$-G^{0}_{\frac{1}{2}\mathrm{en}} = \frac{1}{Z} RT \ln K_{\mathrm{N}}$$

is plotted as ordinate against the affinity per ammonia molecule

$$-G^{0}_{\mathrm{NH_3}} = \frac{1}{N} RT \ln K_{\mathrm{N}}$$

as abscissa (*vide* ref. 3, p. 91 and ref. 1, p. 7).

In the ammine systems in question, the nickel- and cobolt ions have the characteristic co-ordination number 6 and octahedral configuration, while the zinc- and cadmium ions, like the cupric ion, though less pronounced, have the characteristic co-ordination number 4. In this connection it is notable, however, that the cupric ion in its compounds with 4 ligand groups has planar square configuration, while the zinc- and cadmium ions have tetrahedral configuration (ref. 3, p. 101). This difference in configuration is also of interest in connection with the possibilities arising relative to the binding of the 3rd ethylenediamine molecule. In the case of the zinc- and cadmium ions the uptake of this molecule is of necessity accompanied by a re-arrangement to octahedral configuration, but in case of the cupric ion it is also possible that the 3rd ethylenediamine molecule may be bound to the planar di-ethylenedia-

mine complex by only one of its amino groups. Such a configuration in the case of the tri-ethylenediamine complex seems a priori rather improbable, but finds strong support in a number of facts. Thus it is in excellent agreement with the circumstance that the cupric ion, according to J. Bjerrum's previous investigations [6, 7], even in liquid ammonia does not appear to bind more than 5 ammonia molecules. On the whole, the configuration is supported by the appearance of the absorption spectra (see Fig. 2).

Thus it is very characteristic that the absorption maximum in visible light is displaced evenly towards blue at the taking up of the first four molecules monammine, respectively 2 molecules diamine, whereupon additional binding of one molecule ammonia or ethylenediamine displaces the absorption maximum in the opposite direction. Finally, it is a very important argument in favour of the configuration in question that the constant for the taking up of the 3rd ethylenediamine molecule ($k_3 = 0.10$)* here determined is substantially smaller than the corresponding constant in the ammonia system ($k_3 = 0.3$) and that the affinity constant determined by Rosenblatt [8] for the uptake by the di-ethylenediamine complex of one ammonia molecule

$$k_{2,1} = \frac{[\mathrm{Cu\ en_2(NH_3)^{++}}]}{[\mathrm{Cu\ en_2^{++}}]\ [\mathrm{NH_3}]}$$

likewise is about 1/3 (*cf.* ref. 3, p. 110). The main point of what has just been said is that the small value of k_3 relative to $k_{2,1}$ makes it rather improbable that there occurs a re-arrangement to octahedral configuration at the uptake to the 3rd ethylenediamine molecule, inasmuch as only a value of $k_3 > k_{2,1} \simeq k_5$ makes this re-arrangement energetically possible relative to the simple attachment of the ethylenediamine by only one end of its molecule.

There is nothing unique in the fact that ethylenediamine is bound by one of its amino groups only. Thus the silver ion, with the characteristic coordination number 2 and linear configuration, has only an extremely slight tendency to chelate-complex formation, as evident from the fact that the diethylenediamine complex has a stability similar to that of the diammonia complex (*cf.* ref. 3, p. 93). The mono-ethylenediamine complex, though, has a substantially greater complexity than the mono-ammonia complex and contains undoubtedly to a predominant degree chelate-bound diamine [9], and it is not until the binding of the 2nd ethylenediamine molecule that the ring is opened, resulting in an elongated complex where both ethylenediamine molecules are bound by one amino group only.

* Wrongly assuming that this constant was about 30 times larger, J. Bjerrum has previously believed it necessary to draw the opposite conclusion (ref. 3, p. 110).

It is reasonable to assume *a priori* that conditions are analogous in the cuprous-ethylenediamine system, a contention which is supported qualitatively by the fact that the equilibrium between cuprous-, cupric-complex and copper, which in ammoniacal solution is strongly in favour of the cuprous, in ethylenediamine containing solution is completely displaced in favour of the cupric step. Randles [10] has, by direct analysis, investigated this equilibrium in solutions containing an excess of ethylenediamine, and by combining his data with the present measurements K_2 in the cuprous system is roughly estimated (see Table 1). It is worthy of note that this constant is of the same order of magnitude as the corresponding constant in the cuprous ammonia system which previously, at the same temperature, has been found to be $10^{10.59}$, and this circumstance strongly supports the truth of the assumed configuration for the di-ethylenediamine-cuprous complex.

In the following, mention is first made of some hydrogen electrode determinations of the acid-base constant of ethylenediamine at high concentrations of ethylenediamine. Then follow the potentiometric determinations of the complexity constants of the cupric-ethylenediamine system, and, finally, the extinction measurements of the equilibrium between the di- and tri-ethylenediamine-cupric complexes.

SOME DETERMINATIONS OF THE ACID-BASE CONSTANT OF ETHYLENEDIAMINE

In order to follow the complex formation of metal ions with ethylenediamine by measurement of the hydrogen ion concentration of the solutions it is necessary to know the acid-base constants of the ethylenediamine under corresponding conditions. These constants have previously [1] been found to have the following values at 25°

$$k_{enH_2^{++}} = \frac{[H^+]\,[enH^+]}{[enH_2^{++}]} = 10^{-7.49}, \quad K_{enH^+} = \frac{[H^+]\,[en]}{[enH^+]} = 10^{-10.17}$$

in salt solutions with the following molar total concentrations:

$C_{KNO_3} = 1.00$, $C_{BaCl_2} = 0.100$, $C_{HNO_3} = 0.100$, and at not too high ethylenediamine concentration.

With increasing concentration of ethylenediamine k_{enH^+} showed a tendency to decrease. For the purpose of investigating this phenomenon, the hydrogen electrode measurements of Table 2 were carried out, the hydrogen ion concentration being determined by measurement relative to a standard acid solu-

tion having the composition: $C_{HNO_3} = 0.00499$, $C_{KNO_3} = 1.10$, $C_{BaCl_2} = 0.100$. The hydrogen electrode does not function in acid nitrate containing solutions and the glass electrode functions but poorly in alkaline solution with pH>10. For these reasons the standard nitric acid solution was measured with the glass electrode against a standard acetate solution ($E_{St-HNO_3} - E_{St-Ac}$), and the standard acetate solution in turn by means of a hydrogen electrode against the ethylenediamine solutions. With hydrogen electrode the following combinations was measured in a water thermostat at 25°:

— Pt, H_2	Ethylenediamine solution	KCl sat.	1 *N* KCl, Hg_2Cl_2	+ Hg
— Pt, H_2	0.1000 *N* HAc 0.1000 *N* NaAc	KCl sat.	1 *N* KCl, Hg_2Cl_2	+ Hg

and the respective potentials of the measured cells, converted to the hydrogen pressure 760 mm Hg (E (corr.) and E_{St-Ac} (corr.) respectively) and reckoned with sign in accordance with Luther, are recorded in Table 2. Both glass

Table 2. The acidic dissociation constant k_{enH^+} at high concentrations of the free base in 1.3 N salt solution at 25°.

$C_{KNO_3} = 1.00$, $C_{BaCl_2} = 0.100$

$E_{St-HNO_3} - E_{St-Ac} = 0.1393$ V, E_{St-Ac} (corr.) $= -0.5589$ V, $-\log [HNO_3]_{St}$ (corr.) $= 2.307$

No.	C_{HNO_3}	C_{en}	$E_{corr.}$	$-\log [H^+]$	$-\log[OH^-]$	$[OH^-]$
1	0.0992	0.2485	— 0.8960	10.368	3.48	0.00033
2	0.1003	0.4970	— 0.9221	10.810*	3.04	0.00091
3	0.0995	0.9665	— 0.9444	11.187	2.66	0.0022
4	0.1006	1.949	— 0.9686	11.596	2.25	0.0056
5	0.0995	2.870	— 0.9859	11.889	1.96	0.0110

No.	$\bar{n}_{en}$	$\log \frac{\bar{n}_{en}}{1-\bar{n}_{en}}$	$-\log k_{enH^+}$	$-\Delta \log k_{enH^+}$	$f_{en} \cdot F$	[en]
1	0.4005	— 0.175**	10.191	0.021	1.05	0.149
2	0.2036	— 0.593	10.217	0.047	1.11	0.396
3	0.1052	— 0.929	10.258	0.088	1.23	0.865
4	0.0545	— 1.240	10.356	0.186	1.54	1.84
5	0.0385	— 1.398	10.491	0.321	2.09	2.76

* Glass electrode measurements in this solution gave $-\log[H^+] = 10.798$, a value which is but very little below that determined by means of the hydrogen electrode.

** If it is desired to correct for the presence of a small amount of enH_2^{++} there should here, in the calculation of $-\log k_{enH^+}$, be a further addition of -0.002 to $-\log[H^+]$.

electrode measurements and hydrogen electrode measurements as well as the preparation of the ethylenediamine solutions were carried out as previously described. From the data obtained, E_{St-HNO_3} is found to be $-0.5589 + 0.1393 = -0.4196$, and the true hydrogen ion concentration of the solutions measured is consequently given by the relation:

$$-\log [H^+] = \frac{-0.4196 - E \text{ (corr.)}}{0.0591} - \log [HNO_3]_{St} \text{ (corr.)}$$

The corr. added to $-\log [HNO_3]_{St}$ indicates that the concentration of free acid in the standard solution is corrected for a small basic impurity of the salts (*cf.* ref. 1, p. 12). The hydroxyl ion concentration of the particular solutions is calculated from the hydrogen ion concentration, the ionic concentration product of the water being put at $10^{-13.85}$, *i. e.* the value one calculates from N. Bjerrum and Unmack's interpolation formula for potassium chloride solutions at 25° and the ion normality 1.3.

The constant in question $-\log k_{enH+}$ is with sufficient approximation (*vide* footnote of the table) calculated from the relation:

$$-\log k_{enH+} = -\log [H^+] + \log \frac{\bar{n}_{en}}{1 - \bar{n}_{en}}$$

where

$$\bar{n}_{en} = \frac{C_{HNO_3} + [OH^-] - [H^+]}{C_{en}}$$

is the number of hydrogen ions bound per total ethylenediamine.

It seems quite obvious *a priori* to identify the change in the value $10^{-10.17}$ for k_{enH+} caused by the ethylenediamine with a change in the activity coefficient expression $f_{en} \cdot f_{H+}/f_{enH+}$, but in that case one ignores completely any effect in connection with the diffusion potential. If, on the other hand, it is assumed that the most predominating ions — the potassium and the nitrate ions — solely and separately to the same degree take care of the electricity transport, a simple calculation [12] shows that $-\triangle \log k_{enH+}$ is identical with the variation in $\log \frac{f_{en} \cdot f_{K+}^{\frac{1}{2}}}{f_{enH+} \cdot f_{NO_3^-}^{\frac{1}{2}}}$.

In the table the calculated activity coefficient expression is denoted by $f_{en} \cdot F$ and the most plausible conclusion that one can make from the figures is that f_{en} increases rather strongly with increasing ethylenediamine concentration when the concentration of the free base is greater than about

0.1 molar. In this connection it is also of some interest to draw a comparison with the conditions in strong ammonia-ammonium nitrate solutions where the corresponding function and f_{NH_3} both increase in a uniform manner with the ammonia concentration (*cf.* ref. 3, p. 141).

POTENTIOMETRIC DETERMINATION OF THE COMPLEXITY AND HEAT OF FORMATION OF THE DI-ETHYLENEDIAMINE CUPRIC ION

A copper amalgam electrode was employed in measuring a series of cupric-ethylenediamine solutions with more than two molecules ethylenediamine per cupric ion against a weak nitric acid cupric nitrate solution in the same salt medium at 18° and at 25°. As reference electrodes were employed two mutually well conforming 1 *N* calomel electrodes of which the older one had been used in the hydrogen electrode measurements just mentioned. Both calomel electrodes were prepared according to Gjaldbæk, in electrode vessels of the model introduced by Lewis, Brighton and Sebastian.

The experimental details were like those previously described.[7] It should be mentioned, though, that the measurements were more easily carried out since the copper amalgam already was so close to being in equilibrium with the copper solutions that it was unnecessary to shake the electrodes with contents in the thermostat before the measurement. Of course, it was necessary to work in a nitrogen atmosphere because metallic copper is rapidly dissolved in ethylenediamine solutions in the presence of free oxygen. The copper amalgam, which is a suspension of so-called γ-phase with about 25 % copper in almost pure mercury [13], was prepared as previously described [7] and was kept under a layer of weakly acidic cupric nitrate solution. The volume of the electrode vessels was about 50 ml and differed from those previously described by having the one-way tap on the side tube replaced by a three-way tap as used by Biilmann and Lund [14]; moreover, the ascending tube of the syphon was omitted. In the preparation of an electrode the amalgam was not added until the copper solution had been made free of oxygen by leading nitrogen through, the nitrogen being introduced through the syphon tube and the flow being continued for a while afterwards until all oxygen had been driven out of the apparatus. The nitrogen was obtained from a cylinder and purified like the hydrogen for the hydrogen electrodes, by being led over red-hot copper (*cf.* ref. 1, p. 11). Immediately before a measurement, the syphon tube was filled with copper solution by forcing a small amount of nitrogen in through the three-way tap, and after closing this tap, letting the excess pressure be equalized through the tap of the syphon tube. The intermediate liquid was a saturated potassium chloride solution. After immersion

of the electrode in this solution, the diffusion potential was established automatically at a certain height in the syphon tube when the tap of this tube was opened. In this way it was possible to reproduce the individual measurements with an accuracy of about 0.1 millivolt.

Table 3. The normal oxidation potentials Cu,Hg → $Cu(H_2O)_4^{++}$ and Cu,Hg → $Cu(en)_2^{++}$ (relative to the normal calomel electrode) in 1 N KNO_3 at 18° and 25° C from measurements of the cells:

No. 1:

—						+
Cu,Hg	$C_{Cu(NO_3)_2}$, C_{HNO_3}, 1.05 *N* KNO_3		KCl sat.	1 *N* KCl, Hg_2Cl_2		Hg

No. 2—6:

—						+
Cu,Hg	$C_{Cu(NO_3)_2}$, C_{en}, C_{HNO_3}	1.00 *N* KNO_3	KCl sat.	1 *N* KCl, Hg_2Cl_2		Hg

No.	$C_{Cu(NO_3)_2}$	C_{HNO_3}	E (18°)	0.02887 logC_{Cu++}	$E°$ (18°)
1	0.00983	0.0025	— 0.0103	— 0.0579	+ 0.0476
			E (25°)	0.02957logC_{Cu++}	$E°$ (25°)
1	0.00983	0.0025	— 0.0113	— 0.0593	+ 0.0480

	$C_{Cu(NO_3)_2}$	C_{HNO_3}	C_{en}	[en]	E (18°)	0.02887 log$\frac{C_{Cu++}}{[en]^2}$	$E°$ (18°)
2	0.00972	0.0499	0.1698	0.1004	— 0.5457	— 0.0005	— 0.5452
3	0.00972	0.0499	0.2319	0.1625	— 0.5591	— 0.0125	— 0.5466
4	0.00972	0.0499	0.3239	0.2545	— 0.5711	— 0.0238	— 0.5473
5	0.00972	0.0499	0.4724	0.4030	— 0.5834	— 0.0353	— 0.5481
6	0.00972	0.0499	0.7094	0.6400	— 0.5978	— 0.0469	— 0.5509
					E (25°)	0.02957 log$\frac{C_{Cu++}}{[en]^2}$	$E°$ (25°)
2	0.00972	0.0499	0.1698	0.1004	— 0.5460	— 0.0005	— 0.5455
3	0.00972	0.0499	0.2319	0.1625	— 0.5597	— 0.0128	— 0.5469
4	0.00972	0.0499	0.3239	0.2545	— 0.5721	— 0.0244	— 0.5477
5	0.00972	0.0499	0.4724	0.4030	— 0.5847	— 0.0362	— 0.5485
6	0.00972	0.0499	0.7094	0.6400	— 0.5993	— 0.0480	— 0.5513

Table 3 summarizes the measurements. Of the directly measured potentials E the normal potential copper amalgam-cupric ion (relative to the calomel electrode) is calculated by means of the expression

$$E = E° + \frac{RT}{2F \cdot \log e} \log [Cu^{++}]$$

and the normal potential copper amalgam di-ethylenediamine-cupric ion by means of the expression

$$E = E^\circ + \frac{RT}{2F \cdot \log e} \log \frac{[\mathrm{Cu\ en_2^{++}}]}{[\mathrm{en}]^2}$$

In the acid solution the cupric ion concentration is identified by the total copper concentration, and in case of the ethylenediamine solutions the calculation is carried out with the assumption that the copper solely is present as di-ethylenediamine-cupric complex and that the concentration of $\mathrm{enH^+}$ is identical with the added nitric acid concentration. It is evident from the other measurements in the present paper that these assumptions are sufficiently satisfied. When the amalgam-cupric-ethylenediamine normal potential is not entirely constant but shows a slight fall with increasing ethylenediamine concentration it is presumably to a large extent because the activity of the free ethylenediamine shows an increase which is more pronounced than that of the concentration (comp. ref. 7, p. 18).

If the normal potentials from solutions 1 and 2 are combined, log K_2 for the reaction

$$\mathrm{Cu^{++} + 2\ en \rightleftharpoons Cu\ en_2^{++}}$$

in 1 N $\mathrm{KNO_3}$ is calculated to be

$$\frac{0.0476 + 0.5452}{0.02887} = \underline{20.53 \text{ at } 18^\circ}, \text{ and } \frac{0.0480 + 0.5455}{0.02957} = \underline{20.07 \text{ at } 25^\circ}$$

From the change in log K_2 with the temperature one calculates $-\Delta H$ or the decrease in the heat content for the same reaction to be 26.1 kcal. The heat evolution at the formation of the corresponding tetrammine complex is 19.7 kcal (*cf.* ref. 3, p. 79).

THE MAGNITUDE OF THE STABILITY OF THE CUPROUS-ETHYLENEDIAMINE COMPLEX

Randles[10] has, at 25°, investigated the equilibrium between cuprous-cupric complex and metallic copper in the presence of an excess of ethylenediamine, *i. e.* under conditions where both the cupric and cuprous copper must be assumed to be present as di-ethylenediamine complex. Randles' measurements are rather inaccurate, and one must take into account that the copper activity of copper amalgam is somewhat smaller than that of pure copper, but, regarded simply as an estimate, one finds:

$$K_{Cu,Hg} = \frac{[Cu\ en_2^{++}]\ [en]^2}{[Cu\ en_2^{+}]^2} = 0.4 \cdot 10^5$$

For the corresponding constant for the equilibrium between the copper-aquo ions one may put at 25° (ref. 7, p. 23)

$$K_{Cu,Hg} = 1.63 \cdot 10^6$$

If these equilibrium constants are combined with the values for the normal potentials determined by means of the solutions 1 and 2 the corresponding cuprous normal potentials

$$E^\circ\ (Cu,Hg \rightarrow Cu^+) = + 0.0480 + 0.02957\ \log\ (1.63 \cdot 10^6) = + 0.232$$
$$E^\circ\ (Cu,Hg \rightarrow Cu\ en_2^+) = - 0.5455 + 0.02957\ \log\ (\ 0.4 \cdot 10^5) = - 0.409$$

are calculated, and with the aid of these constants one calculates in turn the cuprous-complexity constant log K_2 to be

$$\frac{0.232 + 0.409}{0.05914} \sim 10.8 \text{ at } 25^\circ$$

CONVERSION OF THE NORMAL POTENTIALS TO THE NORMAL HYDROGEN ELECTRODE

The potential of the 1 N calomel electrode against the normal hydrogen electrode is known from the literature and calculated on the basis of the data given by N. Bjerrum and Unmack (ref. 11, pp. 34, 81) this potential is calculated to be 0.2844 at 18° and 0.2831 at 25°. If, at the respective temperatures these values are added to the normal potentials found (E°) relative to the calomel electrode one gets the normal potentials relative to the normal hydrogen electrode (E°_H). In this calculation it is tacitly assumed that the saturated potassium chloride solution eliminates the diffusion potential between the copper solution in 1 N KNO_3 and the 1 N calomel electrode. Finally it is an assumption applying to the whole calculation that the calomel electrodes have the correct potential. This assumption is supported by the circumstance that the potential between the hydrogen electrode in standard-acetate and the 1 N calomel electrode (with potassium chloride as intermediate liquid), which in the present paper is found to be — 0.5589 at 25° (see Table 2), is calculated to be — 0.5579 on the basis of the data given by Clark [15] and Bjerrum and Unmack [11]. Moreover, the potential of the following two cells:

+ Cu, Hg	$C_{Cu(NO_3)_2} = 0.1165$, $C_{HNO_3} = 0.01$	$2\ N\ NH_4NO_3$	NH_4NO_3 2.1 N	1 N KCl, Hg_2Cl_2	— Hg

$E = 0.0214$

+ Cu, Hg	$C_{Cu(NO_3)_2} = 0.1165$, $C_{HNO_3} = 0.01$	$2\ N\ NH_4NO_3$	KCl sat.	1 N KCl, Hg_2Cl_2	— Hg

$E = 0.0254$

was measured at 18°. The potential 0.0214 found for the upper one of these cells is in fairly good agreement with J. Bjerrum's [7] previously determined mean value 0.0209 for this combination. The potential of the second cell shows the importance of inserting a saturated potassium chloride solution. The diffusion potential must be assumed to be the smallest in the combination with potassium chloride inserted, and the previously determined normal potentials in 2 N NH_4NO_3 (ref. 7, p. 64) should probably be corrected accordingly.

DETERMINATION OF THE CONSECUTIVE COMPLEXITY CONSTANTS IN THE CUPRIC-ETHYLENEDIAMINE SYSTEM

Here measurements were made at 25° of a series of cupric nitrate-ethylenediamine solutions, employing both glass and copper amalgam electrodes. The investigations involved solutions having the composition: $C_{Cu(NO_3)_2} = 0.1$ $C_{HNO_3} = 0.1$, $C_{KNO_3} = 1.00$ and varying ethylenediamine concentration, *inter alia* in order to be able to apply the previously determined values for the acid-base constants of ethylenediamine $k_{enH_2}{}^{++} = 10^{-7.49}$ and $k_{enH^+} = 10^{-10.17}$ to the calculation of $\bar{n}_{en}$ and the concentration of free ethylenediamine from the experimentally determined hydrogen ion concentration. In the determination of this concentration, use was made of the already (p. 302) mentioned standard nitric acid solution while the cupric ion concentration of the particular solutions were determined by measuring relative to a copper standard solution having the following concentrations: $C_{Cu(NO_3)_2} = 0.09742$, $C_{HNO_3} = 0.0025$, $C_{KNO_3} = 1.10$. Measured against the calomel electrode a copper amalgam electrode in this solution

+ Cu, Hg	$C_{Cu(NO_3)_2} = 0.09742$, $C_{HNO_3} = 0.0025$	1.1 N KNO_3	KCl sat.	1 N KCl, Hg_2Cl_2	— Hg

had the potential 0.0198 at 25°, and relative to a weaker copper solution in an analogous salt medium

Table 4. Calculation of the consecutive complexity constants on the basis of measurements with glass electrode and copper amalgam electrode at 25° C.

Calc. with $-\log k_{enH_2}{}^{++} = 7.49$ and $-\log k_{enH}{}^{+} = 10.17$.
Solutions of the following composition were measured:

$$C_{Cu(NO_3)_2} = 0.09716\,,\ C_{HNO_3} = 0.1001\,,\ C_{KNO_3} = 1.00$$

No.	C_{en}	$-\log[H^+]$	$\bar{n}_{en}$	$\bar{n}$	$-\log[en]$	$\log k_1$	$-E_{Cu,Hg}$	$-\log a_{Cu}{}^{++}$	$\log k_1$
1	0.0683	3.791	2.000	0.190	11.379	10.741	0.0027	0.090	10.737
2	0.0866	3.996	2.000	0.379	10.969	10.731	0.0063	0.212	10.759
3	0.1020	4.128	1.999	0.520	10.705	10.685	0.0097	0.327	10.739
4	0.1229	4.282	1.999	0.754	10.396	10.736	0.0158	0.533	10.746
						$\log K_2$			
5	0.1423	4.436	1.998	0.954	10.088	20.03	0.0236	0.797	10.746
6	0.1579	4.569	1.998	1.135	9.822		0.0313	1.058	10.725
						$\log k_1$			
7	0.1761	4.696	1.997	1.303	9.569	10.704	0.0411	1.389	10.765
8	0.2037	4.916	1.996	1.589	9.129	10.716	0.0594	2.008	10.750
9	0.2132	5.010	1.996	1.679	8.941	10.747	0.0687	2.322	10.775
					Mean	10.720		Mean	10.749
									$\log K_2$
10	0.2535	7.063	1.728	2.014	4.909		0.3011	10.182	20.00
11	0.3002	9.023	0.962	2.019	2.171		0.4648	15.718	20.06
								Mean	20.03
						$\log k_2$			$\log k_2$
					Mean	9.310		Mean	9.281

$$+\ \text{Cu,Hg}\ \Big|\ C_{Cu(NO_3)_2} = 0.0974,\ C_{HNO_3} = 0.0025\ \Big|\ 1.1\ N\ KNO_3\ \Big|\ \text{KCl sat.}\ \Big|\ 1.1\ N\ KNO_3,\ C_{Cu(NO_3)_2} = 0.0197,\ C_{Ba(NO_3)_2} = 0.0800,\ C_{HNO_3} = 0.0025\ \Big|\ -\ \text{Cu,Hg}$$

the potential 0.0210, in good agreement with the theoretical value 0.0205 for this concentration cell. Table 4 records $E_{Cu,Hg}$, the copper amalgam potential in the complex solution measured relative to the standard copper solution, and $a_{Cu}{}^{++}$, *i. e.* the fraction of the total copper that is present as cupric ion is consequently given by the relation

$$E_{Cu,Hg} = 0.02957\ \log \frac{a_{Cu}{}^{++} \cdot 0.09716}{0.09742}$$

where 0.09716 refers to the total copper concentration of the measured complex solutions.

The consecutive constants were determined in two different ways: (1) from our knowledge of the formation curve, calculated as previously described (ref. 1, p. 8), and (2) by means of the values, determined for α_{Cu++} in connection with our knowledge of —log [en].

As for (1): The first consecutive constant was calculated in this case with the aid of the previously (ref. 3, p. 133) derived formula

$$k_1 = \frac{(2 - \bar{n})\ [\text{en}]^2\, K_2 - \bar{n}}{(\bar{n} - 1)\ [\text{en}]}$$

For the gross constant K_2 there was inserted the value that was calculated from —log [en] in the midpoint of the formation curve for $\bar{n} = 1$. For this $\bar{n}$-value —log [en] was determined by graphical interpolation to be = 10.015, whence follows log $K_2 = 20.03$.

As for (2): Here the first consecutive constant was calculated with the aid of the relation

$$\frac{1}{\alpha_{Cu^{++}}} = 1 + k_1\ [\text{en}] + K_2\ [\text{en}]^2$$

For solutions 10 and 11 in Table 4, the di-ethylenediamine complex is the only complex present so that K_2 can be directly calculated by means of the expression

$$K_2 = \frac{1}{\alpha_{Cu^{++}} \cdot [\text{en}]^2}$$

On this basis one calculates log K_2 to be 20.00 and 20.06 respectively, *i. e.* in average the same value as that calculated from —log [en] in the midpoint of the formation curve, and this value of log K_2 is therefore used in the calculation of values of log k_1 recorded in the last column of Table 4.

Table 4 summarises all of the worked-up material. If one compares the values for log k_1 calculated in the two different ways and the mean values for log k_1 and log k_2 it is seen that all of the measurements made are in good agreement.

EXTINCTION MEASUREMENTS AND DETERMINATION OF THE THIRD CONSECUTIVE CONSTANT

Table 5 shows some determinations of the molar extinction coefficient in various cupric-ethylenediamine solutions. The molar extinction coefficient is calculated in accordance with the expression:

$$\varepsilon = \frac{\log \frac{I_0}{I}}{C_{Cu} \cdot d}$$

where I_0 and I denote the intensity of the incident and the transmitted light respectively, C_{Cu} the total copper concentration, and d the thickness of the absorbing layer in cm. The measurements were carried out by means of a König-Martens Spetrophotometer equipped with the »grosse Beleuchtungseinrichtung». The smallest possible slit opening was used, and as a rule the measurement gave the mean extinction coefficient for a range of wavelengths of the order of magnitude of $\pm$ 4 mμ (*vide* ref. 6, p. 6).

According to the magnitude of the 2nd consecutive complexity constant the formation of the di-ethylenediamine complex is completed at a concentration of free ethylenediamine as low as about 10^{-7}, and the extinction coeffi-

Table 5. Molar extinction coefficients of some cupric ethylenediamine solutions at 25°.

With potassium nitrate (1 *N*)

No.	$C_{Cu(NO_3)_2}$	C_{en}	C_{HNO_3}	[en]	ε_{670}	ε_{650}	ε_{630}	ε_{590}
10 (Table 4)	0.09716	0.2535	0.1001	$10^{-4.91}$	17.49	23.78	31.35	50.50
1	0.09716	0.3181	0.1001	$10^{-1.62}$	17.38	23.30	31.28	50.47
2	0.00972	0.05347	0.0499	$10^{-4.36}$	16.91	22.91	30.58	49.58
4 (Table 3)	0.00972	0.3239	0.0499	0.2545	18.24	24.48	32.15	—
6 (Table 3)	0.00972	0.7094	0.0499	0.6400	21.78	28.21	36.09	54.39
3	0.05034	1.736	0.0505	1.585	29.11	35.79	43.76	59.62
5	0.00983	1.635	0.0505	1.565	29.20	35.85	43.97	59.84
7	0.00476	1.649	0.0503	1.589	30.00	36.57	44.54	58.46
8	0.00983	3.256	0.0505	3.186	44.31	51.94	60.25	72.41
Without potassium nitrate								
9	0.00983	3.272	0.0505	3.202	40.52	47.66	56.72	69.54
11	0.00570	7.101	0		75.03	83.10	90.74	96.98
12	0.00729	9.208	0		84.86	95.02	103.5	106.4
13	0.00977	12.18	0		87.38	98.31	107.0	108.6

cients of Table 5 clearly show that this complex thereafter is predominant over a wide range of concentration. Not until the concentration of free ethylenediamine exceeds 0.1 molar does the extinction coefficient in the red part of the spectrum begin to show noticeable growth which thereafter becomes increasingly stronger with the base concentration. Comparison of solutions 3, 5 and 7 show, however, that Beer's law applies rather accurately to solutions with the same concentration of ethylenediamine. This excludes the formation of polynuclear complexes like for example

$$\mathrm{en_2^{++}CuNH_2CH_2CH_2NH_2Cu^{++}en_2}$$

A graphical presentation of how the extinction coefficient changes with both ethylenediamine concentration and wavelength is shown in the upper left of Fig. 2. It will be seen that the absorption maximum shows both an increase and a displacement towards red at the same time as the curves converge towards a limiting curve at very high concentrations of ethylenediamine. It is seen, moreover, that the absorption curves all pass through one and the same point of intersection, which shows that one has to do with a system of only two absorbing complexes (*cf.* ref. 6, p. 7). It therefore seems logical to assume that the limiting curve at high ethylenediamine concentrations represents the absorption curve for the tri-ethylenediamine complex, and with this assumption a calculation has been made of the 3rd consecutive constant at each of the 4 wavelengths in Table 5. The result of this calculation is summarized in Table 6. The individual values of k_3 are calculated by means of the expression

$$k_3 = \frac{\varepsilon_{\mathrm{NoX}} - \varepsilon_{\mathrm{No2}}}{(\varepsilon_{\mathrm{No13}} - \varepsilon_{\mathrm{NoX}})\ [\mathrm{en}]}$$

where $\varepsilon_{\mathrm{NoX}}$ denotes the extinction coefficient of the solution in question at the particular wavelength. The very small extinction differences $\varepsilon_{\mathrm{No4}} - \varepsilon_{\mathrm{No2}}$ and $\varepsilon_{\mathrm{No6}} - \varepsilon_{\mathrm{No2}}$ are, in order to obtain values for k_3 at all usable, determined by direct measurement of the particular solutions relative to solution 2. The concentration of free ethylenediamine is in all cases with sufficient accuracy calculated by means of the expression

$$[\mathrm{en}] = C_{\mathrm{en}} - C_{\mathrm{HNO_3}} - 2C_{\mathrm{Cu(NO_3)_2}}$$

The basis for the calculation of k_3 is essentially less favourable at wavelength 590 mμ than at the three other wavelengths, and for this reason the values calculated at this wavelength are not included in the general mean value

Table 6. Values for the third consecutive complexity constant calculated from the extinction coefficients given in Table 5.

No.	$C_{Cu(NO_3)_2}$	[en]	670	650	630	590	k_3 (mean)
			1 *N* potassium nitrate				
4	0.00972	0.2545	0.076	0.084	0.083	—	0.081
6	0.00972	0.6400	0.116	0.118	0.121	(0.139)	0.118
3	0.05034	1.585	0.132	0.130	0.131	(0.130)	0.131
5	0.00983	1.565	0.135	0.133	0.136	(0.135)	0.135
7	0.00476	1.589	0.144	0.139	0.141	(0.112)	0.141
8	0.00983	3.186	0.200	0.197	0.199	(0.198)	0.199
			Without potassium nitrate				
9	0.00983	3.202	0.157	0.153	0.162	(0.159)	0.157

determination in Table 6. It will be seen that the mean values thus calculated for k_3 increase rather strongly with the ethylenediamine concentration, and that the calculated constant in dilute solution is smaller than in 1 *N* potassium nitrate at the same ethylenediamine concentration. The trend of the calculated values for k_3 agrees qualitatively with the fact that f_{en} increases with the ethylenediamine concentration (compare with $f_{en} \cdot F$ in Table 2) as well as with increasing salt concentration (*cf.* ref. 3, p. 129). The value of k_3 determined by means of solution 4 is, for reasons of calculation, rather uncertain, but if log k_3 is plotted against the concentration of free ethylenediamine a determination can be made, graphically utilizing the whole material, of k_3 in 1 *N* KNO_3 at small ethylenediamine concentrations. In this way k_3 is found to be, on the average, about 0.10.

COMPARISON OF THE EXTINCTION CURVES OF THE CUPRIC-ETHYLENEDIAMINE COMPLEXES WITH THE CORRESPONDING CURVES FOR THE AMMONIA- AND PYRIDINE COMPLEXES

In order to establish the absorption curve for the mono-ethylenediamine complex, determinations were made, at a series of wavelengths, of the molar extinction coefficient of solutions 3, 4 and 5 in Table 4 as well as that of a pure copper nitrate solution in 1 *N* KNO_3. The relative distribution of the copper in the solutions is calculated with the aid of the values determined for k_1 and K_2 and is, along with the calculated complex distribution at a number of other ethylenediamine concentrations, recorded in Table 7. The experimentally determined extinction curves, and the values of the light absorption

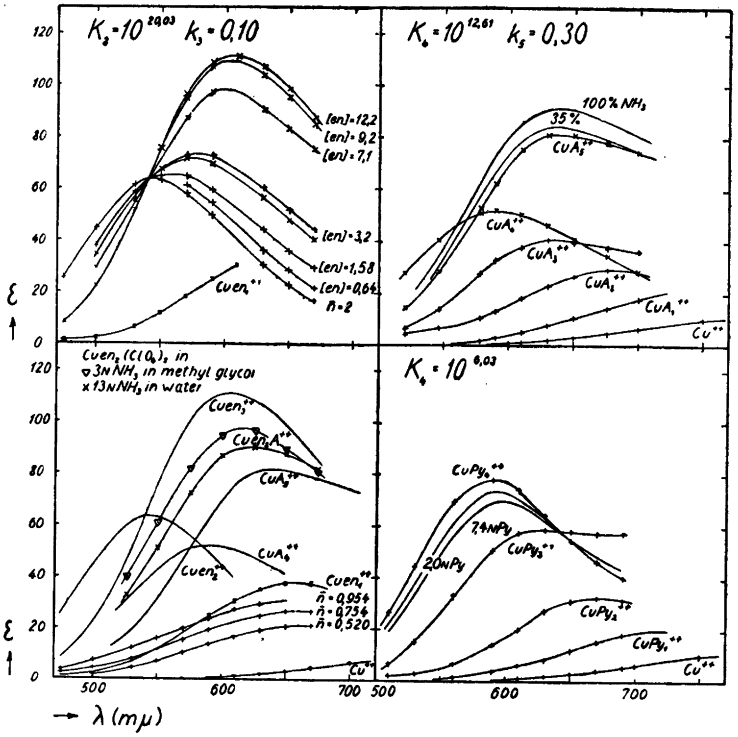


Fig. 2. Extinction curves for cupric complexes with ethylenediamine, ammonia and pyridine.

Upper left: Curves for cupric nitrate in 1 *N* KNO_3 (+ points) and without salt added (× points) at various concentrations of free ethylenediamine. The highest of the concentrations [en] = 12.2 corresponds very closely to ethylenediamine hydrate.

Lower left: Curves for 3 mixed solutions having $\bar{n} = 0.520$, 0.754 and 0.954 (+ points) as well as a curve for the mono-ethylenediamine complex determined with the aid of these curves. The last mentioned curve is partly drawn at the upper left, and the scattering of the calculated points is indicated by a special signature. The graph moreover contains curves for Cu en_2^{++} ($\bar{n} = 2$), Cu en_3^{++} ([en] = 12.2) as well as for $Cu(NH_3)_4^{++}$ and $Cu(NH_3)_5^{++}$ and the mixed complex Cu $en_2(NH_3)^{++}$. The curves for the latter ion are determined by graphical interpretation of Rosenblatt's measurements [8].

Upper right: J. Bjerrum's [6,7] curves for the cupric ammonia ions in 2 *N* NH_4NO_3 (+ points) and in ammonia solutions without added salt (× points) as well as for the cupric complex in 35 % and 100 % NH_3.

Lower right: Estimated values for the pyridino cupric complexes in 0.5 *N* $PyHNO_3$-pyridine solutions as well as the values for cupric nitrate in the same salt medium for [Py] = 2.0 and 7.4. The latter concentration corresponds to 590 g of pyridine per liter.

of the mono-ethylenediamine complex calculated therefrom by means of the relation

$$\varepsilon = \alpha_0 \varepsilon_{Cu^{++}} + \alpha_1 \varepsilon_{Cu\ en_1^{++}} + \alpha_2 \varepsilon_{Cu\ en_2^{++}}$$

and from our knowledge regarding $\varepsilon_{Cu^{++}}$, $_{Cu\ en_2^{++}}$ and the α-coefficients, are shown graphically in the lower left of Fig. 2. The calculation is carried out for each one of the mixed solutions measured, and the scattering of the points computed is marked by a special signature. The curve for the mono-ethylenediamine complex intersects the curves for the experimental mixtures, and the points computed for the pure complex are for this reason partly plotted in the upper left of the figure. Table 8 records the mean values found, together

Table 7. Distribution of the different complexes in 1 N KNO_3 at 25°.

$$\alpha_n = \frac{[Cu\ en_n^{++}]}{C_{Cu^{++}}}, \quad \bar{n} = \Sigma n \alpha_n$$

Calculated with $k_1 = 10^{10.72}$, $K_2 = 10^{20.03}$

No.	—log [en]	α_0	α_1	α_2	α_3	$\bar{n}$
3 (Table 4)	10.705	0.482	0.498	0.020	0	0.520
4 »	10.396	0.305	0.643	0.053	0	0.754
5 »	10.088	0.167	0.714	0.119	0	0.954
	9.00	0.006	0.327	0.667	0	1.661
	8.00	0	0.047	0.952	0	1.952
	2.00	0	0	0.999	0.001	2.001
	1.00	0	0	0.990	0.010	2.010

Table 8. Estimated molar extinction coefficients for the cupric ethylenediamine ions at 25° C.

Cu^{++} Extinction coefficients of the solution: $C_{Cu(NO_3)_2} = 0.09742$, $C_{HNO_3} = 0.00025$, $C_{KNO_3} = 1.10$.
$Cu\ en^{++}$ Calculation from the extinction coefficients of sols. no. 3, 4 and 5 in Table 4.
$Cu\ en_2^{++}$ Extinction coefficients of solution no. 2 in Table 5.
$Cu\ en_3^{++}$ Extinction coefficients of solution no. 13 in Table 5.

Wavelengths in mμ

	670	650	630	610	590	570	550	530	500	475
Cu^{++}	4.51	3.14	2.01	1.21	0.75	0.40	0.21	0.10	0.03	—
$Cu\ en^{++}$	37.5	37.5	35.0	30.6	24.7	18.0	11.7	6.4	2.3	1.4
$Cu\ en_2^{++}$	16.91	22.91	30.58	(39.0)	49.58	57.94	63.05	61.23	44.34	25.19
$Cu\ en_3^{++}$	87.38	98.31	107.0	(110.8)	108.6	96.09	75.37	51.77	22.32	8.65

with the directly determined extinction coefficients for the aquo-cupric ion and the di- and tri-ethylenediamine complex.

The absorption curves for the cupric-ammonia complexes have previously been determined (ref. 6, p. 52, *cf.* also ref. 7, p. 55), and the absorption curves for the cupric-pyridine complexes have recently been determined by J. Bjerrum in an investigation not yet published. The pyridine investigation was carried out in an aqueous 0.5 *N* $PyHNO_3$, and the consecutive formation constants for the tetra-pyridino complex were found, at 25°, to be:

$$k_1 = 10^{2.41},\ k_2 = 10^{1.88},\ k_3 = 10^{1.14},\ k_4 = 10^{0.60}$$

The absorption curves for the pure ammonia- and pyridine complexes are shown at the right in Fig. 2. It will be seen how the absorption maximum is displaced evenly towards blue from the aquo-cupric ion to the tetrammine complexes, at the same time as the magnitude of the absorption increases in the same sequence (*cf.* ref. 3, p. 196). The displacement towards blue per amino group is very nearly the same for the ammonia- and the pyridine complexes, but somewhat smaller than in the case of the mono- and di-ethylenediamine complexes, a circumstance which presumably is associated with the chelate binding of the diamine (*cf.* ref. 16). The special character of the pentammine- and tri-ethylenediamine complexes is reflected in the circumstance that the absorption maximum of these complexes, relatively to the respective tetrammine complexes, is shifted towards red. But it is seen that the tetrapyridine spectrum does not at all suffer a corresponding change even at very high pyridine concentrations. This is hardly due to the relatively small complexity of the tetrapyridino-cupric ion, but rather to steric reasons. In this connection it is worthy of note that hexapyridino complexes of the luteo type apparently cannot be prepared*, and one cannot therefore be sure that the existing molecular compounds with 6 molecules pyridine [17] have all their pyridine co-ordinated to the metal ion.

In a strongly ammoniacal solution of Cu $en_2(ClO_4)_2$ the copper is predominantly present as the mixed complex Cu $en_2(NH_3)^{++}$ [8]. In the lower left part of Fig. 2 the absorption curves for 2 such solutions are compared with the absorption curves for the pentammine- and tri-ethylenediamine complex. It will be noticed that the curves for the 3 complexes are located relative to one another in such a way that one must assume that the three complexes all have the same constitution. Hence the optical data are in every respect in support

* One of the authors (J. Bjerrum) has tried in vain to do so.

of the idea that the tri-ethylenediamine complex actually is a pentammine complex with the amino molecule last taken up bound only by one of its amino groups.

SUMMARY

The consecutive formation constants of the di-ethylenediamine cupric ion are determined with the aid of glass- as well as copper amalgam-electrode, and the 3rd consecutive complexity constant from extinction measurements made by means of a König-Martens spectrophotometer (*vide* Table 1).

The following is a survey of the normal-oxidation potentials established in connection with the electrometric measurements in 1 *N* KNO_3 and converted to the normal hydrogen electrode.

Electrode reaction	E°_H (18°)	E°_H (25°)
$Cu(amalg.) + 2\ en = Cu\ en_2^{++} + 2\ e^-$	— 0.2608	— 0.2624
$Cu(amalg.) + 2\ en = Cu\ en_2^{+} + e^-$		c. — 0.126
$Cu(amalg.) + 4\ H_2O = Cu(H_2O)_4^{++} + 2\ e^-$	+ 0.3320	+ 0.3311
$Cu(amalg.) + 2\ H_2O = Cu(H_2O)_2^{+} + e^-$		c. + 0.515

In connection with the optical measurements a determination was made of the extinction curves for the mono-, di- and tri-ethylenediamine cupric ion, and a comparison with the corresponding spectra of the cupric-ammonia- and the cupric-pyridine complexes. The material as a whole is in strong support of the idea that the tri-ethylenediamine complex is a pentammine complex with the ethylenediamine molecule last taken up bound by only one of its amino groups.

REFERENCES

1. Bjerrum, J., and Andersen, P. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **22** (1945) no. 7.
2. Carlson, G. A., Mc Reynolds, J. P., and Verhoek, F. H. *J. Am. Chem. Soc.* **67** (1945) 1333.
3. Bjerrum, J. *Metal ammine formation in aqueous solution*, Copenhagen (1941).
4. Job, P. *Ann. chim.* [10] **9** (1928) 190.
5. Werner, A., and Spruck, W. *Z. anorg. Chem.* **21** (1899) 201.
6. Bjerrum, J. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **11** (1932) no. 10.
7. Bjerrum, J. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **12** (1934) no. 15.
8. Rosenblatt, F. *Z. anorg. Chem.* **204** (1932) 351.
9. Bjerrum, J. *Sjätte nordiska kemistmötet*, Lund (1947) 112.
10. Randles, J. E. B. *J. Chem. Soc.* (1941) 802.

11. Bjerrum, N., and Unmack, A. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **9** (1929) no. 1.
12. Guggenheim, E. A. *J. Phys. Chem.* **34** (1930) 1758.
13. Katoh, N. *Z. physik. Chem.* **B 6** (1929) 27.
14. Biilmann, E., and Lund, H. *Ann. chim.* [9] **19** (1923) 137.
15. Clark, W. M. *The determination of hydrogen ions* 3rd. ed., London (1928) p. 672.
16. Linhard, M. *Z. Elektrochem.* **50** (1944) 224.
17. Pfeiffer, P. *Organische Molekülverbindungen* 2nd ed., Stuttgart (1927).

Received February 14, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 319—332

On Identification and Determination of Polycyclic Hydrocarbons by Ultraviolet Absorption and Chromatographic Adsorption

NILS HELLSTRÖM

Statens Provningsanstalt, Stockholm, Sweden

In a previous investigation of fir-stump tars and oils originating from them, a chromatographic method was worked out which, combined with treatment with solvents, made it possible to separate a mixture of pure hydrocarbons from these raw materials [1]. One of the greatest difficulties to overcome was to purify the neutral oils, which were obtained as the intermediate products, from a neutral material containing oxygen. The chromatographic separation was used as the only means by which the separation could be achieved. For this purpose the following adsorbents were employed: Frankonit KL, Tonsil ACA with hexane as a solvent. The purification was carried out by pouring a certain amount of the material dissolved in hexane through the column and then eluting with hexane. Several different fractions were taken out, and after the solvent had been driven off, the residual substances obtained were examined by means of ultraviolet spectra. It was thus found that a certain separation of hydrocarbon occured, and retene, dihydroretene and octahydroretene were likely to be present. In this experiment, however, the solutions used were rather concentrated and the number of fractions taken out was relatively low. Only a »continuous» curve for the amount of the eluated material versus the amount of used solvent was obtained. It was expected that experiments with more diluted solutions and improved methods of measurement would yield more information about the composition of the material from a qualitative as well as a quantitative point of view.

The earlier experiments undertaken by Drumond and co-workers [2] with marine oils showed that circumstances in chromatographic analysis may be very complicated, they may for instance, depend upon relative concentrations.

They showed, however, that more saturated hydrocarbons passed through more easily than less saturated ones. However, it proved to be possible to separate closely related hydrocarbons from one another [3]. Mair and Forziati [4] separated aromatics, benzene etc., from low boiling paraffins and naphthalenes, at about the same time as the present author carried out the above-mentioned investigations. It should also be mentioned that Willingham [5] succeeded in separating one higher cycloparaffin hydrocarbon from the corresponding aromatic compounds, as well as a paraffin from an aromatic hydrocarbon with about the same molecular weight as that of the paraffin. Further, Walker and Mills [6] found during their investigation of linseed oil that is was possible to separate the oils in fractions with 7, 6, 5 and 4 double bonds in the molecule, when hexane and aluminum oxide were used.

As our knowledge of the adsorption of hydrocarbons is far from perfect and information regarding the properties of various solvents is unsatisfactory, it was necessary for the planned investigation of the hydrocarbons contained in tars and oils to make some test experiments with authentic hydrocarbons and to try out different adsorbents. There are two possible types of adsorption analysis: frontal eluation and displacement analysis. An account is given below of experiments using frontal analysis.

The study was carried out in an apparatus constructed after Claeson's design [7] by *LKB-Produkter AB*, in Stockholm.

EXPERIMENTAL SECTION

Adsorbent, solvent and hydrocarbons investigated

The following adsorbents were used: Tonsil ACA obtained with the help of Dr Guy S:son Frey, Super Filtrol, activated clays (BW), Terrana D F, Hyflo Super Cel., obtained by Eng. Bertil Knutsson, Silikagel commercial from AB Eka. The solvent used for the experiments was heptane.

Hydrocarbons used for the test experiments were naphthalene pro analysi [8], and phenanthrene pro analysi [9], which have hitherto shown all signs of homogeneity. In another connection the present author has pointed out that the purification of retene from some impyrity * presents a hard task which can, however, be overcome by recrystallization combined with formation of pictrate or styfnate etc. [1] Only pure retene m. p. 100.5° was used. Dihydroretene was prepared according to Virtanen [10] and had m. p. 62—63° C.

* Add. to the corr. The presence of dihydroretene is lately denied by *K. J. Karrman* (*Svensk Kem. Tid.* **60** (1948) 18), but the reasons which led the present author to assume dihydroretene to be the »impurity» are only partly published [1] and further discussion will be given later.

Moreover a few fractions of intensely refined tar lubricating oils, were investigated.

The intensely refined tar lubricating oil formerly described by the name R L [1] was distilled in Klitt's apparatus [12], and the following fractions were obtained at 1.7 mm: up to 130°, 8 %; from 130—140°, 11 %; from 140—147', 5 %, and from 147—151°, 53 % yield. The latter fraction contained 89.5 % C and 10.8 % H and had n_D^{20} 1.5500. The residue partly crystallized after distillation.

The specific rotation of the original oil was found to be $[\alpha] = + 30.4°$ in benzene and + 31.8° in abs. alcohol. The corresponding values for the above mentioned main fraction were: 40.0 and 41.0° respectively.

In the same way as in the earlier experiments with rosin acids, the oil was isomerised with Pd-charcoal according to the method of Diels [14], for 3 h at 190°. After the catalyst had been removed by successive washing with ether and filtering, the main fraction was distilled at 138—148° and 0.3 mm pressure. The product obtained contained 89.2 % C, 10.7 % H and had n_D^{20} 1.5490 and D_4^{20} 0.9802. The ultraviolet spectrum of the original oil gives very little information. This is also true of the spectrum of the main fraction. The absorption curve of the latter is shown in Fig. 1. The shape of the curve indicates napththalene structure and that the concentration of the corresponding hydrocarbon may be 10—20 %[16]. Fig. 1 gives the curve of the isomerized oil fraction, which is not essentially different from the curve of the non-isomerized oil. The dien number of the oil was investigated in the same manner as was worked out and used on the crystallized rosin acids from fir-stump tar, but the percentage of dien content proved to be very low. According to one experiment at 130° less than 1 % is present. Evidently the treatment with Pd-charcoal does not have any particularly significant influence on the hydrocarbons.

The main fraction from the distillation of the original oil was sulphonated with conc. sulfuric acid at 70° C. By salting out, a dark-green sticky mass was obtained, which was then dried at high temperature. Extraction with ether resulted in a rather manageable product, containing 5.7 % Na. Next, the product was treated with acetone containing some water and the undissolved part was crystallized from acetone-water solution. The crystals were then washed with ether and dried in the air. Two different crystallized fractions were then worked up, dissolved in 50 % alcohol and their respective ultraviolet spectra determined. As it is evident from Fig. 1, the benzene structure is present. The above results indicate that the hydrocarbons possess naphthalene as well as benzene structure. On the other hand, the diene, styrene an phenanthrene structures are missing, at least to a measureable extent, so as

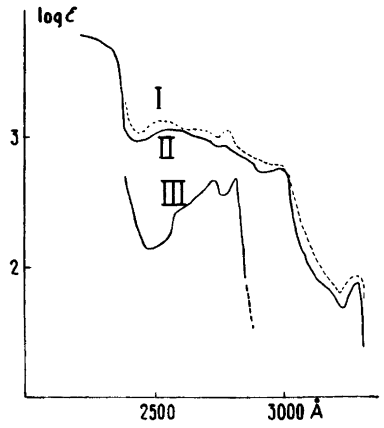


Fig. 1. Ultraviolet absorption of oil before (I) and after isomerization (II) and the sulphonate with benzene structure (III).

their corresponding and very characteristic maxima and typical curve shape are not seen in the absorption curves obtained [15, 16]. The elementary analyses of the hydrocarbon most closely approximates the formulas $C_{18}H_{26}$- or $C_{19}H_{28}$ and, presumably, this hydrocarbon dominates and has benzene structure.

The above mentioned sulphonated salt with benzene structure is not identical with a sulphonated salt with benzene structure from a hydrogenated retene. For instance the corresponding *p*-toluidine salts have been prepared. They have approximately the same melting points: 225—228° and 223—225° respectively, their mixture, however, has a melting point. 15—20° lower. Since the C_{18} hydrocarbon is present in the hydrogenated retene, then the C_{19} hydrocarbon ought to be present in the latter case. The sodium sulphonate was, however, practically inactive and the yield was rather poor.

It has previously [1] been pointed out, that the partially refined lubricating oil contains one or more oxygen compounds which cannot be removed by distillation. It was also stated that they could be removed together with other contaminitants by intense refining, using a special process, *e. g.*, by treatment with stronger H_2SO_4. Thus, it is highly probable that on treating the tar lubricating oil with H_2SO_4, not only the oxygen compounds but also a part of the hydrocarbons are sulphonated. Consequently the working up of the oil involves difficulties and the yields obtained are poor.

Experiments with clays etc.

As an example, an account is given below of some experiments in which a clay, Tonsil A. C. A., was used as adsorbent and heptane as solvent. Frontal analyses were performed using the fraction discussed above. When, for

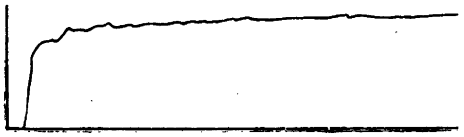


Fig. 2. Frontal analysis of main fraction of intensely refined oil.

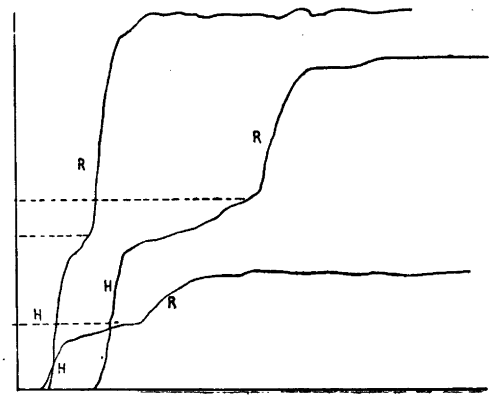


Fig. 3. Frontal analysis of main fraction of intensely refined oil and retene. H head front of oil, R retene step, dotted lines give the calculated values for the oil alone.

instance, 0.60 % solution and filter 1.250 π^7 were used a curve was obtained which is shown in Fig. 2. This curve, as well as a number of others not shown here, indicates that the front is well developed and that the first rise of refractive index is followed by another step. This indicates the presence of smaller amounts of hydrocarbons, being much more strongly adsorbed than those hydrocarbons indicated by the head step. As it was suspected that this hydrocarbon would be replaced by retene, an experiment was undertaken with the fraction of the tar lubricating oil and retene in the same solution. Fig. 3 shows three experiments with various concentrations of tar lubricating oil and filter sizes and of retene. The diagram shows that between the head-front (H) and the retene-front (R) there is a plateau which is flatter at low concentrations of retene. To learn to what extent the separation really occurs between retene and the hydrocarbons of the tar oil, an experiment with this fraction was carried out as follows. The solution 0.765 % of oil and 0.317 % of retene was passed through the apparatus and changes of refractive index were read off direct while the liquid leaving the apparatus was taken out in small fractions and then weighed. From this data a curve was drawn and the fractions were combined as shown in Fig. 4.

The solvent was removed from these fractions over a boiling water bath, and suitable amounts of residues were dissolved in absolute alcohol for examination of their ultraviolet spectra. The results obtained are shown in Fig. 5. The spectra of the hydrocarbons which are represented in Fig. 4, in fractions 1—3, are rather similar and all of them have the typical benzene structure showing double maxima at 2.700—2.800 Å. The hydrocarbons represented by the fourth section in Fig. 4, gave an absorption curve which shows the typical

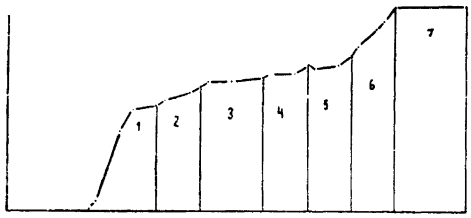


Fig. 4. Frontal analysis of main fraction of intensely refined oil and retene performed to give fractions 1—7.

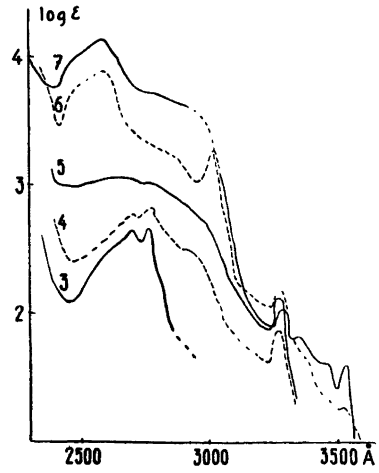


Fig. 5. Ultraviolet absorption of fractions 3—7 from frontal analysis, fig. 4.

naphthalene structure maximum at 3.250 Å. Only the hydrocarbons in fraction 6, show the typical retene maximum at 2.600 Å. However the maximum at 3,000 Å [1] is only slightly apparent. This indicates that sections 1—3 contain hydrocarbons with benzene structure, 4 contains benzene and naphthalene structures, 5 contains these plus an insignificant amount of compounds with phenanthrene structure, and 6 and 7 contain these plus compounds with phenanthrene structure (retene). It suggests that the hydrocarbons are adsorbed in the following order: 3<5<6 F. Unfortunately, the separation was not so sharp as was desired. This can be due partly to the presence of smaller amounts of some compounds whose adsorption isotherms are such that a sharper separation is impossible, or because the number of isomerides and homologues is too large, or both. An attempt to obtain more distinct steps in adsorbtion curves by changing the absolute and relative concentrations unfortunately failed.

A number of active clays were tried as adsorbent for the tar lubricating oil. In these experiments, however, the separations obtained were not sharper than the ones described above. The results of some of the experiments, performed in different ways, are collected in Table 1.

In this case the final step height was adjusted to $n = 100$, and concentration 1 %. The table indicates that the oil fraction in question gives the maximal step height about 11.1—11.4 units. The lower values for step heights are caused by the use of more adsorbent than could be saturated with the oil present in the 50 ml of solution pressed through the filter.

Table 1. Frontal analysis of tar oil and different adsorbents.

Adsorbent	Filter π	Conc. %	Corr. step-height cm
Tonsil ACA, non-dried	1250	0.82	11.1
» » dried 105°	250	1.47, 0.58	11.3, 11.4
Frankonit KL	500	0.72, 0.75	10.9, 10.5
Hyflo	1250	0.79	11.4
Terrana	1250	0.78	11.2
Super Filtrol	1250	0.76	10.4
» »	2500	0.75	8.7
Silikagel, EKA	500	0.84	11.2
» »	1250	0.84	10.8

The retention volumes in these cases are so small that they cannot be used accurately for calculating constants etc.

It has been assumed above that retene displaces the hydrocarbons in the oil, and that all the hydrocarbons should be present in the steps preceding the retene step. In a series of experiments, shown in Table 2, it was found

Table 2. Frontal analysis of tar oil and retene.

Fraction of tar lubricating oil %	Retene %	Maximum step-height Found cm	Calc. cm
0.795	0.402	16.0	15.8
0.760	0.750	20.5	21.4
0.765	0.312	14.1	14.1
0.870	0.525	19.6	18.9
0.395	0.208	8.6	8.0
0.770	0.320	13.2	14.2
0.785	0.323	14.3	14.4
1.180	0.284	12.4	18.2
0.800	0.324	8.3	15.1

possible to calculate the final step height, if the concentrations of oil and retene were known.

For the calculations the factors 11.3 for the oil and 17.0 for retene have been used. The latter was obtained in the experiment with retene only. Agreement is quite satisfactory.

It is also possible to estimate from such a curve the amount of oil used, as it is evident from the experiments shown in Fig. 3, where the dotted lines indicate the concentrations of oil in the test solutions used. It is noteworthy

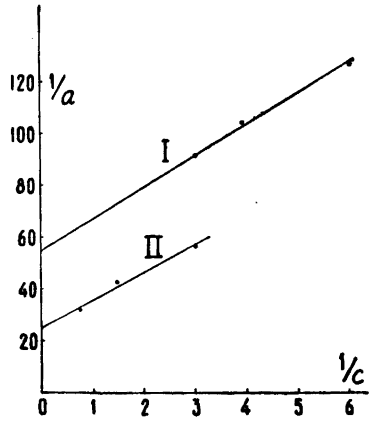


Fig. 6. Adsorption isotherms for phenantrene (I) and retene (II).

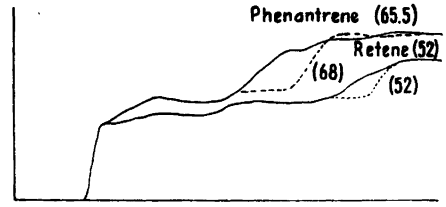


Fig. 7. Frontal analysis (sensitivity given in brackets) of phenantrene and retene (dotted lines) and these hydrocarbons and tar oil.

that the step height for the oil is almost independent of the ratio of oil to retene.

As it was of interest to try out phenanthrene in a mixture with the tar lubricating oil, test experiments with retene and phenanthrene were first carried out. Super Filtrol, which had previously been heated in small portions (50 g) to 200°, was used as adsorbent. Comparative test experiments with non-dried Super Filtrol gave about the same results in frontal analysis; for displacement development with certain reagents, it is necessary to use dried Super Filtrol. Thus, for better comparison in the future, the use of dried Super Filtrol has been adopted throughout this investigation. The results are collected in Fig. 6. From these experiments also the value 17.0 has been obtained for the retene step height, calculated for sensitivity $n = 100$ and concentration 1 %.

From Fig. 6 it is evident that the isotherms for retene and phenanthrene are nearly parallel. For frontal analysis with two components the following relationship holds [7]:

$$C_{1,2} - \left(1 - \frac{v_1^0}{v_2^0}\right) C_{1,1} = \frac{k_1}{k_2} \cdot C_{1,2}$$

When $k_1 = k_2$ then $v_1^0 = v_2^0$. That is, retention volumes in frontal analyses are alike, if reversible adsorption occurs between phenenthrene and retene, the separation is not obtained. In the experiment with a mixture of phenanthrene and retene, only one pronounced step was observed in the adsorption curve.

It appeared that dihydroretene and retene are not separated in frontal analysis and therefore the calculated retene may include dihydroretene.

The behavior of a phenanthrene and tar oil mixture is a little different from retene, as is evident from the experiment shown in Fig. 7. It shows that — as was expected — the phenanthrene step lies ahead of the retene step. The figure shows, however, that the step heights of the benzene compounds, are practically the same. It may also be noted that the retene step in the curve from the experiment with the mixture of retene and tar oil, has a retention volume which does not differ much from the one given by retene alone. In this figure the curve for retene alone (the dotted line), was shifted along the concentration axis so that the maximum values of the curves coincide.

From Fig. 8, it is evident that the phenanthrene step comes at definitely smaller retention volume than that for phenanthrene alone. Moreover, the step denoting hydrocarbons of naphthalene structure is missing. Hence, one can conclude that »naphthalenes» do not separate from phenanthrene. It might be possible to determine the hydrocarbons with benzene structure by adding phenanthrene, but it seems to be impossible to aquire any information about the concentration of »naphthalene» in this way.

It is of interest to note that the step heights for the hydrocarbons with benzene and naphthalene structures, respectively, within the limits of experimental error, are unchanged in the presence of retene or phenanthrene (this was shown by numerous experiments in which the relative as well as absolute concentrations were varied, but they can only be mentioned in the present report).

These experiments show clearly that it may be possible to obtain a picture of the quantitative composition of the tar oil by adding the proper amounts of retene or phenanthrene and performing frontal analysis.

Experiments with various tar lubricating oils

The previously described preparation of hydrocarbon (neutral oil fractions [1]) containing oxygen, has been used for the following experiments. It has been found that the oxygen containing contamitants are completely adsorbed on dried Super Filtrol. Some of the neutral oil fractions originated from refined tar lubricating oils and some from unrefined ones. The experiments have been carried out as follows. At first the frontal analysis adsorption curves were determined for the various neutral oils, and then the curves were repeated with admixture of suitable amounts of retene or phenanthrene. The concentration of neutral oil was 0.79 % in all experiments. The results are given in Fig. 8 for oil DL. That added retene is present to a concentration of 16 %, is

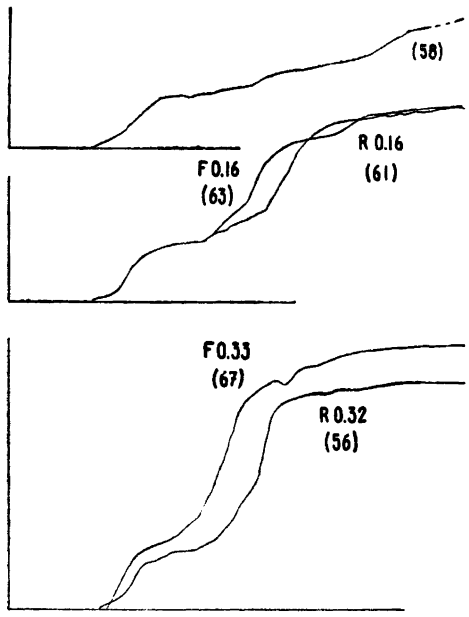


Fig. 8. Frontal analysis (sensitivity given in brackets) of a tar oil, DL, a) alone b) with phenantrene, resp. retene in 0.16 % solution and c) the same but in 0.32 % solution.

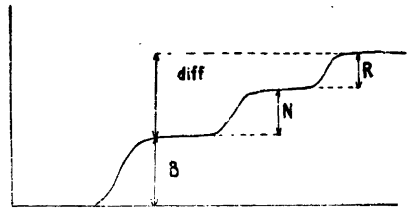


Fig. 9. Principal diagram for frontal analysis. B denotes hydrocarbons with benzene structure N hydrocarbons with naphtalene structure and R retene.

denoted by the number 0.16 and that the sensitivity in the experiment was 61 by (61) etc. The results of the experiment with phenanthrene (F) are given in a similar way.

All of the curves from experiments with the oils possess two more pronounced steps and, as a rule, in low retene concentrations, it is possible to estimate retene step height (see Fig. 7, the dotted lines). The curves may be used for quantitative calculation in the following way. The height B in Fig. 9 corresponds to the compound with benzene structure, the step height N to the compound with naphthalene structure, and the step height R to retene. To calculate concentrations of the hydrocarbons from the step heights one must know the refractive index of a standard solution of each. To learn how variations of the values for the factors affect the final results the following values for the factors have been used: benzene-hydrocarbon, 10.0 and 11.0, naphthalene-hydrocarbon and retene, 17.0 and 18.5. From the step heights and these factors, the concentrations of hydrocarbons with benzene structure, naphthalene structure, and retene, have been calculated. Their sum is considerably lower than the weighed quantities of tar oil. The difference F. between these values is due to the presence of impurities. From the table, showing the results of all these calculations, it is seen that the variations in F,

Table 3. Compositions of tar oil according to frontal analysis.

	% B[1])	% R[2]/	% N[3])	% F[4]
RL	75,68	13,14	9,9	(— 4),8
			8,10	3,1
RO	37,34	22,24	19,20	15,24
			17,20	18,22
RN	38,36	33,36	11,14	10,20
			9,16	14,18
DL	47,43	18,19	20,23	11,19
			19,24	14,15
DO	39,36	19,20	19,20	18,24
			18,22	23,24
DN	33,30	32,34	13,20	10,19
			17,23	13,18
T	24,23	15,18	14,14	38,44
			11,16	39,42

[1] Calculated from stepheight B
[2] R
[3] Diff — R
[4] 100 — B — Diff.

depending upon different numerical values for factors, are not great. Thus, it may be concluded that it is possible to obtain in this way a fairly accurate picture of the quantitative composition of the oils. Some information concerning the content of impurities in some neutral oils has been previously gained by direct quantitative analyses of larger samples. The comparison of these values and the values obtained by adsorption analysis are shown in the following table.

Table 4. Impurities in tar oil according to frontal analysis.

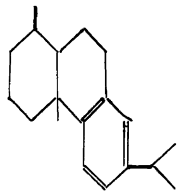
Oil	Previously determined	Present values
DO	28 %	18, 24, 23, 24 %
DN	20 »	10, 19, 13, 18 »
T	41 »	38, 44, 39, 42 »

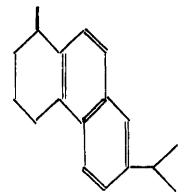
The agreement is remarkably good, particularly when the systematic errors appear in the values for precentage of impurities. It may be possible to regard these results as accurate to at least to 4—5 units.

SUMMARY AND DISCUSSION

Measurement of hydrocarbons in neutral oil fractions of tar lubricating oils has been studied by means of chromatographic adsorption and ultraviolet absorption. Several clays have been employed as adsorbent and heptane as solvent. The adsorption studies have been performed in Claesson's apparatus [7], and frontal analysis has been used. The results have been checked by control experiments in which phenanthrene and retene have been added to the samples. Ultraviolet absorption spectra of small fractions of the eluate have been measured. From the absorption curves it is concluded that components of the oils possess benzene, naphthalene and phenanthrene structures. Structures with double bonds, as in the case of abietic acid, styrene etc., were not detected. Their presence is not excluded, but is very doubtful whether such double bonds occur, as they should be easily disproportioned into the just mentioned structures under the conditions which predominate in the oils production.

The hydrocarbons contained in these tar lubricating oils originate from resin acids. Hence, the following hydrocarbons seem to be present: dehydroabietene and tetrahydroretene



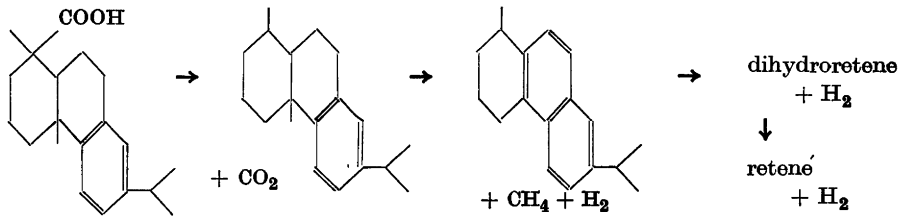


and further retene.

For calculating concentrations of these hydrocarbons the retention volumes could not be used, partly because the adsorption affinity of the hydrocarbon with benzene structure was too weak, when compared with retene adsorption, and partly because the particular step front characterizing the hydrocarbon with naphthalene structure was indistinct. On the other hand, the step heights might be used for calculating concentrations, as it is shown above. With certain reservations, the composition of the neutral oils investigated are given in Table 3.

From the latter it is evident that the intensely refined oil RL, as was to be expected, contains the smallest amounts of impurities (oxygen compounds) and relatively low quantities of retene (dihydroretene) and hydrocarbons with naphthalene structure. On the other hand it is clearly shown that the hard-cracked oils DN and RN respectively, contain great amounts of retene. It is also noteworthy that the content of hydrocarbons with naphthalene structure is not considerably large. This seems to imply that this stage of cracking has passed relatively easily.

It has been stated out earlier that dehydroabietic acid his been the main constituent of the original resin acids, and therefore the following scheme of cracking seems to be most probable:



It is also illuminating that the quantities of impurities in Table 4 are relatively small in these hard-cracked oils. For the significance of the intensity of refining the reader is referred to the paper by Frey.[17]

Frey has determined the retene content of oils not identical with but similar to those investigated here. He got definitly smaller amounts of retene, but further discussion is better postponed until the nature of the »impurity» following retene is elucidated.

The author wishes to acknowledge grants from *Statens Tekniska Forskningsråd* for the purchase of the instruments and from the Lubricating Oil Committe of the Royal Swedish Academy of Engineering Sciences to support this investigation. Mr. Haldor Ottar, Miss Blenda Carlsson and Mr. Henryk Borgiel were of helpful assistance during the investigations which were carried out at *Kungl. Tekniska Högskolan.*

REFERENCES

1. Hellström, N. *Trans. Roy. Inst. Technol. Sweden* (1948) no. 21.
2. Drummund, J. C., *et al. Biochem. J.* **29** (1935) 456; *Analyst* **60** (1935) 23, 382.
3. Strain, H. *Chromatographic adsorption analysis* (1945); *Analyst* **71** (1946) 251.
4. Mair, B. J., and Forziati, A. F. *Nat. Bur. Standards Research J.* **32** (1944) 165; *Cf.* Mair, B. J., and White, J. D. *Ibid.* **15** (1935) 51; Mair, B. J. *Ibid.* **34** (1945) 435.
5. Willingham, Ch. B. *Ibid.* **22** (1939(321.
6. Walker, F. T., and Mills, M. R. *J. Soc. Chem. Ind.* **66** (1942) 125, **62** (1943) 106.

7. Claesson, S. *Dissertation* Uppsala (1946).
8. »Bakers Analyzed».
9. Merck, Darmstadt.
10. Virtanen, A. I. *Ber.* **53** (1920) 1885.
11. Morton, R. A., and de Goureia, A. J. A. *J. Chem. Soc. London* (1934) 911.
12. Schroeter, G. *Ber.* **57** (1924) 2025.
13. Klit, A. *Studier over fractioneret Destillation* Uppsala (1943).
14. Diels, O. *Ber.* **58** (1925) 1231.
15. *Cf. e. g.* Morton, R. A., and de Goureia, A. J. A. (*l. c.*).
16. *Cf. e. g.* Morton, R. A., and de Goureia, A. J. A. (*l. c.*), Mohler, H. *Das Absorptionsspektrum der chemischen Bindung* (1943).
17. Frey, G. S:son *Tek. Tid.* (1945).

Received February 20, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 333—342

The Light Absorption of Sheep Hemoglobin and Myoglobin Compounds in the Soret region

A Spectrophotometric Method for the Simultaneous Determination of Hemoglobin and Myoglobin

MARGIT BEZNÁK

The Biochemical Department of the Medical Nobel Institute, Stockholm, Sweden

Myoglobin was first crystallized by Theorell[1] in 1932; its possible physiological function was discussed by Millikan[2] (1939). Its quantitative estimation has been attempted several times. The latest of these is the detailed study of de Duve[3] (1948), who used the differences in the light absorption of the CO compounds of hemoglobin and myoglobin at three well-chosen wave-lengths (575.7 mμ = an isobestic point; 568 and 583.4 mμ where the CO-myoglobin has equal extinction coefficients, while those of the CO-hemoglobin differ widely from each other) for estimating the relative amount of the two compounds in the same solution. For a detailed discussion of the literature see de Duve's article [3].

The present work was made on myoglobin prepared from sheeps' hearts, at the suggestion of Professor H. Theorell. Sheep were selected as experimental animals in order to determine the myoglobin content in normal hearts and during experimental cardiac hypertrophy.

In the present investigation, the absorption of different sheep hemoglobin and myoglobin preparations is studied in the Soret region (between 400—450 mμ) and a method for the possible estimation of hemoglobin and myoglobin in the same solution suggested on the basis of the differences observed.

Fig. 1. The light absorption curves of myoglobin from sheeps' hearts precipitated with different ammonium sulphate saturations. All precipitates are redissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 174 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

○——○ *75— 85 % saturation.*
●——● *85—100 » »*
×——× *85— 90 » »*
△——△ *90—100 » »*

METHODS

Preparation of myoglobin

Sheep myoglobin was prepared as described by Theorell [1, 4]. It was possible to obtain it only in amorphous form, though the pure amorphous preparation contained a small quantity of very irregular crystals. It was found that a 75 % ammonium sulphate saturation always removed all the hemoglobin present in the heart extracts and a further fractionation proved the remaining material to be spectroscopically homogeneous. (This was the case even in those preparations where the hearts were not washed free of blood.) Fig. 1 shows the light absorption curves of a heart extract with which most of the absorption curves in the Soret region were carried out. It is to be seen that, in equal concentrations referred to hematin, the precipitates from 75 % ammonium sulphate saturation onwards give identical absorption curves, proving the homogeneity of the material. (All precipitates were redissolved in *M*/100 phosphate buffer, pH 7.)

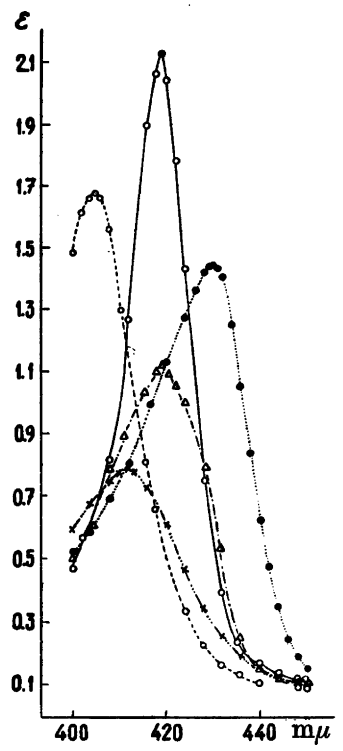


Fig. 2. The light absorption curves of sheep hemoglobin compounds in the Soret region. Hemoglobin is dissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 10.8 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

○— — —○ *Met-compound*
×—···—× *Oxy-* »
△—·—·—△ *CN-* »
○————○ *CO-compound*
●·····● *Reduced compound*

Preparation of hemoglobin

This was done by washing the centrifuged corpuscles of sheep blood (citrated blood) three times with Ringer solution, laking them with distilled water and centrifuging off the cellular residue.

Preparation of the different compounds

All dilutions were made with *M*/100 phosphate buffer, pH 7.

Reduced compounds. Solid $Na_2S_2O_4$ was added to a suitably diluted hemoglobin or myoglobin solution.

Oxy-compounds. Oxygen from a bomb was bubbled through the reduced solution for different times.

CO-compounds. Pure, washed CO was bubbled through a suitably diluted solution for about 5 minutes, solid $Na_2S_2O_4$ added while the bubbling was continued for another 5 minutes. The cuvettes, on the bottom of which a very small amount of $Na_2S_2O_4$ was placed, were filled and the readings taken immediately.

Met-compounds. One drop (about 0.04 ml) of a 0.5 % $K_3Fe(CN)_6$ solution was added to 3 ml of the suitably diluted solutions.

Cyanide-compounds. To the above solutions of the met-compounds, 0.3 ml 0.1 % KCN solution was added.

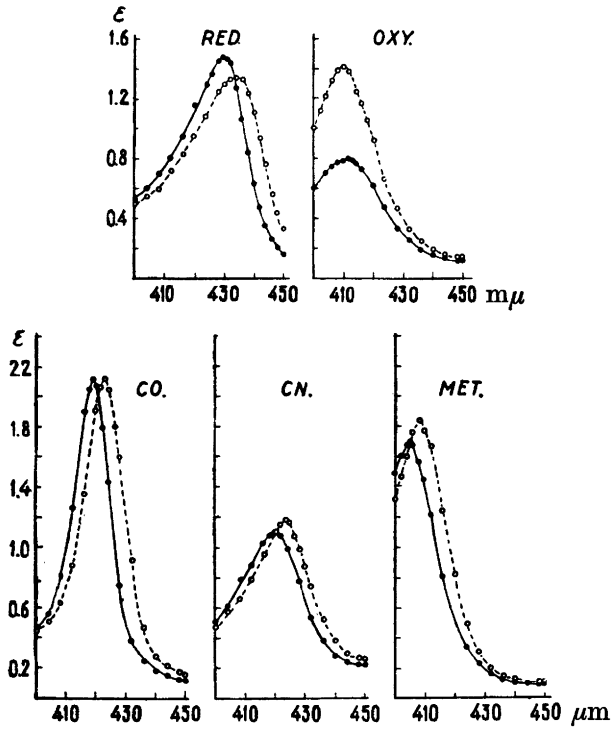


Fig. 3. The light absorption curves of sheep hemoglobin and myoglobin in the Soret region. Both hemoglobin and myoglobin are dissolved in M/100 phosphate buffer, pH 7. The data plotted above were obtained on solutions containing 10.8 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

●——● *Hemoglobin* ○- - -○ *Myoglobin*

Pyridine-hemochromogen. To 3 ml of a suitably diluted solution 0.3 ml *N*.NaOH was added, then 1 ml pyridine. After mixing, solid $Na_2S_2O_4$ was added in excess, and readings taken immediately. All concentrations were calculated on the basis that a millimolar solution of pyridine protohemochromogen gives an extinction coefficient of 31.0 at 557 mμ.

Apparatus

The Beckmann spectrophotometer was used. The width of the cuvette, *d*, was 1 cm in all cases.

EXPERIMENTAL RESULTS AND DISCUSSION

Fig. 2 shows the light absorption curves of sheep hemoglobin compounds in the Soret region. It is to be noted that not only the wave length where each compound has its absorption maximum is characteristic and different

from the other, but there is also a marked difference in the value of the extinction coefficients. The CO-compound has a much higher peak than any other compound, *e. g.* cyan-ferri-hemoglobin which has its maximum at the same wave-length, shows only half the absorption of the CO-compound. A very striking difference is found between the reduced and oxy compounds. Not only is the peak of the oxy compound shifted to lower wave-lengths, but also its extinction coefficient is about half that of the reduced compound.

Having thus established the differences between the sheep hemoglobin compounds, it seemed interesting to investigate the absorption of similar myoglobin compounds. Fig. 3 shows the absorption curves of sheep hemoglobin and myoglobin in equal concentrations, with regard to hematin. It is seen that the absorption peak of myoglobin is in all cases shifted by a few mμ towards longer wave-lengths as compared to those of the respective hemoglobin compounds. The value of the extinction coefficients is equal in all hemoglobin and myoglobin compounds, with the one exception of the oxy compounds. In this case the light absorption of the oxy-myoglobin compound is about twice as high as that of oxy-hemoglobin, or, more correctly, the bubbling through of oxygen does not decrease the extinction coefficient of the reduced compound in the case of myoglobin, but strongly diminishes it (to about half its value) in the case of hemoglobin. These results are summarized in Table 1.

Table 1. The absorption maxima and the extinction coefficients of sheep hemoglobin and myoglobin compounds.

	Hemoglobin		Myoglobin		Hemoglobin		Myoglobin	
	No.	mμ	No.	mμ	No.	$\varepsilon = \log \frac{I_0}{I} . 10^3$	No.	$\varepsilon = \log \frac{I_0}{I} . 10^3$
Reduced	24	430	9	434	6	1423 $\pm$ 20	6	1323 $\pm$ 37
CO	7	419	7	423	6	2175 $\pm$ 55	6	2172 $\pm$ 54
CN	3	420	2	423	2	1126 $\pm$ 39	2	1204 $\pm$ 43
Oxy	28	412	15	410	7	778 $\pm$ 19	7	1403 $\pm$ 35
Met	2	405	2	408	2	1690 $\pm$ 4	2	1867 $\pm$ 62

All data are calculated for 10.8 μM/*l* hematin.
No. = number of determinations.

The most striking difference between hemoglobin and myoglobin, therefore, was the difference in the value of the extinction coefficients of the oxy compounds. It was found in 20 experiments that the extinction coefficient on the top of the Soret bands of the oxy-hemoglobin compound was 53 $\pm$

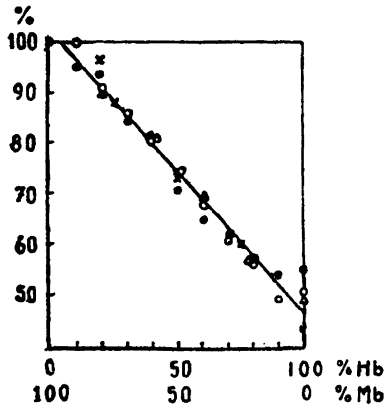


Fig. 4. Abscissa: The percentual hemoglobin and myoglobin content of a solution.

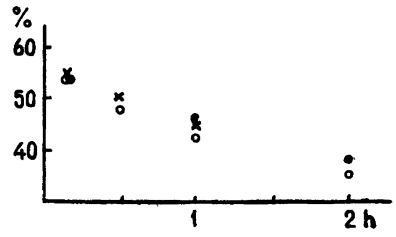
Ordinate: $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}} \times 100.$

Dilution with M/100 phosphate buffer, pH 7.

1.9 % of the reduced compound, whereas the absorption of the oxy-myoglobin was 105 $\pm$ 1.1 % of the reduced myoglobin in 16 experiments. Thus it seemed possible to estimate the amount of myoglobin and hemoglobin present in a given solution by determining the absorption maxima of its reduced and oxidised forms, thus finding the percentual decrease in the absorption of the oxy compound. Evidently, the more hemoglobin the solution contained, the greater would be this decrease and vice versa. Solutions of pure sheep hemoglobin and myoglobin were, therefore, prepared, suitably diluted with *M* 100 phosphate buffer, pH 7, and mixed in different proportions. The absorption of each mixture was determined in the reduced form, and again after bubbling through oxygen for 15 minutes. The decrease in the height of the absorption was calculated and the amount of hemoglobin and myoglobin present in that particular mixture established by the pyridine hemochromogen method. The results thus obtained are represented in Fig. 4, where the abscissa shows the percentual proportion of hemoglobin and myoglobin, the ordinate the percentual decrease in absorption of the oxy-form as compared to that of the reduced form, of the mixture in question. The different signs show estimations with different solutions. It is seen from this figure that a straight line is thus obtained. For example, if the extinction coefficient of a suitably diluted mixture after oxygenation is 80 % of that of the reduced form, the mixture will contain 40 % hemoglobin and 60 % myoglobin.

A few experiments were carried out to see how the time of oxygenation and the concentration of the solution would influence the absorption of the oxy compound. It was found that in the case of myoglobin neither its concentration, nor the time of oxygenation had any marked influence on the absorption of the oxy-compound, this being in all cases 104—106 % of that

Fig. 5. The influence of the time of oxygenation on the value $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}} \times 100$ *of sheep hemoglobin.*
Abscissa: Time of oxygenation.
Ordinate: $\frac{\varepsilon \text{ oxy compound}}{\varepsilon \text{ reduced compound}}$



of the reduced form. In the case of hemoglobin, however, the absorption of the oxy compound decreased with the time of the oxygenation (Fig. 5). Also the concentration of the hemoglobin seemed to have some influence, the absorption of the oxy compound showing a tendency to decrease more slowly in the case of more concentrated solutions. This, however, was more irregular than the influence of time. In view of these findings, the above factors were carefully standardised. The oxygen was allowed to bubble through the solution at such a rate that the individual bubbles were just countable and yet did not flow together in a stream. The time of oxygenation was exactly 15 minutes in all cases. The dilution of the solutions was selected in such a way that the maximal absorption of the reduced compound should give an extinction value of about 1.2—1.5. The data shown in Fig. 4 were obtained by using all these precautions.

A few experiments were carried out in order to estimate the myoglobin and hemoglobin contents of heart extracts. The heart muscle, after dissecting it free of connective tissue and fat, and after washing it with Ringer solution in 2 of the experiments, was homogenised in a Turmix blendor with about the same amount of distilled water, and left in the ice-chest overnight. 0.25

Table 2. The percentual proportion of hemoglobin and myoglobin in sheep heart extracts.

Extract	Hemoglobin				Myoglobin			
1*	16,	14			84,	86		
2**	26,	30,	30,	24	74,	70,	70,	76
3	37,	40			63,	60		
4	38,	42			62,	58		
5	38,	42,	38,	38	62,	58,	62,	62
6	38,	32,	34,	30	62,	68,	66,	70

* Heart washed thoroughly free of blood with Ringer through the aorta.

** Heart washed incompletely free of blood.

3—6 Unwashed heart muscle.

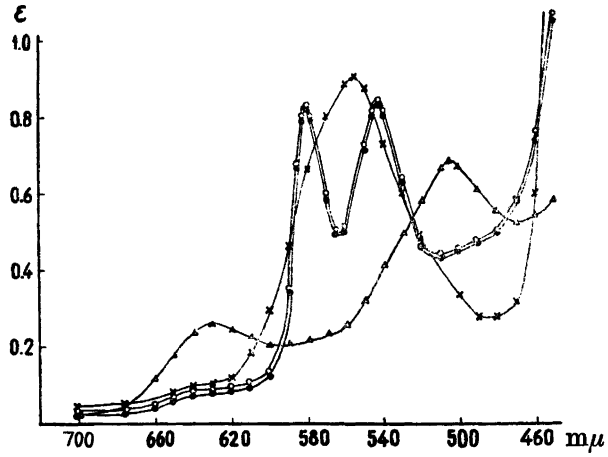


Fig. 6. The light absorption curve of met-, oxy-, and reduced sheep myoglobin. All dilutions are made with M/100 phosphate buffer, pH 7. The data plotted were obtained on solutions containing 67 μM/l hematin; $\varepsilon = \log \frac{I_0}{I}$.

△——△ *The original met myoglobin solution*
×——× *After reduction with solid* $Na_2S_2O_4$
○——○ *After oxygenation for 10 minutes*
●——● *After oxygenation for 60 minutes*

volume % of a 20 % lead acetate solution was then added, the precipitate centrifuged off and the excess lead removed by the addition of solid Na_2HPO_4. (The pH was controlled during all these operations as described by Theorell [1].) A small quantity of these extracts was suitably diluted with *M*/100 phosphate buffer, pH 7. Solid $Na_2S_2O_4$ was added and the extinction of the reduced form determined in a part of it, while oxygen was bubbled through the other part for 15 minutes, then its extinction read. The maximal extinction of the oxy-compound, expressed in percent of that of the reduced compound, was then calculated, and the corresponding percentual hemoglobin content read from the line in Fig. 4. These values are found in Table 2. The parallel determinations mean parallels made on the same extracts. There was no attempt to make determinations on the same heart muscle, extracted differently. As no care was taken to extract the myoglobin quantitatively, the data have not been re-calculated in gs. Thus it is not stated f. i. how many gs of myoglobin 1 kg of wet or dry heart muscle contained, only what percentage of the total hemin content of a particular extract was found in the form of myoglobin and hemoglobin respectively.

In order to check the purity of the oxy-compounds, the absorption curve was determined in the visible region. The myoglobin as prepared from sheep heart, was a met-compound; after reduction and oxygenation the oxy-compound was formed, which was, however, probably not quite free from the met-form. Fig. 6 illustrates these results. It is seen from this figure that a small elevation is present around 630 mμ, characteristic of the met-compound. As no such elevation was seen in the oxy-hemoglobin, the method here described warrants certain precautions. It is impossible to state definitely at present what part of the phenomenon — *i. e.* that the absorption of oxy-myoglobin is unchanged as compared to that of the reduced form — is caused by the small contamination by the met-form not present in hemoglobin. As, however, the contamination with the met-form must — as seen from Fig. 6 — be very small, and as extracted myoglobin used by other authors, was probably never entirely free from it, we feel justified in calling attention to the very big differences found in the Soret bands of oxy-myoglobin and oxy-hemoglobin. Further work is, however, needed to ascertain the general validity of the method here described.

SUMMARY

1. The absorption of sheep myoglobin and hemoglobin in the Soret region is determined.

2. The absorption maxima of the CO, reduced, CN, and Met compounds of myoglobin are shifted with 2—4 mμ to longer wave-lengths, as compared with those of hemoglobin.

3. The absorption maximum of oxy-hemoglobin is about half of that of oxy-myoglobin, reduced myoglobin and reduced hemoglobin.

4. The difference between the extinction coefficients of oxy-myoglobin and oxy-hemoglobin, as compared to that of their respective reduced forms, is suggested as a possible method for estimating simultaneously hemoglobin and myoglobin in the same solution.

5. Some possible sources of error in this estimation are discussed.

6. A few determinations in sheep heart extracts (washed and unwashed) are described.

Grateful acknowledgement is made to Professor H. Theorell, for his hospitality and for suggesting the present investigation. My heartiest thanks are also due to Dr. R. K. Bonnichsen, for his constant interest and help during the course of this work.

My deepest gratitude is offered to the Swedish Institute *(Svenska Institutet för Kulturellt Utbyte med Utlandet)* for its generous invitation, making my stay in Stockholm possible for 3 months.

LITERATURE

1. Theorell, H. *Biochem. Z.* **252** (1932) 1.
2. Millikan, G. H. *Physiol. Rev.* **19** (1939) 501.
3. de Duve, Ch. *Acta Chem. Scand.* **2** (1948) 264.
4. Theorell, H., and de Duve, Ch. *Arch. Biochem.* **12** (1947) 113.

Received February 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 343—351

Determination of Small Amounts of Tin in Alloys

Isolation by Adsorption of Stannic Acid on Manganese Dioxide

S. KÜHNEL HAGEN, NIELS HOFMAN-BANG and POUL GJERTSEN

Laboratoriet for Metalundersøgelser, Nordiske Kabel- og Traadfabriker Akts., København, Denmark

Isolation of small percentages of tin from alloys has been, and is still, something of a problem. When a bronze with a low tin content is dissolved in nitric acid, the stannic acid will be obtained in partly disperse and partly colloidal form, so that complete filtering-off is impossible. Furthermore iron and possibly also chromium shows a tendency to prevent the colloidal stannic acid from coagulating. The presence of phosphorus will also be troublesome. Certainly, there are chemical micro-methods for the isolation and determination of tin, but they are inadequate in cases, where the amounts of other elements present are disproportionately much larger than the amount of tin. Exemplifying methods are precipitation with phenylarsonic acid [1], as also precipitation with cupferron [2].

Blumenthal [3] has investigated a method for isolating very small amounts of antimony in copper. The copper is dissolved in nitric acid, after which the solution is made only slightly acid with nitric acid and diluted to a considerable volume. Through addition of manganous nitrate and potassium permanganate a precipitate consisting of manganese dioxide is formed, which will adsorb most (96—97 %) of the antimonic acid present. By repeating the precipitation in the filtrate, a practically complete isolation of antimony is obtained. Blumenthal states, that in all likelihood tin and bismuth will also be precipitated quantitatively.

In »Analyse der Metalle» [4] Blumenthal's method is referred to in respect to the determination of tin.

Park [5] estimates spectrographically traces of antimony, tin, bismuth and molybdenum in copper after co-precipitation with manganese dioxide. As in »Analyse der Metalle» the precipitate is dissolved in hydrochloric acid and

bromine by boiling, until the solution is reduced to a considerably smaller volume.

During experimentation in our laboratory in this way it proved difficult to avoid loss of tin, as also antimony (*cf.* Table 1, IV and V). The loss of tin and antimony depends on the concentration of hydrochloric acid. After the method in »Analyse der Metalle», as well as the method of Park, a subsequent precipitation with hydrogen sulphide is used with regard to further purification.

We have made a few experiments in order to compare the volatility of tin, antimony and arsenic in circumstances similar to those used in »Analyse der Metalle» when dissolving manganese dioxide plus stannic acid or antimonic acid. a) A solution of arsenic is made by dissolving 4.917 g arsenic trioxide in a solution of 15 g sodium hydroxide in water and diluting to 1000 ml. The arsenic content is determined by titration with 0.1 *N* potassium bromate. b) 6.111 g pure antimony is dissolved in 30 ml conc. sulphuric acid. The solution is diluted to 1000 ml with water and at the same time with 150 ml conc. hydrochloric acid. The antimony content is determined by titration (in hot solution) with 0.1 *N* potassium bromate. c) 11.3 g cryst. stannous chloride is dissolved in 100 ml conc. hydrochloric acid, diluted with water, oxidized with hydrogen peroxide and finally diluted with water to 1000 ml. The tin content is determined as usual by reduction with aluminium and titration with potassium iodate.

All the experiments given in Table 1 are carried out in a beaker. When carried out in an Erlenmeyer flask, the loss will be somewhat smaller. 25 ml (representing about 1.25 millimole of respectively arsenic, antimony and tin) of the stock solutions are used in each experiment. The results show, that the procedure in »Analyse der Metalle» might bring about a considerable loss of antimony, but no loss or only a small loss of tin. Nevertheless, when, for instance, dissolving lead with a small percentage of tin by boiling with nitric acid and treating as in »Analyse der Metalle», only a part of the tin will be found — in all likelihood due to formation of metastannic acid, which will not dissolve in hydrochloric acid plus bromine. Of course this metastannic acid could be filtered off and fused with sodium peroxide, but that is an unpleasant and time-wasting complication.

Fainberg and Troitzkaja [6] have described a procedure for the determination of tin in electrlytic copper using Blumenthals method and the tin-titration method of Clarke [7]: To the slightly acid (with nitric acid) solution of the electrolytic copper is added manganous sulphate and potassium permanganate. The manganese dioxide precipitate, which has adsorbed all stannic acid present is boiled with nitric acid, so that all the tin will be at hand as

Table 1. Experiments showing the amounts lost of arsenic, antimony and tin by boiling — in a beaker — solutions acidified with hydrochloric acid as in »Analyse der Metalle«.

		Percentage volatilized		
		As	Sb	Sn
I	25 ml solution is diluted to 100 ml (6 *N* as to hydrochloric acid) and boiled down to 40 ml. Titration	84.5 91.6	0 0	0 0
I a	The same procedure as in I except that 10 ml conc. sulphuric acid is added before boiling	95.6 98.0	6.5 6.5	0 0
II	25 ml solution is diluted to 60 ml (4 *N* as to hydrochloric acid). Boiling at constant volume (addition of water) for 1 hour. Titration	6.0 14.3	0 0	0 0
II a	The same procedure as in II except that 10 ml conc. sulphuric acid is added before boiling	80.0 79.1	4.4 3.0	0 0
III	25 ml solution is diluted to 125 ml (9 *N* as to hydrochloric acid) 2 g sodium sulphite is added, after which the solution is boiled down to 50 ml. Titration ..	99.0 99.2	1 1	0 0
IV	25 ml solution is diluted to 150 ml (8 *N* as to hydrochloric acid), 10 ml bromine water added. Boiling down to 50 ml. After this 1 g sodium sulphite and 25 ml water is added. Boiling down to 50 ml. Titration	84.0	4.7 4.0	0.7 1.4
V	25 ml solution is diluted to 100 ml (6 *N* as to hydrochloric acid), 20 ml bromine water added. Boiling down to 50 ml. After this 2 g sodium sulphite and 75 ml conc. hydrochloric acid is added. Boiling down to 50 ml. Titration ..	99.5	0.5 0.5	0

metastannic acid. After filtering and drying the precipitate is fused in a porcelain crucible with sodium peroxide. The melt is dissolved in water, and the tin is determined by reduction with lead foil in carbon dioxide atmosphere and titration with 0.01 *N* iodine solution with starch as indicator. The method yields good results, but it is tedious and particular.

We have, as will appear later, made a closer investigation of the adsorption of stannic acid on manganese dioxide and at the same time altered the determination of tin in this precipitate, so that a fairly speedy and convenient method is available.

PROCEDURE

An appropriate solution with known tin-content is produced by dissolving about 50 mg Banka tin in hot conc. sulphuric acid; the sulphur hereby formed is removed by gently heating the sides of the flask. The solution is diluted with water to 100 ml in a volumetric flask. From this solution 10 ml is taken out with a pipette — for each experiment.

10 ml tin-solution (about 5 mg tin) is diluted to 600 ml in a beaker with redistilled water (in all experiments redistilled water, condensed in a silica condenser, is used). 10 ml conc. nitric acid and one drop of methyl red is added, after which neutralization with conc. ammonium hydroxide takes place. The final degree of acidity is imparted by addition of 2 ml conc. nitric acid. The precipitate of manganese dioxide is now generated by adding 5 ml 0.4 *M* manganous nitrate and 5 ml 0.04 *M* potassium permanganate solution. The permanganate is added while vigorously stirred, so that a homogeneous solution is obtained before formation of the precipitate. The beaker is now while stirring, heated, to boiling point, or at least until the manganese dioxide is aggregated in large flakes. The hot suspension is filtered on a soft analytical filter paper, and the precipitate is washed with hot water 6—8 times. Into the beaker used for the precipitation is poured 5 ml conc. nitric acid and a couple of drops of 30 % hydrogen peroxide. Hereby the small amount of manganese dioxide, covering the bottom and the sides of the beaker, is dissolved, and the possibly adsorbed stannic acid will become suspended in the solution *, which — without further cleaning of the beaker — is transferred to a 300 ml Erlenmeyer flask. The filter paper with the manganese dioxide precipitate is pushed into the flask and 10 ml conc. sulphuric acid added. By heating until sulphur trioxide fumes are generated the paper gradually becomes oxidized. At the beginning of this destruction it is advisable to almost cover the mouth of the flask with a watch-glass to avoid splashes escaping. As a rule it is sufficient for complete destruction to add twice a few drops of fuming nitric acid, each time heating until fumes of sulphur trioxide are formed. The solution will now in addition to sulphuric acid, manganous sulphate and stannic sulphate contain some nitrosylsulphuric acid, which can be broken down by twice adding 10 ml dist. water, each time heating until all water is evaporated.

The tin titration is carried out in a test tube 230 × 40 mm fitted with a three-hole stopper carrying in one hole a gas inlet tube extending to the bottom of the test tube, in the second a vertical outlet tube, 70—80 cm in length, and in the third hole a small glass stopper, which can be removed when the tip of the burette is to be inserted. The solution

* For the possible adhesion of SnO_2 to the bottom of the beaker see by »Results» exp. no 5.

of stannic and manganous sulphate in 10 ml conc. sulphuric acid is diluted with 25 ml of water, cooled, and poured into the titration test tube. The flask is washed with a cold mixture of 25 ml of water and 15 ml conc. hydrochloric acid. As a reducing agent a piece of aluminium sheet 70 × 7 × 0.4 mm (about 0.5 g) is used. This aluminium is spectrographically found to be tin free and furthermore, when a blank analysis is carried out as described, no tin is found. After addition of the aluminium the stopper is inserted and a slow current (2—3 bubbles per second) of carbon dioxide is started through the solution (carbon dioxide from a pressure cylinder is sufficiently pure).

In the course of about ½ hour all aluminium will be dissolved, and the stannic ions have been reduced to free tin, which again is brought into solution by heating the test tube in a steam bath of with a pilot flame (without boiling). Finally, the tube is cooled in cold water after increasing the current of carbon dioxide to prevent back pressure.

The solution — now containing stannous ions — is titrated with potassium iodate or potassium bromate solution with starch plus potassium iodide as an indicator. Hereby the stannous ions will be oxidized to stannic ions, while iodate and bromate are reduced to iodide and bromide. 1 ml standard solution is equal to about 2 mg tin.

When the solution is cold, the stopper in the third hole is removed, and 5 ml of a solution of starch and potassium iodide added from a pipette. The outlet capillary tip of the burette is now inserted — the tip extending to below the surface of the solution, and the solution is titrated to a faintly blue colour. The carbon dioxide current is used for stirring. The indicator liquid is made of 50 ml fresh 1 % starch solution and 4 g potassium iodide. Just before use 10 ml of this solution is mixed with 1 ml 4 n sulphuric acid and a small spatula of sodium bicarbonate, in order to remove most of the oxygen dissolved. A 5 ml burette with 1/100 ml divisions, the tip drawn down to a capillary, is used.

The titre of the iodate or bromate solution must be determined by direct reduction and titration of a known amount of the solution of tin in sulphuric acid, because the theoretical titre is a little too low due to oxygen dissolved in the standard solution.

We have obtained the procedure detailed above through several unsuccessful experiments not described.

RESULTS

Experiments nos. 1, 2, 3 and 4 are carried out exactly as given in the procedure above. As Table 2 shows, all tin is found again.

In experiment no. 5 10 ml solution containing 6.61 mg tin, 9 ml water and 1 ml conc. sulphuric acid is boiled for 1 ½ hour with nitric acid in the beaker later used for the manganese dioxide precipitation. The volume is kept at about 100 ml by adding, from time to time, conc. nitric acid. As a result of this treatment some of the colloidal stannic acid will become disperse; furthermore a thin, nearly invisible film of stannic acid, covering the bottom of the beaker, will be produced. After boiling the solution is diluted with water, neutralized with ammonium hydroxide and an excess of 2 ml conc. nitric acid added, after which the manganese dioxide precipitation takes place. The tin content in the manganese dioxide precipitate is found to be 2.80 mg. The bottom of the beaker is now covered with 10 ml conc. sulphuric acid, which

Table 2. Determination of tin under diverse conditions.

No.	Tin present	Tin found	
1	4.86 mg	4.81 mg	As above
2	4.86 »	4.84 »	
3	6.61 »	6.60 »	
4	6.61 »	6.61 »	
5	6.61 »	6.57 »	Prolonged boiling of the stannic acid solution
6	7.39 »	7.33 »	Ferric ions present
7	7.39 »	7.34 »	
8	7.39 »	7.40 »	Ferric ions present
9	7.15 »	7.04 »	Cupric ions present
10	7.15 »	7.07 »	
11	5.30 %	5.33 %	Bronze
12	5.30 »	5.36 »	
13	2.88 mg	2.85 mg	Lead ions present
14	2.89 »	2.89 »	
15	7.54 »	7.36 »	Nitric acid present
16	7.54 »	7.22 »	
17	6.71 »	6.63 »	Nitric acid present
18	6.71 »	6.60 »	

is kept at boiling point for about 15 minutes. The tin in this solution is determined and found to be 3.77 mg. The total amount of tin found will thus be 6.57 mg — only little less than the 6.61 mg added.

It appears from this experiment, that, when dissolving low tin content alloys in boiling nitric acid, a considerable amount of the tin can be lost by adsorption to the bottom of the beaker. That production of the stannic acid film only takes place on the bottom, through which the heat is conducted, can

be shown by mixing 10 ml solution containing about 5 mg tin and 0.5 ml conc. sulphuric acid with 100 ml conc. nitric acid and heating in a beaker for about 30 minutes with a pilot flame. Where the flame has touched the bottom of the beaker, a just visible spot will be found.

Experiments nos. 6 and 7 show, that it is possible to separate small amounts of tin from large amounts of ferric ions. 1 g of ferrous ammonium sulphate is oxidized with conc. nitric acid. 7.39 mg tin is added and manganese dioxide precipitation carried out as above. For sake of further purification the manganese dioxide precipitation is repeated. The precipitate is dissolved in nitric acid and hydrogen peroxide, after which the excess of the latter is removed by adding, in drops, permanganate solution. The second precipitation can now be carried out exactly as the first.

Experiment no. 8 is similar to nos. 6 and 7, except that 10 g ferrous ammonium sulphate is added instead of 1 g, and only one manganese dioxide precipitation has taken place. In this case also the result is excellent. Separation of stannic acid from ferric ions has a special interest, because the latter to a considerable degree hinders the precipitation of stannic acid [8] when carried out in the usual way.

Experiments nos. 9 and 10 show separation of stannic acid from cupric ions. 3 g of very pure electrolytic copper is dissolved in nitric acid, stannic sulphate solution is added, after which the procedure is as above. That the results are a little low may be explained by inclusion of free tin in copper. When the free tin in the titration tube is dissolved, a small lump of free copper will remain, and it seems likely that the tin not found is included in this lump.

In experiments nos. 11 and 12 a bronze with the tin content known in advance is analyzed. The tin content is determined as stannic oxide by treating with ammonium iodide [9]. About 1 g bronze is dissolved in nitric acid, 10 ml conc. sulphuric acid is added, and the solution is gently heated until fumes of sulphuric acid are given off. Some water is now added and — after cooling — the solution is transferred to a volumetric flask and diluted to 100 ml. With a pipette 10 ml is taken out and treated as above. As the results show, there is good accordance between the two methods. The low residue in the second column are probably due to a small iron contents in tin bronze, which hinders the total precipitation of SnO_2 when carried out in the usual way by treating with nitric acid alone.

Experiments nos. 13 and 14 show separation of stannic acid from large amounts of lead ions. The tin is added as a zinc alloy containing 1.029 % tin, because adding of stannic sulphate solution would give rise to a precipitate of lead sulphate. The alloy is dissolved in diluted nitric acid, and lead nitrate derived from 25 g Tadanac lead is added, after which precipitation and deter-

mination is carried out as above. The tin contained in the lead is determined by running a blank analysis on 50 g of Tadanac lead. The tin content is found to be 0.00012 and 0.00015 %. Before the titration it is convenient to cool the solution to only 30—35° C to prevent precipitation of lead sulphate and lead iodide (some lead will always be adsorbed to the manganese dioxide). The higher temperature has not any noticeable influence on the results.

In experiments nos. 15 and 16 50 ml conc. nitric acid was added just before the manganese dioxide precipitation. The beaker was heated for ½ hour in a steambath because the high acidity hinders the precipitation. The results show, that not all stannic acid is adsorbed.

Experiments nos. 17 and 18 are similar to nos. 15 and 16, except that the manganese dioxide precipitation is carried out while stirring with a spatula. The results are better but not good.

From the experimental results here detailed, it is evident, that isolation of small amounts of tin as stannic acid by adsorption on and inclusion in, manganese dioxide is an excellent method, one single precipitation usually being sufficient. When titrating, potassium iodate as well as potassium bromate can be used.

If tin has to be isolated from a solution containing chloride, the latter must be expelled as chlorine by boiling with a large excess of conc. nitric acid. In this case the stannic acid possibly adhering to the bottom of the beaker must be dissolved in hot conc. sulphuric acid. This procedure is not quite reliable, when considerable amounts of chloride are present. A small percentage of the tin may be volatilized when boiling with conc. nitric acid.

As arsenic is partly and antimony is nearly completely precipitated together with tin by the mentioned procedure, and as these elements to a certain extend interfere with the titration of tin, they must be separated when present in considerable amounts.

SUMMARY

On the basis of the fact — observed by Blumenthal [3] — that colloidal and despersoid antimonic acid is readily adsorbed on and included in a manganese dioxide precipitate, a convenient and rapid method for the isolation and determination of small percentages of tin is worked out.

Separation of small amounts of tin from large amounts of ferric ions, cupric ions and lead ions is investigated (compare with Table 2). If dissolution of an alloy requires boiling with nitric acid, the bottom of the beaker must be treated with hot conc. sulphuric acid, because some of the stannic acid present may adhere as a film.

REFERENCES

1. Knapper, I. S., Craig, K. A., and Chandlee, G. C. *J. Am. Chem. Soc.* **55** (1933) 3945.
2. Mack, M. v., and Hecht, F. *Mikrochim. Acta* **2** (1937) 227.
3. Blumenthal, H. *Z. anal. Chem.* **74** (1928) 33.
4. *Analyse der Metalle* Vol. 1, Berlin (1942).
5. Park, B. *Ind. Eng. Chem., Anal. Ed.* **6** (1934) 189.
6. Fainberg, S. Y., and Troitzkaya, M. I. *Zavodskaya Lab.* **3** (1934) 126 (Ref. *C. A.*).
7. Clarke, S. C. *Analyst* **56** (1931) 82.
8. Hillebrand, W. F., and Lundell, G. E. F. *Applied inorganic analysis* New York (1929) p. 235.
9. Caley, E. R., and Burford, M. G. *Ind. Eng. Chem., Anal. Ed.* **8** (1936) 114.

Received February 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 352—358

The Electrolyte and Solvent Influence upon the Reaction between Persulphate and Iodide

II. The Reaction Entropies and Heats of Activation

ATTE MERETOJA

The Chemical Institute, University of Helsinki, Finland

The variation of the specific rate of a reaction in solution with temperature, provided that the temperature range is not wide, is represented by the Arrhenius equation

$$k = Ae^{-E/RT} \tag{1}$$

where E is the experimental activation energy and A is the frequency factor. According to the theory of absolute reaction rates by Eyring [1] this relationship is to be expressed, supposing the transmission coefficient to unity, by the equation

$$k = \frac{\mathrm{k}T}{\mathrm{h}} e^{-\Delta G^{\ddagger}/RT} = \frac{\mathrm{k}T}{\mathrm{h}} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \tag{2}$$

where $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the free energy, heat content and entropy of activation, respectively. In comparing the Eqs. (1) and (2) with one another we obtain

$$\Delta H^{\ddagger} = E - RT \tag{3}$$

$$A = \frac{e\mathrm{k}T}{\mathrm{h}} e^{\Delta S^{\ddagger}/R} \tag{4}$$

In a previous investigation [2] I have derived, on the basis of Born's equation, expressions which give the dependence of the specific rate, experimental activation energy and frequency factor, respectively, on the dielectric constant of the solvent, referred to zero ionic strength. The equations are:

$$\log\ k^0 = \log\ k_{D_\infty} - \frac{z_A z_B e^2}{2.3kT\varrho D} \tag{5}$$

$$E_D^0 = E_{D_\infty} + \frac{z_A z_B e^2 R}{k\varrho D} \tag{6}$$

$$\log\ A_D^0 = \log\ k_{D_\infty} + \frac{E_{D_\infty}}{2.3RT} = \text{a constant at constant temperature} \tag{7}$$

$$E_C^0 = E_D^0 + \frac{z_A z_B e^2 RT}{k\varrho D^2} \cdot \frac{dD}{dT}\left(1 + \frac{D}{\varrho}\frac{d\varrho}{dD}\right) \tag{8}$$

$$= E_{D_\infty} + \frac{z_A z_B e^2 R}{k\varrho D}\left(1 + \frac{T}{D}\frac{dD}{dT} + \frac{T}{\varrho}\frac{d\varrho}{dT}\right)$$

and

$$\log\ A_C^0 = \log\ A_D^0 + \frac{z_A z_B e^2}{2.3\ k\varrho \mathrm{D}^2} \cdot \frac{dD}{dT}\left(1 + \frac{D}{\varrho}\frac{d\varrho}{dD}\right) \tag{9}$$

By the subscripts C and D are designated the constants in a medium of fixed composition and in one of fixed dielectric constant, respectively. The terms with subscript D_∞ are those in the hypothetical solvent in which all the electrostatic effects vanish ($D = \infty$).

The Eq. (2) may be put in the form

$$\ln\ k^0 = \ln \frac{\mathbf{k}T}{\mathbf{h}} - \frac{\Delta G^{\ddagger}}{RT} \tag{10}$$

and the comparison with Eq. (5) gives

$$\Delta G^{\ddagger} = RT\ \left(\ln \frac{\mathbf{k}T}{\mathbf{h}} - \ln\ k_{D_\infty}\right) + \frac{z_A z_B e^2 R}{\mathbf{k}\varrho D} \tag{11}$$

The two terms on the right-hand side may be identified with two types of free energy changes [3], *viz.*, $\Delta G_0^{\ddagger}$ for the non-electrical reaction and $\Delta G_D^{\ddagger}$ as the dielectric effect; thus,

$$\Delta G_0^{\ddagger} = RT\ \left(\ln \frac{\mathbf{k}T}{\mathbf{h}} - \ln\ k_{D_\infty}\right) = \text{a constant at constant temperature} \tag{12}$$

and

$$\Delta G_D^{\ddagger} = \frac{z_A z_B e^2 R}{\mathbf{k}\varrho D} \tag{13}$$

The differentiation of these free energy terms with respect to temperature, and taking into account Eqs. (7) and (8), gives

$$\Delta S_0^{\ddagger} = -\left[\frac{\partial(\Delta G_0^{\ddagger})}{\partial T}\right]_p = R\ (\ln\ A_D^0 - \ln\frac{e\mathbf{k}T}{\mathbf{h}}) = \text{a constant at constant temperature} \tag{14}$$

and

$$\Delta S_D^{\ddagger} = -\left[\frac{\partial(\Delta G_D^{\ddagger})}{\partial T}\right]_p = \frac{z_A z_B e^2 R}{\mathbf{k}\varrho D^2}\left(\frac{\partial D}{\partial T}\right)_p\left(1 + \frac{D}{\varrho}\frac{d\varrho}{dD}\right) \tag{15}$$

or, according to Eq. (9),

$$\Delta S_D^{\ddagger} = R\ (\ln\ A_C^0 - \ln\ A_D^0) \tag{15'}$$

for the non-electrical and dielectric contributions to the entropy of activation at zero ionic strength.

The total entropy of activation $\Delta S^{\ddagger}$ is the sum of $\Delta S_0^{\ddagger}$ for the non-electrical reaction and of $\Delta S_D^{\ddagger}$ for the dielectric effect, hence it is seen that

$$\Delta S^{\ddagger} = R\ (\ln\ A_C^0 - \ln\frac{e\mathbf{k}T}{\mathbf{h}}) \tag{16}$$

as given by Eq. (4).

If the thermodynamic relationship $\Delta H = \Delta G + T\Delta S$ is utilized, it follows from Eqs. (12)—(15) that the contributions to the heat of activation are

$$\Delta H_0^{\ddagger} = E_{D\infty} - RT = \text{a constant at constant temperature} \tag{17}$$

and

$$\Delta H_D^{\ddagger} = \frac{z_A z_B e^2 R}{\mathbf{k}\varrho D}\ (1 + \frac{T}{D}\frac{dD}{dT} + \frac{T}{\varrho}\frac{d\varrho}{dT}) \tag{18}$$

or, according to Eq. (8),

$$\Delta H_D^{\ddagger} = E_C^0 - E_{D\infty} \tag{18'}$$

Hence,

$$\Delta H^{\ddagger} = E_C^0 - RT \tag{19}$$

From Eqs. (6) and (13) there follows the interesting result

$$E_D^0 = E_{D\infty} + \Delta G_D^{\ddagger} \tag{20}$$

which indicates the dependence of the experimental activation energy in media of fixed dielectric constants on the dielectric constant of these media. Thus the standard contribution $E_{D\infty}(= \Delta H_0^{\neq} + RT)$ designates the bond-stretching energy and the dielectric contribution $\Delta G_D^{\neq}$ the repulsion energy of Hinshelwood, Laidler and Timm [4].

The abovementioned Eqs. (11), (13), (15) and (18) are valid only for reactions between two ions with charges $z_A e$ and $z_B e$. If one or both of the reactants are dipoles, $z_A e$ and $z_B e$ in these equations should be replaced with corresponding dipole expressions, in which case the Eqs. (15′) and (18′) are, however, still in force and can be applied to the measurement results. The $E_{D\infty}$ value should be obtained on extrapolating from the E_D values in regard to $1/D$ to zero, after which the non-electrical and dielectric contributions may be determined without a knowledge of the ϱ values and their variation with the dielectric constant.

In the first part of this investigation there were derived also equations for the influence of ionic strength upon the Arrhenius parameters A_C and E_C. These equations (17 and 20 on page 23) are to be applied directly to Eq. (2) and we have

$$(\Delta G_D^{\neq})_{\sqrt{\mu}} = (\Delta G_D^{\neq})_{\mu=0} - 4.6RT \frac{z_A z_B A\sqrt{\mu}}{1 + a\sqrt{\mu}} + B\mu, \tag{21}$$

$$(\Delta H_D^{\neq})_{\sqrt{\mu}} = (\Delta H_D^{\neq})_{\mu=0} - 2.3RT \left(1 + \frac{T}{D}\frac{dD}{dT}\right) \frac{3 z_A z_B A\sqrt{\mu}}{1 + a\sqrt{\mu}} \left(1 - \frac{a}{3}\frac{\sqrt{\mu}}{1 + a\sqrt{\mu}}\right) - B'\mu \tag{22}$$

and

$$(\Delta S_D^{\neq})_{\sqrt{\mu}} = (\Delta S_D^{\neq})_{\mu=0} - 2.3R \frac{z_A z_B A\sqrt{\mu}}{1 + a\sqrt{\mu}} \left[1 + 3\frac{T}{D}\frac{dD}{dT} - \frac{a\sqrt{\mu}}{1 + a\sqrt{\mu}} \left(1 + \frac{T}{D}\frac{dD}{dT}\right)\right] - B''\mu \tag{23}$$

or applied to the reaction under consideration in aqueous solution of 30° C:

$$(\Delta G_D^{\neq})_{\sqrt{\mu}} = 5\,300 - \frac{2835\sqrt{\mu}}{1 + 0.75\sqrt{\mu}} + 88\ \mu \tag{21′}$$

$$(\Delta H_D^{\neq})_{\sqrt{\mu}} = -2\,400 + \frac{1638\sqrt{\mu}}{1 + 0.75\sqrt{\mu}} \left(1 - \frac{0.25\sqrt{\mu}}{1 + 0.75\sqrt{\mu}}\right) - 400\ \mu \tag{22′}$$

$$(\Delta S_D^{\neq})_{\sqrt{\mu}} = -25.4 + \frac{14.75\sqrt{\mu}}{1 + 0.75\sqrt{\mu}} \left(1 - \frac{0.092\sqrt{\mu}}{1 + 0.75\sqrt{\mu}}\right) - 1.61\ \mu \tag{23′}$$

Table 1. The persulphate-iodide reaction in aqueous methyl alcohol solutions at zero ionic strength (30° C). The free energy, heat content, and entropy of activation and their non-electrical and dielectric contributions.

Wt. % alcohol	$D_{30°}$	$\Delta G_0^{\ddagger}$ kcal/ mole	$\Delta H_0^{\ddagger}$ kcal/ mole	$\Delta S_0^{\ddagger}$ E. U.	$\Delta G_D^{\ddagger}$ kcal/ mole	$\Delta H_D^{\ddagger}$ kcal/ mole	$\Delta S_D^{\ddagger}$ E. U.	$\Delta G^{\ddagger}$ kcal/ mole	$\Delta H^{\ddagger}$ kcal/ mole	$\Delta S^{\ddagger}$ E. U.
0	76.7	12.2	14.5	+ 7.6	5.3	— 2.4	— 25.4	17.5	12.1	— 17.8
10.2	72.3	12.2	14.5	+ 7.6	5.7	— 1.5	— 23.6	17.9	13.0	— 16.0
20.7	67.3	12.2	14.5	+ 7.6	6.0	— 0.7	— 22.1	18.2	13.8	— 14.5
26.6	64.5	12.2	14.5	+ 7.6	6.2	+ 0.4	— 19.0	18.4	14.9	— 11.4
31.7	62.0	12.2	14.5	+ 7.6	6.3	+ 1.1	— 17.1	18.5	15.6	— 9.5
43.1	56.6	12.2	14.5	+ 7.6	6.6	+ 2.3	— 14.1	18.8	16.8	— 6.5
57.5	49.8	12.2	14.5	+ 7.6	6.8	+ 3.8	— 9.9	19.0	18.3	— 2.3

EXPERIMENTAL RESULTS

Table 1 presents the contributions of free energy, heat content, and entropy of activation for reaction between persulphate and iodide ions in various aqueous methyl alcohol solutions containing 0—57.5 weight per cent alcohol. The non-electrical contributions $\Delta G_0^{\ddagger}$, $\Delta H_0^{\ddagger}$ and $\Delta S_0^{\ddagger}$ are fundamental values for the reaction under consideration. It would be of interest to know the corresponding values for other reactions of different types. However, only few reactions in solution have hitherto been measured with such an accuracy and with such an extensive variation of the temperature and dielectric constant that a determination of $E_{D\infty}$, especially, either making use of Eq. (6) or graphically, is possible. However, after my calculations according to experimental results of several authors, it seems that $\Delta H_0^{\ddagger}$ has a value of the same

Table 2. The effect of ionic strength μ (KNO_3) on the free energy, heat content, and entropy of activation.

$\sqrt{\mu}$	$(\Delta G_D^{\ddagger})\sqrt{\mu}$ kcal/ mole	$(\Delta H_D^{\ddagger})\sqrt{\mu}$ kcal/ mole	$(\Delta S_D^{\ddagger})\sqrt{\mu}$ E. U.	$\Delta G^{\ddagger}$ kcal/ mole	$\Delta H^{\ddagger}$ kcal/ mole	$\Delta S^{\ddagger}$ E. U.
0	5.3	— 2.4	— 25.4	17.5	12.1	— 17.8
0.05	5.2	— 2.3	— 24.7	17.4	12.2	— 17.1
0.1	5.0	— 2.3	— 24.0	17.2	12.2	— 16.4
0.2	4.8	— 2.2	— 23.0	17.0	12.3	— 15.4
0.3	4.6	— 2.1	— 22.2	16.8	12.4	— 14.6
0.5	4.3	— 2.1	— 21.1	16.5	12.4	— 13.5
0.7	4.0	— 2.1	— 20.3	16.2	12.4	— 12.7
1.0	3.8	— 2.2	— 19.6	16.0	12.3	— 12.0
1.2	3.6	— 2.2	— 19.3	15.8	12.3	— 11.7
1.5	3.5	— 2.3	— 19.2	15.7	12.2	— 11.6

order as for the reaction under consideration, *viz.* 14—15 kcal, for a number of reactions of different types, as for fading of bromphenolblue [5], hydrolysis of ethyl benzoate [6], dealdolization of diaceton alcohol [7], Cannizzaro reaction [8] and for reaction between ethylene chlorhydrin and hydroxyl ion [9], wherein $\Delta S_0^{\ddagger}$ also, at least for the three first mentioned reactions, has the value appr. + 7 E. U. (cal/degree · mole).

Table 2 gives the dependence of the various contributions on the ionic strength of the solution. The heat of activation is, as is perceivable from these values, only slightly dependent on the ionic strength, and the variation of the free energy change with the ionic strength is to a great extent caused by the variation of the entropy change.

SUMMARY

Provided that the specific rate of a reaction has been measured in different solvents by varying the temperature and dielectric constant of the solvent, the parameters of the Arrhenius equation can be calculated both for solvents of constant composition or of fixed dielectric constant. If the measurements furthermore, have been performed in sufficiently dilute solutions or by variation of the ionic strength, the values of these parameters referred to zero ionic strength can be calculated. According to the expressions derived above, the non-electrical and dielectric contributions to the free energy, heat content, and entropy of activation then can be determined without a knowledge of the radii of the reactants, or of their variation with solvent, or of any other conceptions.

The equations giving the influence of the ionic strength of the solution on the free energy, heat content, and entropy of activation of ionic reactions have been derived on the basis of the Debye-Hückel theory.

REFERENCES

1. Eyring, H. *Cf.* Glasstone, S., Laidler, K. J., and Eyring, H. *The theory of rate processes* New York (1941).
2. Meretoja, A. *Ann. Acad. Scient. Fenn.* A II **24** (1947).
3. La Mer, V. K., and Kamner, M. L. *J. Am. Chem. Soc.* **57** (1935) 2662.
   La Mer, V. K. *J. Franklin Inst.* **225** (1938) 709.
   Amis, E. S., and La Mer, V. K. *J. Am. Chem. Soc.* **61** (1939) 905.
4. Hinshelwood, C. N., Laidler, K. J., and Timm, E. W. *J. Chem. Soc.* (1938) 848. *Cf.* also Meretoja (*l. c.*).
5. Amis, E. S., and La Mer, V. K. (*l. c.*).
6. Fairclough, R. A., and Hinshelwood, C. N. *J. Chem. Soc.* (1937) 538.
7. La Mer, V. K., and Miller, M. L. *J. Am. Chem. Soc.* **57** (1935) 2674.
8. Tommila, E. *Ann. Acad. Scient. Fenn.* A LIX 8 (1942).
9. Winström, L. O., and Warner, J. C. *J. Am. Chem. Soc.* **61** (1939) 1205.

Received February 17, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 358—364

The Kinetics of the Cannizzaro Reaction

The Free Energy, Heat Content, and Entropy of Activation

ATTE MERETOJA and EERO TOMMILA

The Chemical Institute, University of Helsinki, Finland

In a previous paper [1] one of us (E. T.) reported the results of his investigation of the kinetics of the Cannizzaro reaction with benzaldehyde and several substituted benzaldehydes in aqueous methanol. The paper contains the parameters of the Arrhenius equation

$$k = Ae^{-E/RT} \tag{1}$$

obtained for solutions of constant solvent composition (E_C and A_C). As the second of the authors (A. M.) has stated [2], the parameters of the Arrhenius equation in isodielectric solutions (E_D and A_D) may be got by a graphical method from such results. The values for benzaldehyde thus obtained are now given in Table 1.

Plotting the values of E_D against $1/D$ and through extrapolation with regard to $1/D$ to zero (Fig. 1) one obtains for the bond-stretching energy [3] (for the complex Y, see later) the value

$$E_{D\infty} = 15.1 \text{ kcal/mole}$$

Theoretically, in very dilute solutions log A_D should be constant [2]; we see that this condition is rather well fulfilled, although the k-values are not extrapolated to zero concentration. The influence of the concentration on k is in this case, of course, only very small owing to the fact that one of the reactants is a neutral molecule. From the values for E_D in Table 1 and from equation [4]

$$E_D = E_{D\infty} + \Delta G_D^{\ddagger} \tag{2}$$

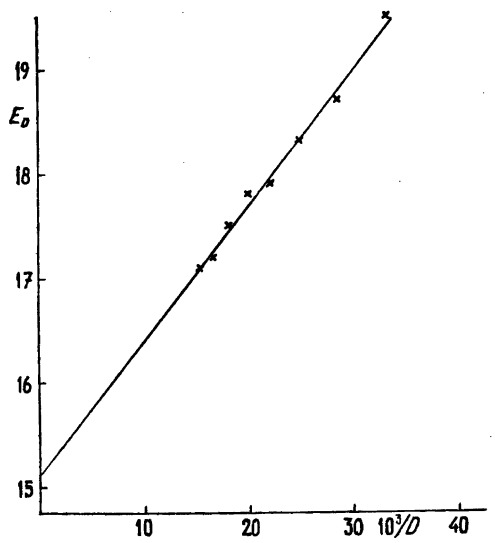


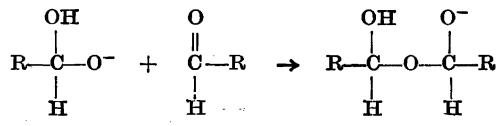
Fig. 1. Plotting of E_D against 1/D.

where $\Delta G_D^{\ddagger}$ is the repulsion energy, we see that the latter energy is always only a rather small fraction of the total energy E_D of the reaction.

Table 1. The reaction between benzaldehyde and NaOH in isodielectric water-methanol mixtures. (k is the trimolecular rate constant in $ml^2 \cdot mole^{-2} \cdot sec^{-1}$, E_D kcal).

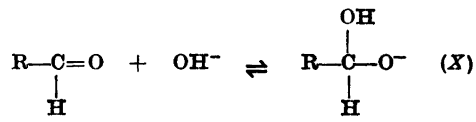
D	$k_{40}°$	$k_{60}°$	$k_{80}°$	E_D	log A_D
65	31.6	166	—	(17.1)	(13.44)
60	19.1	100	363	17.2	13.26
55	12.0	67.6	282	17.5	13.30
50	8.13	44.7	209	17.8	13.34
45	5.50	31.6	145	17.9	13.24
40	3.72	22.9	105	18.3	13.35
35	2.57	16.2	77.6	18.7	13.46
30	—	11.5	60.3	(19.5)	(13.85)
				Mean	13.32

It is now generally accepted [1,5,6,7] that the rate-determining step of the reaction is



(3)

and that before this there exists the following equilibrium:



(4)

the corresponding equilibrium constant being

$$K = \frac{[\mathrm{RHC(OH)O^-}]}{[\mathrm{RCHO}][\mathrm{OH^-}]} \tag{5}$$

The rate of the formation of the critical complex Y is thus proportional to the product $[\mathrm{RHC(OH)O^-}]$ [RCHO] and as net result there follows a reaction formally of third order:

$$\frac{dY}{dt} = k'K[\mathrm{RCHO}]\ [\mathrm{RCHO}]\ [\mathrm{OH^-}] = k[\mathrm{RCHO}]^2[\mathrm{OH^-}] \tag{6}$$

where k' is the rate constant of the bimolecular reaction (3), and k the experimentally found trimolecular constant. The values given in Tables 1 and 2 are obtained from k-values for different temperatures in different solvents. As k is the product of the real bimolecular rate constant k' and of the equilibrium constant K, $k = k'K$, the next task is to eliminate the effect of K and state the details of this bimolecular reaction (3). Because, however, nothing is known about the absolute values of K in conditions prevailing during the experiments, conclusions of only general nature can be drawn.

In connection with the experimental determination of the trimolecular rate constant k at different temperatures and in various solvents, the dependence of the equilibrium constant K on these factors was also measured. As

$$\log\ k = \log\ k' + \log\ K \tag{7}$$

we can generally write

$$2.303RT^2\ \frac{\partial \log\ k}{\partial T} = 2.303RT^2\ \frac{\partial \log\ k'}{\partial T} + 2.303RT^2\ \frac{\partial \log\ \mathrm{K}}{\partial T} \tag{8}$$

or consequently, taking Eq. (1) and the van't Hoff reaction isochore into consideration:

$$E_C = E'_C + W_{p,C} \tag{9}$$

which shows that the experimental activation energy, E_C, is the sum of the activation energy of the true bimolecular reaction (3), E'_C, and of the heat, $W_{p,C}$, of the equilibrium reaction (4). From Eqs. (1), (7) and (9) it follows directly:

$$\log A_C = \log A'_C + \log A_{p,C} \tag{10}$$

or, according to the theory of absolute reaction rates, that

$$\Delta S^{\ddagger} = \Delta S^{\ddagger\prime} + \Delta S^{\ddagger}_p \tag{11}$$

For isodielectric conditions the following equations, respectively, are obtained [4]:

$$E_D = E'_D + W_{p,D} \tag{12}$$

$$\Delta S^{\ddagger}_0 = \Delta S^{\ddagger\prime}_0 + \Delta S^{\ddagger}_{p,0} \tag{13}$$

Thus the non-electrical contribution $\Delta S^{\ddagger}_0$ in the Table 2 is also the sum of the corresponding constants for the reactions (3) and (4).

According to Meretoja [4], by means of the values of A_C, E_C, A_D, E_D, and the constant $E_{D\infty}$, the free energy, heat content, and entropy of activation, as well as their non-electrical and dielectric contributions can be calculated. These values for benzaldehyde in different methanol-water mixtures are given in Table 2. In so far as W_p and $\Delta S^{\ddagger}_p$ are negative, the activation energy E'_C and the frequency factor A'_C of the reaction (3) are greater than the experimental values, which would explain the unexpected low activation energy of

Table 2. The free energy, heat content, and entropy of activation and their non-electrical and dielectric contributions for the Cannizzaro reaction with benzaldehyde in different methanol-water mixtures. ΔG and ΔH in kcals, ΔS in E. U:s.

Wt. % alcohol	$D_{60°}$	$\Delta G^{\ddagger}_0$	$\Delta H^{\ddagger}_0$	$\Delta S^{\ddagger}_0$	$\Delta G^{\ddagger}_D$	$\Delta H^{\ddagger}_D$	$\Delta S^{\ddagger}_D$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
0	66.6	14.5	14.5	0	1.4	— 5.0	— 19.3	15.9	9.5	— 19.3
8.5	62.9	14.5	14.5	0	1.7	— 4.4	— 18.4	16.2	10.1	— 18.4
17.6	59.2	14.5	14.5	0	1.9	— 3.9	— 17.5	16.4	10.6	— 17.5
26.9	55.2	14.5	14.5	0	2.2	— 2.7	— 14.7	16.7	11.8	— 14.7
36.2	51.3	14.5	14.5	0	2.5	— 2.2	— 14.1	17.0	12.3	— 14.1
45.9	47.2	14.5	14.5	0	2.7	— 1.3	— 12.0	17.2	13.2	— 12.0
55.8	43.1	14.5	14.5	0	2.9	— 0.4	— 9.9	17.4	14.1	— 9.9
71.2	36.5	14.5	14.5	0	3.2	+ 0.6	— 7.8	17.7	15.1	— 7.8
87.9	29.8	14.5	14.5	0	3.5	+ 2.2	— 4.0	18.0	16.7	— 4.0
100	26.0	14.5	14.5	0	3.9	+ 5.9	+ 5.9	18.4	20.4	+ 5.9

the Cannizzaro reaction for many aromatic aldehydes (*cf.* 1, pp. 50, 51), as well as the fact that the value of the nonelectrical entropy $\Delta S_0^{\ddagger}$ in Table 2 happens to be equal to zero.

The assumption that W_p and $\Delta S_p^{\ddagger}$ are negative is very reasonable, because it is quite natural to believe that the extent of the dissociation

$$RHC(OH)O^- \rightarrow RCHO + OH^- \qquad (14)$$

increases with temperature, or that K decreases as the temperature rises.

As already mentioned, concerning the value of K nothing certain is known. However, taking into consideration that E_C' for reaction (3) must be equal to or greater than 10 200 cal,[1] we get

$$\log A_C' - \log k' \geqq \frac{10\,200}{2.303RT} = 6.70$$

As furthermore, for bimolecular reactions, the steric factor[8] $P \leqq 1$ or $A (= PZ) \leqq 2.6 \cdot 10^{14}$ (when the kinetic collision number Z is given in absolute system), we obtain at 60° C for benzaldehyde in aqueous solution:

$$\log k' = \log k - \log K = 2.30^{1} - \log K \leqq \log A_C' - 6.70 = \log 2.6 \cdot 10^{14} - 6.70 = 7.72,$$

and therefore

$$\log K \geqq -5.42$$

or

$$K \geqq 4 \cdot 10^{-6} \text{ ml.mole}^{-1}$$

The value obtained for K is so great that the complex X in Eq. (4) must be a true molecule in equilibrium with its parent substances, and not a simple collision pair with a short life time of the order of a collision duration.

If the second stage of the reaction is not much slower than the first, different kinetic velocity laws are possible. This is probably the case, *e. g.*, with nitrobenzaldehydes[1] and furfurol[9].

The values of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for the Cannizzaro reaction with a number of substituted benzaldehydes in 46 wt. per cent aqueous methanol and in

50 per cent dioxane are given in Table 3. It is interesting to note that although $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for various aldehydes are very different, the values of $\Delta G^{\ddagger}$ show much smaller differences. Also in both the solvents the $\Delta G^{\ddagger}$-values for the same aldehyde are about the same, but $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are very different. Furthermore, a comparison of the values for benzaldehyde in water given in Table 2 with those in Table 3 shows that the values in 50 per cent dioxane are about the same as in water. The influence of methanol on the reaction is much more powerful.

Table 3. The energy and entropy quantities for several substituted benzaldehydes in 46 wt. % methanol and in 50 % dioxane (25—100° C).

Solvent	46 % Methanol			50 % Dioxane		
	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
Benzaldehyde	17.2	13.2	— 12.0	17.6	9.9	— 20.6
m-Bromobenzaldehyde	14.6	11.7	— 8.8			
m-Chlorobenzaldehyde	14.9	12.2	— 8.0	15.3	6.9	— 22.5
p- »	15.7	14.2	— 4.6	16.3	7.6	— 23.4
o- »	16.5	18.6	+ 6.3	16.7	8.1	— 23.1
p-Methyl benzaldehyde	18.2	14.0	— 12.7			
p-iso-Propyl »	18.1	15.7	— 7.2			
p-Methoxy »	18.8	15.8	— 9.1			
m-Nitrobenzaldehyde	13.4	17.1	+ 11.1			
p- »	13.4	20.1	+ 21.9			
o- »	14.4	26.5	+ 36.2			
p-Dimethylaminobenzaldehyde	23.5	23.0	— 1.4			

SUMMARY

The free energy, heat content, and entropy of activation for the Cannizzaro reaction with benzaldehyde and a number of substituted benzaldehydes in various solutions, calculated on the basis of the experimental values found by Tommila, and Eitel and Lock, are given, as well as the non-electrical and dielectric contributions to these quantities for benzaldehyde in different methanol-water mixtures. This new interpretation brings strong evidence for the previously proposed mechanism for the reaction.

REFERENCES

1. Tommila, E. *Ann. Acad. Scient. Fenn.* **A LIX** 8 (1942).
2. Meretoja, A. *Ibid.* **A II** 24 (1947).

3. Hinshelwood, C. N., Laidler, K. J., and Timm, E. W. *J. Chem. Soc.* (1938) 848; *cf.* also Meretoja, A. (*l. c.*).
4. Meretoja, A. *Acta Chem. Scand.* **2** (1948) 352.
5. Eitel, A., and Lock, G. *Monatsh.* **72** (1939) 410.
6. Alexander, E. R. *J. Am. Chem. Soc.* **69** (1947) 289.
7. Geissman, T. A. *Organic reactions* Vol. II, New York (1944) p. 94.
8. Hinshelwood, C. N., and Winkler, C. A. *J. Chem. Soc.* (1936) 371.
9. Eitel, A. *Monatsh.* **74** (1942) 124.

Received March 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 365—380

Enzymatic Breakdown of Polymetaphosphate. II

B. INGELMAN and H. MALMGREN

The Institutes of Biochemistry and Physical Chemistry, University of Upsala, Sweden

In a previous paper [1] we have shown that polymetaphosphates of very high molecular weight are broken down by enzymes from some microorganisms such as *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium expansum*. We were able to show that the enzymatic breakdown of polymetaphosphate proceeds through the breaking of scattered -P-O-P- links in the polymetaphosphate chains, so that comparatively large fragments are at first formed; the hydrolysis does not occur by the removal of one or a few orthophosphoric acid groups at a time from the ends of the polymetaphosphate chains. We used preparations of polymetaphosphate having molecular weights of more than one million. Thus in our experiments a colloid of purely inorganic character was broken down by enzymatic means. (Concerning the earlier investigations on metaphosphatase see the references in our previous paper [1].)

We have continued our investigations by working out a convenient method of determining the enzyme activity of enzymes degrading polymetaphosphates. Hence it has become possible for us to investigate the influence of various factors on the velocity of the enzymatic breakdown of the polymetaphosphate with good accuracy. In these experiments we have used enzyme preparations from *A. niger*. We have also investigated the enzyme from *A. niger* regarding some of its physico-chemical properties.

Investigations on the occurrence of enzymes degrading polymetaphosphates in other organisms than those mentioned above are being carried out at present. The results will be published in a separate paper.

A non dialysable, high molecular substance of high phosphorus content has been isolated from *A. niger;* it has been described in a preliminary not [2]. The isolation of this substance, which is very like the polymetaphosphates, will be described in more detail in a future paper (where references will also be given to work dealing with the importance of the metaphosphate for micro-

organisms and substances of high phosphorus content from such micro-organisms).

The polymetaphosphates used as substrates in our investigations are designated as K 10, K 11, K 12, K 14 and K 15 of which all except K 15 has been described in previous papers [1, 3-6]. These polymetaphosphates are prepared by heating primary potassium orthophosphate. n in the formula has

$$n\,KH_2PO_4 \rightarrow (KPO_3)_n + n\,H_2O$$

magnitudes of the order 2,000—20,000. For the experiments the potassium metaphosphate is dissolved in buffer solutions containing an excess of sodium ions, whence the polymetaphosphate in solutions may be regarded as a sodium salt. (The potassium metaphosphate is insoluble in pure water.) Some data of the substances used, *viz.* the temperature of the synthesis, the intrinsic viscosity (defined as the limiting value $(\eta_{sp}/c)_{c\rightarrow 0}$) and the molecular weight have been collected in Table 1. The intrinsic viscosity was determined in 0.4 *N* NaCl. The molecular weights determined (in 0.4 *N* NaCl) by means of sedimentation in the ultracentrifuge and diffusion refer to the original $(KPO_3)_n$. The preparations are of course polydisperse. (Some of the molecular weight determinations were carried out some years ago [4], whereas the intrinsic viscosity measurements have been performed this year. It may happen that the polymetaphosphate preparations have altered a little during the storage.)

For the enzyme preparation we have used cultures of *A. niger* van Tieghem (no. 594 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council) [cf. 7]. The mould was cultivated at 28° C on a medium of the following composition: 10 g glucose, 0.2 g K_2HPO_4, 0.4 g $NaNO_3$, 0.05 g $MgSO_4$, 7 H_2O and 100 ml water. The time of cultivation has varied but generally the enzyme preparation was begun after about 7 days growth.

The enzyme solutions have been prepared in the following manner: After addition of a suitable quantity of acetic acid — sodium acetate buffer (pH = 5.4; ionic strength = 0.1) the mould was ground up in a »Waring blender» and allowed to stand over night at a few degrees above zero. The mass was then filtered and the solution obtained dialysed against distilled water for some days. The solution was then evaporated at $\sim 35°$ *in vacuo* to about 1/20th of the original volume. After this the solution was dialysed against the buffer to be used in the experiments. Before being used the solution was centrifuged and filtered. In spite of a comparatively low nitrogen content these solutions have such a large enzyme activity that they must be diluted many times with a suitable buffer before being used for the study of the

Table 1. Some physico-chemical data of the polymetaphosphate preparations.

Preparation	Temperature of synthesis °C	Intrinsic viscosity	Mol. wt.
K 10	290	0.48	460,000
K 11	445	1.13	2,000,000
K 12	260	0.73	280,000
K 14	495	0.80	1,300,000
K 15	500	0.92	1,200,000

polymetaphosphate degradation. The enzyme solutions contain not only the enzyme degrading polymetaphosphate but also, of course, other high molecular products from the mould, for instance an enzyme degrading cellulose; this enzyme can destroy the cellophane bags used for dialysis. Sometimes it has been necessary, therefore, to use bags of nitrocellulose for dialysis of the concentrated enzyme solutions. Several different enzyme preparations have been used in our experiments. We have started experiments to fractionate the impure enzyme solution in order to obtain the enzyme for the degradation of polymetaphosphate in a purer state.

Calculations of the enzyme activity from viscosity measurements

In our previous work we followed the enzymatic breakdown of the polymetaphosphate mainely by means of viscosity measurements. As these polymetaphosphate solutions have a comparatively high viscosity it is easy to follow the first phase of the breakdown by viscosity measurements. Therefore in this work we have used the same method; the viscosity measurements have been performed in a capillary viscosimeter according to Ostwald. The viscosity of the polymetaphosphate solution has been measured relative to that of the buffer solutions. From the relative viscosity (η/η_o) the specific viscosity has been calculated ($\eta_{sp} = \eta/\eta_o - 1$). All viscosity measurements in this work have been performed in a thermostat at 25.0°C.

When calculating the enzyme activity it is particularly valuable to determine the velocity of the breakdown during its first phase. However the derivative $d\eta_{sp}/dt$ at $t = 0$ is not easy to determine with sufficient accuracy, and is very dependent on the degree of polymerisation of the subtrate and the ionic strength; hence it can only be used as a measure of the breakdown velocity

under very well-defined conditions. Therefore we have used the method of calculation described below.

A somewhat analogous degradation, namely the breakdown of starch by α-amylase has been described by, among others, Hultin [8]. He has deduced and applied the following formula for the enzyme activity A:

$$A = c^2 \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{1}$$

where c is the substrate concentration, η_{sp} the specific viscosity and t the time. We have tried to apply this formula for the polymetaphosphatase activity but in our case conditions are more complicated. The viscosity of a polymetaphosphate solution is not only dependent on the concentration of the colloid but also on the concentration and species of the low molecular salts in the solution. On addition of salt to a polymetaphosphate solution the viscosity decreases, at first very rapidly, then more moderately, but without approaching a limiting value before the salting out of the polymetaphosphate occurs [5]. Kern [9] has proposed to regard the specific viscosity of a colloidal electrolyte as the product of two factors, the ionic factor and the macromolecular factor allowing for the charge effect and the degree of polymerization of the colloid respectively

$$\eta_{sp}/c = J \cdot (\eta_{sp}/c)_m \tag{2}$$

where J is the ionic factor and $(\eta_{sp}/c)_m$ is the macromolecular factor. Kern's work concerns polyacrylic acid and its sodium salt, but assuming Eq. (2) to be valid for the polymetaphosphates also, it is suggested to write Hultin's formula (Eq. (1)) in the following form for the present.

$$A = J \cdot c^2 \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{3}$$

However, it is very difficult to determine the ionic factor J. As an approximation we may assume J to be independent of the substrate concentration and constant during the first phase of the enzymatic breakdown and proportional to the spec. viscosity at $t = 0$ $((\eta_{sp})_{t=0})$, but if we do so we must not forget that $(\eta_{sp})_{t=0}$ is dependent not only on the substrate concentration but also on the molecular size and shape. Hence it follows that if we replace J by $(\eta_{sp})_{t=0}$ the

measurements of the enzyme activities must be carried out on the same substrate and at the same substrate concentration if the activities are to be compared. Of course it is desirable to evaluate the absolute value of J, or of least a relative one, for all polymetaphosphate preparations and all concentrations and in different salt solutions but this work — if possible to perform — must be carried out later. As a relative measure of the enzyme activity in comparison experiments (the same substrate and substrate concentration) we use a quantity z defined by Eq. (4)

$$z = (\eta_{sp})_{t=0} \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{4}$$

The z-values are calculated from the curves which are obtained when the values of $(\eta_{sp})_{t=0}/(\eta_{sp})_{t=t}$ are plotted against t.

(It may be pointed out that if Eq. (2) is valid and the ionic factor can be regarded constant during the first phase of the breakdown in a certain experiment we may write

$$\frac{(\eta_{sp})_{t=0}}{(\eta_{sp})_{t=t}} = \frac{[(\eta_{sp})_m]_{t=0}}{[(\eta_{sp})_m]_{t=t}} \tag{5}$$

The factor J cancels out by division of the equations corresponding to the times $t = 0$ and $t = t$ respectively. The quantities $(\eta_{sp})_{t=0}$ and $[(\eta_{sp})_m]_{t=0}$ are constants for each experiment. However, it must be emphasized that the application of the equation of Kern and the assumption that the ionic factor J is constant during the first phase of the breakdown have rather weak foundations.)

The z-values are usually suitable as a relative measure of the enzyme activity (the substrate and the substrate concentration being the same), as the z-values are rather independent of the variations in the salt concentrations which may occur. At varying salt concentrations and ionic strengths, but other factors such as substrate concentration and enzyme concentration pH etc. being constant, the $d\ (1/\eta_{sp})/dt$-values differ very much whereas the z-values differ but slightly. This is illustrated by experiments described below. (See Table 2). However, the determinations ought to be carried out at salt concentrations as high as possible but not too near the point of salting out.

When plotting $1/\eta_{sp}$ or $(\eta_{sp})_{t=0}/\eta_{sp}$ against t we usually get a linear relation. This is a support for the assumption that the ionic factor is rather constant during the first phase of the breakdown. Some typical curves are shown in Figs. 1 and 2 illustrating the change of the spec. viscosity with time for various

enzyme concentrations and the corresponding $(\eta_{sp})_{t=0}/\eta_{sp}$-values as a function of t. (t is the time that has elapsed from the addition of the enzyme to the beginning of the viscosity measurements + half the time of outflow). The experiments in question are described below.

Experiments at different ionic strengths

As has been mentioned already the viscosity of a polymetaphosphate solution is very dependent on the ionic strength and the species of the low molecular salts present. However, even if the original η_{sp} values and the decrease of η_{sp} with time differ very much with the ionic strength, almost the same z-values are obtained within the μ-interval convenient for these experiments (if enzyme and substrate concentration, pH, etc., are constant). This is illu-

Table 2. Experiments at different ionic strengths.

Substrate	Ionic strength	$(\eta_{sp})_{t=0}$	$d\left(\frac{1}{\eta_{sp}}\right)/dt \cdot 10^4 \text{min}^{-1}$	$z \cdot 10^4 \text{min}^{-1}$
K 11	0.30	0.62	416	258
in acetate	0.20	1.62	159	258
pH = 4.6	0.10	3.72	67	250
	0.05	7.14	31	220
K 15	0.22	2.20	133	292
in phosphate	0.15	3.00	97	292
pH = 6.4	0.09	5.20	51	263

strated by the following experiments. To samples of 5 ml of 0.5 % polymetaphosphate solutions in buffers of different ionic strengths was added 1 ml of enzyme solution. (Different enzyme preparations in the two series.) The measurements were made at 25.0° C. The results are collected in Table 2.

As is seen from the table the z-values are rather constant in each series, whereas the $d(1/\eta_{sp})/dt$-values vary greatly with the ionic strength. This is a good support for the use of the z-values in comparison experiments where the salt concentration may vary a little. However, there seems to be a tendency for the z-values to decrease with decreasing ionic strength. For our purposes this fact has no importance as our measurements are generally carried out at ionic strengths where z varies very little with μ. Wheather the equation of Kern is valid or not, the z-values have proved to be useful in comparison measurements of the enzyme activity at varying salt concentrations.

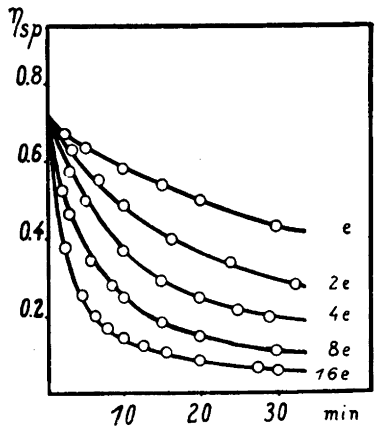


Fig. 1. Variation of η_{sp} with time during the enzymatic breakdown. Different concentrations of the enzyme. The curves refer to experiment no. I in Table 3.

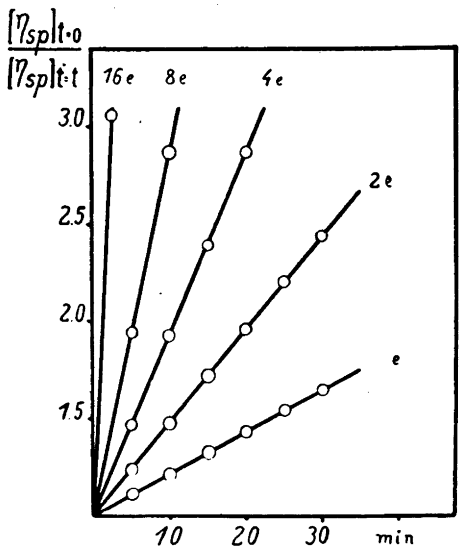


Fig. 2. Variation of $(\eta_{sp})_{t=0}/(\eta_{sp})_{t=t}$ with time. Different concentrations of the enzyme. The curves refer to experiment no. I in Table 3.

Experiments at different enzyme concentrations

If the enzymatic breakdown of the polymetaphosphate occurs in the same manner as assumed in the deduction of Hultin's formula, one can expect a direct proportionality between the z-values obtained and the enzyme concentrations if all other factors such as pH, substrate concentration, etc., are constant. We have performed some series of measurements in order to investigate if there exists such a relation under the experimental conditions used by us. These measurements were made at 25.0° C in an acetate buffer of ionic strength 0.3 and pH 5.4. To samples of 5 ml of a 0.5 % polymetaphosphate solution in this buffer was added 1 ml of enzyme solutions of different dilutions in the same buffer.

The curves of one such typical series are shown in Figs. 1 and 2 where the solution of the lowest enzyme concentration is designated as e and the solutions of higher enzyme concentrations as multiples of e. The z-values of this series are obtained from the slopes of the straight lines in Fig. 2. The results of the measurements of some series are collected in Table 3. Different enzyme stock solutions have been used in the three series. In the table are given the

Table 3. Experiments at different enzyme concentrations.

Expt. no.	Relative enzyme concentration	$z \cdot 10^4$ min^{-1} (experimental value)	Method of least squares $z \cdot 10^4$ min^{-1}
I	16 e	3690	3680
	8 e	1800	1840
	4 e	945	920
	2 e	480	460
	e	220	230
	6 e′	190	187
	4 e′	123	125
II	2 e′	61	62
	e′	27	31
	24 e″	1600	1590
III	12 e″	768	794
	2 e″	134	132
	e″	60	66

determined z-values as well as the z-values calculated from the experimental data according to the method of least squares on the assumption that z is a linear function of the enzyme concentration.

As is seen from the table the values agree rather well and it may be justified to regard the z-values as a relative measure of the enzyme concentration under similar conditions (the same substrate and the same substrate concentration etc.). Of course, in this case, the $d(1/\eta_{sp})/dt$-values might be used as well as the z-values, for these differ only by the constant factor $(\eta_{sp})_{t=0}$ provided that the saltconcentration is exactly the same. However, comparatively small changes in the ionic strength generally have so great an influence on the specific viscosity that the z-values are to be preferred in comparison measurements of the activity. Thus we have shown that if the concentrations of the polymetaphosphate and the enzyme are chosen in an interval convenient for measurements, as in our case, z is proportional to the enzyme concentration.

EXPERIMENTS AT DIFFERENT SUBSTRATE CONCENTRATIONS

In Eq. (1) the concentration of the substrate is involved as a squared factor. However, Eq. (1) is derived on the assumption that Staudinger's formula is valid, which does not seem to be strictly true for the polymetaphosphates.

In order to investigate whether Eq. (3) is valid for the enzymatic breakdown of polymetaphosphate we write Eq. (3) in the more common form represented by Eq. (6).

$$A = J \cdot c^n \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{6}$$

Writing this equation in a logarithmic form we have

$$\log \frac{A}{J} = \log \left(\frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \right) + n \log c \tag{7}$$

At different substrate concentrations in the same buffer the contribution to the ionic strength from the dissociated polymetaphosphate cannot be expected to be the same. For the present we neglect this contribution to the ionic strength and assume J constant at different substrate concentrations for a certain polymetaphosphate preparation when the salt concentration is constant. Under this assumption we have determined the exponent n in Eq. (6) by measuring the $d(1/\eta_{sp})/dt$-values for solutions of various substrate concentrations, the enzyme concentration and the salt concentration etc. being constant. The experiments were performed in acetate buffers of ionic strength 0.3 and pH 5.4. Four series of measurements have been carried out on different polymetaphosphate preparations. Different enzyme preparations of varying concentrations have been used in these four series, but of course the same preparation within a given series. The experimental c- and $d(1/\eta_{sp})/dt$-values have been put in Eq. (7) and n has been calculated according to the method of least squares. The results of these measurements are collected in Table 4.

The n-values obtained are less than 2. However, it must be remembered that in some respects there are large differences between the properties of starch and polymetaphosphate. As is seen from Table 4 n is about 1.7 in all the series. The variation in the n-values is perhaps to be ascribed to differences in the molecular shape and size and polydispersity of the substrates (in addition to experimental errors). If the contribution of the polymetaphosphate to the ionic strength at varying substrate concentration cannot be neglected, the difference of the n-values from 2 may be a consequence of the dependence of J on the substrate concentration rather than a deviation from Eq. (3). However, the experimental material does not yet permit the drawing of final conclusions.

Table 4. Experiments at different substrate concentrations.

Substrate	Substrate concentration (%)	$d(1/\eta_{sp})/dt \cdot 10^4$ min^{-1}	n
K 10	0.125	13370	1.64
	0.250	3940	
	0.500	1270	
	0.750	860	
	1.000	380	
K 11	0.167	960	
	0.333	320	1.75
	0.667	86	
K 12	0.500	2200	1.62
	0.750	1180	
	1.000	710	
K 15	0.104	3380	
	0.208	1010	1.85
	0.416	260	
	0.624	120	
	0.832	75	
		mean value: $n = 1.71$	

Experiments with different polymetaphosphate preparations

Although the reaction mechanism of the enzymatic breakdown of the polymetaphosphate is probably the same irrespective of the polymetaphosphate preparation used, the $d(1/\eta_{sp})/dt$-values of various preparations differ somewhat, even if the experimental conditions such as pH, substrate and enzyme concentrations, ionic strength, etc., are identical. This is illustrated by some experiments, the data of which are collected in Table 5. These investigations were carried out in acetate buffer of pH 5.4 and ionic strength 0.3 (expt. I) and phosphate buffer of pH 5.7 and ionic strength 0.3 of which 0.2 was from NaCl (expt. II). The solutions contained 0.5 % of polymetaphosphate. To 5 ml of each of these solutions was added 1 ml enzyme solution (different enzyme preparations in the two series) and $d(1/\eta_{sp})/dt$ was determined in the usual manner.

Table 5. Experiments with different polymetaphosphate preparations.

Preparation		$(\eta_{sp})_{t=0}$	$d\left(\frac{1}{\eta_{sp}}\right)/dt \cdot 10^4$ min^{-1}
Expt. I	K 12	0.49	667
	K 14	0.55	291
	K 15	0.74	337
	K 11	0.82	306
Expt. II	K 10	0.42	298
	K 12	0.80	430
	K 14	0.87	258
	K 15	1.13	282
	K 11	1.24	311

As is seen from the Tables 5 and 1, it is difficult to find any relation between the molecular constants and the $d(1/\eta_{sp})/dt$-values. In neither of the two series are these values constant, but they do not differ very much with the exception of the value for K 12. It is possible that in addition to experimental errors the molecular size, shape, and polydispersity of the different preparations are responsible for the variation in the values, because the ionic factor J might depend not only on the salt concentration but also on the molecular properties mentioned above. K 12 seems to give somewhat higher $d(1/\eta_{sp})/dt$-values than the other preparations, which might be due to its polydispersity. K 12 probably contains a fraction of relatively low molecular polymetaphosphate, which fraction does not satisfy the assumptions in the derivation of the Hultin formula, where all the substrate is considered to be sufficiently high molecular. That would mean that the »effective concentration» is less than the analytical one and therefore the experiments with K 12 cannot be compared with the others.

The unit of the enzyme activity will not be given yet, as we cannot determine the ionic factor J in Eq. (3). In future perhaps it will be possible to choose an arbitrary J_o value for a certain preparation under well defined conditions and then measure J relative to this J_o under other conditions.

Experiments at different pH-values

In our preliminary investigations it was demonstrated that the enzyme from *A. niger* degrading polymetaphosphate, has its optimal activity at an acid pH. However, a more accurate determination of the influence of the pH

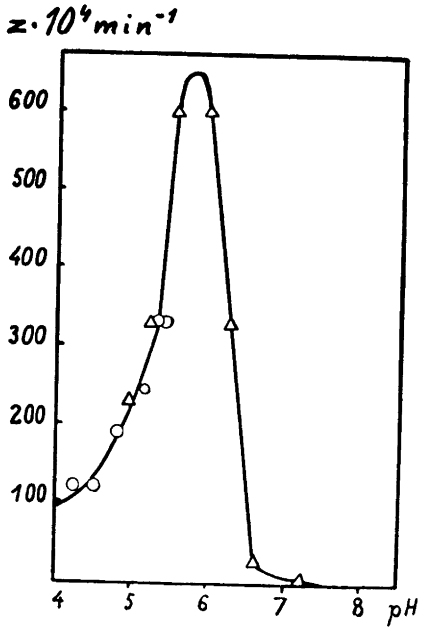


Fig. 3. The influence of pH upon the activity. O = *acetate buffers.* △ = *phosphate buffers.*

on the activity was not made at that time and therefore we have now carried out a series of measurements on the variation of z with the pH. The experiments have been performed in the following manner. The polymetaphosphate (preparation K 14) was dissolved in sodium acetate-acetic acid and sodium phosphate buffers of different pH but of the same ionic strength, 0.3 (0.1 from the buffer substances and 0.2 from NaCl). The solutions contained 0.5 % of the polymetaphosphate. To samples of 5 ml of these solutions was added 1 ml of an enzyme solution and the breakdown was followed as usual. The enzyme used in these measurements was taken from a freshly prepared stock solution which was diluted 10 times with water. As the determinations were performed in a rather short time the enzyme activity of the stock solution may be considered constant. (The activities of our freshly prepared enzyme solutions usually remain constant for several days if the solutions are stored in a refrigerator.) In very acid medium the z-values are to be corrected for the spontaneous breakdown of the substrate, but in the pH-range of our measurements these corrections are mostly rather negligible and have only been applied at the lowest pH values. In Fig. 3 the z-values obtained are plotted against the pH. As is seen from the figure the maximum at about pH 5.7 is rather pronounced. It may also be pointed out that the z-values in acetate and phosphate buffers of the same pH agree very well, whereas the $d(1/\eta_{sp})/dt$-values in dif-

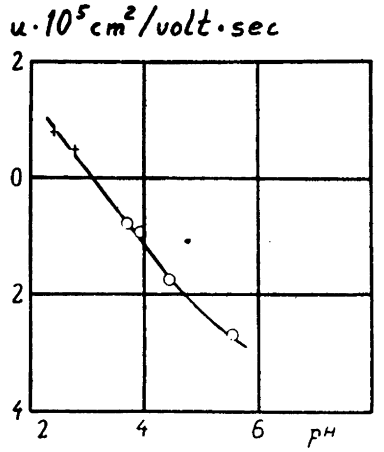


Fig. 4. Variation of the electrophoretic mobility of the enzyme with pH at a temperature of 0°.0 C. ○ = *acetate buffers.* + = *citrate buffers.*

ferent buffers of the same pH may differ very much. A curve where $d(1/\eta_{sp})/dt$ is plotted as a function of pH shows irregularities depending on the fact that the contribution of the ionic factor to the viscosity is not the same in different buffers.

SOME PHYSICO-CHEMICAL PROPERTIES OF THE ENZYME

Electrophoretic mobility and the isoelectric point

The electrophoretic mobility of the enzyme has been studied in the Tiselius apparatus (provided with two pairs of short cells [10]). However it was not possible to use the Svensson optical method of registering the electrophoretic movement because the concentration of the enzyme was too low to give a sufficient increment of refractive index. Therefore we measured instead the enzyme activity of the solutions in the different cells of the Tiselius apparatus after the electrophoretic runs as well as the activity of the original enzyme solution used to fill the apparatus. Thus, for instance, if after the experiment the activity of the solution in the upper cell on the ascending side (the activity measured after stirring) is half the activity of the original enzyme solution, the mobility was calculated on the assumption that the boundary had moved half the length of the upper cell, the dimensions of which are known.

A series of electrophoretic experiments has been performed at different pH-values in acetate and citrate buffers of ionic strength 0.1. (The enzyme solutions dialysed against citrate buffers and that dialysed against acetate buffer of pH 4.5 were made 0.3 % with respect to saccharose in order to get a

more stable boundary, as the enzyme concentration is very low.) The electrophoretic experiments were carried out at 0.0° C; the current strength was 15—20 mA. The activity of the samples taken out after the experiments has been determined at 25° C under identical conditions, which in this case means that 0.5 % solutions of polymetaphosphate in an acetate buffer of pH 5.4 and ionic strength 0.3 were used for the activity measurements, which were performed as described above. As electrophoretic mobility u we have here adopted the mean value of the mobilities of the ascending and the descending side. In the experiments in citrate buffers the mobility has been corrected for the rather high viscosity of these buffers. Fig. 4 shows the mobility as a function of the pH. As is seen this enzyme has its isoelectric point at a rather acid pH, about 3.1. Thus the isoelectric point differs by more than 2 pH-units from the pH of the optimal activity.

Determination of the sedimentation constant

We have determined the sedimentation constant of the enzyme in the Svedberg ultracentrifuge. However, it has not been possible to use the ordinary optical method (»the scale method») for recording the sedimentation, as the enzyme was not pure and the concentration too low to use such a method. These difficulties have been overcome by the use of a separation cell instead of the ordinary ultracentrifuge cell and we have succeeded in determining the sedimentation constant s with a fairly good accuracy. (Concerning the construction and the theory of the separation cell see [11, 12]). After the centrifugation runs the solutions in the upper and the lower parts of the cell were taken out separately and the activity of both solutions was measured, and, of course, also that of the original solution. From these data s was calculated. For details concerning the sedimentation in the ultracentrifuge we refer to the book of Svedberg and Pedersen [12]. It may be mentioned here that it is rather difficult to take out all the substance sedimented to the bottom part of the cell. Hence the s value obtained from the upper part of the cell is to be regarded more reliable than that calculated from the increase of the concentration in the lower part of the cell. Some runs were carried out in acetate buffer of pH 5.4 and ionic strength 0.3; centrifugal field 250,000 times gravity (59,000 r. p. m.). Four of the s-values obtained (upper part of the cell) agree very well: 3.18, 3.11, 3.15 and 3.22 S (Svedberg units). (In two cases we get lower s-values because of convections during the stopping of the rotor.) As a mean value we adopt $s_{20} = 3.2$ S. (We are indebted to Mr. E. Hellman for carrying out the numerical calculations of the sedimentation constant.)

Determination of the diffusion constant

The diffusion constant of the enzyme has been determined according to »the porous-disk method»[13, 14] as being the most convenient in this case. As »porous-disk» we have used a »Jena glass filter G4». The apparatus was calibrated by means of a 0.1 *M* KCl solution the diffusion coefficient of which is known [15]. The experiments were performed at 0.1° C in acetate buffer of pH 5.4 and ionic strength 0.1. The activity measurements necessary for the calculations were carried out as described before. The diffusion constant thus obtained was corrected to pure water of 20.0° C. $D_{20} = 8.8 \times 10^{-7}$ cm²/sec.

The molecular weight of the enzyme

In Svedberg's wellknown formula of the molecular weight [12] the partial spec. volume V of the substance is also involved. It has not been possible to make a direct determination of V but as the partial spec. volume of proteins in most cases is about 0.73 within a small range of variation we assume this value to be valied for this enzyme also. On this assumption the molecular weight was calculated to be about 33,000.

SUMMARY

The enzymatic breakdown of polymetaphosphate of very high molecular weight with an enzyme extract from *Aspergillus niger* has been studied by viscosity measurements. A convenient method of determing the enzyme activity has been worked out. The influence of enzyme concentration, substrate concentration, ionic strength and pH on the breakdown velocity has been studied. Some physico-chemical properties of the enzymes degrading polymetaphosphate has also been investigated.

The authors wish to thank Prof. T. Svedberg and Prof. A. Tiselius for the privilege of carrying out this work in their laboratories. We are grateful for the valuable technical assistance rendered by Miss. A. Karlsson and Mrs. M. Hårsta. We also whish to thank Fil.lic. E. Hultin for valuable discussions.

REFERENCES

1. Ingelman, B., and Malmgren, H. *Acta Chem. Scand.* **1** (1947) 422.
2. Ingelman, B. *Acta Chem. Scand.* **1** (1947) 776.
3. Lamm, O., and Malmgren, H. *Z. anorg. Chem.* **245** (1940) 103.
4. Malmgren, H., and Lamm, O. *Z. anorg. Chem.* **252** (1944) 255.
5. Mamlgren, H. *Acta Chem. Scand.* **2** (1948) 147.

6. Malmgren, H. *Acta Chem. Scand.* (to be published).
7. Thom, C., and Raper, K. *A manual of the Aspergilli.* Baltimore (1945).
8. Hultin, E. *Acta Chem. Scand.* **1** (1947) 269.
9. Kern, W. *Z. physik. Chem. A* **181** (1938) 283.
10. Svensson, H. *Arkiv Kemi, Mineral. Geol.* **A 22** (1946) no. 10.
11. Tiselius, A., Pedersen, K. O., and Svedberg, T. *Nature* **140** (1937) 848.
12. Svedberg, T., and Pedersen, K. O. *The ultracentrifuge* Oxford (1940).
13. Northrop, J., and Anson, M. L. *J. Gen. Physiol.* **12** (1928—29) 543, **20** (1937) 575.
14. McBain, J. W., and Liu, T. H. *J. Am. Chem. Soc.* **53** (1931) 59.
15. Lamm, O. *Nova Acta Regiae Soc. Sci. Upsaliensis* **IV 10** (1937) no. 6.

Received March 20, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 381—384

Short Communications

Quaternary Derivatives of some Anaesthetic, Spasmolytic, Analgesic and Antihistaminic Agents

KAI ARNE JENSEN, MARTIN LAURIDSEN AND JENS ANDERS CHRISTENSEN

Research Laboratory of A/S Ferrosan, Copenhagen, Denmark

Cholinesterase is inhibited by a great variety of drugs [1], among these atropine and other drugs with spasmolytic activity. Certain local anaesthetics [2] and some analgetics [3], like dolantin (demerol, pethidin), in larger doses antagonize cholinesterase too, but it is uncertain whether this action is of any importance for their pharmacological effects, although Thimann [4] has put forward the theory that local anaesthetics compete with acetylcholine for the receptor substances of nerve endings.

As acetylcholine is a quaternary ammonium compound it would be anticipated that quaternary ammonium compounds should inhibit cholinesterase more strongly than tertiary, at any rate if their activity is due to a drug-receptor combination. As a matter of fact it has been shown that the spasmolytic activity of scopolamine and some other spasmolytic drugs is enhanced by quaternization [5]. On the other hand Ehrlich [6] found that the methoiodide of cocaine and Löfgren and Fischer [7] that the ethochloride of xylocaine were devoid of anaesthetic activity.

On the whole very few quaternary derivatives of drugs, that are tertiary amines, have been described. For example we have not been able to find other quaternary derivatives of local anaesthetics than the two above mentioned. With a view to studying the effect of quaternization somewhat more systematically we have prepared quaternary derivatives of some of the common local anaesthetics, of the analgesic (and spasmolytic) drugs dolantin and amidone and of the antispasmodic drug trasentin.

In this connexion we have also prepared quaternary derivatives of some antihistaminic agents, although in this case there is no reason for supposing that antagonism with acetylcholine should be of any importance for their activity.

The pharmacological effects of the new quaternary compounds are being studied by Dr. Kjerulff-Jensen and Dr. Grandjean of this laboratory. The following, purely preliminary, results were obtained:

1. Quaternary compounds derived from local anaesthetics have only weak anaesthetic effect, but at any rate in the case of derivatives of nupercaine, the activity is not completely abolished.

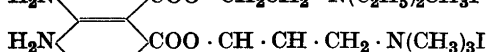
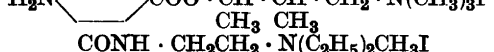
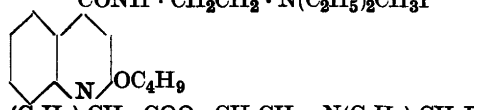
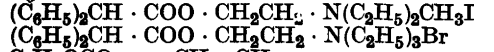
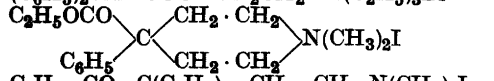
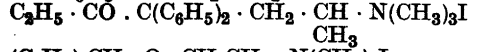
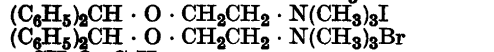
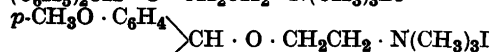
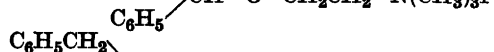
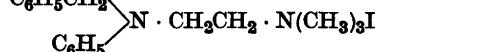
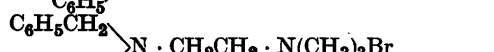
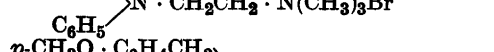
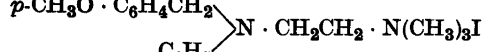
2. Quaternary compounds derived from antispasmodics retain the antispasmodic effect.

3. Quaternary derivatives of the analgesic drugs amidone and dolantin seem to be without or almost without analgesic effect.

4. Quaternary derivatives of antihistaminic drugs retain their antihistamine effect and in some cases the toxicity is lowered.

The compounds could be prepared by dissolving the tertiary compounds in ethanol and adding alkyl halogenide; in cases where the tertiary amine was on

Table 1. The melting points and analyses of the prepared compounds.

Structure	Quaternary deriv. of	M. p. °C	% N calc.	% N found	Recryst. from	Solubility in water
	Procaine	138	7.41	7.25	A	s.
	Tutocaine	170	7.15	6.92	A + E	s.
	Nupercaine	114	8.65	8.69	Ac + E	s.
	Trasentin	117-123	3.09	3.12	Ac + E	sl. s.
	Trasentin	155	3.32	3.30	A + E	v. s.
	Dolantin	196	3.60	3.55	A	s.
	Amidone	165	3.10	3.11	Ac + E	s.
	Benadryl	194	3.53	3.47	A	sl. s.
	Benadryl	197	3.99	3.93		s.
	—	144	3.28	3.18	A	sl. s.
	Antergan	158	7.07	6.93	A	sl. s.
	Antergan	196	8.02		A	v. s.
	—	184	6.58	6.57	A	sl. s.
	Pyribenzamin	d.	10.58	10.18	A	sl. s.
	Neoantergan	131	9.82	10.02	A	s.

Abbreviations: A = alcohol, E = ether, Ac = acetone, sl. s. = slightly soluble (< 1 : 50), s. = soluble (1 : 10—1 : 50), v. s. = very soluble (> 1 : 10). The analyses were performed by the micro-Kjeldahl method. Micro-Dumas determinations consistently gave too high values, presumably due to the formation of methane (resp. ethane) which is not completely oxidized. We are indebted to Mr. O. Rosenlund Hansen for carrying out the analyses.

hand as hydrochloride, this was decomposed by sodium hydroxide, the amine extracted with ether and after drying with sodium sulphate the alkyl halogenide was added to the ethereal solution. After standing for from 24 hours to several days at ordinary temperature or in the refrigerator a crystalline precipitate had generally formed. The ethobromide of trasentin was prepared by heating a benzene solution of trasentin and ethyl bromide in a closed vessel at 100° for 8 hours.

Paper Chromatography of Primary Aromatic Amines

BIRGER EKMAN

Laboratory of Clinical Chemistry and Department of Physiological Chemistry, University of Lund, Sweden

The method of Consden, Gordon and Martin [1] for the analysis of amino acids by paper chromatography has been applied for the separation, identification and quantitative determination of various other substances, for instance sugars (Partridge [2], Flood, Hirst and Jones [3]), organic acids (Lugg and Overell [4]), adrenaline and associated compounds (James [5]), flavin nucleotides (Crammer [6]).

We have used this method for the analysis of primary aromatic amines. The procedure and the apparatus have been in principle the same as that of Consden *et al.*[1]. For the development of the spots we have diazotized the amines with sodium nitrite in acid solution. By coupling the resulting diazonium salts with ethyl-α-naphtylamine a characteristic red-violet colour is developed. Mixtures of benzene, amyl and methyl alcohol saturated with water have been found most suitable as solvents with regard to the differentiation of various amines and have the advantage of a short running time (4—6 hours at room temperature).

Procedure. Spots comprising the samples and containing 10—20 γ of the amines no. 1—13 and 100—200 γ of no. 14—17 in Table 1. are placed in the usual way on the paper. According to Edman [7] we have used the quick-filtering paper no. OB (50 × 50 cm), supplied by J. H. Munktells Pappersfabriksaktiebolag, Grycksbo, Sweden. After running the paper, it is dried at about 80° C till most of the organic solvents have disappeared. The paper is then sprayed with a 0.2 % sodium nitrite solution in 0.1 *N* HCl. After drying for some minutes at 50—80°, the paper is sprayed with a solution of 0.2 % ethyl-α-naphtylaminehydrochloride in conc. ethyl alcohol. The spots generally turn coloured immediately and are not influenced by heating. It is, however, easier to observe spots only faintly coloured, if the paper is dry.

Results. The R_F values for 17 primary aromatic amines run in various combinations of benzene, amyl and methyl alcohol, with or without the addition of hydrochloric acid or ammonia, are given in Table 1. Mixtures of amines are easily separated.

The compounds were as a rule recrystallized from ethanol. The compounds derived from tutocaine, trasentin, amidone and nupercaine were purified by dissolving in ethanol, resp. acetone and precipitating with ether.

The quaternary compounds are white, beautifully crystalline compounds, slightly soluble in alcohol and almost insoluble in ether and benzene. The bromides are easily soluble in water, the iodides, however, are slightly soluble also in water.

The methoiodide of trasentin is unstable when moist: It rapidly turns brown and deliquesces. When pure and dry it is quite stable.

The melting points and analyses of the quaternary derivatives are presented in Table 1.

1. Bernheim, F. *The interaction of drugs and cell catalysts* Minneapolis (1942).
2. Ammon, I., and Zipf, K. *Klin. Wochschr.* **20** (1941) 1176; Adriani, J., and Rovenstine, E. A. *Anesthesia & Analgesia* **20** (1941) 109.
3. Bernheim, F. *(l. c.)*
4. Thimann, K. V. *Arch. Biochem.* **2** (1943) 87.
5. Nyman, E. *Acta Physiol. Scand.* Suppl. X (1942).
6. Fränkel, S. *Arzneimittelsynthese* 5. Aufl. Berlin (1921) p. 337.
7. Löfgren, N., and Fischer, I. *Svensk Kem. Tid.* **58** (1946) 219.

Received July 9, 1948.

Paper Chromatography of Primary Aromatic Amines

BIRGER EKMAN

Laboratory of Clinical Chemistry and Department of Physiological Chemistry, University of Lund, Sweden

The method of Consden, Gordon and Martin [1] for the analysis of amino acids by paper chromatography has been applied for the separation, identification and quantitative determination of various other substances, for instance sugars (Partridge [2], Flood, Hirst and Jones [3]), organic acids (Lugg and Overell [4]), adrenaline and associated compounds (James [5]), flavin nucleotides (Crammer [6]).

We have used this method for the analysis of primary aromatic amines. The procedure and the apparatus have been in principle the same as that of Consden *et al.*[1]. For the development of the spots we have diazotized the amines with sodium nitrite in acid solution. By coupling the resulting diazonium salts with ethyl-α-naphtylamine a characteristic red-violet colour is developed. Mixtures of benzene, amyl and methyl alcohol saturated with water have been found most suitable as solvents with regard to the differentiation of various amines and have the advantage of a short running time (4—6 hours at room temperature).

Procedure. Spots comprising the samples and containing 10—20 γ of the amines no. 1—13 and 100—200 γ of no. 14—17 in Table 1. are placed in the usual way on the paper. According to Edman [7] we have used the quick-filtering paper no. OB (50 × 50 cm), supplied by J. H. Munktells Pappersfabriksaktiebolag, Grycksbo, Sweden. After running the paper, it is dried at about 80° C till most of the organic solvents have disappeared. The paper is then sprayed with a 0.2 % sodium nitrite solution in 0.1 *N* HCl. After drying for some minutes at 50—80°, the paper is sprayed with a solution of 0.2 % ethyl-α-naphtylaminehydrochloride in conc. ethyl alcohol. The spots generally turn coloured immediately and are not influenced by heating. It is, however, easier to observe spots only faintly coloured, if the paper is dry.

Results. The R_F values for 17 primary aromatic amines run in various combinations of benzene, amyl and methyl alcohol, with or without the addition of hydrochloric acid or ammonia, are given in Table 1. Mixtures of amines are easily separated.

The compounds were as a rule recrystallized from ethanol. The compounds derived from tutocaine, trasentin, amidone and nupercaine were purified by dissolving in ethanol, resp. acetone and precipitating with ether.

The quaternary compounds are white, beautifully crystalline compounds, slightly soluble in alcohol and almost insoluble in ether and benzene. The bromides are easily soluble in water, the iodides, however, are slightly soluble also in water.

The methoiodide of trasentin is unstable when moist: It rapidly turns brown and deliquesces. When pure and dry it is quite stable.

The melting points and analyses of the quaternary derivatives are presented in Table 1.

1. Bernheim, F. *The interaction of drugs and cell catalysts* Minneapolis (1942).
2. Ammon, I., and Zipf, K. *Klin. Wochschr.* **20** (1941) 1176; Adriani, J., and Rovenstine, E. A. *Anesthesia & Analgesia* **20** (1941) 109.
3. Bernheim, F. *(l. c.)*
4. Thimann, K. V. *Arch. Biochem.* **2** (1943) 87.
5. Nyman, E. *Acta Physiol. Scand.* Suppl. X (1942).
6. Fränkel, S. *Arzneimittelsynthese* 5. Aufl. Berlin (1921) p. 337.
7. Löfgren, N., and Fischer, I. *Svensk Kem. Tid.* **58** (1946) 219.

Received July 9, 1948.

Table 1. R_F values for 17 primary aromatic amines run in various solvents.

Solvents:					
% methyl alcohol	40	35	35	35	30.8
» amyl alcohol	20	17.5	17.5	17.5	15.2
» benzene	20	35	35	35	46
» water	20	12.5	12.5 (2 *N* HCl)	12.5 (4%NH_3)	8
R_F values for:					
1. sulphatiazole	0.86	0.78	0.53	0.64	0.64
2. sulphanilamide	0.78	0.73	0.45	0.69	0.53
3. sulphapyridine	0.87	0.82	0.53	0.73	0.72
4. diaminodiphenylsulphone	0.89	0.84	0.80	0.84	0.79
5. *p*-aminobenzoic acid	0.88	0.56	0.79	0.41	0.71
6. *o*- » »	0.90	0.61	0.82	0.53	0.84
7. *m*- » »	0.81	0.42	0.78	0.45	0.76
8. *p*-aminosalicylic »	0.79	0.44	0.79	0.46	0.71
9. sulphanilic »	0.74	0.73	0.50	0.50	0.35
10. α-naphtylamine	0.92	0.95	0.92	0.95	0.98
11. β »	0.92	0.93	0.89	0.95	0.93
12. *m*-phenylendiamine	0.62	0.75	0.35	0.71	0.50
13. *p*- »	0.25	0.75	0.28	0.70	0.46
14. *m*-toluidine	0.89	0.85	0.87	0.86	0.83
15. *o*- »	0.88	0.88	0.86	0.89	0.87
16. aniline	0.88	—	0.71	0.91	0.84
17. *p*-aminophenol	0.51	0.82	0.33	0.74	0.46

So we have, for instance, observed that on standing in alcoholic solution *p*-aminophenol with an R_F value of 0.31 (in benzene, amyl and methyl alcohol and 2 *N* HCl) gives oxidation products with R_F values of 0.26, 0.41, 0.63, and 0.73. A mixture of sulphanilamide, sulphatiasole, sulphapyridine and *p*-aminobenzoic acid could be differentiated in four spots distinctly separated after running the paper first with 46 % benzene, 15.2 % amyl alcohol, 30.8 % methyl alcohol and 8 % water and then at cross angles with 20 % benzene, 20 % amyl alcohol, 40 % methyl alcohol and 20 % *N* HCl.

Other organic solvents, for instance phenol, acetone and ethyl acetate have less favourable dissolving properties and the spots are drawn out. Combinations with pyridine have no advantages and run very slowly.

Discussion. The above method was intended for the analysis of aromatic amines present in urines from patients with tumors in the digestive tract or in the urine bladder (Ekman and Strömbeck [8]). Further it will be used in studies of the metabolism of cancerogenic substances which yield aromatic amines in the body, for instance azotoluene and acetaminofluorene. The method may also be of value in investigations of the antagonism between *p*-aminobenzoic acid and sulphapreparations in bacterial metabolism.

1. Consden, R., Gordon, A. H., and Martin, A. J. *Bioch. J.* **38** (1944) 224.
2. Partridge, S. M. *Nature* **158** (1946) 270.
3. Flood, A. E., Hirst, E. L., and Jones, J. K. N. *Nature* **160** (1947) 86.
4. Lugg, J. W. H., and Overell, B. T. *Nature* **160** (1947) 87.
5. James, W. O. *Nature* **161** (1948) 851.
6. Crammer, J. L. *Bioch. J.* **42** (1948) v.
7. Edman, P. *Arkiv Kemi, Mineral., Geol.* **A 22** (1945) no. 3.
8. Ekman, B., and Strömbeck, J. P. *Acta Physiol. Scand.* **14** (1947) 43.

Received August 27, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 385—399

The Catalysis by Certain Metal Ions in the Bromination of 2-Carbethoxycyclopentanone

KAI JULIUS PEDERSEN

The Chemical Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, Denmark

In a previous paper [1], the effect of cupric and barium ions on the bromination of ethyl acetoacetate was examined. The two α-hydrogen atoms are replaced by bromine by two first order reactions which are both catalysed by bases in general (*e. g.* by water molecules or acetate ions). The rate-determining step is, in both changes, a proton transfer from the ester to the base. It was shown that the proton transfer from ethyl acetoacetate is catalysed by cupric ions while barium ions have only an insignificant influence. In order to explain this catalysis, it was assumed that a small amount of a complex between the cupric ion and the keto-form of ethyl acetoacetate exists in the solution. In such a complex, the proton is bound much more loosely than in the free ester, and the rate of transfer of protons to the base is, therefore, much greater.

In continuation of this examination, a study was made of the effect of different metal ions on the velocity of bromination of 2-carbethoxycyclopentanone,

$$\overline{|\quad|}\,(CH_2)_3COCHCOOC_2H_5 + Br_2 \rightarrow \overline{|\quad|}\,(CH_2)_3COCBrCOOC_2H_5 + H^+ + Br^- \qquad (1)$$

This β-keto-ester was chosen instead of ethyl acetoacetate because it has only one α-hydrogen atom for which reason no subsequent bromination takes place. The computation of the velocity constant is therefore both easier and more accurate. The kinetic behaviour of the two esters is, otherwise, qualitatively the same.

MATERIALS AND PROCEDURE

Adipic acid was prepared from cyclohexanol by the procedure described in *Org. Syntheses* [2], except that stirring was made by a vigorous current of air instead of a mechanical stirrer. It is uncertain whether it is for this reason that the yield was greater and the product purer than stated in *Organic Syntheses*. By direct crystallisation was obtained 71 per cent of the theoretical amount (melting point 149.5° C), and by evaporation of the mother liquor was further gained 4 per cent (m. p. 147—149°).

Diethyl adipate was prepared from adipic acid and ethyl alcohol by the method of Micovic[3]. The yield was 84 per cent of the theoretical amount.

2-Carbethoxycyclopentanone was prepared by the following procedure which has been worked out by Professor Hakon Lund [4] who has kindly communicated it to me.

12 g of sodium is fused under toluene in a 500-ml round-bottomed flask. The flask is closed, shaken vigorously until the sodium solidifies to tiny balls, and is allowed to cool without the admission of air. It is then opened, and the toluene is decanted from the sodium which is washed by decantation with dry ether. 200 ml of dry ether and 75 g of diethyl adipate are now added. The flask is provided with a reflux condenser, and 1 ml of absolute alcohol is added to start the reaction. Now and then, the flask is shaken, especially when the reaction mixture gets thick. It is left till the next day when 35 ml of glacial acetic acid is added. The biggest lumps are mechanically disintegrated. When no more reaction is visible, sufficient water for dissolving the sodium acetate formed is added in small portions while the flask is cooled in ice water. The ether layer is washed with water and dried. The ether is evaporated, and the ester distilled under reduced pressure. The yield of ester boiling within 1° (boiling point 105° C/12 mm) at constant heat supply is 53 g or more than 90 per cent of the theoretical amount.

The 2-carbethoxycyclopentanone prepared in this way was purified by the following procedure. 80 g of crystalline cupric sulphate and 100 g of the ester were dissolved in water. The solution was neutralized with 2 molar ammonia (about 320 ml) which was added dropwise and with stirring. A precipitate of the cupric compound of the ester separated out. It was filtered and washed with water and with alcohol. It was then recrystallised from 96 per cent alcohol. Light-green crystals consisting of an alcoholate were obtained. They were dried at room temperature and, finally, at 100° C until all alcohol had evaporated. A mixture of 100 g of the dry cupric compound and 250 ml of 20 per cent hydrochloric acid was shaken and extracted once with 200 ml of ether and twice with 100 ml portions. The extract was dried with anhydrous sodium sulphate. The ether was removed by distillation. The ester was distilled through a Vigreux still-head in an all-glass apparatus. When the heat supply was kept constant, nearly all the preparation distilled at constant temperature (113° C/20 mm).

The inorganic substances used for the measurements were the purest commercial preparations, usually »for analysis». *Cupric nitrate, lead nitrate,* and *sodium perchlorate* were further recrystallised. In the *nickel nitrate* (for analysis), no cobalt, iron or heavy metals could be detected. Standard solutions of *zinc, cadmium,* and *manganous nitrate* were prepared by mixing equivalent amounts of solutions of the corresponding sulphates (for analysis) and barium nitrate (for analysis). A standard solution of *beryllium perchlorate* was prepared from solutions of beryllium sulphate and barium perchlorate. The beryllium sulphate had been prepared from pure beryllium oxide, and the barium perchlorate from barium hydroxide and perchloric acid (both for analysis). The solvents

for the bromination were prepared from standard solutions of the different substances. When necessary, the concentrations of the standard solutions were checked by analysis.

The procedure for the kinetic measurements was the same as in the previous paper [1]. It was sometimes noticed that a platinum electrode which had been in use for some time gave inaccurate results because it did not respond sufficiently quickly to changes in the redox potential of the solution. Two or three electrodes were therefore used alternately, and the measurement was approved only when different electrodes gave concordant results.

MEASUREMENTS IN SOLUTIONS CONTAINING STRONG ACIDS

A study was made of the effects of a series of salts on the rate of bromination in solvents containing either hydrochloric, nitric, or perchloric acid. In the presence of strong acid, the reaction always satisfied the first order law. The velocity constant k^* was calculated by means of the formula

$$k^*t = \log\ c - \log\ (c - \frac{x}{2})$$

where t is the time in minutes from the start of the reaction to the complete disappearance of the bromine, c the initial concentration of ester, and x the initial equivalent concentration of bromine. As velocity constant for a solution was taken the average of, as a rule, from 6 to 8 determinations with different initial concentrations of bromine. Table 1 illustrates the computation of k^* from the experimental data for two of the solutions.

Table 1. Bromination of 2-carbethoxycyclopentanone at 18.0° C.

0.003472 molar ester
0.0200 molar KBr
0.0300 molar HNO_3

Electrode	t	$\frac{x}{2}10^3$	k^*
a	3.18	0.799	0.03573
b	5.30	1.226	0.03570
a	7.10	1.536	0.03573
b	9.77	1.916	0.03567
a	12.46	2.226	0.03571
b	16.85	2.593	0.03540
b	18.81	2.723	0.03542
		Mean	0.03562

0.003529 molar ester
0.0200 molar KBr
0.0300 molar HNO_3
0.04085 molar $Cu(NO_3)_2$

Electrode	t	$\frac{x}{2}10^3$	k^*
c	1.411	1.472	0.1661
d	1.898	1.840	0.1686
d	2.224	2.011	0.1647
d	2.432	2.161	0.1692
c	2.919	2.387	0.1679
c	3.063	2.455	0.1687
c	3.090	2.476	0.1700
		Mean	0.1679

Table 2. Bromination of 2-carbethoxycyclopentanone (initial concentration 0.0035 molar) at 18.0° C in the solutions A to F containing, in addition, the substances given in the table (concentration c_S molar). k^* is the velocity constant, and k_S^* the increase of k^* per mole/liter of the substance.

		k^* (calc.)
A.	0.0200 *M* KBr	0.03542
B.	0.0200 *M* KBr + 0.0300 *M* HNO_3	0.03562
B_1.	0.0200 *M* KBr + 0.100 *M* HNO_3	0.03609
B_2.	0.0200 *M* KBr + 0.0100 *M* HNO_3	0.03549
C.	0.0200 *M* KBr + 0.0300 *M* HCl	0.03575
D.	0.0200 *M* KBr + 0.0300 *M* $HClO_4$	0.03527
D_1.	0.0200 *M* KBr + 0.100 *M* $HClO_4$	0.03492
E.	0.0200 *M* NaBr	0.03545
F.	0.0200 *M* NaBr + 0.0300 *M* $HClO_4$	0.03530

	c_S	k^*	k_S^*
	Aluminium nitrate		
B_1	0.050	0.03553	— 0.0112
B_1	0.100	0.03416	— 0.0193
B	0.100	0.03455	— 0.0107
B_2	0.100	0.03563	+ 0.0014
B_1 [a]	0.050	0.03364	— 0.0136
	Barium chloride		
C	0.100	0.03704	0.0129
C	0.200	0.03787	0.0106
C	0.300	0.03860	0.0095
	Beryllium perchlorate		
D_1	0.0200	0.03425	— 0.0340
D_1	0.0500	0.03320	— 0.0344
D	0.0500	0.03367	— 0.0320
	Cadmium nitrate		
B	0.0200	0.03685	0.062
B	0.0600	0.03994	0.072
	Calcium chloride		
C	0.0502	0.04042	0.0930
C	0.1003	0.04490	0.0912
C	0.2013	0.05401	0.0907

	c_S	k^*	k_S^*
	Cupric nitrate		
B	0.00513	0.0517	3.14
B	0.01028	0.0680	3.15
B [b]	0.01028	0.0677	3.12
B_1	0.01028	0.0693	3.23
B	0.02057	0.1021	3.23
B	0.04085	0.1679	3.24
	Diaquotetramminecobaltic nitrate		
B	0.0200	0.03552	— 0.005
B_1	0.0200	0.03574	— 0.018
	Hexamminecobaltic nitrate		
B	0.0200	0.03470	— 0.046
	Hydrochloric acid		
A	0.030	0.03578	0.0120
A	0.100	0.03659	0.0117
A	0.200	0.03760	0.0109
A	0.300	0.03868	0.0109

[a] The solution contained, in addition, 0.150 *M* KNO_3.

[b] The initial concentration of ester was 0.0070 *M* instead of 0.0035 *M*.

Table 2 (continued)

	c_S	k^*	k_S^*
	Lanthanum nitrate [c]		
B	0.00497	0.0427	1.43
B	0.00996	0.0498	1.43
B	0.02001	0.0642	1.43
B	0.02139	0.0661	1.43
B_1	0.02139	0.0659	1.39
B [d]	0.02139	0.0652	1.41
	Lead nitrate		
B	0.0250	0.04194	0.253
B	0.0499	0.04845	0.257
B	0.1000	0.06054	0.249
	Magnesium perchlorate		
D	0.0400	0.03741	0.0535
D	0.1000	0.04058	0.0531
D	0.2000	0.0458	0.0527
	Manganous nitrate		
B	0.0194	0.03818	0.132
B	0.0583	0.04473	0.156
B_1	0.0583	0.04480	0.149
	Nickel nitrate		
B	0.00993	0.0566	2.11
B	0.01995	0.0783	2.14
B_1	0.01995	0.0787	2.14
B	0.04963	0.1425	2.15
B	0.09974	0.2475	2.12
	Nitric acid		
A	0.030	0.03562	0.0067
A	0.100	0.03591	0.0049
A	0.200	0.03661	0.0060
A	0.300	0.03739	0.0066
	Perchloric acid		
E	0.030	0.03522	— 0.0077
E	0.100	0.03500	— 0.0045
E	0.230	0.03444	— 0.0044
E	0.300	0.03410	— 0.0045
	Potassium bromide		
B	0.030	0.0350	— 0.021
	Potassium chloride		
C	0.100	0.03502	— 0.0073
C	0.200	0.03420	— 0.0078
	Potassium nitrate		
B	0.100	0.03454	— 0.0108
B	0.200	0.03322	— 0.0120
	Sodium chloride		
C	0.100	0.03514	— 0.0061
C	0.200	0.03456	— 0.0060
	Sodium nitrate		
B	0.100	0.03464	— 0.0098
B	0.200	0.03350	— 0.0106
	Sodium perchlorate		
F	0.100	0.03289	— 0.0241
F	0.200	0.03090	— 0.0220
	Zinc nitrate		
B	0.01980	0.04605	0.527
B	0.04962	0.06241	0.540
B_1	0.04962	0.06198	0.522
B	0.0991	0.0896	0.545

[c] Lanthanum nitrate purified through the oxalate was used only for the first three determinations.

[d] The solution contained 0.05 *M* KBr instead of 0.02 *M* KBr.

Table 2 gives a general view of the results obtained. The 23 substances examined are arranged alphabetically. The solvents to which they were added are denoted by the letters from A to F. Their composition and the corresponding velocity constant are given at the top of the table. When $\triangle k^*$ is the in-

crease of k^* due to the addition of the substance S in the concentration c_S molar, the effect of S may be expressed by the ratio $k_S^* = \triangle k^*/c_S$. For most of the substances examined k_S^* is independent of c_S. Only four of the substances, namely cupric, nickel, lanthanum, and zink nitrate, have considerable effects, from 90 to 15 per cent increase in 0.01 molar solution. Moderate increases of the rate are found for the following five salts: lead nitrate, manganous nitrate, cadmium nitrate, calcium chloride, and magnesium perchlorate. The rest of the substances examined have very small, positive or negative, effects.

In some cases, the same substance was examined in solvents with different concentrations of strong acid. If the effect of the salt were partly a basic catalysis by a hydroxy-metal ion formed by dissociation of the hydrated metal ion, k_S^* would decrease when the concentration of strong acid is increased. A decrease of k_S^* was found for aluminium nitrate and diaquotetramminecobaltic nitrate in which cases it is known that hydroxy-metal ions are formed. For the other salts, no or only an insignificant change of k_S^* was found.

Table 3.

S	k_S^* found	k_S^* calc.	S	k_S^* found	k_S^* calc.
HCl	0.0111	0.0110	$NaNO_3$	— 0.0103	— 0.0105
HNO_3	0.0061	0.0067	$NaClO_4$	— 0.0227	— 0.0222
$HClO_4$	— 0.0046	— 0.0055	KCl	— 0.0076	— 0.0075
NaCl	— 0.0060	— 0.0062	KNO_3	— 0.0116	— 0.0118

In Table 3 are presented the average values of k_S^* found for the three strong acids and their sodium and potassium salts. The table shows that k_S^* is an additive property of the ions. If we arbitrarily take the effect of the potassium ion as zero, the best agreement is obtained when we for the other ions involved choose the values given in Table 4. From these values we calculate k_S^* given in the last column of Table 3.

The molar effects of the other ions examined may now be calculated from the experimental data together with the molar effects of the anions when it is assumed that no complex formation between the metal ions and the anions present takes place. This assumption is supported by the fact that k_S^* in general is independent of c_S. There is however one case where it does not hold. It is known that cadmium ions to a considerable extent form complexes with bromide ions [5]. Data for the calculation of the cadmium-ion concentration in the two solutions examined are not available. It may only be very

Table 4. Bromination of 2-carbethoxycyclopentanone at 18.0° C in solutions containing strong acid.

$$k^* = 0.03500 + \Sigma k_I^* c_I$$

The values in parentheses are approximate.

I	k_I^*	I	k_I^*
Cu^{++}	3.17	Be^{++}	0.014
Ni^{++}	2.15	Al^{+++}	(0.01)
La^{+++}	1.47	$Co(NH_3)_4(H_2O)_2^{+++}$	(0.01)
Zn^{++}	0.55	Na^+	0.0013
Pb^{++}	0.28	K^+	*0.0000*
Mn^{++}	0.17	$Co(NH_3)_6^{+++}$	— 0.01
Cd^{++}	(0.1—0.2)		
Mg^{++}	0.111	Cl^-	— 0.0075
Ca^{++}	0.106	NO_3^-	— 0.0118
Ba^{++}	0.028	Br^-	— 0.021
H^+	0.0185	ClO_4^-	— 0.0235

roughly estimated that, in the first solution, nearly one half and, in the second, about one fourth of the cadmium ions are bound to bromide ions. When a complex formation of this magnitude is taken into account, we find that the molar effect of the cadmium ion is between 0.1 and 0.2. The molar effects of all the ions examined are given in table 4. The velocity constant in a solution of these ions may be expressed by the formula

$$k^* = 0.03500 + \Sigma k_I^* c_I \qquad (2)$$

where k_I^* is the molar effect and c_I the concentration of the ion I, and the summation includes all the ions present in the solution. The velocity constants for the solvents A to F given at the top of Table 2 have been calculated by means of this formula. They have been used for the calculation of k_S^* given in the table. The velocity constants for the solvents B, B_1, C, and F have also been measured directly. They may be found in the table under the headings nitric, hydrochloric, and perchloric acid.

MEASUREMENTS IN ACETATE BUFFER SOLUTIONS

In the previous part of this paper, we have examined the effects of a series of metal ions on the rate of bromination in solutions containing strong acids. In this part, we shall study the effects of two of them, cupric and nickel ions, when the reaction takes place in acetate buffer solutions.

When cupric ions are present, the following differential equation holds

$$\frac{d\,(c-\frac{x}{2})}{dt} = \left[k_r + k_{Cu^{++}}\, c_{Cu^{++}} + k_{Ac^-}\, c_{Ac^-} + k_{Cu^{++}\,Ac^-}\, c_{Cu^{++}}\, c_{Ac^-}\right](c-\frac{x}{2}) \quad (3)$$

In this equation, k_r includes k_0, the velocity constant in pure water, and the effects of all the ions present except the cupric and the acetate ion. Owing to the production of hydrogen ions by the bromination (scheme 1) and to complex formation between cupric and acetate ions, both the acetate- and cupric-ion concentration change during the reaction. These changes are approximately proportional to the concentration of hydrogen bromide formed $(=\frac{x}{2})$. We, therefore, may write

$$c_{Ac^-} = c_{Ac^-(\infty)} + p(c-\frac{x}{2}) \quad (4)$$

$$c_{Cu^{++}} = c_{Cu^{++}(\infty)} - q(c-\frac{x}{2}) \quad (5)$$

When these expressions are introduced into equation 3, we obtain by integration

$$k_{(\infty)}{}^* t = \frac{n+l}{2n}\log\frac{\frac{n-l}{2m}+(c-\frac{x}{2})}{\frac{n-l}{2m}+c} + \frac{n-l}{2n}\log\frac{\frac{n+l}{2m}-(c-\frac{x}{2})}{\frac{n+l}{2m}-c} - \log\frac{c-\frac{x}{2}}{c} \quad (6)$$

where

$$k_{(\infty)}{}^* = k_r{}^* + k_{Cu^{++}}{}^*\, c_{Cu^{++}(\infty)} + k_{Ac^-}{}^*\, c_{Ac^-(\infty)} + k_{Cu^{++}Ac^-}{}^*\, c_{Cu^{++}(\infty)}\, c_{Ac^-(\infty)} \quad (7)$$

$$l = p\,[k_{Ac^-} + k_{Cu^{++}Ac^-}\, c_{Cu^{++}(\infty)}] - q\,[k_{Cu^{++}} + k_{Cu^{++}Ac^-}\, c_{Ac^-(\infty)}] \quad (8)$$

$$m = pqk_{Cu^{++}Ac^-} \quad (9)$$

$$n = \sqrt{l^2 + 4k_{(\infty)}\, m} \quad (10)$$

p and q are found by introducing $c_{Ac^-(0)}$ and $c_{Cu^{++}(0)}$ corresponding to $t = 0$ and $x = 0$ into equation 4 and 5. In computing $k_{(\infty)}{}^*$ by means of equation 6, it is necessary to use a method of successive approximations since $k_{(\infty)}{}^*$ enters into both sides of the equation.

In the calculation of the acetate and cupric ion concentrations corresponding to the times 0 and ∞, we have used values found in a previous paper [6]

for the dissociation constant of acetic acid, K_{HAc}, and for the complexity constants

$$K_1 = c_{Cu++}\, c_{Ac^-}/c_{CuAc^+} \text{ and } K_2 = c_{Cu++}\, c_{Ac^-}{}^2/c_{CuAc_2}$$

They may be expressed by the following formulae where μ is the ionic strength of the solution

$$-\log K_{HAc} = 4.7595 - 0.996\sqrt{\mu} + 1.15\mu - 0.8\mu^2 + 0.035\, c_{HAc} - 0.25\, c_{Ac^-} \quad (11)$$

$$\log K_1 = 2.164 - 1.992\sqrt{\mu}\,/\,(1 + 1.8\sqrt{\mu}) \quad (12)$$

$$\log K_2 = 3.205 - 2.988\sqrt{\mu}\,/\,(1 + 2\sqrt{\mu}) \quad (13)$$

When the buffer solution contains nickel instead of cupric ions, we may use the same system of formulae if only Cu^{++} is replaced by Ni^{++}, but here no data are available for the calculation of the degree of complex formation. The following observation indicates, however, that no, or only a very slight, complex formation between nickel and acetate ions takes place in dilute solutions. When 25 ml of each of the four solutions (a) 0.1 *M* $Ni(NO_3)_2$, (b) 0.1 *M* $Ni(NO_3)_2$ + 0.1 *M* HAc, (c) 0.1 *M* $Ni(NO_3)_2$ + 0.1 *M* HAc + 0.2 *M* NaAc, and (d) 0.1 *M* $Ni(NO_3)_2$ + 0.1 *M* HAc + 0.5 *M* NaAc were placed in test tubes of the same size, no difference in colour could be seen by the naked eye in the first three solutions while it was just possible to distinguish a displacement in solution (d).

The solutions used for the kinetic measurements were much more dilute (see Table 8). It therefore seems to be permissible to neglect the complex formation between nickel and acetate ions. When the nickel ion concentration is constant throughout the reaction we obtain instead of equation 6

$$k_{(\infty)}{}^* t = \log \frac{\dfrac{k_{(\infty)}}{p\,(k_{Ac^-} + k_{Ni^{++}Ac^-}\, c_{Ni^{++}})} + (c - \dfrac{x}{2})}{\dfrac{k_{(\infty)}}{p\,(k_{Ac^-} + k_{Ni^{++}Ac^-}\, c_{Ni^{++}})} + c} - \log \frac{c - \dfrac{x}{2}}{c} \quad (14)$$

In order to see which influence a possible complex formation may have, the velocity constants have been computed both on the assumption that complex formation may be neglected (formula 14) and on the assumption that an ion $NiAc^+$ with a complexity constant $K_1 = 10$ is formed (formula 6).

The acetate-ion catalysis. Measurements were carried out in four acetate buffer solutions of the compositions given in Table 5. The experimental data

Table 5. Bromination of 2-carbethoxycyclopentanone at 18.0° C in acetate buffer solutions. The initial molar concentrations of the solutions are given in the first five lines of the table.

	I	II	III	IV
HAc	0.0200	0.0200	0.0400	0.0200
NaAc	0.00598	0.01000	0.01000	0.01600
$NaNO_3$	0.0740	0.0700	0.0700	0.0640
KBr	0.0200	0.0200	0.0200	0.0200
Ester	0.003623	0.003623	0.003623	0.003623
$K_{HAc}\ 10^5$	2.80	2.79	2.78	2.77
$c_{H^+(0)}\ 10^5$	9.2	5.5	11.0	3.4
$c_{H^+(\infty)}\ 10^5$	25.1	10.1	18.4	5.2
$c_{Ac^-(0)}$	0.00607	0.01006	0.01011	0.01603
$c_{Ac^-(\infty)}$	0.00261	0.00648	0.00656	0.01243
$k_{(\infty)}{}^*$	0.06236	0.1036	0.1045	0.1669
$k_r{}^*$	0.03466	0.0347	0.0347	0.0348
$k_{Ac^-}{}^*$	10.61	10.63	10.64	10.63

for one of the solutions are presented in more detail in Table 6. The velocity constant $k_{(\infty)}{}^*$ has been calculated by means of formula 14 ($c_{Ni^{++}} = 0$). $k_{Ac^-}{}^*$ given in the last line of Table 5 has been computed from $k_{(\infty)}{}^*$ by means of the equation $k_{(\infty)}{}^* = k_r{}^* + k_{Ac^-}{}^*\ c_{Ac^-(\infty)}$, where $k_r{}^*$ has been calculated from the data in Table 4. There is a good agreement between the values of $k_{Ac^-}{}^*$ found for the four solutions. The average is $k_{Ac^-}{}^* = 10.63$.

The combined cupric- and acetate-ion catalysis. Measurements were carried out in five acetate buffer solutions containing cupric nitrate. The compositions of the solutions are given in Table 7. The experimental data for one of the

Table 6. Experimental data.

Solution IV (Table 5)			Solution IX (Table 7)		
t	$\frac{x}{2}10^3$	$k_{(\infty)}{}^*$	t	$\frac{x}{2}10^3$	$k_{(\infty)}{}^*$
0.902	1.224	0.1671	0.835	1.471	0.2176
1.240	1.527	0.1630	0.849	1.486	0.2170
1.522	1.777	0.1650	1.091	1.791	0.2212
1.976	2.120	0.1680	1.162	1.860	0.2201
2.062	2.170	0.1675	1.566	2.209	0.2173
2.296	2.315	0.1687	1.576	2.223	0.2184
2.684	2.515	0.1693	1.975	2.486	0.2158
	Mean	0.1669	2.129	2.578	0.2161
				Mean	0.2179

Table 7. Bromination of 2-carbethoxycyclopentanone at 18.0° C in acetate buffer solutions containing cupric nitrate. The initial molar concentrations of the solutions are given in the first six lines of the table.

	V	VI	VII	VIII	IX
HAc	0.1000	0.02000	0.02000	0.02000	0.01000
NaAc	0.00996	0.00598	0.01000	0.01000	0.00997
$NaNO_3$	0.0500	0.0540	0.0500	0.0600	0.0500
KBr	0.0200	0.0200	0.0200	0.0200	0.0200
$Cu(NO_3)_2$	0.01023	0.01023	0.01023	0.005115	0.01023
Ester	0.003603	0.003623	0.003623	0.003584	0.003603
K_{HAc} 10^5	2.80	2.83	2.82	2.80	2.82
K_1	56.8	56.4	56.8	57.4	56.8
K_2	412	408	412	418	412
$c_{H+(0)}$ 10^5	39.0	13.7	8.0	6.7	4.1
$c_{H+(\infty)}$ 10^5	61.5	38.3	15.1	12.5	8.8
$c_{Ac^-(0)}$	0.007130	0.004088	0.006931	0.008256	0.006880
$c_{Ac^-(\infty)}$	0.004691	0.001717	0.004377	0.005273	0.004325
$c_{Cu++(0)}$	0.007161	0.008258	0.007225	0.003401	0.007241
$c_{Cu++(\infty)}$	0.008022	0.009318	0.008143	0.003892	0.008163
$\mu_{(0)}$	0.1047	0.1068	0.1046	0.1019	0.1046
$\mu_{(\infty)}$	0.1068	0.1092	0.1066	0.1030	0.1066
$k_{(\infty)}$*	0.2182	0.1278	0.2108	0.1671	0.2179
k_r*	0.03468	0.03462	0.03468	0.03469	0.03468
$k_{Cu++}{}^* c_{Cu++(\infty)}$	0.02543	0.02954	0.02581	0.01234	0.02588
$k_{Ac^-}{}^* c_{Ac^-(\infty)}$	0.04987	0.01825	0.04653	0.05605	0.04597
$k_{Cu++Ac^-}{}^* c_{Cu++(\infty)} c_{Ac^-(\infty)}$	0.1082	0.0454	0.1038	0.0640	0.1114
k_{Cu++Ac^-}* 10^{-3}	2.88	2.84	2.91	3.12	3.16

solutions are presented in more detail in Table 6. The velocity constant $k_{(\infty)}$* has been calculated by means of formula 6. It may be expressed as a sum of four terms by means of equation 7. The first three terms may be calculated from the data given earlier in this paper. When they are subtracted from $k_{(\infty)}$*, we find the last term of the expression, and from this again k_{Cu++Ac^-}* given in the last line of table 7. The values of k_{Cu++Ac^-}* found for the first three and for the last two of the solutions agree well among themselves, but the latter group has given somewhat higher results than the former. The average of all five values is 2.98×10^3. k_{Cu++Ac^-}* is the bimolecular velocity constant for a reaction between two ions of opposite sign. According to Brønsted [7], it will therefore decrease with increasing ionic strength. The ionic strengths of the solutions examined are, however, nearly the same. The differences are so small that they can have no importance for the constancy of k_{Cu++Ac^-}*.

The combined nickel- and acetate-ion catalysis. Measurements were carried out in four acetate buffer solutions containing nickel nitrate. The compositions of the solutions are given in Table 8. The experimental data for one of the solutions are presented in more detail in Table 9. The velocity constant $k_{(\infty)}$* has been calculated (a) on the assumption that complex formation between nickel and acetate ions may be neglected, and (b) on the assumption that the ion $NiAc^+$ is the only complex formed and that its complexity constant is $K_1 = 10$. When $k_{(\infty)}$* is expressed by an equation analogous to equation 7, we find the values of $k_{Ni^{++}Ac^-}$* given in table 8. While $k_{(\infty)}$* increases only 1 per cent when we make assumption (b) instead of (a), $k_{Ni^{++}Ac^-}$* increases considerably. There is a moderately good agreement between the values of $k_{Ni^{++}Ac^-}$*

Table 8. Bromination of 2-carbethoxycyclopentanone at 18.0° C in acetate buffer solutions containing nickel nitrate. The initial molar concentrations of the solutions are given in the first six lines of the table.

	X	XI	XII	XIII
HAc	0.0200	0.0600	0.0200	0.0200
NaAc	0.00996	0.00996	0.005955	0.005955
$NaNO_3$	0.0500	0.0500	0.0540	0.0340
KBr	0.0200	0.0200	0.0200	0.0200
$Ni(NO_3)_2$	0.009934	0.009934	0.009934	0.01987
Ester	0.003603	0.003603	0.003591	0.003603
	a) Complex formation neglected:			
μ	0.1099	0.1100	0.1100	0.1198
$K_{HAc}\ 10^5$	2.82	2.81	2.83	2.86
$c_{H^+(0)}10^5$	5.6	16.7	9.3	9.4
$c_{H^+\ (\infty)}10^5$	10.3	26.9	25.2	25.6
$c_{Ac^-(0)}$	0.01002	0.01013	0.006048	0.006049
$c_{Ac^-(\infty)}$	0.006460	0.006626	0.002616	0.002608
$k_{(\infty)}$*	0.2238	0.2243	0.1275	0.1855
k_r*	0.03467	0.03467	0.03463	0.03460
$k_{Ni^{++}} * c_{Ni^{++}}$	0.02136	0.02136	0.02136	0.04272
$k_{Ac^-} * c_{Ac^-(\infty)}$	0.06867	0.07043	0.02781	0.02772
$k_{Ni^{++}Ac^-} * c_{Ni^{++}} c_{Ac^-(\infty)}$	0.0991	0.0978	0.0437	0.0805
$k_{Ni^{++}Ac^-} * 10^{-3}$	1.54	1.49	1.68	1.55
	b) Complexity constant $K_1 = 10$:			
$c_{Ac^-(0)}$	0.009185	0.009296	0.005536	0.005101
$c_{Ac^-(\infty)}$	0.005914	0.006079	0.002405	0.002220
$c_{Ni^{++}(0)}$	0.009098	0.009089	0.009413	0.01890
$c_{Ni^{++}(\infty)}$	0.009379	0.009364	0.009701	0.01944
$k_{(\infty)}$*	0.2257	0.2264	0.1286	0.1880
$k_{Ni^{++}Ac^-} * 10^{-3}$	1.95	1.88	2.04	1.88

Table 9. Experimental data for solution X (Table 8).

t	$\frac{x}{2}10^3$	$k_{(\infty)}$ *a)	$k_{(\infty)}$ *b)
0.921	1.630	0.2170	0.2193
1.121	1.889	0.2234	0.2255
1.265	2.036	0.2239	0.2259
1.461	2.215	0.2246	0.2266
1.767	2.541	0.2258	0.2275
1.832	2.500	0.2269	0.2286
2.345	2.776	0.2253	0.2268
	Mean	0.2238	0.2257

a) Complex formation between nickel and acetate ions neglected.
b) It has been assumed that the complexity constant of the ion $NiAc^+$ is 10.

found for the four solutions. The average is 1.57×10^3 when complex formation is neglected, while it is 1.94×10^3 when it is assumed that $K_1 = 10$. The values agree only slightly better among themselves in case (b) than in case (a), and there is no reason to expect a better agreement for another value of K_1. The constancy of the colour of a dilute nickel nitrate solution when acetate ions are added, seems to show that no, or only a very small, complex formation takes place. We therefore consider 1.57×10^3 to be the most reliable value of the constant $k_{Ni^{++}Ac^-}$*.

DISCUSSION

According to the theory developed in earlier papers [8,9], the rate-determining step in the bromination of β-keto-esters is a proton transfer from the ester to the catalysing base (*e. g.* water or acetate ions). It has now been found that certain metal ions accelerate both the water reaction and the acetate-ion catalysis. The effect of the metal ions may be interpreted as a catalysis of the proton transfer from the ester to the base. As seen from Table 10, cupric ions increase the velocity of proton transfer from ethyl acetoacetate to water molecules and to acetate ions by nearly the same factor, while, in the bromination of 2-carbethoxycyclopentanone, cupric and nickel ions have effects on the transfer to acetate ions which are, respectively, 3.1 and 2.4 times as great as their effects on the transfer to water molecules. In order to explain the metal-ion catalysis, it is assumed that a small amount of a complex between the metal ion and the keto-form of the ester exists in the solution. In such a complex, the proton is bound much more loosely than in the free ester, and the

Table 10. Comparison of the effects of metal ions ($Me^{++} = Cu^{++}$ or Ni^{++}) on the velocity of proton transfer to water molecules and to acetate ions.

$r_0 = k_{Me^{++}}/k_0$ and $r_{Ac^-} = k_{Me^{++}Ac^-}/k_{Ac^-}$

	k_0*	k_{Ac^-}*	Me^{++}	r_0	r_{Ac^-}	$\frac{r_{Ac^-}}{r_0}$
Ethyl acetoacetate [1]	0.01855	8.21	Cu^{++}	145	139	1.0
Carbethoxycyclopentanone	0.03500	10.63	Cu^{++}	90.6	280	3.1
			Ni^{++}	61.4	148	2.4

rate of transfer of protons to the base is, therefore, much greater. While no other experimental facts indicate the existence of complexes between metal ions and the keto-forms of β-keto-esters, it is known that their (enolic) anions, and other anions of a similar structure, combine with certain metal ions. Mellor and Maley [10] have recently determined the stabilities of the complexes formed between the anion of salicylaldehyde and eight divalent metal ions in the solvent 50 per cent dioxane — 50 per cent water. They found that the stability constant of the complex decreases in the order: Pd^{++}, Cu^{++}, Ni^{++}, Pb^{++}, Co^{++}, Cd^{++}, Zn^{++}, Mg^{++}. The catalysis by six of these ions has been examined in this paper. It was found that the effect decreases in the same order, except that Zn^{++} comes between Ni^{++} and Pb^{++}.

SUMMARY

The rate of bromination of 2-carbethoxycyclopentanone was measured in solutions containing strong acids and, in addition, different electrolytes. It was found that certain metal ions catalyse the reaction. The catalytic effect decreases in the order: Cu^{++}, Ni^{++}, La^{++}, Zn^{++}, Pb^{++}, Mn^{++}, Cd^{++}, Mg^{++}, Ca^{++}. The rest of the ions examined: H^+, Na^+, K^+, Ba^{++}, Be^{++}, Al^{+++}, $Co(NH_3)_6^{+++}$, $Co(NH_3)_4(H_2O)_2^{+++}$, Cl^-, Br^-, NO_3^-, and ClO_4^-, have only very small, positive or negative, effects.

The reaction was also studied in acetate buffer solutions with and without added cupric or nickel nitrate. The catalytic effects of the acetate, cupric, and nickel ion were determined.

The velocity of the base-catalysed bromination of β-keto-esters is a measure of the rate of proton transfer from the ester to the base (*e. g.* water or acetate ion). It follows from the measurements in this and a previous paper [1], that certain metal ions catalyse the proton transfer. In order to explain this catalysis, it is assumed that a small amount of a complex between the metal ion

and the keto-form of the ester exists in the solution. In such a complex, the proton is bound much more loosely than in the free ester, and the rate with which it is transferred to a base is, therefore, much greater.

I wish to express my thanks to the head of the laboratory, Professor Niels Bjerrum, for his kind interest in my work.

REFERENCES

1. Pedersen, K. J. *Acta Chem. Scand.* **2** (1948) 252.
2. *Org. Syntheses*, Coll. Vol. I, p. 18.
3. Micovic, V. M. *Bull. soc. chim.* [5] **4** (1937) 1661.
4. Lund, H. Private communication (1935).
5. Leden, I. *Potentiometrisk undersökning av några kadmiumsalters komplexitet* Lund (1943).
6. Pedersen, K. J. *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.* **22** (1945) no. 12.
7. Brønsted, J. N. *Z. physik. Chem.* **102** (1922) 169.
8. Pedersen, K. J. *J. Phys. Chem.* **38** (1934) 581.
9. Pedersen, K. J. *Trans. Faraday Soc.* **34** (1938) 237.
10. Mellor, D. P., and Maley, L. *Nature* **159** (1947) 370.

Received March 17, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 400—407

The Crystal Structure of CdSb and ZnSb

KARL ERIK ALMIN

Institute of Inorganic and Physical Chemistry, University of Stockholm, Sweden

Attempts to solve the crystal structure of the intermetallic compounds CdSb and ZbSb have previously been made by Halla and Adler [1], Halla, Nowotny and Tompa [2] and Ölander [3]. Of them, only Ölander was successful in approximately determining the dimensions of and the number of atoms in the elementary cell for CdSb, but without Weissenberg photographs a determination of the space group was difficult. The dimensions of the elementary cell given by Halla and coworkers do not agree at all with those given by Ölander or those found in this investigation and are certainly wrong. In an electrochemical investigation Ölander [4] also found that at 240°—290° C there is not more than one stable intermediate phase in the Cd-Sb system between 16.0 and 86.5 atomic % Cd.

In this investigation the alloys were prepared by melting Cd (Zn) together with Sb in evacuated silica tubes in such proportions as to obtain alloys containing 50.0 atomic % Sb. The powder photographs of CdSb (Table 5) are those taken by Ölander [3]. Powder photographs of ZnSb (Table 6) were obtained from filings of the alloy, annealled at 400° C in evacuated silica tubes. The filings were allowed to cool rather slowly after the annealing. The powder photographs which were taken with Cr-K radiation in Phragmén focussing cameras, are very rich in lines, and it was not possible to determine the indices of the reflections until the structure was solved by means of Weissenberg photographs.

In order to obtain single crystals, small fragments of the alloy were annealed at 400° C in evacuated silica tubes for one week and allowed to cool rather slowly. The Weissenberg photographs of these fragments were very sharp, but in spite of taking photographs of several crystals it was not possible to avoid strong absorption effects in parts of them. There is no doubt that the absorption depends on the shape of the crystals used.

From the photographs it was found that the two compounds are isomorphous and orthorhombic with the following lattice dimensions:

	a	*b*	*c*
CdSb	6.471 Å (6.458 kX)	8.253 Å (8.236 kX)	8.526 Å (8.509 kX)
ZnSb	6.218 » (6.205 »)	7.741 » (7.725 »)	8.115 » (8.099 »)

If there are 16 atoms in the elementary cell the calculated density is 6.83 for CdSb and 6.36 for ZnSb. These values agree with the values given in the litterature: 6.92 for CdSb [1] and 6.383 for ZnSb [5]. From the photographs the following three groups of systematic absences are found:

$0kl$ where k is odd
$h0l$ where l is odd
$hk0$ where h is odd

This is characteristic of the space group D_{2h}^{15}—P/bca.

In the attempts to solve the crystal structure, the structure problem of CdSb was attacked first. In this case, it was possible to calculate the intensities assuming all the atoms to be of the same kind, as the Cd and Sb atoms have nearly the same scattering power. From the intensity calculations it was easily found that the positions 4 (a) and 4 (b) of the space group could not be occupied by atoms. It therefore only remained to place both the Cd atoms and the Sb atoms in the eightfold position 8 (c), provided that D_{2h}^{15} is the correct space group. The parameter values were found by the method of trial and error. As the intensities of the reflections $h00$, $0k0$ and $00l$ are determined by only two unknown parameters it was possible to find probable but rather approximate values of the atomic coordinates from those reflections. Somewhat better values were obtained when the other reflections were taken into account.

Having obtained an approximately correct structure for CdSb, a more accurate evaluation of the atomic coordinates of ZnSb was made. In this case it was possible to distinguish between the two kinds of atoms. The structure cannot be disordered, because if it were, the intensities of the reflections would be approximately the same as in the photographs of CdSb Ölander's [4] determination of the degree of disorder in CdSb confirms this statement. At 265° C the degree of disorder in CdSb amounts to $5 \cdot 10^{-3}$ %. After the atomic coordinates in ZnSb were ascertained a more exact determination of the parameters in CdSb was made. However, it was not possible to obtain perfect agreement between the observed and the calculated intensities for the reflections in the Weissenberg photographs (Tables 1—4). An attempt to refine

the parameters with Fourier projections was made, but the peaks were not sharp enough to allow a more accurate determination of the atomic coordinates than I had arrived at by means of the method of trial and error. There is no doubt that the discrepancies depend on the strong absorption mentioned above. However, the powder photographs (Tables 5—6) show a very good agreement between the observed and the calculated intensities.

The following parameter values were obtained:

	x_1	y_1	z_1	x_2	y_2	z_2
CdSb	0.136	0.072	0.108	0.456	0.119	— 0.128
ZnSb	0.142	0.081	0.111	0.461	0.103	— 0.122

Table 1. CdSb. Weissenberg photograph. Reflections 0kl. Cu-K radiation.

l \ k	0		2		4		6		8		10	
0			m	9.9	st	19.4	m	11.8	—	0.0	—	0.1
1			—	0.7	w	3.7	m	7.8	—	0.1	st^+	8.4
2	vvw	0.7	—	0.3	—	0.0	vvw	0.3	vw	0.3	—	0.0
3			—	0.1	w^+	6.9	m	8.1	vw	0.4	vst	11.1
4	vst	50.0	m	5.2	m^+	13.6	m	8.5	—	0.0		
5			m^+	2.9	—	0.2	m	4.7	—	0.0		
6	vw	2.4	w	1.2	—	0.0	m	1.8	st	2.1		
7			w	0.4	m^+	5.7	m	6.2	w	0.4		
8	st	18.5	vw	1.6	m	7.3	m	3.6				
9			st	4.8	w	0.4						
10	m	2.8	m	1.6								

Table 2. CdSb. Weissenberg photograph. Reflections h0l. Cu-K radiation.

h \ l	0		2		4		6		8		10	
0			vvw	0.7	vst	50.0	vw	2.4	m	18.5	w	2.8
1			vw	4.4	w^+	1.5	vw	1.2	m^-	2.6	—	0.1
2	m	8.3	—	0.1	w	5.5	vw	0.3	w	3.3	w	0.4
3			—	0.4	vw	0.8	—	0.5	m^-	1.6	w	0.9
4	m	3.5	vw	0.5	vw	2.0	w	2.8	vvw	0.3		
5			st	28.2	vw	0.7	m^+	17.6	m^-	1.6		
6	vw	0.7	—	0.1	vw	0.5	w^+	0.4				
7			m	7.9								
8	vw	0.4	vw	0.2								

Table 3. ZnSb. Weissenberg photograph. Reflections 0kl. Cu-K radiation.

l \ k	0		2		4		6		8	
0			st	9.6	st	11.0	st	17.6	vvw	1.0
1			vw	0.5	m⁻	1.9	w	0.9	vw	0.2
2	w	0.8	vvw	0.2	—	0.1	vvw	0.3	vvw	0.1
3			st⁻	1.7	m	3.5	vw	1.0	m	0.7
4	st	31.6	st	5.3	st	7.4	vst	13.3	vw	0.7
5			vvw	0.0	vvw	0.1	w	0.5	—	0.0
6	m	3.0	w	0.7	vw	0.6	m	1.8	st	0.4
7			m⁺	1.5	st	3.0	w	0.8		
8	st	11.5	m	1.4	m⁺	3.6	vvst	6.9		
9			m	0.5	w	0.1				
10	vst	4.0	st	1.0						

Table 4. ZnSb. Weissenberg photograph. Reflections h0l. Cu-K radiation.

h \ l	0		2		4		6		8		10	
0			w	0.8	vst	31.6	m	3.0	vst	11.5	vst	4.0
1			st	7.7	m	0.9	m	2.8	st⁻	1.6	m	0.7
2	w	1.6	vvw	0.01	m	0.9	w	0.03	w	0.6	m	0.03
3			vvw	0.1	w	0.2	—	0.02	m	0.4		
4	st	4.1	w	0.2	st⁻	2.7	m	1.3	st⁻	1.0		
5			vst	17.2	m⁻	1.0	vvst	10.7	vvst	2.5		
6	m	3.0	vvw	0.1	m⁺	2.3	m	0.5				
8			m	1.8								

In this table suffix 1 refers to Sb and suffix 2 to Cd and Zn. The accuracy of the parameter values amounts to $\pm$ 0.005.

The intensities are calculated from the formula $I = p \cdot S^2$ where p is the frequency factor and S is the structure factor. β reflections have been omitted if they do not coincide with an α reflection. The following abbreviations are used in the tables: st = strong, m = medium, w = weak and v = very. Reflections in the powder photographs with $\sin^2\Theta < 0.23$ must not be compared with reflections of higher glancing angles as the inner and the outer parts of the photographs are taken in different cameras.

As far as could be ascertained this type of crystal structure has not been found previously. Each atom is surrounded by four other atoms. One of them is of the same kind as the central atom, and the other three are of the

*Table 5. CdSb. Powder photograph. Cr-K radiation.**

hkl	$\sin^2\Theta$ calc	$\sin^2\Theta$ obs	*I* obs	*I* calc
020	0.0771	0.0781	vw	18.7
021	0,0951	—	—	2.3
112 β	0.1015	0.1023	m	22.2
102	0.1035			17.7
112	0.1228	0.1237	st$^+$	111
200	0.1253	0.1253	w	14.8
121	0.1264	0.1275	st	58.9
210	0.1446	—	—	0.7
022	0.1493	—	—	1.0
211	0.1627	0.1642	st	88.2
122	0.1806	0.1819	w	24.2
202	0.1975	—	—	0.2
220	0.2024	—	—	0.1
113	0.2130	0.2134	vw	5.9
212	0.2168	—	—	1.3
221	0.2205	0.2212	w	48.8
131	0.2228	0.2241	vw	29.8
123 β	0.2238			4.8
004 β	0.2393	0.2400	w	19.1
023	0.2395			0.4
041 β	0.2704	0.2717	w	2.9
123	0.2708			24.2
222	0.2746	—	—	0.1
132	0.2769	0.2777	st$^+$	198
004	0.2888	0.2895	st$^-$	95.4
230	0.2987	0.2995	w	18.9
232 β	0.3066	0.3079	st	1.0
213	0.3073			119
040	0.3083			36.5
231	0.3168	—	—	1.0
311	0.3193	0.3200	st	89.2
104	0.3201			5.9
041	0.3263	0.3272	w	14.6
114	0.3394	—	—	0.6
302	0.3542	0.3551	vw	1.3
322 β	0.3565			1.6
141	0.3576	0.3590	m$^-$	35.6
240 β	0.3584			5.5
223	0.3648	0.3657	m	41.2
024	0.3658	0.3672	m	21.0
133	0.3672			21.5
232	0.3709	0.3720	vw	5.0
312	0.3735	—	—	0.4
321	0.3771	0.3784	m$^+$	69.2
042	0.3804	—	—	0.0
124	0.3972	0.3980	vw	3.7
142	0.4118	—	—	0.5
204	0.4141	0.4148	w	22.5
400 β	0.4144			1.4
115 β	0.4147			0.4
143 β	0.4149			2.4
410 β	0.4303	0.4335	m$^-$(br)	4.4
323 β	0.4310			9.1
322	0.4313			7.8
214	0.4334			0.4
240	0.4336			27.7
241	0.4516	—	—	2.7
233	0.4612	—	—	0.1
125 β	0.4625	0.4645	m$^-$	9.8
313	0.4637			44.2
043	0.4707	0.4715	w$^+$	28.9
304 β	0.4718			0.6
331	0.4735	—	—	1.8
224	0.4912	—	—	0.1
215 β	0.4924	0.4950	w	4.4
243 β	0.4926			0.6
421 β	0.4930			10.1
044 β	0.4934			11.1
134	0.4935			4.2
400	0.5014	0.5026	w$^+$(br)	7.2
250 β	0.5017			9.1
115	0.5018			2.2
143	0.5020			12.1
341 β	0.5028			3.9
242	0.5058	—	—	0.1
410	0.5206	0.5215	m	21.9
323	0.5215			45.7
332	0.5276	0.5286	w	9.3
025	0.5282			11.6

* In the powder photographs of CdSb is has been impossible to identify the following three linies: 0.2598 (vw), 0.2836 (vw) and 0.4197 (vw).

hkl	$\sin^2\Theta$ calc	$\sin^2\Theta$ obs	*I* obs	*I* calc
151	0.5310	—	—	3.0
411	0.5387	—	—	7.2
430 β	0.5577			6.8
153 β	0.5582	0.5593	m	1.9
125	0.5596			48.8
304	0.5708			3.2
431 β	0.5726			2.7
060 β	0.5733	0.5711	vvw	4.9
402	0.5735			2.0
420	0.5784	0.5804	vw	14.1
116 β	0.5788			11.1
152	0.5852	—	—	0.0
234	0.5875	0.5886	w	22.1
061 β	0.5881			6.4
314	0.5900	—	—	2.8
412	0.5928	—	—	0.0
215	0.5958			21.8
243	0.5960			3.2
421	0.5965	0.5966	st	50.4
044	0.5970			55.4
244 β	0.5970			8.5
250	0.6070	0.6072	m	45.7
341	0.6083			19.5

hkl	$\sin^2\Theta$ calc	$\sin^2\Theta$ obs	*I* obs	*I* calc
333	0.6178	—	—	3.7
251	0.6250	0.6250	m-	39.4
144	0.6283	—	—	0.7
324	0.6478	—	—	1.7
006	0.6497	—	—	4.9
422	0.6506	—	—	1.2
404 β	0.6530			1.7
225	0.6536	0.6540	m	32.3
145 β	0.6536			7.8
135	0.6559			19.7
342	0.6625	0.6624	w	24.5
154 β	0.6626			0.8
430	0.6748	0.6747	w	33.9
153	0.6754			9.7
252	0.6792	—	—	1.8
106	0.6810	—	—	5.1
413	0.6830	0.6846	vvw	2.7
441 β	0.6841			5.5
431	0.6928	0.6929	w^+	13.3
060	0.6936			24.4
116	0.7003	0.7000	w^+	55.3

second kind. The four surrounding atoms form a deformed tetrahedron. It is possible to regard the lattice as a very strongly deformed diamond lattice. The interatomic distances may be seen from the following table:

CdSb		ZnSb	
Cd — Cd	2.99 kX	Zn — Zn	2.58 kX
Cd — Sb	2.80 »	Zn — Sb	2.66 »
	2.81 »		2.69 »
	2.91 »		2.74 »
Sb — Sb	2.81 »	Sb — Sb	2.81 »

These distances seem to be plausible. If the coordination number is assumed to be four, the following distances can be calculated by Pauling's [6] method:

Cd — Cd	2.80 kX	Zn — Zn	2.57 kX
Cd — Sb	2.85 kX	Zn — Sb	2.67 kX
	Sb — Sb 2.89 kX		

Table 6. ZnSb. Powder photograph. Cr-K radiation.

hkl	$\sin^2\Theta$ calc	$\sin^2\Theta$ obs	*I* obs	*I* calc	*hlk*	$\sin^2\Theta$ calc	$\sin^2\Theta$ obs	*I* obs	*I* calc
020	0.0876	0.0862	vw	18.3	232	0.4128	0.4142	m	2.7
021	0.1076	—	—	2.1	321	0.4132			29.8
102	0.1137	0.1132	w	28.7	042	0.4303	—	—	0.2
112	0.1356	0.1350	m	53.6	124	0.4405	—	—	1.8
200	0.1358			2.8	204	0.4548	—	—	3.8
121	0.1415	0.1405	w	48.8	142	0.4643	0.4642	vvw	4.9
210	0.1577	—	—	6.7	322	0.4730	—	—	1.8
022	0.1674	—	—	1.0	214	0.4767	0.4761	vvw	8.2
211	0.1777	0.1778	w	60.9	240	0.4864	—	—	4.7
122	0.2013	0.2014	vw	17.1	241	0.5063	0.5094	w	8.0
221 β	0.2016			4.6	313	0.5069			34.0
202	0.2156	—	—	0.0	233	0.5124	—	—	0.1
220	0.2235	—	—	0.0	331	0.5228	—	—	0.4
113	0.2353	—	—	0.0	043	0.5300	0.5304	vw	14.7
212	0.2375	—	—	0.7	224	0.5424	—	—	0.2
221	0.2434	0.2440	m	22.8	400	0.5433	—	—	8.5
123 β	0.2493	0.2503	vw	3.8	134	0.5501	0.5511	vw	5.8
131	0.2511			3.0	243 β	0.5514			2.0
023	0.2670	0.2681	vvw	7.3	115	0.5542	0.5552	vw	4.0
123	0.3010	0.3020	w	19.0	044 β	0.5544			6.0
222	0.3032	—	—	0.0	143	0.5639	0.5641	vw	13.5
132	0.3109	0.3116	vst	136	410	0.5653			9.5
004	0.3189	0.3208	m	59.8	242	0.5661	0.5675	vw	0.0
230	0.3330			29.6	250 β	0.5661			2.7
223 β	0.3337	0.3335	m	5.0	323	0.5727	0.5739	vw	13.7
141 β	0.3350			6.9	332	0.5826			22.4
024 β	0.3367			4.2	251 β	0.5826	0.5828	w	4.7
213	0.3372	0.3380	st	85.0	143 β	0.5826			2.4
312 β	0.3373			0.7	411	0.5852	—	—	0.0
311	0.3475	0.3495	st	72.9	025	0.5860	—	—	0.1
040	0.3506			21.4	151	0.6016	—	—	0.0
104	0.3529	—	—	3.5	125	0.6199	0.6216	w	43.8
231	0.3530	—	—	0.1	402	0.6231			1.0
041	0.3705	0.3706	vvw	7.5	304	0.6246	—	—	0.7
114	0.3748	—	—	1.7	420	0.6310	—	—	6.7
302	0.3854	—	—	0.4	412	0.6450	—	—	0.2
240 β	0.4028			0.9	314	0.6465	—	—	1.4
223	0.4029	0.4040	m	24.8	421	0.6509	0.6529	m	40.1
141	0.4045			34.6	234	0.6520			38.7
024	0.4066	0.4074	vw	21.2	215	0.6561	—	—	14.6
312	0.4073			3.5	152	0.6614	—	—	8.0
133	0.4106	—	—	2.0	341	0.6761	—	—	13.9
243	0.6658	—	—	9.9	333	0.6822	—	—	0.4
044	0.6695	0.6695	w	30.1	250	0.6836	0.6848	vw	13.7
061 β	0.6697			0.7	315 β	0.6840			13.5

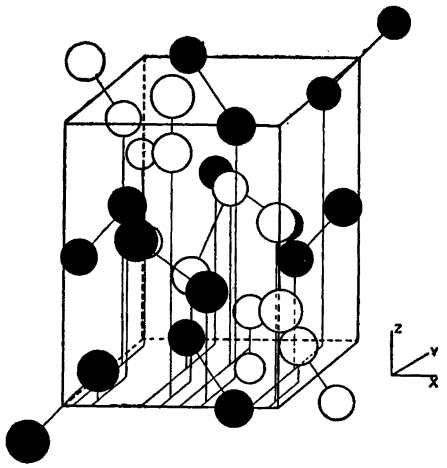


Fig. 1. The crystal structure of CdSb and ZnSb. ● *Sb,* ○ *Cd or Zn.*

Only the distance between the Cd atoms in CdSb differs rather much from the calculated value.

The structure is reproduced in Fig. 1.

SUMMARY

The intermetallic compounds CdSb and ZnSb are isomorphous, crystallize in the space group $D_{2h}^{15} - P/bca$, and their orthorhombic cells contain eight formula units. The lattice dimensions, the atomic coordinates and the interatomic distances are determined and may be seen from the tables in the text.

Finally, I wish to thank Professor A. Ölander for his encouraging interest in my work and Mr Aage Kivikanto who has performed part of the experimental work.

REFERENCES

1. Halla, F., and Adler, J. *Z. anorg. u. allgem. Chem.* **185** (1928) 184.
2. Halla, F., Nowotny, H., and Tompa, H. *Z. anorg. u. allgem. Chem.* **214** (1933) 196.
3. Ölander, A. *Z. Krist.* **91** (1935) 243.
4. Ölander, A. *Z. physik. Chem. A* **173** (1935) 284.
5. Cooke, A. *Am. J. Sci.* (Sill.) 2, **18** (1855) 234, **20** (1855) 212.
6. Pauling ,L. *J. Am. Chem. Soc.* **69** (1947) 542.

Received March 23, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 408—413

Diffusion Experiments on Gramicidin S, Tyrocidine, and Gramicidin

KAI O. PEDERSEN and R. L. M. SYNGE

Institutes of Physical Chemistry and Biochemistry, University of Uppsala, Sweden

In connection with work on gramicidin S, tyrocidine and gramicidin, it was of interest to get an approximate idea of the molecular weights of these peptides. As it could be supposed that these would be of the order of some thousands, we decided to measure the diffusion constants for these peptides.

For an arbitrary molecule we have

$$f = \frac{RT}{D}$$

where f is the frictional coefficient per mol., R the gas constant ($8.314 \cdot 10^7$ ergs per °C per mol.), T the absolute temperature, and D the diffusion constant. For a spherical and unhydrated molecule the frictional coefficient, f_0, may be expressed in the following way

$$f_0 = 6\pi\eta N\left(\frac{3MV}{4\pi N}\right)^{1/3}$$

where η is the viscosity of the solution (in poises), N the Avogadro number, M the molecular weight and V the specific volume of the solute. By combining these two formulae and solving for M, we get

$$M\left(\frac{f}{f_0}\right)^3 = \frac{R^3T^3}{162\,\pi^2N^2\eta^3} \cdot \frac{1}{D^3V} = \frac{k}{D^3V}$$

where k is a constant depending upon the temperature and the solvent. For water at a temperature of 20° C, k has a value of $24.14 \cdot 10^{-15}$. For an un-

hydrated spherical particle $f/f_0 = 1$, in all other cases $f/f_0 > 1$. This means that diffusion experiments will furnish us with an upper limit for the molecular weight, if effects due to association or dissociation are disregarded.

Various solvents were used in the experiments, which were done at 20° C, but in order to get comparable values, the diffusion constants were all reduced to water at 20° C by means of the usual equation

$$D_1 = D_2 \cdot \frac{\eta_2}{\eta_1}$$

The relative viscosities of the various solvents used in the experiments were determined in an Ostwald viscosimeter using water as the reference substance. Potassium chloride was used with the peptide hydrochlorides studied, in order to eliminate effects due to diffusion potentials.

Table 1. Relative viscosity of solvents used in the diffusion experiments. Temperature: 20.0° C.

Solvent	Outflow time (in sec)				Average	Density	η/η_0
Water	46.7	46.8	46.7	46.6	46.7	0.998	1
0.1 *M* KCl in 50 per cent (v/v) ethanol	94.7	94.2	94.6	94.6			
	94.5	94.8	94.6	94.9	94.6	1.062	2.16
Water	108.3	108.8	108.8	108.9	108.7	0.998	1
50 per cent (v/v) ethanol	326.6	326.1	326.0	326.0	326.2	0.928	2.78
70 » » » »		300.7	300.5	300.7	300.6	0.884	2.49
0.2 *M* KCl in 50 per cent ethanol		317.4	317.2	317.6	317.4	0.935	2.73

The diffusion was followed by means of the Lamm scale method [1]. The diffusion constant was calculated both according to the »area method» and according to the »moment method»[2]. In the first case the diffusion constant is defined in the following way

$$D_A = \frac{A^2}{4\pi t H^2}$$

where A denotes the area between the curve and the x-axis, H is the maximum height of the curve and t is the time after the formation of the boundary. In the second case the diffusion constant is calculated from the expression

$$D_m = \frac{m_2}{2\,t\,A}$$

where m_2 is the second moment of the curve about the vertical axis through the arithmetic medium of the curve.

The first experiments were carried out in the ordinary Lamm diffusion cell [1] with inner part of stainless steel. The peptides were dissolved in 50 per cent (v/v) aqueous acetic acid solutions 0.1 M in respect of potassium chloride. The diffusion cell was allowed to stand with this solution for some days before the diffusion experiments were started in order to remove any material from the cell that might disturb the measurements. Silicone was substituted for the wool-fat generally used as sealing-grease in this cell. Only a few, however, of the 18 experiments of this series could be used; all the other measurements were disturbed in some way or another by leakage and had to be rejected.

In the second series of experiments aqueous ethanol was used as solvent. As at that time the first Claesson diffusion cell [3] was put into use, this cell was used for the experiments S 20—S 28. It was found easier to make leak-free, although it sometimes gave trouble. As sealing-grease between the mirror-glass windows and the middle metal part of the cell wool-fat, several times extracted with acetone, was used. Recently it has been found that the Claesson cell worked exceedingly well when the utmost care was taken to make the surfaces of the metal part completely plane, and when, instead of ordinary mirror glasses, windows of specially ground plane parallel optical glass were used.

The length of the diffusion experiment varied from 20 hours to 40 hours.

The gramicidin S, tyrocidine, and gramicidin used in the diffusion experiments were the same preparations as those used by Synge and Tiselius [4]. The tyrocidine components I, II and III were prepared as described in that paper.

DISCUSSION

The general agreement between the results in acetic acid and ethanol solutions suggests that the molecular state of the peptides in the different solvents may not be greatly different and that the diffusion data may provide information concerning the molecular weights of the peptides. All the values lie, as might be expected, between those found for simpler peptides and those for proteins of low molecular weight.

Table 2. Diffusion data for gramicidin S, tyrocidine, and gramicidin.

Substance	Experiment no.	$D_m \cdot 10^7$	$D_A \cdot 10^7$	Concentration g per 100 ml solution	Solvent
Gramicidin S (HCl)	S23A	25.9	27.0	1	0.2 *M* KCl in
	S23B	24.4	25.3	1	50 per cent ($^v/_v$)
	S23C	25.9	27.0	1	aqueous ethanol
	S25B	27.1	27.2	0.5	
	S25C	26.4	27.1	0.5	
	S4	26.3	25.5	1	0.1 *M* KCl in
	S2	27.1	25.1	1	50 per cent ($^v/_v$)
	S6	29.0	30.2	0.5	aqueous acetic
	S18	24.5	25.8	0.3	acid
Tyrocidine (HCl)	S26 II	18.4	17.4	1	0.2 *M* KCl in
(unfractionated)	S20B	20.4	19.6	0.5	50 per cent ($^v/_v$)
	S20A	20.5	19.2	0.5	aqueous ethanol
	S20C	22.7	21.6	0.5	
	S3	20.8	19.8	1	0.1 *M* KCl in
	S1	20.0	19.7	1	50 per cent ($^v/_v$)
	S14	20.4		0.7	aqueous acetic
	S5	18.3	19.3	0.5	acid
Tyrocidine fraction					
I »colorless»	S24C	21.2	22.9	0.5	0.2 *M* KCl in
II »pink»	S28A	20.3	21.8	0.5	50 per cent ($^v/_v$)
	S28C	20.9	21.5	0.5	aqueous ethanol
	S28B	20.6	21.7	0.5	
III »blue»	S27B	16.5	20.2	0.5	
	S27A	17.9	20.1	0.5	
Gramicidin	S21A	18.4	19.2	1	70 per cent ($^v/_v$)
	S21B	17.8	18.3	1	aqueous ethanol
	S21C	16.2	18.2	1	
	S22A	18.7	19.4	0.5	
	S22B	18.8	19.2	0.5	
	S22C	19.7	19.4	0.5	

The X-ray [5] and chemical [6] data on *gramicidin S* indicate that it must be either a cyclopentapeptide (MW of free unhydrated base 570) or a cyclodecapeptide (MW of free unhydrated base 1140). If a spherical unhydrated mole-

cule is assumed having partial specific volume 0.81 (calc. according to Cohn and Edsall) [7], the diffusion constants correspond to molecular weights in the range (1080—1880). This result suggests that gramicidin S has the cyclodecapeptide structure. It would be necessary to postulate considerable departure from the spherical form or extensive solvation or association to reconcile the diffusion data with the cyclopentapeptide structure. The cryoscopic data for gramicidin S in camphor [8] are the only other available indications and they gave a molecular weight of 1060—1340, which appears to be in agreement with our results. Employing the arithmetic mean of our values for gramicidins ($D_{20} \cdot 10^7 = 26.5$), f/f_0 is 1.12 for the cyclodecapeptide molecule.

If the same degree of asymmetry/solvation is assumed for *tyrocidine* as for gramicidin S and the partial specific volume is taken to be 0.75, the diffusion data suggest a molecular weight in the range (1900—5100). This agrees reasonably with a molecule such as that postulated by Hotchkiss [9] — a 19-peptide with a molecular weight of 2473 for the free unhydrated base. The different components of »tyrocidine»[4] separated by adsorption on charcoal and differing in tryptophan content do not differ much from one another in their diffusion characteristics, and are evidently, as suggested by the chemical evidence, rather similar substances. The small differences in their diffusion constants may turn out to be real; it is perhaps significant in this connection that of all the materials here studied only unfractionated »tyrocidine» gave evidence of polydispersity, D_m being systematically, though only slightly, higher than D_A. The differences in D for the different tyrocidine components could be attributed to association effects, increasing with increasing tryptophan content.

The diffusion of *gramicidin* has only been studied in 70 per cent (*v/v*) ethanol. Here, making the same assumptions as with tyrocidine, but assuming partial specific volume 0.80, the MW would lie in the range (2800—5000). This is in fair agreement with the MW determinations based on the vapour pressure lowering in methanol and the diffusion in butanol. Cryoscopic determinations in camphor, phenol and cyclohexanol give lower values (for references see Hotchkiss [9]). It is hoped to report before long new stoichiometric and crystallographic data bearing on this problem.

SUMMARY

1) The diffusion constants for gramicidin S, tyrocidine components and gramicidin have been determined in aqueous acetic acid and ethanol solutions.

2) From the diffusion constants maximum values for the molecular weights of the peptides have been calculated.

3) The results suggest that gramicidin S has the cyclodecapeptide structure. The values for tyrocidine components lie in the range 1900—5100 and those for gramicidin in the range 2800—5000.

The authors wish to thank Professor Svedberg for the facilities put at their disposal. The expences in connection with the investigation were defrayed from a grant from the Rockefeller Foundation. One of the authors (R. L. M. S.) took part in this work while on study leave from the Lister Institute of Preventive Medicine, London, England.

REFERENCES

1. Lamm, O. *Nova Acta Reg. Soc. Scient. Upsal.* **IV, 10** (1937) no. 6.
2. Gralén, N. *Kolloid.-Z.* **95** (1941) 188.
3. Claesson, S. *Nature* **158** (1946) 834.
4. Synge, R. L. M., and Tiselius, A. *Acta Chem. Scand.* **1** (1947) 749.
5. Crowfoot, D., and Schmidt, G. M. J. To be published.
6. Consden, R., Gordon, A. H. P., Martin, A. J., and Synge, R. L. M. *Biochem. J.* **41** (1947) 596.
7. Cohn, E. J., and Edsall, J. T. *Proteins, amino acids and peptides.* American Chemical Society Monograph no. 90 (1943).
8. Belozersky, A. N., and Paskhina, T. S. *Biokhimiya* **10** (1945) 344; *Lancet* **2** (1944) 716.
9. Hotchkiss, R. D. *Advances in Enzymol.* **4** (1944) 153.

Received February 28, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 414—425

Beitrag zur Kenntnis der Natriummetaphosphate. II

W. TEICHERT

Ångvärmeinstitutet, Ingeniörsvetenskapsakademien, Stockholm, Schweden

Im Anschluss an eine kürzlich veröffentlichte Untersuchung über Natriummetaphosphate [1] wurde die Hydratation der Trimetaphosphorsäure und der Polymetaphosphorsäuren studiert, um möglicherweise an Hand der Spaltprodukte Rückschlüsse auf die Konstitution der Metaphosphat-Anionen ziehen zu können.

Die analytischen Methoden zur Bestimmung der bei der Hydratation entstehenden Anionen nebeneinander sind jedoch ziemlich umständliche Operationen und mit Mängeln behaftet, die eine exakte Bestimmung kaum zulassen sondern nur Näherungswerte ergeben. Es erschien daher zweckmässiger, einen anderen Weg zu gehen, besonders weil man es hierbei teilweise mit äusserst instabilen Gebilden zu tun hat.

Die verschiedenen Phosphorsäuren unterscheiden sich in Bezug auf den Verlauf der pH-Kurven bei der potentiometrischen Titration mit Lauge. In Fig. 1 sind die Titrationskurven von Meta-, Ortho- und Pyrophosphorsäure wiedergegeben. Metaphosphorsäure ergibt eine Kurve, welche charakteristisch ist für eine starke Mineralsäure mit nur *einer* Dissociationskonstante für sämtliche H^+-Ionen, während die Kurve der Ortho- und Pyrophosphorsäure zwei deutlich ausgeprägte Potentialsprünge aufweist. Der zweite Potentialsprung mit einem Äquivalenzverhältnis $Na_2O : P_2O_5 = 2 : 1$ liegt bei Orthophosphorsäure bei pH $\approx$ 9,5 und bei Pyrophosphorsäure bei pH $\approx$ 10,8. Pyrophosphorsäure hat darüber hinaus noch einen schwachen Potentialsprung bei pH $\approx$7 und einem Äquivalenzverhältnis $Na_2O : P_2O_5 = 1{,}5 : 1$.

Der Verlauf der Hydratation und Spaltung der Metaphosphorsäuren wurde mit Hilfe von Vergleichstitrationen an Modellösungen, d. h. Mischungen von frisch-hergestellter 0,1 *N* Metaphosphorsäure, 0,1 *M* Ortho- und 0,05 *M* Pyrophosphorsäure, studiert, da es sich schon bei der ober genannten Untersuchung erwiesen hatte, dass bei der Hydratation der Metaphosphorsäuren nur teilweise Orthophosphat gebildet wird, und der höhere pH-Wert des

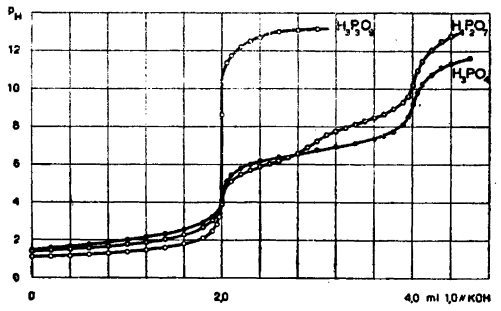


Fig. 1. Potentiometrische Titration von 0,1 N $H_3P_3O_9$, 0,1 M H_3PO_4 und 0,05 M $H_4P_2O_7$.

zweiten Potentialsprunges bei der potentiometrischen Titration von hydratisierter Metaphosphorsäure deutete darauf hin, dass der grössere Anteil der Spaltprodukte auf den totalen Phosphorgehalt der Lösung bezogen von Pyrophosphorsäure ausgemacht wurde. Dies geht auch aus Bell's [2] Untersuchungen an den Natriumsalzen dieser Säuren hervor. Bei der Hydratation von Trimetaphosphorsäure wurde für die gebildete Orthophosphorsäure analytisch ein Endwert von ca 33 % des totalen Phosphorgehaltes gefunden, während bei einer Polymetaphosphorsäure der entsprechende Wert bei ca 42 % lag. Auf Grund des pH-Verlaufs bei der potentiometrischen Titration von Metaphosphorsäuren bei verschiedenen Zeitpunkten nach der Darstellung war also anzunehmen, dass die Differenz zu 100 % in Form von Pyrophosphorsäure vorliegt, zumal da diese Säure bei Raumtemperatur ziemlich beständig ist und nur langsam in Orthophosphorsäure umgewandelt wird.

Parallel mit den Titrationen von Metaphosphorsäuren mit verschiedenem Alterungsgrad wurden Modellösungen titriert, welche ausser dem durch kolorimetrische Phosphat-Bestimmung ermittelten Orthophosphorsäuregehalt in Form von 0,1 *M* Lösung das diesem Wert entsprechende Volumen 0,05 *M* Pyrophosphorsäure enthielten, während der Rest zu 100 % aus frich-bereiteter Metaphosphorsäure bestand. Das Verhältnis Meta- : Ortho- : Pyrophosphorsäure wurde für die verschiedenen Zeitpunkte aus den Zeit-Konzentrationsdiagramm für die Bildung von Orthophosphorsäure, welches in Fig. 2 wiedergegeben ist, ermittelt.

Zur Herstellung dieser Säuregemische wurde die Ionenaustausch-Methode angewendet, und zwar wurden die den Säuren entsprechenden analysen-reinen Natriumsalze eingewogen, in dest. Wasser gelöst, durch eine mit H^+-Ionen gesättigte Ionenaustauscherschicht geschickt und die erhaltenen Säuren unmittelbar mit 1,0 *N* KOH potentiometrisch titriert.

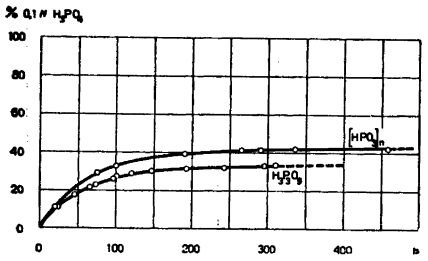


Fig. 2. Bildungsgeschwindigkeit von Orthophosphorsäure bei der Hydratation von Trimeta- und Polymetaphosphorsäure.

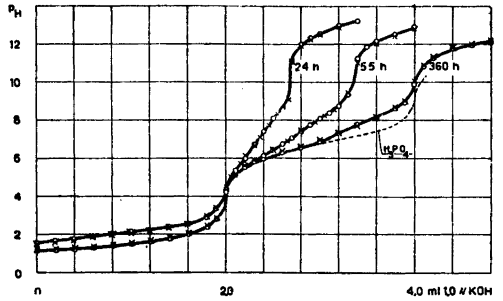


Fig. 3. Vergleichstitrationen von Trimetaphosphorsäure verschiedenen Alterungsgrades und der entsprechenden Modellösungen. o *0.1 N Trimetaphosphorsäure bei verschiedenem Alterungsgrad.* x *Modellösung (0,1 N $H_3P_3O_9$ + 0,1 M H_3PO_4 + 0,05 M $H_4P_2O_7$).*

TRIMETAPHOSPHORSÄURE

Zum Vergleich mit den für Trimetaphosphorsäure bei verschiedenen Alterungsgraden erhaltenen Titrationsdiagrammen wurden Modelllösungen titriert, deren Zusammensetzung aus dem für das entsprechende Alter dieser Säuren bestimmten Orthophosphorsäuregehalt berechnet wurden unter der Annahme, dass der Anteil der gebildeten *Pyrophosphorsäure* ca doppelt so gross ist wie der für *Orthophosphorsäure* analytisch bestimmte Wert. Die procentuale Zusammensetzung dieser Lösungen geht aus Tabelle 1 hervor.

Tabelle 1. Zusammensetzung der Modellösungen.

Alter der Trimetaphosphorsäure h	0,1 *N* $H_3P_3O_9$ %	0,1 *M* H_3PO_4 %	0,05 *M* $H_4P_2O_7$ %
24	66,4	11,2	22,4
55	33	22	45
360	0	35	65

Tabelle 2 gibt ein Beispiel einer derartigen Vergleichstitration von hydratisierter Trimetaphosphorsäure und der entsprechenden Modellösung. Fig. 3 zeigt den Potentialverlauf dieser Titrationen graphisch. Bei sämtlichen Titrationen wurden 20 ml Säure vorgelegt und mit 1,0 *N* KOH titriert. Die zweite Decimale der pH-Werte ist visuell geschätzt.

Tabelle 2. Potentiometrische Titration von $H_3P_3O_9$ (55 h).

$H_3P_3O_9$		Modellösung	
ml 1,0 *N* KOH	pH	ml 1,0 *N* KOH	pH
0	1,20	0	1,22
0,4	1,31	0,2	1,30
0,8	1,50	0,6	1,46
1,2	1,75	1,0	1,67
1,6	2,15	1,4	2,00
1,8	2,55	1,8	2,71
1,9	2,94	1,9	3,19
2,0	4,30	2,0	4,40
2,1	5,10	2,1	5,10
2,2	5,50	2,2	5,60
2,4	6,08	2,3	5,89
2,6	6,70	2,5	6,38
2,7	7,01	2,65	6,86
2,8	7,38	2,8	7,49
3,0	8,04	2,95	7,88
3,2	8,69	3,05	8,18
3,3	9,33	3,15	8,48
3,4	11,20	3,3	9,41
3,5	11,80	3,4	11,00
3,8	12,50	3,6	11,96
4,0	12,90	3,8	12,40

Die in Fig. 3 wiedergegebenen potentiometrischen Titrationen zeigen, dass die für hydratisierte Trimetaphosphorsäuren erhaltenen Kurvenzüge praktisch identisch sind mit denjenigen, welche für die entsprechenden Modellösungen ermittelt wurden. Die Hydratation der Metaphosphorsäure geht also parallel mit einer Spaltung, und die Zerfallsprodukte stehen in einem ganz bestimmten Mengenverhältnis zu einander. Es ergibt sich hieraus weiterhin, dass man mit Hilfe der potentiometrischen Titration die Zerfallsgeschwindigkeit bestimmen kann, welche durch die Verschiebung des zweiten Potentialsprunges bei pH $\approx$ 10,2 zum Ausdruck kommt. Der procentuale Anteil an unzersetzter Metaphosphorsäure in einem derartigen Gemisch errechnet sich nach Gleichung (1). Hierin bedeutet a den Laugenverbrauch bis zum ersten Potentialsprung und b die Differenz zwischen ersten und zweiten Potentialsprung.

$$\frac{(a - b) \cdot 100}{a} = \% \, H_3P_3O_9 \qquad (1)$$

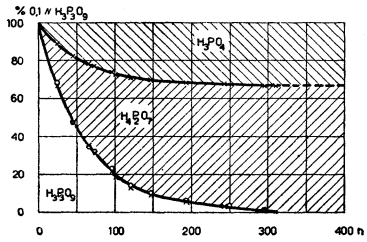


Fig. 4. Spaltung von Trimetaphosphorsäure in Ortho- und Pyrophosphorsäure bei Raumtemperatur.
o .... *% 0,1 N Trimetaphosphorsäure, best. d. potentiometr. Titration*
x .... *% 0,1 M Orthophosphorsäure (kolorimetr.) + 0,05 M Pyrophosphorsäure (ber.)*

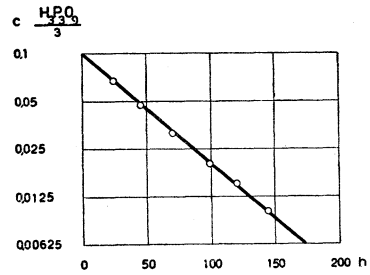


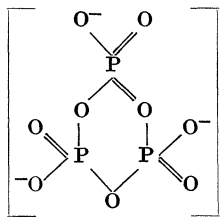
Fig. 5. Zerfallsgeschwindigkeit von Trimetaphosphorsäure.

Fig. 4 gibt den auf diese Weise ermittelten Zerfall der Trimetaphosphorsäure bei Raumtemperatur und die kolorimetrisch bestimmte Bildungsgeschwindigkeit von Orthophosphorsäure und der zugehörigen Pyrophosphorsäure wieder. Es zeigt sich, dass der Zerfall von $H_3P_3O_9$ parallel geht mit der Bildung von Ortho- und Pyrophosphat-Anionen. Auf Grund des nach Gleichung (1) bestimmten Koncentrationsabfalles wurde für Trimetaphosphorsäure die Zerfallskonstante graphisch aus dem in Fig. 5 wiedergegebenen Koncentrations-Zeit-Diagramm ermittelt. In Fig. 5 ist der Verlauf von log c gegen die Zeit aufgetragen, und man ersieht aus dem Verlauf der Kurve, dass es sich hierbei um eine Reaktion erster Ordnung handelt. Die Halbwertszeit ist praktisch konstant und beträgt im Mittel = 43,5 h. Die Zerfallskonstante ergibt sich bei Raumtemperatur nach Gleichung (2).

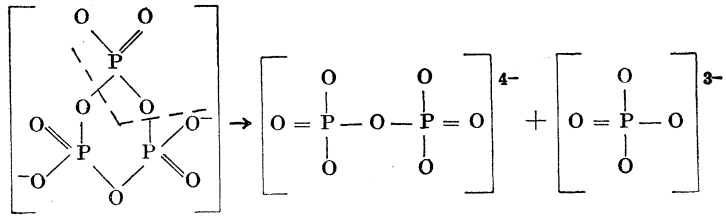
$$k = \frac{-2{,}303 \log \frac{1}{2}}{t_{\frac{1}{2}}}$$
$$k = \frac{0{,}693}{43{,}5} = 0{,}0159 \text{ h}^{-1} \qquad (2)$$

Konstitution des Trimetaphosphat-Anions

Von den für das Trimetaphosphat-Anion möglichen Konstitutionsformeln steht die Ringstruktur etwa in Form eines Sechs-Ringes mit drei koordinativ 4-wertigen Phosphor-Atomen am besten mit den Eigenschaften dieses Anions in Uebereinstimmung.



So haben z. B. sämtliche H^+-Ionen der Säure die gleiche Dissociationskonstante. Das Anion zeigt *keine* ausgeprägte Tendenz zur Bildung stabiler Komplexverbindungen mit Schwermetall-Ionen. Aus wässriger Lösung kristallisiert das Natriumsalz als Hexahydrat. Bei der Aufspaltung des Ringes bildet sich Ortho- und Pyrophorsäure gemäss Gleichung (3). In der von Caglioti [3] und Mitarbeitern für das Natriumtrimetaphosphat-Hexahydrat gefundenen Kristallstruktur bilden je drei $(PO_3)^-$Gruppen ebenfalls eine Einheit in Form eines derartigen P_3O_9-Sechs-Ringes.



(3)

POLYMETAPHOSPHORSÄUREN

Die aus Grahamschen Salzen durch Ionenaustausch dargestellten Polymetaphosphorsäuren weisen bezüglich ihres Verhaltens bei der potentiometrischen und konduktometrischen Titration mit Lauge grosse Ähnlichkeit mit Trimetaphosphorsäure auf. Es liegt daher die Annahme nahe, dass es sich bei der Spaltung dieser Säuren um eine analoge Reaktion handelt.

Auf Grund der kolorimetrisch ermittelten Bildungsgeschwindigkeit von Orthophosphorsäure bei der Hydratation einer Polymetaphosphorsäure, welche aus einem auf ca 750° C erhitzten Grahamschen Salz dargestellt worden war, mit einem Endwert von ca 42 % 0,1 *M* $H_3P\,0_4$, wurde unter der Annahme, dass der Rest aus 0,05 *M* $H_4P_2O_7$ besteht, parallel mit der Metasäure von verschiedenem Alterungsgrad die entsprechenden Modellösungen potentiometrisch titriert. In Tabelle 4 sowie in Fig. 6 sind derartige Vergleichstitrationen wiedergegeben, aus welchen hervorgeht, dass es auch bei der Hydratation von Polymetaphosphorsäure möglich ist, den Reaktionsverlauf an Hand

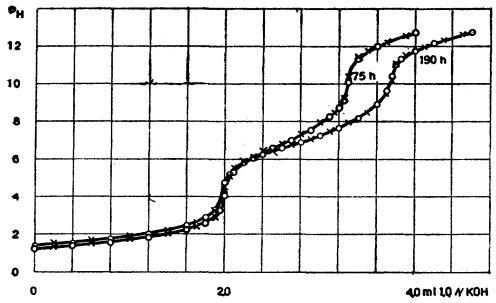


Fig. 6. Vergleichstitrationen einer Polymetaphosphorsäure verschiedenen Alterungsgrades und der entsprechenden Modellösungen.
o *0,1 N Polymetaphosphorsäure bei verschiedenem Alterungsgrad.*
x *Modellösung (0,1 N $[HPO_3]n$ + 0,1 M H_3PO_4 + 0,05 M $H_4P_2O_7$)*

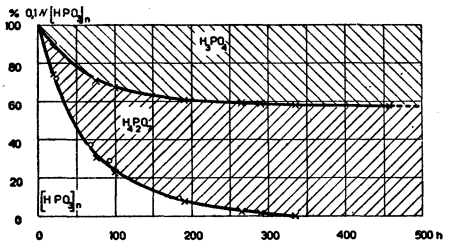


Fig. 7. Spaltung einer Polymetaphosphorsäure in Ortho- und Pyrophosphorsäure bei Raumtemperatur.
o .... *% 0,1 N Polymetaphosphorsäure, best. d. potentiometr. Titration.*
x .... *% 0,1 M Orthophosphorsäure (kolorimetr.) + 0,05 M Pyrophosphorsäure (ber.)*

solcher Modellversuche zu rekonstruieren. Fig. 7 zeigt den durch potentiometrische Titration ermittelten Zerfall einer Polymetaphosphorsäure und die Bildungsgeschwindigkeit von Ortho- und Pyrophosphorsäure.

Tabelle 3. Zusammensetzung der Modellösungen.

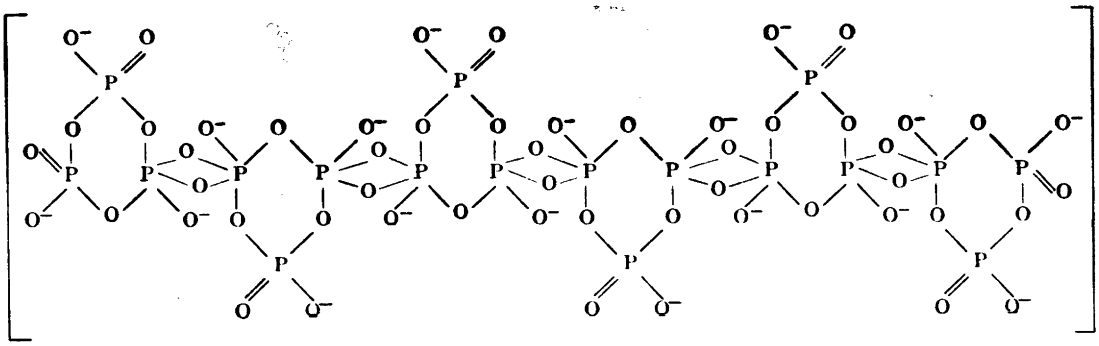
Alter der Polymetaphosphorsäure h	0,1 *N* $[HOP_3]_n$ %	0,1 *M* $H_3P\,O_4$ %	0,05 *M* $H_4P_2O_7$ %
75	35	27	38
190	12	37	51

Der Umstand, dass bei der Hydratation einer Polymetaphosphorsäure der Anteil der gebildeten Orthophosphorsäure ca 42 % des gesamten in der Lösung vorhandenen Phosphors ausmacht, also einen höheren Wert erreicht als bei Trimetaphosphorsäure, deutet darauf hin, dass beim Zerfall der hoch-polymeren Anionen die Spaltungsreaktion einen etwas anderer Verlauf nimmt. Anderseits muss man auf Grund der reversiblen Umwandlung von Trimetaphosphat in Polymetaphosphat vermuten, dass das Trimetaphosphat-Anion die Elementargruppe des Polymetaphosphat-Anions darstellt, zumal da sich

Tabelle 4. Potentiometrische Titration von $[HPO_3]_n$ (75 h).

$[HPO_3]_n$		Modellösung	
ml 1,0 *N* KOH	pH	ml 1,0 *N* KOH	pH
0	1,29	0	1,31
0,4	1,40	0,2	1,35
0,8	1,60	0,6	1,52
1,2	1,86	1,0	1,73
1,6	2,27	1,4	2,02
1,8	2,61	1,7	2,38
1,9	2,95	1,9	2,90
2,0	4,08	2,05	5,12
1,95	3,30	2,0	4,54
2,1	5,30	2,2	5,83
2,3	6,04	2,4	6,40
2,5	6,60	2,6	6,82
2,7	7,00	2,8	7,32
2,9	7,55	3,0	8,00
3,1	8,27	3,15	8,50
3,2	8,75	3,25	9,31
3,25	9,15	3,3	10,38
3,3	10,10	3,4	11,45
3,4	11,35	3,5	11,77
3,6	12,00	3,7	12,18
4,0	12,75	3,9	12,53

die beiden Säuren auch betreffs der Hydratationsgeschwindigkeit sehr ähnlich verhalten. Nimmt man also an, dass die Polymetaphosphat-Anionen durch eine Aneinanderreihung von Trimetaphosphat-Sechsringen entstehen, kommt man zu folgender Konstitutionsformel:



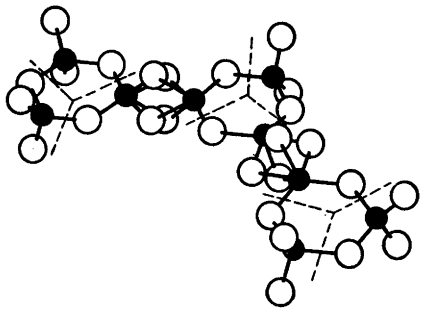


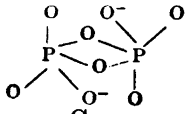
Fig. 8. Vermutlicher räumlicher Bau und Spaltungsschema der Polymetaphosphat-Anionen.

Die Länge der Kette variiert mit der Darstellungstemperatur des Grahamschen Salzes und auch Verzweigungen sind hierbei denkbar. Es muss weiterhin als wahrscheinlich angesehen werden, dass diese Anionen bezüglich der sterischen Anordnungen der Elementargruppen ziemlich unregelmässig aufgebaute Körper darstellen, was durch ihre Tendenz zur Bildung glasartig erstarrender Schmelzen und dem Unvermögen zur Bildung kristalliner Fällungen zum Ausdruck kommt. In Fig. 8 wird gezeigt, wie man sich etwa den räumlichen Aufbau dieser Makro-Anionen und die Spaltung in die Komponenten vorzustellen hat. Bei der Spaltung der Polymetaphosphat-Anionen gehen die koordinativ 4-wertigen P-Atome in Orthophosphat-Ionen über, während je zwei koordinativ 6-wertige P-Atome zu Pyrophosphat hydratisiert werden.

Das Verhältnis der bei der Spaltung gebildeten $(PO_4)^{3-}$ und $(P_2O_7)^{4-}$-Ionen zu einander würde sich dann bei zunehmender Kettenlänge des Polymetaphosphat-Anions zu Gunsten des Pyrophosphats verschieben, was jedoch in diesem Zusammenhang nicht näher untersucht worden ist.

Für eine grössere Anzahl von Polymetaphosphorsäuren, welche aus Grahamschen Salzen mit Darstellungstemperaturen zwischen 650 und 1000° C durch Ionenaustausch dargestellt worden waren, wurde die Zerfallskonstante bei Raumtemperatur bestimmt. Wie aus Fig. 9 hervorgeht, hat die Grösse des Anions kaum einen Einfluss auf die Zerfallsgeschwindigkeit. Die Halbwertszeit für drei verschiedene Polymetaphosphorsäuren wurde zu 51,5 h ermittelt. Für k ergibt sich hieraus ein Wert von 0,0134 h^{-1}.

Konstitution und Komplexbindungsvermögen der Polymetaphosphat-Anionen

Mit Hilfe der im Vorstehenden wiedergegebenen Konstitutionsformel kann eine Erklärung für die Fähigkeit dieser Makro-Anionen zur Bildung stabiler Komplexverbindungen mit den Ionen der Erdalkalimetalle gegeben werden. Die Einlagerung der zweiwertigen Metallionen erfolgt an den Verknüpfungsstellen der einzelnen Trimetaphosphat-Ringe mit einer  Gruppierung. Man kann sich den sterischen Aufbau dieser Gruppe gemäss Fig. 8 ähnlich einem sicherlich deformierten Oktaeder vorstellen mit den beiden Phosphoratomrümpfen in den Spitzen und einer dazwischen senkrecht stehenden, ev. quadratischen Ebene von 4 Sauerstoffionen mit insgesamt 2 überschüssigen negativen Ladungen. Im Schwerpunkt dieses Oktaeders würde dann das zweiwertige Metallion, ionogen an die 2 O^--Gruppen gebunden, eingebaut werden weitgehend abgeschirmt gegen äussere Einwirkung. Die sekundäre Dissociation des Barium-Polymetaphosphat-Komplexes ist bekanntlich so niedrig, dass mit den üblichen für dieses Ion spezifischen Reagentien unter gewöhnlichen Bedingungen eine Fällung nicht zu erzielen ist.

Der kürzlich publizierte Befund, dass das Komplexbindungsvermögen der Polymetaphosphat-Anionen verschieden hoch erhitzter Grahamscher Salze mit zunehmenden Anionengewicht steigt, würde mit dieser Konstitution ebenfalls in Einklang stehen, denn die Anzahl der freien Valenzen an diesen Verknüpfungsstellen im Verhältnis zur Gesamtvalenz des Anions wächst mit steigender Polymerisation. Hieraus folgt, dass man durch konduktometrische Bestimmung des Verhältnisses $Na^+ : X^{2+}$ das mittlere Anionengewicht näherungsweise durch Titration feststellen kann. Die Anzahl der $(P_3O_9)^{3-}$-Gruppen im Makro-Anion lässt sich nach Gleichung (4) berechnen, vorausgesetzt dass diese Konstitutionsformel richtig ist.

$$\frac{2n-2}{n+2} = \frac{X^{2+}}{Na^+} \tag{4}$$

Diese Gleichung gilt unabhängig davon, ob einfache oder verzweigte Kettenstruktur vorliegt. Das verhältnis $X^{2+} : Na^+$ lässt sich durch konduktometrische Titration mit Erdalkali-Acetaten einwandfrei feststellen.

Nephelometrische Titrationen sind für diese Bestimmungen ungeeignet, da die Einstellung des Gleichgewichts in der Nähe des Äquivalenzpunktes ziem-

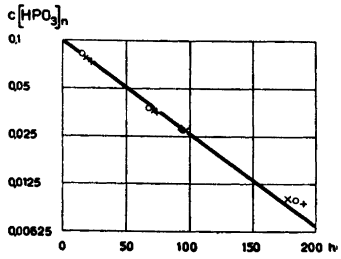


Fig. 9. Zerfallsgeschwindigkeit einiger Polymetaphosphorsäuren.
o ..... *700° C*
+ ..... *800° C*
x ..... *900° C*

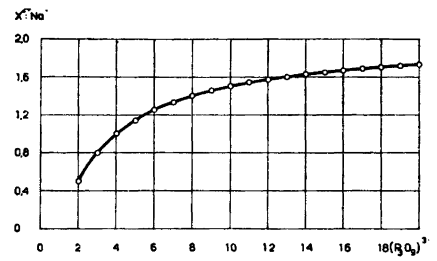


Fig. 10. Abhängigkeit des Komplexbindungsvermögens vom Polymerisationsgrad des Grahamschen Salzes.

lich lange Zeit erfordert und bei lokalem Ueberschuss der Acetatlösung primär entstehende Trübungen sich nur sehr langsam wieder auflösen.

In Fig. 10 ist das berechnete Äquivalenzverhältnis $X^{2+} : Na^{+}$ in Abhängigkeit vom Polymerisationsgrad graphisch wiedergegeben. Aus dem Verlauf des Kurvenzuges geht hervor, dass sich auf diese Weise die Anionengewichte in gewissen Grenzen grössenordnungsmässig ermitteln lassen. Die aus dem titrimetrisch bestimmten Äquivalenzverhältnis graphisch ermittelten Anionen- bzw. Molekulargewichte einiger Grahamscher Salze sind in Tabelle 5 zusammengefasst. Zum Vergleich werden die mit Hilfe der Dialysen-Methode für diese Salze gefundenen Werte aufgeführt, und weisen in Bezug auf die mit diesen Methoden erzielbare Genauigkeit eine zufriedenstellende Uebereinstimmung auf.

Tabelle 5. Anionen- bzw. Molekulargewichte von Grahamschen Salzen.

Darstellungstemperatur °C	Äquivalenzverhältnis $Ba^{2+} : Na^{+}$ best. durch kond. Titr. m. Ba-Acetat	Anionengewicht $(PO_3)^-$ berechnet aus		Molekulargewicht $[NaPO_3]_n$ berechnet aus	
		$Ba^{2+} : Na^{+}$	Dialysenkoeff.	$Ba^{2+} : Na^{+}$	Dialysenkoeff.
650	1,12	1300	1249	1680	1592
750	1,35	1720	1809	2225	2304
850	1,70	4310	—	5560	—

ZUSAMMENFASSUNG

Auf Grund von potentiometrischen Titrationen wurde die Hydratations- und Spaltungsgeschwindigkeit der Trimetaphosphorsäure und der aus verschieden hoch erhitzten Grahamschen Salzen dargestellten Polymetaphosphorsäuren bestimmt. Die bei dem Zerfall auftretenden Spaltungsprodukte lassen den Schluss zu, dass der Struktur des Trimetaphosphat-Anions eine aus je drei Phosphor- und Sauerstoffkernen aufgebauter Sechsring zu Grunde liegt, während die Polymetaphosphat-Anionen Polymerisationsprodukte solcher $(P_3O_9)^{3-}$-Gruppen darstellen. Die auf Grund der experimentellen Befunde vorgeschlagene Konstitution gibt eine Erklärung für die Fähigkeit der Polymetaphosphat-Anionen zur Komplexbildung mit zweiwertigen Metallionen und das Ansteigen des Verhältnisses $X^{2+} : Na^+$ in dem Polymetaphosphat-Erdalkali-Komplex bei steigendem Polymerisationsgrad des Grahamschen Salzes. Durch konduktometrische Titration der Natriumpolymetaphosphat-Lösungen mit Bariumacetat kann die Grösse des Anions in gewissen Grenzen näherungsweise bestimmt werden. Ein Vergleich der konduktometrisch ermittelten Anionengewichte mit den durch Dialyse bestimmten Werten zeigt grössenordnungsmässig eine zufriedenstellende Uebereinstimmung.

LITTERATUR

1. Teichert, W., und Rinman, K. *Acta Chem. Scand.* **2** (1948) 225.
2. Bell, R. N. *Ind. Eng. Chem.* **39** (1947) 136.
3. Caglioti, V., Giacomello, G., und Bianchi, E. *Reale Acc. Ital., Rendiconti*, Ser. VII, 3 (1942) Heft 12.

Eingegangen am 1. Mai 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 426—429

Action of Strong Acids on Acetylated Glycosides

I. Transformation of some Aliphatic Tetraacetyl-β-Glucosides to the α-Form

BENGT LINDBERG

Organisk-kemiska Institutet, Kungl. Tekniska Högskolan, Stockholm, Sweden

While β-glycosides are relatively easy to prepare, there are few good methods for preparation of the corresponding α-glycosides. One of the best is due to Pacsu, who found that acetylated β-glycosides, when treated with stannic chloride [1] or titanium tetrachloride [2] in absolute chloroform, are transformed into α-glycosides. With these reagents, especially with titanium tetrachloride, acetylated β-glycosides of primary and secondary alcohols are easily converted into the α-form.

Glycosides of tertiary alcohols, however, yield acetochlorosugars whilst phenolic glycosides and polysaccarides do not react. Similar transformations have been performed with hydrogen bromide+mercuric bromide in benzene [3] and with concentrated sulfuric acid in acetic anhydride [4]. All the catalysts mentioned are strong acids. It was of interest therefore, to investigate the action of boron trifluoride on acetylated β-glycosides, this being a stronger acid than those mentioned above. Another advantage is its very slight tendency to give up its halogen and consequently it ought to be a more general catalyst for the transformation of β-glycosides than, for example, titanium tetrachloride.

This paper deals with some experiments on aliphatic tetraacetyl-glucosides. Tetraacetyl-α-glucosides of methyl-, ethyl-, isopropyl-, allyl- and benzyl-alcohol have been prepared by treatment of the corresponding β-glucosides with boron trifluoride. Each α-glucoside was also prepared by the action of titanium tetrachloride on the β-glucoside and the resulting products compared and proved identical. Attempts to prepare tetraacetyl-α-tertiary-butylglucoside have not been successful but are being continued.

Absolute chloroform solutions of the β-glucosides were saturated with boron trifluoride and allowed to stand at room-temperature for some time. The time necessary for complete reaction differed considerably. With tetraacetyl-β-isopropylglucoside only 30 minutes was necessary, while the methyl derivative required 24 hours. The reactivity of the different glucosides decreases in the series: isopropyl > ethyl > methyl ≃ allyl ≃ benzyl. An electron repelling group seems to facilate the reaction. Preliminary experiments with tetraacetyl-β(β-chlorethyl)glucoside showed that in this case the reaction proceeds at a slower rate than with the methylglucoside. This is consistent with the observation of the effect of the agluconic group, the inductive effect of chlorine making this group electron attracting.

Table 1. Tetraacetyl-α-glucosides prepared.

Alkyl group	Reaction time in hours with BF_3	M. p.	$[\alpha]_D^{20}$
Methyl	24	100—101°	+ 131°
Ethyl	4	61—61.5°	+ 132°
Isopropyl *	0.5	85.5—86.5°	+ 143°
Allyl *	24	51 —52°	+ 130°
Benzyl	24	109.5—110°.5	+ 142.5°

EXPERIMENTAL PART

Tetraacetyl-α-methylglucoside

A solution of 1 g tetraacetyl-β-methylglucoside (dried over phosphorus pentoxide in a vacuum) in absolute chloroform was saturated with boron trifluoride at room temperature. A slight yellow gelatinous precipitate was formed. The mixture was allowed to stand for 24 hours at room temperature and then shaken with bicarbonate solution. The precipitate disappeared and both phases became colourless.

The chloroform solution was washed with water, dried with calcium chloride, and concentrated under reduced pressure. The residue, 0.98 g, solidified to a white crystalline mass of m.p. 97—98° and $[\alpha]_D^{20}$ + 121° **. After one recrystallization from ethanol the substance had m. p. 100—101° and $[\alpha]_D^{20}$ + 130°.

Tetraacetyl-α-methylglucoside was also prepared by Pacsu's method with titanium tetrachloride [2]. M. p. 100—101°. $[\alpha]_D^{20}$ + 131°. Mixed m. p. of the two preparations was 100—101°.

* These substances are new.

** All m. p. uncorrected. All rotations in about 2 % chloroform solution.

Tetraacetyl-α-ethylglucoside

With boron trifluoride: 1 g tetraacetyl-β-ethylglucoside was treated analogously but the solution was allowed to stand for 4 hours only. Yield 0.91 g crystals of m. p. 60.5—61.5° and $[\alpha]_D^{20}$ + 128°.

With titanium tetrachloride: 5 g tetraacetyl-β-ethylglucoside, 2.5 g titanium tetrachloride and 150 ml absolute chloroform were boiled for 90 minutes on the steam bath. When cold the mixture was shaken with bicarbonate solution, washed with water, dried over calcium chloride and concentrated in a vacuum. The residue was recrystallized from ethanol. Yield 4 g. M. p. 60.5—61°. $[\alpha]_D^{20}$ + 130° Mixed m. p. of the two preparations 60.5—61.5°.

Tetraacetyl-α-isopropylglucoside

With boron trifluoride: 1 g tetraacetyl-β-isopropylglucoside was treated as above. Time 30 minutes. Yield 0.99 g. M. p. 83—84°. $[\alpha]_D^{20}$ + 122°. After two recrystallizations from ethanol its m. p. was 85.5—86.5° and $[\alpha]_D^{20}$ + 142°. The acetyl content was determined according to Clark [5].

$C_9H_{14}O_6(OCCH_3)_4$ (390.2) Calc. Acetyl 44.1 Found Acetyl 44.2

With titanium tetrachloride: 5 g tetraacetyl-β-isopropylglucoside, 2.5 g titanium tetrachloride and 150 ml absolute chloroform were boiled for 60 minutes on the steem bath and the glucoside isolated as above. Yield 3.9 g. M. p. 84—85°. $[\alpha]_D^{20}$ + 135°. After a further recrystallization from ethanol: M. p. 85—86°. $[\alpha]_D^{20}$ + 140°. Mixed m. p. of the two preparations 85—86°.

Tetraacetyl-α-allylglucoside

With boron trifluoride: 2 g tetraacetyl-β-allylglucoside was treated as above. Time 24 hours. Yield 1.6 g of an oil which was dissolved in a small amount of ethanol and allowed to stand in the refrigerator for several days. 1.4 g colourless crystals separated. M. p. 48—50°. $[\alpha]_D^{20}$ + 123°. Recrystallized from ethanol. M. p. 51—52°. $[\alpha]_D^{20}$ + 130°.

$C_9H_{12}O_6(OCCH_3)_4$ (388.2) Calc. Acetyl 44.3 Found Acetyl 43.9

With titanium tetrachloride: 5 g tetraacetyl-β-allylglucoside, 2.5 g titanium tetrachloride and 150 ml absolute chloroform were heated on the steam bath for 5 hours. The mixture was worked up in the usual way. Yield 3.5 g. M. p. 49—51°. $[\alpha]_D^{20}$ + 124°. Mixed m. p. of the two preparations 49.5—51°.

Tetraacetyl-α-benzylglucoside

With boron trifluoride: 2 g tetraacetyl-β-benzylglucoside in 25 ml absolute chloroform was saturated with boron trifluoride and allowed to stand for 24 hours. The solution was washed with bicarbonate and water, dried over calcium chloride and concentrated

under reduced pressure. The viscous residue which slowly solidified, was only partly soluble in ethanol. After extraction with boiling ethanol a brownish-yellow, amorphous, almost optically inactive powder remained. When the ethanol solution was poured into water 0.7 g white crystals of m. p. 99—102° separated. Recrystallized twice from ethanol. M. p. 108.5—109.5° $[\alpha]_D^{20}$ + 140°.

With titanium tetrachloride: 5 g tetraacetyl-β-benzylglucoside, 2.7 g titanium tetrachloride and 70 ml absolute chloroform were heated for 5 hours on the steam bath. The substance was worked up in the usual way. One recrystallication from 75 % ethanol afforded 4.2 g crystals of m. p. 98—99° and $[\alpha]_D^{20}$ + 142°. The substance contained chlorine. Two further recrystallizations removed the chlorine and the substance now had m. p. 109.5—110.5° and $[\alpha]_D^{20}$ + 142.5° Mixed m. p. of the two preparations 108.5—109.5°.

$C_{13}H_{14}O_6$ $(OCCH_3)_4$ (438.2) Calc. Acetyl 39.3 Found Acetyl 39.0

SUMMARY

Five tetraacetyl-α-glucosides, two of which are new, have been prepared from the corresponding β-glucosides by a new method, using boron trifluoride as a catalyst. The glucosides were also prepared with titanium tetrachloride, and the substances prepared by the two methodes were shown to be identical.

A grant from *Statens Naturvetenskapliga Forskningsråd* is gratefully acknowledged.

REFERENCES

1. Pacsu, E. *Ber.* **61** (1928) 137.
2. Pacsu, E. *Ber.* **61** (1928) 1508, *J. Am. Chem. Soc.* **52** (1930) 2563, 2568, 2571.
3. Lindberg, B. *Arkiv Kemi, Mineral. Geol.* **B 18** (1944) no. 9.
4. Lindberg, B. (Unpublished results.)
5. Clark, E. P. *Semimicro quantitative organic analysis* New York (1943).

Received June 14, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 430—439

The Stability of Cytochrome c at Extreme pH Values

KARL-GUSTAV PAUL

Biochemical Department, Medical Nobel Institute, Stockholm, Sweden

In an earlier publication[1] the author has reported an investigation on the oxidation-reduction potential of cytochrome c* in the region pH 4—9. In that paper the existency of two new dissociation constants was shown, one in oxidized c at pH 6.86, and one in reduced c at pH 9.28. From earlier investigations [2] on c is known, however, that some further dissociations occur outside this region, and it would be of interest to confirm these constants by recording the curve E_0' *versus* pH over the complete pH scale. For this reason it was necessary to know the stability of c at different pH values. Generally it takes six hours for a platinum electrode to reach a stable potential in a solution of a partly reduced hemin proteid if no accelerating mediator is present. The aim of this paper is therefore to determine the highest acid and lowest alkaline pH value where no inactivation has taken place after six hours.

c, which has been exposed to pH < 1 or > 12 can not with certainty be distinguished spectroscopically from non-exposed c. A slight shift to the red in the absorption spectrum of reduced, neutralized c is sometimes seen after treatment with strong alkali [3], but never after exposure to even relatively high concentrations of acids [2]. For that reason we considered it necessary to follow the destruction of the enzyme, if it does take place, by measuring its activity in an enzyme system. Not very much has been done in this field, as most investigators have followed the fate of c by the changes in its light absorption. Keilin and Hartree [3], however, using an enzymic method, state that c can stand treatment with 0.3 *M* hydrochloric acid for 18 hours at room temperature without loss of activity and 0.1 *M* potassium hydroxide for 18 hours at the same temperature with a loss of 50 % of the activity.

---

* For the sake of brevity we will call cytochrome c only c in this paper.

METHODS AND PREPARATIONS

The stability experiments were arranged as follows. To a small sample of c in a test tube was added hydrochloric acid or potassium hydroxide to a desired concentration, the tube closed and placed in a waterbath at 20° C. Small samples were withdrawn at certain time intervals and neutralized with potassium hydroxide or hydrochloric acid. The activities of these samples were compared manometrically with the activities of corresponding amounts of untreated c.

We have chosen the succinic acid dehydrogenating system for the determinations of the catalytic activities of the c samples. If the other enzymes in this chain are present in excess of c, the oxygen consumption should vary only with the concentration of active c. We found kidney cortex to be the best source for these enzymes. There are two reasons which support this choice: 1:o Kidney cortex in itself has a low content of c [3], and 2:o. c seems to be easier to remove from this tissue than from for instance heart muscle. The kidney cortex preparations were obtained according to a slight modification of the method of Keilin and Hartree [3]. Slices of about three mm. thickness of pig kidney cortex were ground in a common meatmill and then washed several times with tap water. The residue was homogenized in a »Turmix» together with some crushed ice. The following part of the preparation was carried out at + 5° C. To follow the removal of c from the tissues we washed the homogenate with acetic acid — water mixtures at different pH values. After each acid addition pH was determined with a glass electrode, the suspension centrifuged down (10 min at 3500 r. p. m.) and supernatant and residue investigated spectroscopically, the residue then being used for the washing at the next pH. Parts of the residue were withdrawn immediately after each washing, suspended in *M*/15 phosphate buffer of pH 7.3, and subjected to activity measurements. If the washing with tap water had been very careful, *i. e.* for several hours with running water, nothing unusual was observed. The residues showed after reduction bands at 550—565 mμ and 602—605 mμ, and so did the turbid supernatants but weaker. In the supernatants the band at 550—565 mμ was slightly strengthened at 550 mμ, indicating that the supernatant was enrichened in c in relation to the residue. If, however, the washing with tap water not had been so intensive, some interesting observations could be made. These are collected in Table 1. From Table 1 some conclusions can be drawn.

1. No oxyhemoglobin bands were visible in the insoluble residue after washing at pH 5.2, but the red supernatant after washing the tissue at pH 5.05 obviously contained considerable quantities of what proved to be hemo-

Table 1. Spectroscopical investigation of homogenized kidney cortex (see text). »Dir.» means observation before and »Red.» after addition of dithionite.

pH after add. of acetic acid	Supernatant	Insoluble residue
6.0	Dir. HbO_2-bands Red. Faint band 550—560.	Dir. No bands Red. Faint band 550—560.
5.2	Faintly yellowish-brown. Dir. Faint HbO_2-bands. Red. Band 550—560, reinforcement at 550.	Dir. No bands. Red. Bands 550—565, no reinf. at 550. Faint band at 605.
5.05	Sharply red. clear. Dir. Strong HbO_2-bands. Red. Band 545—565, reinforcement at 550.	Dir. No bands. Red. Band at 550—565, no reinforcement at 550. Strong band 602—605.
4.8	Yellowish-brown like supernatant after pH 5.2. Dir. Faint HbO_2-bands. Red. Faint band at 550.	Dir. No bands. Red. Band at 550—565, no reinforcement at 550. Strong band at 602—605.

globin, since the bands of its CO-compound agreed with those of (human) carbon monoxide hemoglobin. The bands of this oxygenated hemoglobin disappeared upon reduction and were replaced by a broad band at 550—565 mμ.

2. Neither residue showed the c band at 550 mμ after reduction, but in all supernatants this band was found. The absorption spectrum of extracted c from kidney cortex could not be distinguished spectroscopically from that of heart muscle c.

3. In spite of the fact that we never obtained preparations which were free of a blank determination (succinate but not c added), we believe that this residual oxygen uptake in the blanks was due to the presence of traces of c in the homogenates, since the supernatants from pH 4.8 contained c. Obviously c can not be removed completely at a pH where the dehydrogenating system is not completely destroyed. One might call this c »occult», as it can not be seen spectroscopically in the tissue.

4. If residue from pH 5.2 were suspended in phosphate buffer as mentioned above to give a dry weight of about 25 mg/ml and 20 μg of heart muscle c were added to one ml of this suspension, the band at 550 mμ was easily visible after reduction.

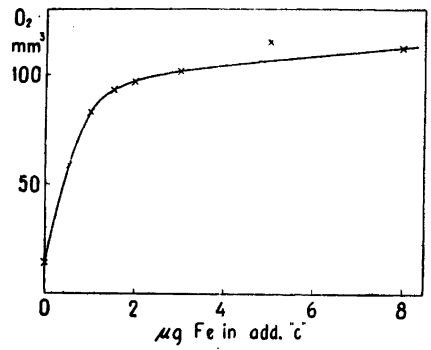


Fig. 1. Calibration curve for kidney cortex preparation number P 11/2. Dry weight 8.1 mg/ml.

With regard to these observations we consider it to be dangerous to decide from spectroscopical data on tissues about the absence of hemin proteids in them or about the spectra and natures of these proteids. Thus we doubt the correctness of stating *from spectroscopy* that kidney cortex and heart muscle c are non-identical. A comparison of washed cow heart muscle and washed kidney cortex confirmed the observation made by Keilin and Hartree [3] that the two distinct bands at 550 and 565 mμ in heart muscle are replaced by one broad band at 550—565 mμ in kidney cortex. If, however, c was extracted from these two organs, we could not spectroscopically find any difference between them. In any case further investigations of this question are required. Bonnichsen found catalases from different organs of one animal to be identical [4].

When the pH of the washings was decreased, the activities progressively declined. Preparations washed at pH 4.8 had almost no activity. Therefore we used washing at pH 5.2 as a standard procedure, since at that pH the majority of c was removed without complete inactivation of the other enzymes in the succinodehydrogenase system. The residue was washed once with distilled water and then suspended in phosphate buffer (*M*/15, pH 7.3). The preparations were stable for 2 days. Before they were used for activity measurements, they were tested with varied amounts of c in order to find the most linear part of the oxygen uptake *versus* added c. A typical curve is shown in Fig. 1.

Dry weights were determined after removal of the buffer salts by dialysis.

The preparations of c which were used for the stability experiments had been prepared according to Keilin and Hartree [5]. This method is a very convenient one even for large scale preparations, but we have never succeeded in reaching an iron content of more than 0.40—0.41 %. The impurities do not contain iron, since the total iron content always agrees with the light absorption values. One electrophoresis at pH 10.5 brings up the iron content to 0.43 %. Other authors had the same experience of the degree of purity

after the ammonia-ammonium sulphate treatment [6]. It may be mentioned here, that when a modification of the usual method of Keilin and Hartree was used (addition of 750 instead of 550 grams of ammonium sulphate per liter of the crude, neutralized trichloroacetic acid extract) an iron content of 0.36—0.37 % was obtained instead of the usual 0.34 %.

A Beckman spectrophotometer type DU, the wavelength accuracy of which is ± 1 mμ, was used for the light absorption measurements.

pH was determined with a hydrogen gas electrode (except in the preparation of homogenate).

The determinations of oxygen uptakes were made in a Warburg apparatus with constant volume manometers. The oxygen uptakes were read after shaking for 30 min at 37° C with a frequency of 78 oscillations per minute. The vessels were filled with: Well: 0.20 ml 2 *M* KOH. Main chamber: 2.00 ml *M*/15 phosphate buffer of pH 7.3 + 0.50 ml kidney preparation + c + water to 3.15 ml. Side bulb: 0.30 ml 10 % sodium succinate.

Iron was determined in the following way, the principle features of which derive from Lorber [8], except the combustion technique *. A sample, containing 5—70 μg iron was boiled in a Pyrex test tube over a free gas flame with 1.0 ml sulphuric acid (sp. g. 1.84) and three drops of 30 % hydrogen peroxide until the color disappeared and reappeared. This generally took 2—5 min. Then the boiling was continued with successive addition of hydrogen peroxide at a rate of one drop every minute until the solution remained colorless. The boiling must be continued ten minutes after the last addition of hydrogen peroxide. This combustion method has been in use in this institute for the last year [1,4], and we have found it to be by far the most reliable and convenient method for iron analyses in hemin proteids. The solution was then cooled in ice water, diluted with a few ml. of water and neutralized with 35 % ammonia. 0.30 ml of 20 % sulphosalicylic acid and more ammonia were added to a yellow color. The solution was then transferred to a 15 ml volumetric flask, diluted with water to the mark and measured in the spectrophotometer at 424 mμ. 1.00 μg iron per ml per 1 cm optical depth gives a density of $\log I_0/I = 0.103$.

RESULTS

It was found that c, treated with very dilute acid or base, accelerated the oxygen uptake more than a corresponding amount of non-treated c did. Even in the experiments with stronger solutions an increased activity could be traced (see discussion). For that reason numerous experiments were made on the influence of addition of potassium chloride to untreated c upon the oxygen uptake (Fig. 2), but in no case we could find that the salt influenced in those concentrations it actually had in the manometer vessels with treated c. At a concentration above 0.01 *M* the respiration was inhibited. The same inhibition was caused by sodium chloride and potassium sulphate at the same concentrations (the concentration of the latter calculated in terms of the potassium ion). Potter [8] observed inhibition by some neutral salts in 0.1 *M* solu-

* Our thanks are due to Dr. Richard Abrams, Institute for Radiobiology, University of Chicago, U. S. A. for informing us about this combustion method.

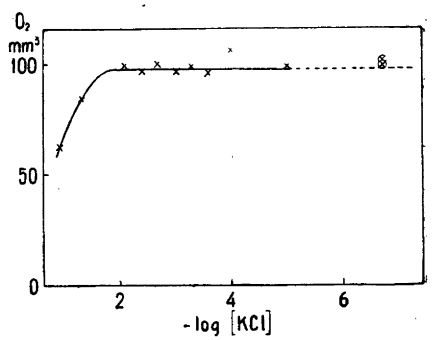


Fig. 2. Influence of KCl upon oxygen uptake. To the main chamber had been added c corresponding to 1.0 µg iron and KCl to give the abscissa concentrations. Three times more of c did not change the shape of the curve, nor that -log[KCl] where inhibition started. Crossed circles: No addition of KCl (place on abscissa quite arbitrarily chosen).

tions. The physiological concentration of total potassium in cells is of that order of magnitude where inhibition just began in our experiments. It must be kept in mind, however, that under the experimental conditions used by us, the contents of the vessels presumably were poorer in c than the intact tissue is, and that a part of the succinate dehydrogenating activity was destroyed during the preparation. In some stability experiments, where the concentration of potassium chloride would have exceeded 0.01 *M* in the manometer vessels, the neutralized sample was dialyzed against distilled water until the chloride reaction became negative.

In some experiments the light absorption was recorded parallel with the activity.

A typical experiment with alkali was carried out as follows. 50.0 ml of 3.32×10^{-4} *M* c and 0.676 ml 6.00 *M* KOH were mixed to give pH 12.77 (0.08 *M* KOH). Samples of 10.0 ml were withdrawn at the time intervals listed in Table 2 and neutralized with 0.133 ml 6.00 *M* HCl. 0.0387 ml of each neutralized sample were used for activity determinations, corresponding to 0.7 µg iron, and compared with 0.0377 ml untreated c (Table 2). Of the neutralized sample 0.30 ml were diluted with 3.50 ml *M*/15 phosphate buffer pH 6.8 for the determinations of the densities of some characteristic bands (Table 2). (Some experiments, not belonging to this investigation were made with the samples, which explains the large volumes used.)

The following is an example of acid treatment of c. 8.00 ml 1.09×10^{-4} *M* c were acidified with 1.14 ml 4.00 *M* HCl to give pH 0.66. After the hours listed in Table 3 samples of 2.00 ml were taken and neutralized with 0.25 ml 4.00 *M* KOH. When all samples had been collected, they were dialyzed against distilled water as was some untreated c. During this procedure, some c passed through the cellophane membranes and some was adsorbed to it, so the concentrations had to be measured by iron analyses. The testing of the kidney preparation was carried out in the same experiment. For the light absorption

Table 2. Changes of activity and light absorption upon treatment with 0.08 M KOH. In the last vessel KCl was added to bring the concentration of the salt to the same value as in the vessels with treated »c». Light absorption determined after reduction. Dry weight of kidney preparation 8.1 mg/ml.

Time for treatm. with 0.08 *M* KOH	Blank	Untreat.	50 min	8 h	20 h	28 h	Untreat. + KCl
Oxygen uptake	58	146	145	126	95.5	76.5	132.5
in μl	—	133.5	148	126.5	101.5	83.5	—
Average	58	140	146.5	126.5	98.5	80	132.5
% remaining activity	—	100	108	83.5	49.5	27	91
Wavelength in mμ for max. of light abs. and dens. at that wavelength		414 1.65 520 0.195 549 0.341	416 1.60 519 0.194 549 0.336	414 1.51 520 0.181 549 0.310	416 1.53 520 0.179 549 0.296	416 1.51 520 0.179 549 0.288	

measurements 0.50 ml of the samples were taken before the dialysis and diluted to 4.00 ml with buffer (Table 3). The results of alkali treatment are given in Fig. 3 and those of acid treatment in Fig. 4. For calculating »per cent remain-

Table 3. Influence of treatment with 0.50 M HCl upon activity and light absorption of cytochrome c. Dry weight of kidney preparation 17.1 mg/ml. The wavelength of the maximal absorption of α-band of the reduced cytochrome was consistently found to be 550 mμ.

Time for treatm. with 0.50 *M* HCl	Blank	Untreated			3 h	6 h	12 h	24 h
μg iron in »c» used to act.detn.	—	0.3	0.7	1.0	0.7	0.7	0.7	0.7
Oxygen uptake in μl	32.5	144.5	250	320	239	226	196.5	163
% activity	—		100		95	89	75.5	60
Density of α-band of red. cytochrome	—		0.325		0.325	0.322	0.323	0.324

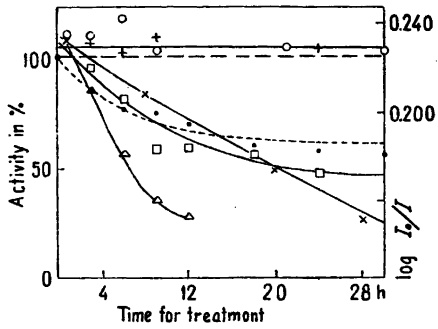


Fig. 3. Stability of »c» at high pH values. ○ 0.01 M KOH (pH 10.81). + 0.03 M KOH (pH 12. 30). □ 0.05 M KOH (pH 12.67). × 0.08 M KOH (pH 12.77). △ 0.10 M KOH (pH 13.04). ● values for log I_0/I at the top of the α-band of reduced »c» (treatment with 0.05 M KOH). ----- curve for a first order reaction with initial concentration corresponding to a density of log $I_0/I = 0.225$, and concentration at infinity corresponding to log $I_0/I = 0.185$, and with $\frac{1}{t} \ln \frac{a}{a-x} = 0.143$. — — »100 % activity». The upper curve is drawn from experiments at both pH 10.81 and pH 12.30.

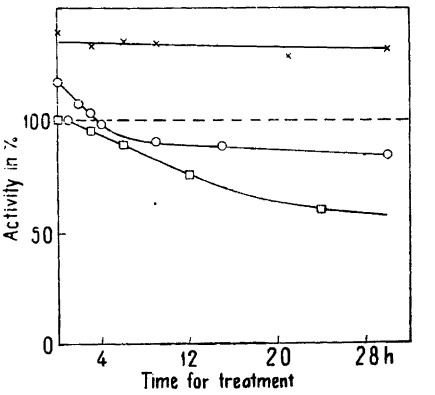


Fig. 4. Stability of »c» at low pH values. × 0.05 M HCl (pH 1.63). ○ 0.10 M HCl (pH 1.25). □ 0.50 M HCl (pH 0.66). — — — — »100 % activity».

ing activity», the oxygen uptake of the blank was subtracted from the values for treated and untreated c, and from the ratio between the two rests was obtained »per cent activity». This is of course not a strictly correct method, since there is no perfect linear relationship between oxygen consumption and amount of c in the vessels, but we believe the errors to be not very large.

DISCUSSION

Figs. 3 and 4 show, that if c is inactivated at a certain pH, this inactivation starts immediately. Therefore, we believe it to be correct to judge from the slopes of the curves »per cent activity» *versus* time, whether c has been stable or not at a given pH. Thus it is seen from Figs. 3 and 4 that c is destroyed at pH 13.04, 12.77, 12.67, 1.25 and 0.66. At pH 1.63 c is just at the lower limit of the stable region, but perfectly stable at pH 12.30.

It is difficult to say how the increase in activity, observed after mild treatment, should be explained. Barron and Kalnitsky[9] recently described the inhibition of succinate dehydrogenase by some heavy metals. However,

we do not believe that the increase in activity observed by us can be explained by the assumption that traces of some heavy metals present as an impurity in our c preparations could be removed by for instance a formation of some complex ion, since the activation occurred at both acidic and alkaline pH, was not reversible and was observed with several different c preparations. Keilin and Hartree [11] criticizing the work of Haas [12, 13] on cytochrome oxidase, have recently showed that proteins exert an unspecifically stimulating effect upon the enzyme system. In our experiments this can hardly be the explanation, since that would mean that destroyed c should be more active than native, and moreover comes that the c, destroyed or not, is quite vanishing regarded upon as a protein in comparison to the masses of inert protein in our kidney preparations (5—10 mg per vessel). The more or less pronounced sigmoid form of the inactivation curves in comparison to the exponential curves for light absorption decrease may be explained by the assumption that the former are composed of two different curves: One curve for inactivation, and one for increasing activity of the remaining, not destroyed c. We must admit, however, that we have not tried to penetrate this problem deeply.

SUMMARY

The stability of cytochrome c has been determined by measuring the activity of samples, which have been kept in hydrochloric acid or potassium hydroxide of various concentrations for various times. Cytochrome c has been found to be perfectly stable from pH 1.6 to pH 12.3.

The density of the absorption band mostly used for concentration determinations of cytochrome c, the α-band, has been followed parallel with the activity. Decrease in activity due to treatment with the acid was not accompanied by a decrease of the band, but during alkali treatment the changes in density and in activity run roughly parallel with each other. In neither case the shape of the spectrum was altered.

Observations made on a homogenate of kidney cortex prove that one can not with certainty judge about the presence or absence of hemin proteids in tissues only from spectroscopical data.

An appearance of a higher activity per μg cytochrome c after its treatment with acid or base has been noted and briefly discussed.

The author wants to express his thanks to Miss Inger Agerberg for her careful and valuable assistance and never failing interest, and to *Stiftelsen Thérèse och Johan Andersson minne* for financial support.

REFERENCES

1. Paul, K.-G. *Arch. Biochem.* **12** (1947) 441.
2. Theorell, H., and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
3. Keilin, D., and Hartree, E. F. *Proc. Roy. Soc. London* (B) **129** (1940) 277.
4. Bonnichsen, R. K. *Arch. Biochem.* **12** (1947) 83.
5. Keilin, D., and Hartree, E. F. *Biochem. J.* **39** (1945) 289.
6. Michel, H. O., and Scheinberg, I. H. *J. Biol. Chem.* **169** (1947) 277.
7. Lorber, F. *Biochem. Z.* **181** (1927) 391.
8. Potter, V. R. *J. Biol. Chem.* **141** (1941) 775.
9. Barron, E. S. G., and Kalnitsky, G. *Biochem. J.* **41** (1947) 346.
10. Keilin, D., and Hartree, E. F. *Biochem. J.* **41** (1947) 500.
11. Haas, E. *J. Biol. Chem.* **148** (1943) 481.
12. Haas, E. *J. Biol. Chem.* **152** (1944) 695.

Received May 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 440—446

On the Determination of Radioactive Hydrogen

LARS MELANDER

Nobel Institute for Physics, Stockholm, Sweden

Several more or less complete descriptions of methods for the quantitative determination of radioactive hydrogen (tritium) have already been published[cf. 1—8]. The apparatus described below involves to a great extent the use of known facts. However, it may be of interest for chemists and biologists to learn how accurate tritium determinations may be made by rather simple means. In contradistinction to most methods previously described, neither the use of a high-vacuum pump, nor that of cooling agents such as liquid air, is needed in the present case.

MAIN PRINCIPLES

The tritium sample, which is assumed to be present as water, is reduced to hydrogen with magnesium turnings (as used by most other authors). A part of the active hydrogen gas is introduced together with methane into a Geiger-Müller tube. Methane has been found to be a good quenching gas for use together with hydrogen in a counter tube connected with a Neher-Pickering quenching circuit. The pulses are registered by a scale of 64 and a mechanical recorder.

The remaining hydrogen may be diluted with inactive hydrogen, and a new measurement made after the first sample has been removed from the counter tube. In this way several activity values are obtained corresponding to decreasing concentrations of tritium, and the agreement between them may be checked in a simple graphical manner. (Figs. 5 and 6, see p. 445).

If the different parts of the apparatus are always flushed several times with pure gases before filling, it is quite satisfactory to use only a good rotary oil pump. All gas transfer within the apparatus takes place from higher to lower pressures and no pumping is necessary for this purpose.

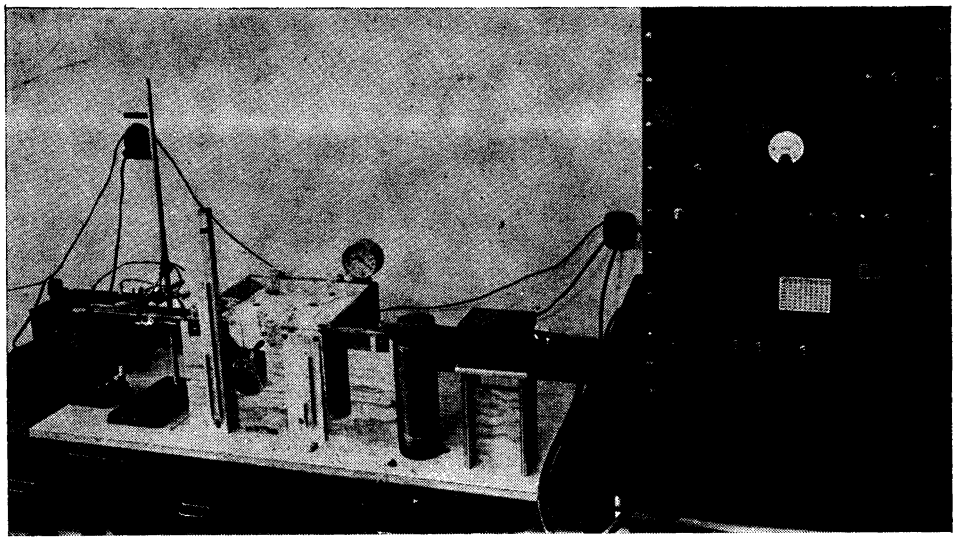


Fig. 1. Photograph of apparatus.

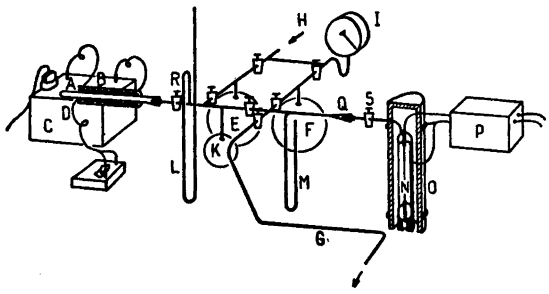


Fig. 2. Schematic drawing (partly in section) of the apparatus. A, furnace tube; B, heating coil; C, transformer; D, thermocouple; E, F, storage bulbs for inactive hydrogen and methane resp.; G, to rotary oil vacuum pump; H, gas inlet; I, metal vacuum gauge; K, bulb for active hydrogen; L, M, mercury manometers; N, Geiger-Müller tube; O, lead shielding; P, pre-amplifier; Q, ground joint; R, S, stopcocks.

APPARATUS

The apparatus is illustrated in Fig. 1, and in Fig. 2 a corresponding schematic drawing (partly in section) is given.

A is a Jena Geräte tube (of about 1 cm diameter and 21 cm length), connected to the apparatus by a ground joint, and closed in the other end. It is heated by means of a Kanthal wire coil, mounted on a Supremax glass tube and protected by a Jena Geräte tube B. In this furnace the reduction is carried out. The transformer C gives the heating current, and the temperature is measured by means of a thermocouple D.

The bulbs E and F are containers (1 litre) for hydrogen and methane respectively. They are evacuated through G, which is connected to a rotary oil pump. The gases are introduced through H and the filling pressure measured by the metal vacuum gauge I. The gases used are ordinary hydrogen from Nordiska Syrgasverken and methane, c. p.,

from the Matheson Co. In the bulb K (250 ml) the active hydrogen is collected and mixed with inactive hydrogen from E. The pressure in K is measured by the mercury manometer L. M is another mercury manometer which serves to measure the filling pressures of the Geiger-Müller tube N.

The Geiger-Müller tube N consists of a copper tube of about 3 cm diameter and 11.5 cm length, ending in two rings of kovar alloy, to which Schönander kovar glass is sealed. The anode is a 0.20 mm tungsten wire. This counter tube is shielded by a lead cylinder O of 1 cm thickness. P is a pre-amplifier, which is connected to a scale and high-voltage supply. Q is a ground joint connecting parts made of different kinds of glass.

EXPERIMENTAL PROCEDURE

About 40 mg of the active water is introduced into a small weighed glass ampoule, the tip of which should be very thin. The ampoule is sealed off and weighed again. (The weight of the water serves as a check on the amount of hydrogen obtained in the reduction.)

The ampoule is introduced into the furnace tube with its tip against the closed end of the latter. A piece of iron and finally a layer of about 12 cm length of magnesium turnings (as used for Grignard reactions) are introduced. The bulbs E and F have previously been filled with hydrogen and methane to almost atmospheric pressure, and now the bulb K and the furnace are evacuated through G. The furnace is heated, and when 575° C is reached, the ampoule is forced against the glass wall at the end of the tube and the tip broken by means of the iron piece, which is operated from the outside with a magnet. The heating coil is slowly moved towards the ampoule and the water slowly evaporated and forced through the magnesium layer. Some air is always present in the ampoule, but it will be taken up by the magnesium. The hydrogen formed is collected in the bulb K, and the course of the reaction is followed on the manometer L. About 15 minutes is sufficient time for the reduction. When the pressure in K rises no further and agrees with that calculated from the weight of the sample and the volumes and temperatures of K and A, the stopcock R is closed. Since the specific activity is to be determined it is of no consequence that some active hydrogen is left in the furnace.

The counter tube, which should have been flushed with methane and evacuated several times, is filled with 10 mm mercury pressure of methane and about 90 mm of the active hydrogen. Finally, the stopcock S is closed. It is advantageous to record the complete characteristic curve and take the activity from the flattest part of the plateau.

After the activity measurement the counter tube is evacuated and flushed with methane. The remaining active hydrogen in K is diluted with inactive hydrogen from E, and a part of the diluted activity is introduced into the counter tube and measured, etc. In this manner several activity values corresponding to different concentrations are obtained in a decreasing series.

The background should be determined with inactive hydrogen before and after the determination of the series of active samples.

PREPARATION OF TRITIUM

As described in several papers, the tritium is easily obtained from beryllium targets used for neutron production in the cyclotron. In the present case some milligrams of

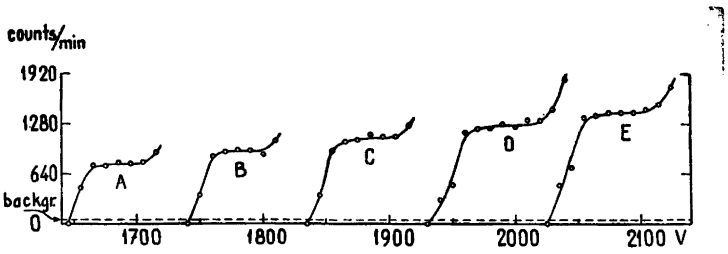


Fig. 3. Characteristic curves of the Geiger-Müller tube corresponding to different hydrogen filling pressures, A, 60.0; B, 73.6; C, 89.0; D, 105.0; E, 119.0 mm (HT +$^{3}_{1}H_2$) with constant specific activity; always 10 mm CH_4.

beryllium filings from an old target, bombarded with 6.5 Mev deuterons, were heated to bright red heat in a current of oxygen in a quartz tube for 1 hour [9]. As it was unnecessary to have an exceptionally high specific activity, the oxygen was moistened in order to provide a carrier for the active water, which was collected in a liquid air trap. Later the water was distilled under reduced pressure into a small storage bottle and some wash water added. The result was about 0.3 g water with a total activity of about $1.7 \cdot 10^7$ disintegrations per minute or a specific activity of 10^6 disintegrations per millimole per minute. If desired, a higher specific activity might have been obtained by the use of less carrier water.

RESULTS

Background. The background has been found to be 53 counts per minute when the hydrogen pressure is 90 mm. Since the effective zone of the counter tube contains about 0.25 mmol hydrogen at 90 mm pressure, it is possible to determine specific activities of an order of magnitude as low as 100 disintegrations per millimole per minute.

Different filling pressures. In order to establish the influence of the filling pressure of hydrogen on the counting characteristic of the Geiger-Müller tube, 10 mm of methane was introduced, followed by an increasing amount of active hydrogen with constant specific activity. Fig. 3 shows the characteristic curves for different hydrogen pressures, and in Fig. 4 the different plateau positions are plotted against the hydrogen pressure. The points in the last plot are found to lie almost on a straight line cutting the activity axis at the background value, as required by the constant specific activity. Hence it is possible to use different hydrogen filling pressures and carry out the calculations on the assumption of proportionality between the measured activity and the true amount of tritium present.

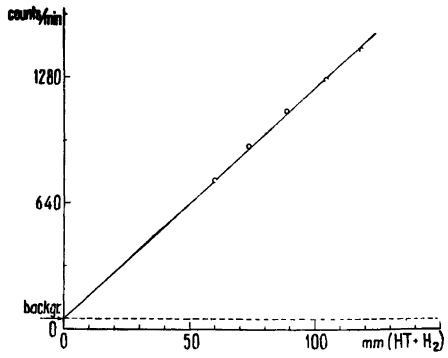


Fig. 4. Measured activity as a function of filling pressure of hydrogen (always 10 mm CH_4, specific activity of hydrogen constant).

In these determinations the background was assumed to be the same for all fillings (that for 90 mm H_2), which is permissible since the background is entirely a wall effect.

Influence of specific activity of hydrogen. Fig. 5 shows the measured activity as a function of the specific activity. The filling pressures of hydrogen were 90 mm. It is seen that the losses (due to the limited resolving power) become appreciable when the counting rate exceeds 6000 counts per minute. Below that counting rate the proportionality is good, as seen in Fig. 6, which is an enlargement of a part of Fig. 5. Simultaneously as the losses become appreciable (the three highest points in Fig. 5) the characteristic curves show a falling instead of rising tendency at high voltages, as pointed out by Kamen [7].

Pulse shape. The shape of the pulses was studied by means of a cathode ray oscillograph. With methane as quenching gas they showed a good aperiodic course. In another counter tube used before that described above, the shape was the same with methane, but with 10 mm alcohol a periodic course was obtained and correspondingly the number of counts registered was 2—3 times that obtained with methane.

Influence of reduction on isotopic ratio. In order to determine whether separation of hydrogen isotopes in the reduction will introduce considerable errors, two water samples of quite different specific activity were reduced and the activities compared (these two measurements were carried out with the old Geiger-Müller tube mentioned in the previous paragraph). For the second experiment a part of the water used in the first one was diluted 51.9 times. The specific activity ratio found was 50.0. (Weighing errors from the dilution have to be taken into account in the comparison of these figures.) Thus it is evident that almost no, if any, error is introduced in these (relative) measurements by isotopic separation.

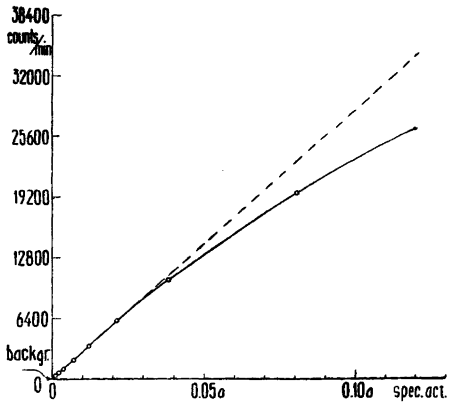


Fig. 5. Measured activity as a function of the specific activity of hydrogen (10 mm CH_4, 90 mm (H_2 + HT), a = spec. act. of original tritium preparation).

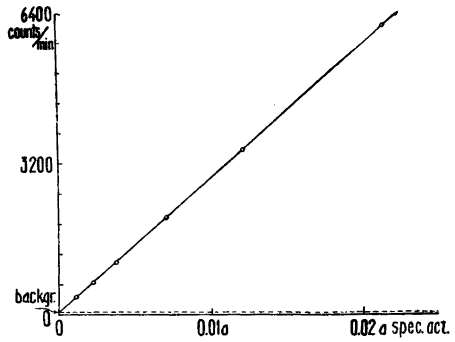


Fig. 6. Measured activity as a function of the specific activity of hydrogen (enlargement of a part of Fig. 5).

From the above results the accuracy in the determination of the specific activity of hydrogen in water is estimated at about $\pm$ 2 %.

SUMMARY

A simple apparatus for the determination of radioactive hydrogen in water is described. The water is reduced by means of magnesium, and the hydrogen introduced into a Geiger-Müller tube (the same method in principle as used by other authors). Methane has been found to be a good quenching gas for use together with hydrogen.

By means of control experiments it has been shown that the measured activity is proportional to the amount of tritium present in the counter tube. This holds independently of the total hydrogen pressure and the absolute amount of tritium over large intervals. It has also been shown that probable separation of isotopes in the reduction introduces no appreciable error.

The accuracy in the determination of tritium in water is estimated at $\pm$ 2 %.

In conclusion I wish to thank the Head of the Institute, Professor Manne Siegbahn, for his kind interest in this work. Mr. Björn Åström, Civ.ing., has constructed the electric equipment, and I also wish to thank him as well as Prof. H. Slätis for valuable discussions.

REFERENCES

1. Allen, M. B., and Ruben, S. *J. Am. Chem. Soc.* **64** (1942) 948.
2. Harman, D., Stewart, T. D., and Ruben, S. *J. Am. Chem. Soc.* **64** (1942) 2294.
3. Norris, T. H., Ruben, S., and Allen, M. B. *J. Am. Chem. Soc.* **64** (1942) 3037.
4. Henriques, F. C. Jr., and Margnetti, C. *Ind. Eng. Chem. An. Ed.* **18** (1946) 420.
5. Eidinoff, M. L. *J. Am. Chem. Soc.* **69** (1947) 2504, 2507.
6. Joris, G. G., and Taylor, H. S. *J. Chem. Phys.* **16** (1948) 45.
7. Kamen, M. D. *Radioactive tracers in biology* New York (1948) Fig. 18.
8. Reid A. F. In the symposium: *Preparation and measurement of isotopic tracers* Michigan (1946).
9. Kamen, M. D. In the same symposium as ref. 8.

Received May 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 447—449

The Azulene from Oil of Elemi

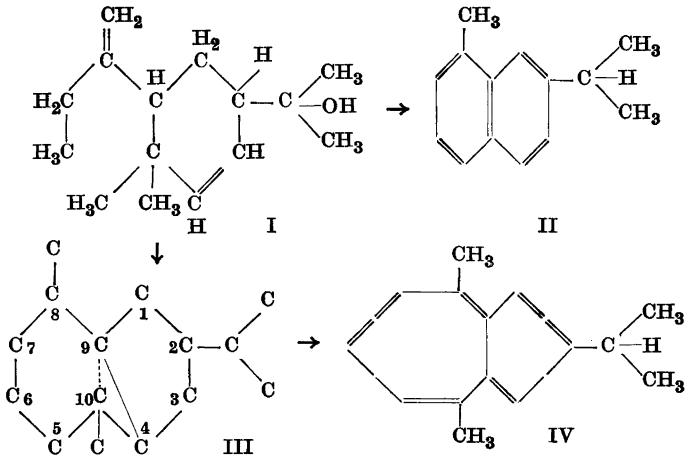
N. A. SÖRENSEN and FRITHJOF HOUGEN

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

The dehydrogenation of terpenes and camphors with sulphur or selenium to aromatic hydrocarbons has rendered most valuable information as to the constitution of the parent compounds. Of course the yields of the dehydrogenation products are frequently very small, and in some instances a definite homogeneous terpene gives rise to at least two different dehydrogenation products, say a naphtalene hydrocarbon and an azulene. So the results must be handled with some care; but on the other side a lucid connection between the two dehydrogenation products and the supposed constitutional formula may be of value.

Investigations in this laboratory on some Norwegian essential oils which gave azulene as well as naphthalene hydrocarbons on dehydrogenation gave rise to some experiments on azulene-donating terpenes, among them elemol.

In spite of the fact that elemol is an easily crystallizing sesquiterpene alcohol, Ruzicka [1], have gained degradation results difficult to connect with a single constitutional formula. Mainly, however, Ruzicka supposes elemol to possess the structure (I)



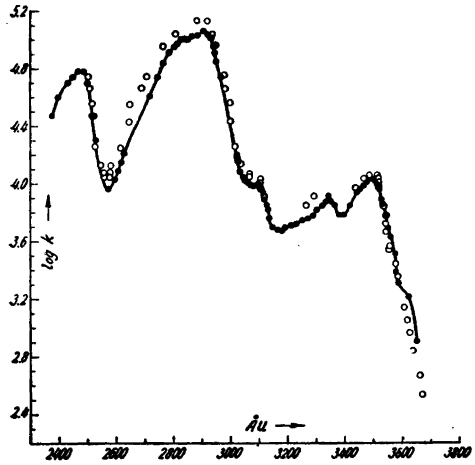


Fig. 1. Ultraviolet absorption of elemazulene and vetivazulene in hexan.
○ *Vetivazulene, Susz, Pfau and Plattner* [4].
● *Elemazulene, present investigation.*
$(k = \frac{ln\ I_0/I}{c \cdot d}$, *c in Mol/l, d = cm).*

Although a monocyclic alcohol, elemol on dehydrogenation with Se gives bicyclic hydrocarbons. Ruzicka got eudalene (II) (1-methyl-7-isopropyl-naphthalene) in 15 % yield besides quite small amounts (about 1 %) of a violet azulene. Ruzicka and Haagen-Smit [2] found this azulene different from the known ones and named it elemazulene. Its constitution remained unknown.

Pfau and Plattner [3], who in 1936 brilliantly elucidated the constitution of the azulene skeleton and some of the natural azulenes and in addition cleared away some insufficiently characterized azulenes, described another violet azulene, vetivazulene with properties similar to elemazulene. Owing to the difference in the m. p. of the picrate: elemazulene 110°, vetivazulene 122°, these two azulenes have so far been considered different.

The revision of the data of the elemazulene compounds which we have carried out puts this supposition to an end. As will be seen from the table the corrected values agree completely with the data of Pfau and Plattner on vetivazulene.

As is seen from Fig. 1, the ultraviolet absorption spectrum of our preparation of elemazulene is identical with the measurements of Susz, Pfau and Plattner [4]. As is known, vetivazulene does not give sharp maxima in visible light, but according to the curves given by Susz *et al.*[4] vetivazulene differs markedly in ultraviolet light from S-guajazulene and azulene.

Although abnormal, the formation of eudalene from Ruzicka's elemol is a very simple process of ring closure. To explain the simultaneous formation of vetivazulene it is necessary to suppose that the ring closure is followed either by a migration of the 9—10 bond to 4—10 or rather more likely by a formation

Table 1. Melting points of the azulene compounds.

M. p.	Vetivazulene Pfau-Plattner [7]	Elemazulene	
		Ruzicka [2]	Present investigation
Azulene	32 — 33°	—	31.5— 32°
» picrate	122°	110°	122.5°
» trotylate	80.5— 81°	—	78.5— 79.5°
» trinitrobenzenate	151.5— 152°	—	154 —155°
» styphnate	—	87°	95 — 96.7°

of a tricyclic skeleton. Both intermediates (III) will generate vetivazulene (IV) in a simple way, well known from the analogous reactions: Ledum camphor → S-guajazulene [5], aromadendrene → S-guajazulene [6].

The bond-formation from 9 to 4 ought to have been favoured through the double bond in 3—4. A bond formation 5—9 would give rise to S-guajazulene as dehydrogenation product.

We suggest that the name elemazulene ought to be dropped. Of course elemazulene was described 7 years before vetivazulene, but these first preparations can not have been quite pure. Further we find it important that vetivazulene has the real skeleton of the dominating sesquiterpene compounds of vetiver oil, α- and β-vetivon [8], whereas elemazulene is rather irregularily related to elemol and elemene.

SUMMARY

Reinvestigations of the azulene from oil of elemi prove this azulene to be vetivazulene.

REFERENCES

1. Ruzicka, L., and van Veen, A. G. *Ann.* **476** (1929) 70.
2. Ruzicka, L., and Haagen-Smit, A. J. *Helv. Chim. Acta* **14** (1931) 1104.
3. Pfau, A. St., and Plattner, Pl. *Helv. Chim. Acta* **19** (1936) 858.
4. Susz, B., Pfau, A. St., and Plattner, Pl. A. *Helv. Chim. Acta* **20** (1937) 469.
5. Komppa, G., and Nyman, G. A. *Compt. rend. trav. lab. Carlsberg* **22** (1938) 272, compare also 3.
6. Radcliffe, C. B., and Short, W. F. *J. Chem. Soc.* (1938) 1201.
7. Pfau, A. St., and Plattner, Pl. A. *Helv. Chim. Acta* **22** (1939) 202.
8. Pfau, A. St., and Plattner, Pl. A. *Helv. Chim. Acta* **22** (1939) 640, **23** (1940) 768.

Received May 19, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 450—454

On the Estimation of Bound Hydroxylamine in Biological Materials

TIHAMÉR Z. CSÁKY*

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The occurence of hydroxylamine in some biological processes has been convincingly shown *e. g.* in the fixation of atmospheric nitrogen by *Azotobacter* and leguminous bacteria [1—4] and in the reduction of nitrates by *B. coli* [5] and *Torula* yeast [6]. Regarding the quantitative study of hydroxylamine the method mostly used for estimation is that of Blom [7]. This very sensitive method is based on the oxidation of hydroxylamine to nitrite in acetic acid medium with iodine and on the estimation of nitrite by means of the colour reaction with sulphanilic acid and α-naphthylamine. The chemically bound hydroxylamine (oxime) is first split off by boiling in 3 *N* sulphuric acid for 6 hours [2, 8].

In this laboratory Blom's quantitative method was recently used in the investigations on the nitrate assimilation of *Torula* yeast [6]. In the course of this work some attention was devoted to the influence of other oxidized nitrogen compounds possibly occuring in the living cells on the estimation of oxime nitrogen. The substances investigated in this respect are: nitrous, hyponitrous acid, and nitrohydroxamic acid. In the following, the results of these experiments are briefly given.

EXPERIMENTAL

Preparations. The following preparations have been used in the experiments:
Hydroxylamine hydrochloride, Merck's preparation *(pro analysi)*,
Sodium nitrite, Baker's analyzed.

* Visiting scientist from the Hungarian Biological Research Institute, Tihany, Hungary.

Sodium hyponitrite and sodium salt of nitrohydroxamic acid were prepared, the former by reducing sodium nitrate with sodium amalgam, the latter by mixing a warm saturated solution of hydroxylamine hydrochloride in alcohol with a concentrated solution of sodium ethylate in absolute alcohol, filtering off the sodium chloride and treating the filtrate with ethyl nitrate [9].

Estimation of free hydroxylamine. In the original Blom's method iodine was used for the oxidation of hydroxylamine to nitrite and sodium thiosulphate for destroying the excess of iodine. Working in acetic acid medium, thiosulphate gives mostly a turbidity which causes great difficulties in the colorimetric measurement. The turbidity can be avoided by using sodium arsenite instead of sodium thiosulphate. In this case the solution is kept excellently clear and the colour can be easily measured in a convenient colorimeter.

The modified reaction is made as follows: 1—5 ml of the solution containing 0.2—0.5 μg hydroxylamine N is placed in a 10 ml calibrated tube and 1 ml of sulphanilic acid (10 g sulphanilic acid dissolved in 1000 ml of 30 % acetic acid by heating on the water-bath) and 0.5 ml iodine solution (1.3 g iodine in 100 ml glacial acetic acid) measured to it. After 3 to 5 minutes the excess of iodine is destroyed with 1 ml of sodium arsenite solution (2 g sodium arsenite in 100 ml distilled water) and after adding 1 ml α-naphthylamine solution (3 g α-naphthylamine dissolved in 1000 ml of 30 % acetic acid) the mixture is made up with distilled water to 10 ml. After 20—30 minutes the colour is developed and measured in Klett-Summerson photoelectric colorimeter using a green filter (500 mμ) against a control containing all reagents but the solution to be tested. The amount of hydroxylamine-N can be read from a calibration curve obtained with pure sodium nitrite or hydroxylamine hydrochloride solution.

Estimation of bound (oxime-) hydroxylamine. A sample of 1 ml of the solution to be tested and 1 ml 6 *N* sulphuric acid is placed in a Pyrex glass test tube with ground-in stopper carrying a capillary tubing **. The solution is boiled in a water-bath for 6 hours, treated with charcoal if it has become coloured and transferred quantitatively into a 10 ml tube. The excess of sulphuric acid is buffered with 3 ml of 35 % sodium acetate and the free hydroxylamine estimated as above.

Effect of boiling in sulphuric acid on nitrous, hyponitrous and nitrohydroxamic acid and hydroxylamine in dilute solution. All these substances give the colour reaction without any treatment with iodine; hyponitrous acid of course

** If the solution contains nitrous, hyponitrous acid or nitrohydroxamic acid, sulphanilic acid has to be added to it before hydrolysis. In this case 1 ml of the solution to be tested is measured into the test tube together with 0.5 ml sulphanilic acid and 0.5 ml 12 *N* H_2SO_4 (*cf.* Table 3).

only in higher concentrations. In order to investigate how these substances influence the determination of bound hydroxylamine they were at first boiled separately in 3 *N* sulphuric acid. 1 ml of the solution was placed in a Pyrex glass tube carrying a capillary tubing together with 1 ml 6 *N* H_2SO_4 and boiled for several hours. After boiling the solution was transferred into a 10 ml calibrated tube, the excess of acid neutralized with 3 ml of 35 % sodium acetate and the colour reaction made as above.

Table 1 shows that nitrous, hyponitrous acid, and nitrohydroxamic acid were totally destroyed after boiling in 3 *N* sulphuric acid for 6 hours giving no traces of colour reaction whereas hydroxylamine was stable against such treatment.

Table 1. Effect of boiling in 3 N sulphuric acid on solutions of sodium nitrite (0.01 mg) sodium hyponitrite (0.05 mg) sodium salt of nitrohydroxamic acid (0.05 mg) and hydroxylamine (0.001 mg).

Time of boiling hours	Colour reaction after boiling (Extinction)			
	Nitrite	Hyponitrite	Nitrohydroxamic acid	Hydroxylamine
0	0.260	0.104	> 1.000	0.061
2		0.010	0.041	
4		0.000	0.000	
6	0.000	0.000	0.000	0.056

Effect of nitrous, hyponitrous and nitrohydroxamic acids on the estimation of hydroxylamine. A sample of 0.5 ml hydroxylamine solution and 0.5 ml of the solution of one of the three oxidized nitrogen compounds were boiled together with 1 ml of 6 *N* sulphuric acid. The concentration of nitrite, hyponitrous or nitrohydroxamic acid was always higher than that of hydroxylamine. Table 2 shows that all the substances investigated destroy hydroxylamine completely when boiled in sulphuric acid for 6 hours.

Table 2. Effect of nitrous, hyponitrous, and nitrohydroxamic acid on very small amounts of hydroxylamine when boiled in 3 N sulphuric acid.

A: 0.45 μg hydroxylamine N + 250 μg sodium salt of hyponitrous acid
B: 0.45 μg hydroxylamine N + 250 μg sodium salt of nitrohydroxamic acid
C: 2.00 μg hydroxylamine N + 100 μg sodium nitrite
boiled in 3 *N* H_2SO_4

Time of boiling hours	Colour reaction after boiling (Extinction)		
	A	B	C
0	0.061	0.061	0.730
6	0.000	0.000	0.009

Table 3 shows the results of the experiments where the concentration of hydroxylamine and of the other nitrogen compounds was the same. In this case the greatest part of hydroxylamine has been destroyed, too. If sulphanilic acid was added before boiling the destruction was smaller.

Table 3. Effect of nitrous, hyponitrous, and nitrohydroxamic acid on hydroxylamine when boiled in equal concentrations in 3 N sulphuric acid.

A : 1.0 μg hydroxylamine N + 1.0 μg nitrohydroxamic acid N
A^1 : 1.0 μg hydroxylamine N + 1.0 μg nitrohydroxamic acid N + sulphanilic acid

B : 2.0 μg hydroxylamine N + 2.0 μg nitrite N
B^1 : 2.0 μg hydroxylamine N + 2.0 μg nitrite N + sulphanilic acid boiled in 3 *N* H_2SO_4

Time of boiling hours	Found N (μg) after boiling			
	A	A^1	B	B^1
0 *	0.60	0.92		1.94
½	0.34	0.81		
1	0.35	0.74	0.32	1.66
2	0.35	0.74		
4	0.35	0.80	0.37	1.66
6	0.34	0.78	0.45	1.55

DISCUSSION

Free hydroxylamine occurs under biological circumstances possibly only in a very small amount as it reacts very easily with keto acids forming oximes. So the estimation of hydroxylamine is connected with that of oximes. As the experiments described above show, the estimation of oximes by splitting off with sulphuric acid is exact only when hydroxylamine or oximes are in the medium but no other oxidized nitrogen compounds. If other oxidized nitrogen compounds are also present they destroy hydroxylamine partially or totally depending on the concentration. The addition of sulphanilic acid before the boiling prevents a part of hydroxylamine from being destroyed. Unfortunately, we have no sensitive methods for detecting nitrite, hyponitrous or nitrohydroxamic ions separately, all three substances giving the colour reaction with sulphanilic acid and α-naphthylamine without oxidation, hyponitrous acid only in higher concentrations.

On the other hand, it is important to know whether in the case of oxime estimation hydroxylamine alone gives the colour reaction after hydrolysis

* Solution kept with sulphuric acid at room temperature for 30—60 minutes.

with sulphuric acid or whether other substances as well give the same reaction. The colour reaction with sulphanilic acid and α-naphthylamine is given only by nitrous, hyponitrous and nitrohydroxamic acid and by hydroxylamine after oxidation with iodine. The experiments show, that all the first mentioned substances are destroyed totally by boiling in 3 *N* sulphuric acid for 6 hours, whereas hydroxylamine is stable against boiling with sulphuric acid. Therefore only hydroxylamine gives the positive Blom's test after hydrolysis.

SUMMARY

The influence of nitrous, hyponitrous and nitrohydroxamic acid on the estimation of oxime nitrogen by Blom's method has been investigated. Nitrous, hyponitrous and nitrohydroxamic acid are totally destroyed by boiling in 3 *N* sulphuric acid for 6 hours giving no traces of colour reaction. Hydroxylamine is stable against boiling with sulphuric acid if it is alone in the solution, whereas it is partly destroyed in the presence of one of the three oxidized nitrogen compounds.

The values obtained in oxime estimation by Blom's method may therefore be too low, but only hydroxylamine gives the positive reaction.

REFERENCES

1. Blom, J. *Zentr. Bakt. Parasitenk. Abt. II* **84** (1931) 60.
2. Endres, G. *Ann.* **518** (1935) 109.
3. Virtanen, A. I., and Laine, T. *Suomen Kemistilehti* **B 9** (1936) 5.
4. Virtanen, A. I. *Cattle fodder and human nutrition* Cambridge (1938) pp. 14—22.
5. Aubel, E. *Compt. rend. soc. biol.* **128** (1938) 45. — Ref. *Ber. ges. Physiol. u. exp. Pharmakol.* **108** (1938) 195.
6. Virtanen, A. I., and Csáky, T. Z. *Nature* **161** (1948) 814.
7. Blom, J. *Ber.* **59** (1926) 121.
8. Laine, T., and Virtanen, A. I. *Die Stickstoffassimilation. Die Methoden der Fermentforschung* Leipzig (1941) p. 2725.
9. Vanino, L. *Handbuch der präparativen Chemie* Bd. I. Stuttgart (1921) p. 313.

Received May 25, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 455—460

2-Quinoxalinealdehyde and some Derivatives

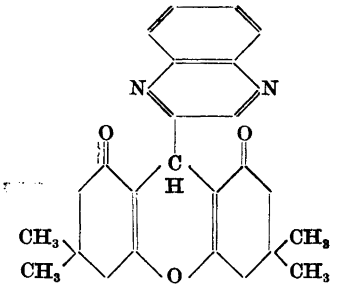
ANDERS KJÆR

The Chemical Laboratory of the University of Copenhagen, Denmark

In connection with an investigation in this laboratory a need arose for 2-quinoxalinealdehyde. This compound was first reported by Borsche and Doeller [1] in 1939, who obtained it by oxidation of 2-methylquinoxaline with selenium dioxide in boiling xylene. Although a 25 % yield is reported for the reaction we have not, in spite of numerous attempts, been able to duplicate this yield. Moreover the yields tend to decrease further by increasing the amounts of the reacting components. The same year Müller and Varga [2] reported 2-quinoxalinealdehyde as a reaction product from the cleavage with lead tetraacetate of 2-(*d-arabo*)-tetrahydroxybutyl-quinoxaline, which in turn was prepared by condensation between *o*-phenylenediamine and fructose. This route to the aldehyde was checked too, but although the oxidative cleavage proceeded smoothly in good yields, the condensation in our hands gave yields quite mediocre. An experiment in which glucose was used in the condensation led to a 9 % yield. Hence an improved method for preparing the 2-quinoxalinealdehyde was deemed desirable and was found in carrying out the oxidation with selenium dioxide of 2-methylquinoxaline in aqueous dioxane at moderate temperature. Using this procedure yields of about 60 % were easily obtained.

For characterization Borsche and Doeller (*l. c.*) prepared the phenylhydrazone and the oxime while Müller and Varga (*l. c.*) report data for the phenylhydrazone and the semicarbazone. Apart from these few notations no observations have been found in the literature as to the properties and reactions of the aldehyde. On account of the somewhat anomalous behaviour of 3-hydroxy-2-quinoxalinealdehyde in forming derivatives, observed by Ohle [3], similar reactions with 2-quinoxalinealdehyde were studied. This investigation, however, did not disclose any irregularities in the reactions of the aldehyde. The hydrazone, azine, 2,4-dinitrophenylhydrazone and anil were formed in

excellent yields. Ohle (*l. c.*) mentions briefly and without any experimental evidence that 2-quinoxalinealdehyde like the 3-hydroxyaldehyde forms an aldazinehydrate, whereas we have been able to obtain only the normal azine as a nicely crystallizing yellow compound. By treatment of this with hot alcoholic potassium hydroxide a change in colour from yellow to red is observed, probably due to a tautomeric shift to the azo-structure as suggested by Ohle. In contradistinction to the hydroxyaldehyde, which with methone primarily gives a normal methonederivative that can be transformed into an anhydride quite readily, we obtain under various conditions always and only an anhydride to which the octahydroxanthene structure is ascribed:



3,6-tetramethyl-9-(2′-quinoxalyl)-
1,2,3,4,5,6,7,8-octahydroxanthenedione-1,8

It was found that 2-quinoxalinealdehyde condensed readily with *p*-aminobenzoic acid and its ethyl ester, *p*-aminobenzamide and sulfanilamide to Schiff bases. The reactions were performed in alcohol solution and gave yields close to the theoretical ones. The azomethines were obtained as nice crystalline, more or less yellow compounds, stable to alkali but quite sensitive to acidic reagents.

Several attempts at reduction of the azomethine double bond were made but none of them were successful. Invariably the reaction mixtures took on intensive blue and violet colours turning red by shaking with air. The occurrence of pigmented solutions by reduction of pyrazine derivatives is known from the literature and is undoubtedly due to at least partial reduction of the - C = N-bonds in the ringskeleton. Catalytic hydrogenation with different types of catalyst and solvent was attempted as well as milder procedures (sodium amalgam a. o.) but in all cases the ring seemed to be attacked more or at least as readily as the double bond in the side chain.

EXPERIMENTAL

2-Quinoxalinealdehyde

A solution of 9.4 g of 2-methylquinoxaline in 45 ml of dioxane was heated to 70°. Under vigorous stirring a solution of 7.3 g of freshly sublimed selenium dioxide in 25 ml of dioxane and 5 ml of water was added dropwise over a 90 min. period. The temperature was raised to 85° and the stirring continued for about four hours at this temperature. After cooling the selenium was filtered off on a glass filter and the dioxane removed from the filtrate *in vacuo*. The residue was steam-distilled and about 3 l of filtrate were collected, saturated with sodium chloride and extracted thoroughly with ether. The etheral solution was dried over anhydrous sodium sulfate and the ether removed at diminished pressure. The crude product (6.2 g) was recrystallized from *n*-hexane and 5 g of large yellow needles were obtained. This product melts at 109°,* and is pure enough for most purposes. By sublimation *in vacuo* well formed and fairly large pale yellow prisms with m. p. 110° were obtained. The aldehyde has a characteristic, not unpleasant, smell and is advantageously kept well protected from light. Even then the preparation does not keep unchanged for a very long time.

2-Quinoxalinealdehyde-hydrazone

To 0.4 ml of hydrazine hydrate dissolved in 2 ml of water was added gradually by shaking a solution of 316 mg of the aldehyde in 2 ml of ethanol. The mixture was heated to boiling and concentrated somewhat. By cooling a crystalline product separated. After recrystallization from water containing a few drops of ethanol the product weighed 270 mg, corresponding to a 79 % yield. Long, pale yellow needles melting under the microscope at 151° after beginning sublimation from 142°. Macro-m. p. 156° under evolution of gas.

$C_9H_8N_4$ (172.2)	Calc.	C 62.77	H 4.68	N 32.54
	Found	» 62.64	» 4.76	» 32.60

2-Quinoxalinealdazine

To 158 mg of the aldehyde dissolved in 5 ml of ethanol was added 172 mg of 2-quinoxalinealdehyde hydrazone. Instantaneously the separation of yellow crystals starts, which after cooling and separation were recrystallized from a large volume of amyl acetate. Thus 256 mg of beautiful, long, yellow needles were obtained (82 % yield). The azine is very slightly soluble in ethanol and sparingly soluble in hot ether and benzene. In cold or hot aqueous alkali no colour change is observed, whereas boiling with ethanolic potassium hydroxide causes a reddish-brown colour, turning yellow again with acid.

On the hot stage under the microscope the azine shows a transformation point at ca. 240°. Sublimation starts at about 260°, and the compound melts at 265°. Macro-m. p. 270° (destr.)

$C_{18}H_{12}N_6$ (312.3)	Calc.	C 69.22	H 3.87	N 26.89
	Found	» 68.73	» 3.83	» 26.86

* All the reported »macro melting points» are uncorrigated and determined in an electrically heated bloc.

2-Quinoxalinealdehyde-2′,4′-dinitrophenylhydrazone

A solution of 79 mg of the aldehyde dissolved in 2 ml of ethanol was added to a hot solution of 100 mg of 2,4-dinitrophenylhydrazine in 6 ml of 2 N hydrochloric acid. A copious precipitate is formed momentarily. Yield after drying: 161 mg (95 %). Recrystallized from boiling toluene. Intensively yellow-orange prisms, melting under the microscope at 234—235°. Macro-m. p. 241° (destr.).

An alcoholic suspension of the derivative gives with traces of alkali a very intensive purple colour, disappearing again by acidification.

$C_{15}H_{10}N_6O_4$ (318.3) Calc. N 24.85 Found N 24.86

2-Quinoxalinealdehyde-anil

By heating together 47.4 mg of the aldehyde and 27.9 mg of freshly distilled aniline in 0.3 ml of ethanol for a short time a crystalline product was obtained. Recrystallized from 0.5 ml of ethanol it consisted of yellow-brownish, flat prisms weighing 54 mg (77 %)

Micro-m. p. 130° Macro-m. p. 133°

$C_{15}H_{11}N_3$ (233.3)	Calc.	C 77.23	H 4.75	N 18.01
	Found	» 76.85	» 4.85	» 17.85

Methone derivative

To a solution of 140 mg of methone in 1 ml of ethanol and 2 ml of water was added 79 mg of the aldehyde in 2 ml of ethanol. The mixture was heated to boiling and one drop of piperidine was added. The separation of crystals starts and after recrystallization from 70 % ethanol there was obtained 92 mg of colourless prisms having the structure indicated above. Under the microscope sublimation takes place from 190° to 225°, the sublimate forming rectangular plates which melt sharply at 231°. Macro-m. p. 229°.

$C_{25}H_{26}O_3N_2$ (402.5)	Calc.	C 74.60	H 6.51	N 6.96
	Found	» 74.59	» 6.49	» 6.99

N-(2-quinoxalylmethylene)-*p*-aminobenzoic acid

A mixture of 500 mg of the aldehyde and 435 mg of *p*-aminobenzoic acid in 5 ml of ethanol was heated to boiling for one hour. After cooling 812 mg of lemon yellow rods had separated. Recrystallized from dioxane. Under the microscope the yellow prims are observed sublimating at 180° to fine, thin needles. These sublimate in turn from 220° until all has disappeared at 240°. Macro-m. p. 285° (destr.)

$C_{16}H_{11}O_2N_3$ (277.3)	Calc.	C 69.30	H 4.00	N 15.15
	Found	» 69.55	» 4.07	» 15.30

Ethyl N-(2-quinoxalylmethylene)-*p*-aminobenzoate

Dissolved in 3 ml of ethanol 316 mg of the aldehyde and 330 mg of ethyl *p*-aminobenzoate were boiled for about 3 hours. The reaction mixture was then taken to dryness *in vacuo* and the residue recrystallized from *n*-hexane. Clusters of yellow needles were obtained by slow cooling, weighing 216 mg. From the mother liquor an additional crop (30 mg) was isolated. Total yield: 40 %.

Micro-m. p. 137° Macro-m. p. 139°

$C_{18}H_{15}N_3O_2$ (305.3) Calc. N 13.76 Found N 13.61

Treatment of the ester with 2 *N* sodium hydroxide at 80° gave the corresponding acid mentioned above.

N^4-(2-quinoxalylmethylene)-aminobenzamide

Heating for a few minutes a solution of 79 mg of the aldehyde and 70.3 mg of *p*-aminobenzamide in 3 ml of ethanol gave 118 mg (85 %) of lemon yellow crystals. Recrystallized from dioxane. Rectangular plates melting under the microscope from 244—252°. Macro-m. p. 245-252° (destr.)

$C_{16}H_{12}N_4O$ (276.3)	Calc.	C 69.55	H 4.38	N 20.28
	Found	» 70.42	» 4.48	» 20.28

N^4-(2-quinoxalylmethylene)-sulfanilamide

Seventy nine milligrams of the aldehyde were heated for a short time to boiling with 86 mg of sulfanilamide in 3 ml of ethanol. A precipitate was formed rapidly but dissolved again by prolonged heating. After concentration to a small volume 102 mg (65 %) of pale yellow crystals were isolated. Recrystallized from a small volume of dioxane. Small prisms melting on the hot stage at 220°. Macro-m. p. 227° (destr.)

$C_{15}H_{12}O_2N_4S$ (312.3)	Calc.	N 17.94	S 10.26	
	Found	» 18.27	» 10.50	

SUMMARY

An improved method for the preparation of 2-quinoxalinealdehyde is described.

Different derivatives and condensation products of the aldehyde are reported.

The work here represented was carried out with means furnished by *Foreningen af Medicinalfabrikker af 1933* (Association of Medicinal Factories).

I am grateful to lektor K. A. Jensen (Ph. D) for his kind interest in this work and to Mr. O. Rosenlund Hansen for his performance of the microanalyses.

REFERENCES

1. Borsche, W., and Doeller, W. *Ann.* **537** (1939) 39.
2. Müller, A., and Varga, I. *Ber.* **72** (1939) 1999.
3. Ohle, H. *Ber.* **76** (1943) 624.

Received May 27, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 461—466

The Distribution of Nicotinic Acid in the Grains of Wheat, Rye, Barley, and Oats

L. H. PULKKI and K. PUUTULA

The Laboratory of OTK Flour Mills, Helsinki, Finland

The nicotinic acid content of cereals and cereal products has not drawn the attention of the investigators to such an extent as the aneurin content but, nevertheless, some information of it is already available. A common feature and at the same time the chief motive of the research work is probably the well-known fact that the white flour contains both of these vitamins, as well as others, less than the whole grain, owing to the low vitamin content of the interior of the endosperm. Many differences are, however, easy to note. Apart from the fact that the absolute quantities of these vitamins are very different, it may be mentioned that the niacin content of various kinds of cereals is not so even as the aneurin content, *e. g.* the grains of rye and oats are poor in niacin compared with wheat and barley.

The germ region and, in particular, scutellum and the tissues near it contain, as known, unexceptionally much aneurin. No corresponding accumulation of niacin has been noted, at least not in wheat, which has been dealt with in many publications. In the literature appeared in the 1940's values have been reported on the niacin content of the different parts of germ and of different germ-containing milling products, varying within the range 0.5—1.8 times the total content of the whole grain [1, 2].

On the basis of the information published on the niacin content of the milling products of wheat it can be concluded that the major part of this vitamin is located in the outer layers of grain or in their proximity. So, for instance, Andrews, Boyd, and Gortner [3], Moran and Drummond [1], and Jones and Moran [4], have obtained for coarse bran niacin values which are approximately five times that of the whole grain. By analyzing the products of a commercial mill it is not possible to decide definitely in which outer layer of grain niacin is chiefly localized. Nevertheless, the fact that the high-fibre

coarse bran invariably contains this substance appreciably more than does the fine bran easily gives rise to the assumption that it would be located mostly in the outermost coverings. For instance, Jones and Moran [4] report for coarse bran the value 296.0 μg/g and for fine bran 188.0 μg/g (13 % moisture basis), while the aneurin contents of the same mill products are 4.95 and 10.38 μg/g, hence the relation of aneurin and niacin is very different.

The authors proved as early as 1941 that the aneurin content of the outer pericarp tissues of wheat is lower than the average content of grain [5, 6]. Later we have made the same finding also in regard to rye [7]. In these experiments, a technique was successfully employed where the material was gradually removed from the grains, beginning from the surface, by means of emery scouring. Principally on account of the fact that the shape of the grain deviates from that of a ball the layers of grain cannot be completely separated in this way, but in examining the experimental data both in the previous works and in the present paper it is easy to see that this technique gives a truer picture, in particular, of the composition of the outer layers of the grain than does the analysis of the products of an industrial mill. As also the distribution of the nicotinic acid in grains, especially in the layers near surface interested the authors, the above technique was adopted to investigation of this vitamin, too. The results obtained in the experiments with wheat, rye, barley, and oats are recorded in the following.

MATERIAL AND METHODS

For the experiments were used the imported wheat Manitoba No. 2 and in addition the following cereals grown in Finland: rye, Toivo (Jokioinen); barley, Binder (Abed); oats, Guldregn II (Svalöf). The material was the same as used in the experiments on the location of aneurin [7].

The samples selected from normally developed pure lots of cereals were freed from foreign substances and broken grains. However, they were not washed owing to the nature of the experiment, although in the light of the previous experiments concerning aneurin it could be presumed that the loss of nicotinic acid in washing would remain fairly negligible [6]. The scouring experiments on oats were performed by using kernels from which the hulls were removed. The grains of other cereals were intact.

A simple machine specially designed for the purpose was used in the scouring. Its construction has been earlier described [7].

The determination of nicotinic acid was performed by the cyanogen bromide method according to the instructions of A.A.C.C. [8]. Instead of Lloyd's reagent, a preparation »Montana B» had to be used as the adsorption substance. This proved to be very suitable. For determination of colour Pulfrich-photometer with filter S 43 was employed.

Crude fibre was determined according to the modification worked out by Puranen and Tomula [9]. It gives very similar results as the ordinary so-called Weende-method (*cf. e. g.* instructions of A.O.A.C. [10]) but is more rapid and convenient.

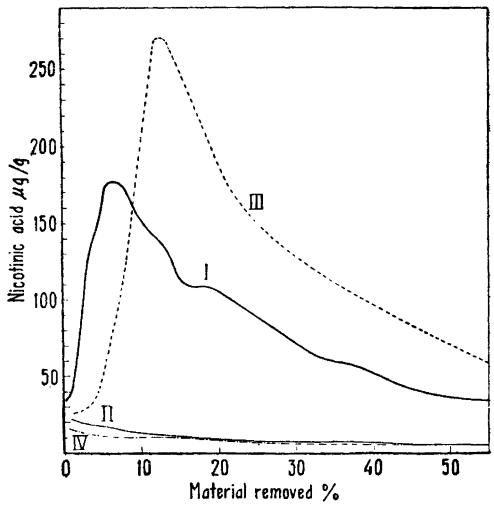


Fig. 1. Effect of scouring treatment on wheat (I), rye (II), barley (III), and hulled oats (IV). Nicotinic acid content (µg/g dry matter) of the material removed in each successive phase.

RESULTS AND DISCUSSION

The curves in Fig. 1 show the niacin content of the substance removed at each stage of scouring, expressed in micrograms per gram of dry matter.

The total niacin contents of the grains used for scouring experiments were: wheat 61 µg/g (calculated from the scouring fractions 58 µg/g); rye 7.8 µg/g (7.4); barley 66 µg/g (65); oats, kernel 6.6 µg/g (6.1).

Furthermore, it should be mentioned that for fairly pure oat hulls removed by hands a value of 5.6 µg/g was obtained. The nicotinic acid contents of the whole oat grains varied from 5.4 to 6.6 µg/g.

In examining the results there are noted at the first glance great differences between different kinds of cereals. This applies as well to the total quantity of nicotinic acid as its distribution in different parts of grain. The niacin content of the grains of wheat and barley is roughly ten times that of rye and oats. In the scouring treatment there is segregated from the outermost layers of wheat (Fig. 1, curve I) and barley (III) material whose niacin content is lower than the average content of grains. The pericarp tissues of rye (II) and oats (IV) contain, again, this substance comparatively more than the tissues in the interior of the kernel. This latter fact is, however, of no great value from the nutrition chemical point of view because the absolute quantities of niacin in rye and oats are so small.

It is interesting to compare the distribution of niacin and aneurin in the grains, in particular, in their outer layers. The results previously reported by the authors on the distribution of aneurin are without any difficulty compa-

rable with those given here, since the material analyzed has been for the major part the same [7, 11]. Other values, too, concerning the composition of the tissues separated at the corresponding phase of scouring, as, for instance, ash, fat, and fibre contents, might well be used in connection with the niacin contents given in the present paper.

It can be seen at the first glance that the distribution of niacin in the grain of rye is very different from that of aneurin. The same applies to oats, too, although the low total quantity of niacin is striking. On the contrary, in wheat and barley the curves for aneurin and niacin markedly resemble each other in shape [11]. The content of both these vitamins in the outer layers is lower than average rising rapidly towards the interior. The nicotinic acid content reaches its maximum much earlier than aneurin, in wheat after removal of about 6 % of the weight of grain, and in barley after removal of about 13 %. The respective figures for aneurin are about 21 % and 18 % [11].

Since the niacin content of the outer layers of both wheat and barley is lower than the average it is possible to prepare by means of a deliberate scouring treatment products whose niacin content is as great or slightly smaller than that of the whole grain. As the outer layers on account of their high fibre content are the least digestible part their removal ought to be advantageous both from the standpoint of baking quality and nutrition physiology. This of course with the proviso that the high fibre content is not desirable for

Table 1. Effect of scouring treatment on wheat, rye, barley, and hulled oats. Relative contents of nicotinic acid and fibre in the kernel after the scouring (niacin and fibre content of the whole kernel = 100).

Material removed %	Wheat		Rye		Barley		Oats (hulled)
	Niacin	Fibre	Niacin	Fibre	Niacin	Fibre	Niacin
1	100.3	94	98.0	95	100.6	97	98.5
2	100.4	87	95.9	89	101.3	92	97.2
3	99.8	81	94.3	84	101.8	86	96.1
4	98.4	76	92.7	79	102.4	81	95.2
6	95	70	90	69	102.0	70	93
8	90	64	87	62	101.0	59	92
10	86	60	85	58	100.0	49	90
15	77	52	81	53	82	31	86
20	70	46	77	51	65	25	82
30	58	35	73	49	42	22	75
40	50	27	68	46	—	—	72
50	45	19	67	45	—	—	69

some special reason and that the outer layers of the grain do not contain certain specially valuable nutrients.

Table 1 gives the relative contents of nicotinic acid and fibre in the bulk of grain after each stage of scouring, expressed in per cent of the content of untreated grain designated as 100.

The crude fibre content does not of course *per se* express the total quantity of the indigestible substance but it may serve as a ratio. It appears from Table 1 that if, for instance, 10 % of the weight of barley grain is carefully removed by scouring, the nicotinic acid content of the remaining bulk of grain is the same as that of the original whole grain, but the crude fibre content only half the original and accordingly, the digestibility much better. The treatment of wheat does not regularly give equally good results but even that may, however, considerably promote the materialization of the hopes the physiologists have fixed to the so-called graham meal. The figures pertaining to rye grain and oat kernel are in this sense of less interest, the total quantity of nicotinic acid being so slight. Reference is made to the previously reported results concerning aneurin.

SUMMARY

The distribution of nicotinic acid in the grains of wheat, rye, barley, and oats has been investigated by means of the scouring method developed by the authors. Niacin was determined colorimetrically according to the cyanogen bromide method. The following contents of niacin were found (all data are expressed on dry matter basis).

The outer pericarp layers of wheat contain niacin less (34 μg/g) than the kernel on the average (58—61 μg/g). Higher concentrations were met in the interior of the bran coverings (maximum 175 μg/g). For other kinds of cereals the following niacin contents were found respectively: Rye, outer pericarp 22 μg/g, whole kernel 7.4—7.8 μg/g; barley, outer layers (chaff) 26 μg/g, whole grain 65—66 μg/g (max. 270 μg/g); hulled oats, outer pericarp 16 μg/g, whole groats 6.1—6.6 μg/g (hulls removed by hand 5.6 μg/g).

REFERENCES

1. Moran, T., and Drummond, J. C. *Lancet* (1945) 698. Rev. Kent-Jones, D. W., and Amos A. J. *Modern cereal chemistry* 4th ed. Liverpool (1947).
2. Bailey, C. H. *The constituents of wheat and wheat products* New York (1944).
3. Andrews, J. S., Boyd, H. M., and Gortner, W. A. *Ind. Eng. Chem., Anal. Ed.* **14** (1942) 663.

4. Jones, C. R., and Moran, T. *Cereal Chem.* **23** (1946) 248.
5. Pulkki, L. H., and Puutula, K. *Biochem. Z.* **303** (1941) 122.
6. Pulkki, L. H., and Puutula, K. *Duodecim* **57** (1941) 425.
7. Pulkki, L. H., and Puutula, K. *Cereal Chem.* **24** (1947) 337.
8. Steele, H. K. *Cereal Chem.* **22** (1945) 448.
9. Puranen, U. H., and Tomula, E. S. *Suomen Kemistilehti* **3** (1930) 85.
10. Association of Official Agricultural Chemists, Washington, D. C. *Official and tentative methods of the A. O. A. C.* 6th ed. (1945).
11. Pulkki, L. H. *The Miller* **77** (1947) 417; *Suomen Kemistilehti* **A 20** (1947) 197.

Received May 27, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 467—473

The Determination of Hexosamines According to Elson and Morgan

GUNNAR BLIX

The Institute of Medical Chemistry, University of Upsala, Sweden

The method for the quantitative determination of hexosamines elaborated by Elson and Morgan [1] is based on the fact that those substances when treated with acetylacetone in alkaline solution are converted into a pyrrol derivative, which gives a coloured condensation product with *p*-dimethylaminobenzaldehyde. Although the colour reaction is not specific the method has proved to be of considerable value in biological work.

Elson and Morgan showed that under certain conditions various sugars and amino acids do not interfere with the colour reaction. Employing the method with protein hydrolysates Boyer and Fürth [2], however, obtained low values. On the other hand Nilsson [3], Sörensen [4] and Meyer *et al.*[5] successfully applied the method to material which contained protein. The explanation of this discrepancy is apparently to be found in the greater amounts of protein split products present in the reaction mixtures of Boyer and Fürth as compared with those of the other investigators. Following the directions given by Nilsson we have frequently observed, however, that lower values were obtained when a greater quantity of material was analysed than when a smaller amount was taken. For example, when 1 ml of blood serum was hydrolysed and one and two ml respectively of the hydrolysate were analysed, the percentage value found in the latter case was generally about 10 % lower than that obtained in the former. In both instances the hexosamine quantities measured were well within the range of the standard curve where a linear relation exists between hexosamine concentration and extinction coefficient. This experience prompted us to make a more detailed study of the method. In the first instance it was considered essential to establish whether or not protein split products influenced the results obtained with the procedure employed. Other details of the procedure were also considered.

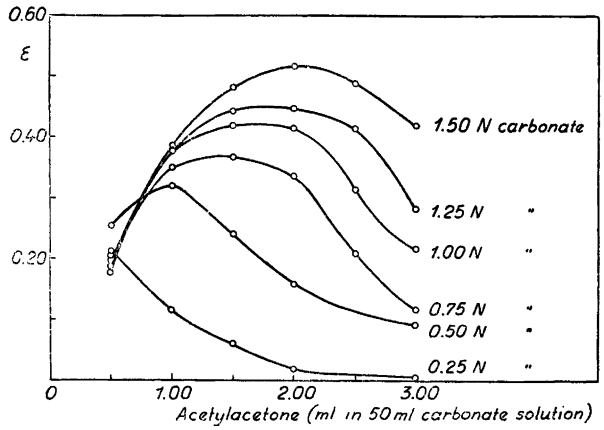


Fig. 1. Variation of extinction value with varying composition of the acetylacetone reagent.

EXPERIMENTAL

Working with solutions of pure glucosamine hydrochloride the following procedure was employed: To 2 ml glucosamine solution were added 2 ml of acetylacetone solution and the mixture heated at + 96° C in a closed Thunberg tube for 20 min. After cooling to room temperature 20 ml 96 % ethyl alcohol and 2 ml of Ehrlich's reagent were added. After standig for 45—60 min the light absorption was measured in a Zeiss' step photometer. Fig. 1 shows the results of a series of experiments in which the composition of the acetylacetone solution was varied. In all these experiments the Ehrlich reagent contained 1.6 g *p*-dimethylaminobenzaldehyde in a mixture of 30 ml concentrated HCl and 30 ml 96 % alcohol. The glucosamine solution used throughout these experiments contained 0.30 mg of the hydrochloride per ml.

The original acetylacetone reagent described by Elson and Morgan is a solution of 1.00 ml acetylacetone in 50 ml 0.50 *N* Na_2CO_3. Using this strength for the carbonate solution the greatest light absorption was, as seen, obtained with 1.00 ml acetylacetone in 50 ml solution. This result agrees with those of earlier investigators. However, during the heating of the reaction mixture free acetic acid is probably formed, and if the amount of acetylacetone is augmented above that originally used, the concentration of the carbonate has also to be increased in order to get maximal extinction. If the strength of both these reagents are somewhat increased, the conditions for the formation of the coloured product become more favourable, *i. e.*, the extinction values rise. A solution of, say, 2.00 ml acetylacetone in 50 ml 1.25 or 1.50 *N* Na_2CO_3 is advantageous not only because a greater amount of the coloured

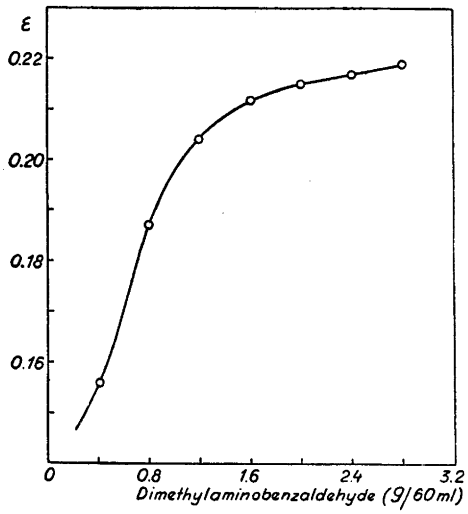


Fig. 2. Variation of extinction value with varying concentrations of p-dimethylaminobenzaldehyde.

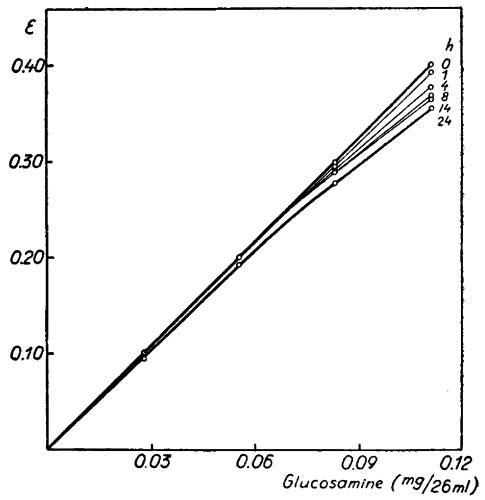


Fig. 3. Influence on extinction value of treatment of the glucosamine with hydrochloric acid in the heat at varying glucosamine concentrations.

product is formed but also because the extinction values are less influenced by small variations in the acetylacetone concentration than are those obtained with the original reagent. With increased concentration of the carbonate the reagent becomes less stable. Whereas the original reagent keeps at + 4° for at least 3 days, the reagents with 1.25 or 1.50 *N* Na_2CO_3 must be made fresh every day. Because of shortage of acetylacetone and of the increasing instability of the reagent with rising carbonate concentration we have in all subsequent determinations used a solution composed of 1.50 ml acetylacetone and 50 ml 1.25 *N* Na_2CO_3.

The results of a series of experiments in which the concentration of the *p*-dimethylaminobenzaldehyde was varied is given in Fig. 2. The reagent contained 1.50 ml of acetylacetone in 50 ml 1.25 Na_2CO_3.

It appears that the value obtained with the original Ehrlich reagent (0.8 g/60 ml) is situated on the edge of the steep slope of the curve. Because of this fact and because the extinction values are somewhat higher with the higher concentrations of the *p*-dimethylaminobenzaldehyde we have found it advisable to change over to an Ehrlich reagent containing 1.6 g of the aldehyde in 60 ml.

After a considerable number of experiments we concluded that the amounts of protein and other foreign substances present in the materials examined by

us (blood serum, joint fluids, cartilage and other tissues) did not cause any error in the analyses, and that the difficulties mentioned earlier were due to an action on the glucosamine of 2 *N* HCl at + 100° C. We had studied earlier the stability of glucosamine under these conditions and found no change in glucosamine concentration up to a heating period of 18 h, but a small decrease was observed in 24 h. These experiments were, however, carried out with one single and relatively low concentration of glucosamine. Fig. 3 shows the results of a number of experiments, where glucosamine in varying concentrations was heated for different lengthes of time with 2 *N* HCl.

In all these experiments 0.50—1.00 ml of solutions of pure glucosamine hydrochloride were heated with 5.00 ml 2 *N* HCl in closed glass bulbs at + 100° C. After cooling, the solution was made up to 10 ml with distilled water. Of this solution 5 ml were carefully titrated with 4 *N* NaOH using methyl red as an indicator. The quantity of 4 *N* NaOH required for the neutralisation was added to the remaining 5 ml of the experimental solution and the mixture then filled up to 8 ml. Of the solution thus obtained 2 ml were heated at + 96° C for 20 min in closed Thunberg tubes with 2 ml of the acetylacetone reagent. After addition of alcohol and Ehrlich's reagent the photometric reading was performed as above described.

When the treatment with acid was excluded the same procedure gave a linear relation between extinction coefficient and glucosamine concentration up to a concentration of about 0.20 mg in 26 ml solution (2 ml test solution + 2 ml acetylacetone solution + 2 ml Ehrlich reagent + 20 ml alcohol). On heating with hydrochloric acid for 1—14 h a deviation from the linear relation begins to appear when the concentration of the glucosamine exceeds 0.07—0.08 mg/26 ml. The course of the deviating curves is not consistent with a monomolecular decomposition reaction. On heating for as much as 24 h the deviation of the curve appears already at lower concentrations and the lower part of the curve seems to indicate a simple destruction. The same results were obtained with solutions which in addition contained protein split products.

Although no certain explanation can be given of the early deviation of the curves obtained with acid-treated glucosamine some further insight in the process was gained in the experiments graphically represented in Fig. 4.

Solutions of pure glucosamine-HCl were treated with acetylacetone in the ordinary way but for varying periods of time. In one series the glucosamine was previously heated for 14 h with 2 *N* HCl, in the other series the glucosamine did not undergo any pretreatment with HCl. As seen from the graph the values in the two series do not differ with the lower concentrations of glucosamine tried (0.04—0.08 mg/26 ml, curve I, II and III). With a somewhat greater

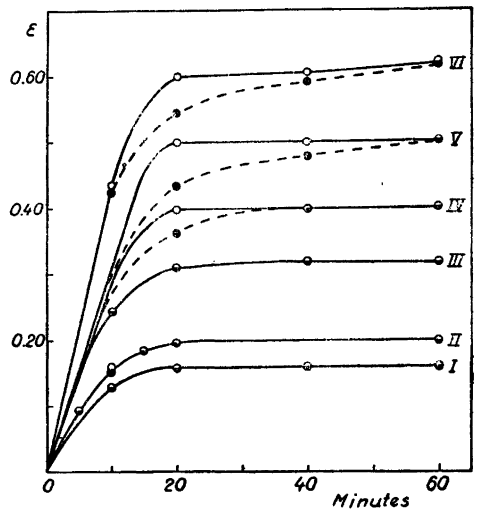


Fig. 4. Variation of extinction value with varying acetylation time.
●— — —● *Glucosamine treated with hydrochloric acid at 100° C for 14 h.*
○———○ *Untreated glucosamine.*
I = 0.042 mg glucosamine/26 ml,
II = 0.053 mg/26 ml,
III = 0.085 mg/26 ml,
IV = 0.106 mg/26 ml,
V = 0.133 mg/26 ml,
VI = 0.170 mg/26 ml.

concentration of glucosamine (0.11 mg/26 ml, curve IV) the acetylation in the HCl-treated sample is not complete within 20 min, but is after 40 min. With still greater glucosamine concentration complete acetylation is attained only after about 60 min (0.13 mg/26 ml, curve V). With 0.17 mg/26 ml glucosamine the extinction value of the acid-treated sample has reached that of the non-treated in 60 min, but both were somewhat short of the expected values ($E = 0.62$ instead of 0.65). Evidently all or a part of the glucosamine in experiments IV—VI is changed in such a way that the acetylation proceeds slower than with the untreated substance. The non-appearence of this change with the lowest glucosamine concentration tried is difficult to explain. A relative change of the same order as in experiments IV—VI should lie outside the experimental error, at least in experiment III. Possibly the change might depend on a reaction of higher order, *e. g.* polymerization reaction.

Whatever may be the explanation of the HCl effect, due regard must be taken to its existence in the practical performance of the method. When carrying out the determination in the manner described in this paper an acetylation time of 20 min. can be used only with glucosamine amounts of 0.09 mg (in 26 ml) or less. Using an acetylation time of 60 min. it is possible to extend the range upwards to about 0.14 mg glucosamine.

For the convenience of the reader the details of the method as used by us are now given:

Reagents

p-Dimethylaminobenzaldehyde solution. — 1.6 g of the substance are dissolved in 30 ml concentrated HCl and 30 ml 96 % alcohol added. A pure sample of the reagent should be used. The solution has then a very pale yellow colour.

Acetylacetone solution. — 0.75 ml of pure acetylacetone is dissolved in 25 ml 1.25 *N* Na_2CO_3. This solution should be made fresh every day.

Procedure

Hydrolysis. — With the materials studied by us (various glucoproteins of animal origin, chondroitin sulfuric acid and hyaluronic acid) a complete liberation of hexosamine has always been attained by heating the material for 14 h in a closed glass bulb at + 100° C with about 2 *N* HCl. Frequently less time is required. When there has been no shortage of the material to be analysed we have used a standard procedure in which the analytical range is 0.25—0.70 mg in the sample taken for analysis. However, it is possible with slight changes to adapt the method for considerably smaller amounts of the hexosamine, down to 5—30 μg.

To weighed amount of the substance or to ½—1 ml of the solution to be analysed are added 5 ml 2 *N* HCl and the mixture is heated for 14 h at 100° (in a boiling water bath or in an air thermostat). We have used 30 ml glass bulbs with ground stoppers held in position by a metal clamp. After hydrolysis the solution (if necessary filtrated) is made up to 10 ml. Of this quantity 5 ml are carefully titrated with 4 *N* NaOH using a drop of methyl red as indicator. The exact amount of NaOH required for neutralisation is added to the remaining 5 ml of the hydrolysate and this solution is made up to 8 ml. With the acetylacetone reagent here used an over-neutralization to pH 7—8 does not invalidate the determination. — As it has turned out that the methyl red does not interfere with the colour reaction with Ehrlich's reagent, the *total* hydrolysate may after addition of a drop of methyl red be directly neutralized and thereafter made up to 8 ml.

Acetylation. — Of the neutralized hydrolysate 2 ml are pipetted into a Thunberg tube (about 185 mm in length and holding 30—35 ml). 2 ml of the acetylacetone reagent are added. The tube is closed and put into a water bath of + 96° for 20 min.

The 2 ml of the glucosamine solution taken should not contain more than 0.09 and not less than 0.03 mg hexosamine. If the acetylation time is extended to 60 min. reliable results are obtained with quantities up to 0.14 mg.

Condensation with p-dimethylaminobenzaldehyde and photometric determination. — After cooling to room temperature with cold water, adding 20 ml 96 % alcohol, *thoroughly mixing* and adding 2 ml of Ehrlich's reagent the light absorption is measured in a Zeiss' step photometer using filter S 53 and a 10 mm cuvette. The reading is made at least 45 min after the addition of Ehrlich's reagent. The light absorption remains constant for 12 h but shows some decrease in 24 h. Of course a photoelectric apparatus, *e. g.* a Beckman photometer, may be used as well. Employing a Beckman photometer it is possible with the standard procedure given here to determine with reasonable accuracy as little as 15—20 μg in 2 ml of neutralized hydrolysate.

When it is possible to use very small amounts of the solution or of the substance to be analysed, *e. g.* 0.1 ml of joint fluid, the hydrolysis can be carried out with 3 ml 2 *N* HCl, suitably in a small Thunberg tube marked at 5 ml. After the hydrolysis the

solution is neutralized and made up to the mark. To 4 ml of this solution are added 4 ml of the acetylacetone reagent and the acetylation is performed as above. After cooling, 16 ml alcohol and 2 ml Ehrlich's reagent are added. The extinction values obtained are identical with those of the standard procedure for the same quantity of hexosamine. This modification has been introduced in this laboratory by Dr. L. Sundblad and proved to be as reliable as the »standard procedure». *

If 50 mm microcuvettes are used instead of the ordinary 10 mm cuvettes amounts of hexosamine as small as 5 μg in the hydrolysed and neutralized sample can be determined using the step photometer.

Within the analytical range an extinction coefficient of 0.100 corresponds to 0.0279 mg glucosamine in the part of the neutralized solution, which is acetylated. This ratio has been obtained as an average value from a series of analyses on two different, analytically pure specimens of glucosamine hydrochloride.

An idea of the accuracy of the method (standard procedure) can be gained from a series of 43 consequtive duplicate determinations of glucosamine in joint fluids (and a few pleural effusions). The average difference between the double analyses was 1.0 %. In one single case the difference was as high as 7.4 %, in no other instance did it exceed 3.2 %. The differences were calculated on the lowest value in each pair of analyses. Each single analysis was carried out separately commencing with an individual sample. The step in the analysis which most often limited the accuracy of the method was without doubt the photometric reading.

SUMMARY

Various factors influencing the formation of the coloured compound in the method of Elson and Morgan had been studied. As consequence of the experiences gained certain changes of the reagents and of the procedure have been proposed. The procedure adopted is given in details. With small variations in the performance the method may be used for the determination of hexosamine in quantities from about 5 μg up to 150 μg.

LITERATURE

1. Elson, L. A., and Morgan, W. T. J. *Biochem. J.* **27** (1933) 1824.
2. Boyer, R., and Fürth, O. *Biochem. Z.* **282** (1935) 242.
3. Nilsson, I. *Biochem. Z.* **285** (1936) 386.
4. Sörensen, K. M. *Compt. rend. trav. lab. Carlsberg* **22** (1938) 487.
5. Palmer, J. W., Smyth, E. M., and Meyer, K. *J. Biol. Chem.* **119** (1937) 491.

Received May 29, 1948.

* With extinction values as low as 0.05—0.10 the blank should contain methyl red in the same concentration as the test.

ACTA CHEMICA SCANDINAVICA 2 (1948) 474—485

A New Method to Determine the Degree of Solvation of Macromolecules

HANNES SIHTOLA and THE SVEDBERG

The Institute of Physical Chemistry, University of Uppsala, Sweden

During the last two decades many physico-chemical investigations have been carried out to elucidate the mutual interaction between the solvent and the solute molecules. As a special problem the solvation of macromolecular substances has been closely studied.

I. INTRODUCTION

In a solution the solvent molecules generally form a solvate mantle around the solute molecules. Some investigators differentiate two kinds of solvation see 1, 2. The solvent molecules close to the solute molecule (»touching» it) are so firmly attached to it — either by secondary valences, for instance hydrogen bonds, or by van der Waal's forces — that they continually follow the movements of the macromolecule, forming a thin layer around it. This is called »primary solvation» (Moll [3]), »proper solvation» (Fikentscher and Mark [4]) or »chemical solvation» (Schulz [5]). In a wider sense the solvation comprises all solvent molecules within the range of the attractive forces from the solute molecule and also the solvent molecules mechanically immobilized in holes, micelles etc. of the high polymer molecules. The volume of the solvated particles in this sense, tentatively calculated from viscosity data, is of quite another order of magnitude, containing as much as several hundred ml of solvent per gram of solute.

Various methods have been employed for the elucidation of the solvation problem. The principles of the more general ones are briefly the following (for a complete review *cf.* Eirich and Mark [1] and Philippoff [2]):

1. Sorption of solvent vapours. The substance is swelled in the solvent vapour at various pressures and temperatures and the degree of solvation is calculated from the increase in weight.

2. Adsorption of solvent from a mixture of solvent and nonsolvent (Methode des nichtlösenden Raumes). The substance is immersed in a mixture, and the amount of solvent taken up is measured by observing the changes in the composition of the mixture before and after the addition of the substance.

3. Thermochemical measurements. From the heat of swelling, solution, and dilution of the substance, as functions of concentration, the degree of solvation can be calculated.

4. Thermodynamic calculations. The thermodynamic quantities free energy, heat content, and entropy of solution can be calculated from osmotic pressure, lowering of vapour pressure, and temperature dependence of the heat of dilution. From these data an approximate estimate of the degree of solvation of the dissolved substance is obtained.

5. Dielectric measurements. The dielectric constant of a solution of a given concentration is connected also with the degree of solvation of the solute molecules.

Furthermore conclusions on the solvation can also be drawn from viscosity data, X-ray diffraction, and in special cases colour change of swelling substances containing an indicator, for instance Cobaltsalts with water.

The first two of these methods are the simplest and the most commonly used. But the disadvantage of them is that the measurements must be made in solutions of high concentrations sometimes near to the jelly state. The method described in this article is similar to that of type two, but it has the advantage of being applicable even for dilute solutions.

II. THE NEW METHOD

The method is based on the possibility to remove the macromolecules with their primary solvate mantles from a solution by sedimentation in the ultracentrifuge (for a description *cf.* the monograph by Svedberg and Pedersen [6]). If the substance is dissolved in a mixture of a solvent and a nonsolvent, the solvent molecules alone forms a primary solvate mantle around the macromolecule and it can be assumed that the number of the solvent molecules taking part in the solvation of the solute molecule is independent of the nonsolvent used. During the ultracentrifugation the macromolecules with their primary solvate mantles are sedimenting in a mixture of solvent and nonsolvent. Thus the sedimentation gives a solute-free layer of solvent-nonsolvent mixture, above the sedimenting boundary, in which, due to the trans-

port of solvent molecules in the solvate mantles of the sedimenting solute molecules, the concentration of the nonsolvent is higher than in the original solvent-nonsolvent mixture. If solvent and nonsolvent have about the same density and the same compressibility the centrifugal field does not cause any separation of the liquids by sedimentation. From the amount of solvent transported by the solute the solvation number (S), *i. e.* the number of solvent molecules per chain unit taking part in the primary solvate mantle, can be calculated. In the first experiments, we tried to analyse the solvent mixture by measuring the refractive index before and after the removal of the solvated macromolecules from it by sedimentation. Reproducible results were not obtained, however, probably because of evaporation from the small amounts of solution available from the experiment (the volumes of the ultracentrifugal cells used were only 0.5—0.6 ml) and the rather small changes in nonsolvent concentration, which can be obtained at the low solute concentrations applicable for this purpose. Likewise, it was not possible to obtain a solute-free sample, owing to the fact that at least a part of the solute, removed from the solution by sedimentation, was again mixed with the solute free layer on stopping the rotor. This disadvantage could have been avoided by using a so-called separation cell, in which a horizontal porous false bottom separates the upper part of the cell from the lower preventing the solutions in both parts from mixing with each other. For lack of a suitable separation cell another method was worked out, based on Lamm's scale method for measuring the refractive index gradient (dn/dx) at any point in the cell *cf.* 6, p. 253. By integration of the gradient curve between any two points we obtain the refractive index difference between these points.

$$n_2 - n_1 = \int_{x_1}^{x_1} \frac{dn}{dx} \cdot dx$$

Thus, the refractive index difference between the solute free solvent mixture layer and the sedimenting solution is directly proportional to the area of the maximum of the sedimentation picture. Since the refractive index of the later can be measured (= the refractive index of the original solution), the refractive index of the former can be obtained, and finally, the amount of solvent removed from the solution together with the solute molecules can be calculated from the refractive index difference between the solute free solvent mixture and the original solvent mixture.

For the application of this method for measuring the solvation, the following assumptions were made:

1. The refractive index of the solvent mixture is calculable from those of the components on a molar ratio basis (interaction between solvent and non-solvent is not taken into account).

2. No nonsolvent molecules are absorbed by the macromolecules in their primary solvate mantles.

3. The refractive index increment of the solute in the mixed solvent is proportional to the concentration and independent of the ratio solvent — nonsolvent.

These assumptions are probably valid to a first approximation for the systems used for this investigation. The proof of their validity would require series of accurate measurements. The first experiments were made without the last assumption. The refractive index of each solution was measured by an interferometer, but evidently due to evaporation of the two liquids, reproducible results were not obtained. The refractive indices of all solutions were later calculated with the refractive index increment of the solute in pure solvent.

In the calculation the following symbols were used:

n_s = the refractive index of the solvent
n_n = » » » » » non-solvent
n_0 = » » » » » solvent mixture
n = » » » » » solution
n_0' = » » » » » solvent mixture after the solute has been removed by centrifuging
Δn = $n - n_0$
$\Delta' n$ = $n - n_0'$
m_s = the molar percentage of solvent in the solvent mixture
m_n = » » » » non-solvent » » »
Δm = number of solvent moles sedimentated in the solvate mantles per 100 moles of original solvent mixture
M_s = the molecular weight of the solvent
M_n = » » » » » non-solvent
M_0 = » mean molecular weight of the solvent mixture
M = » basic molecular weight of the solute investigated (the molecular weight of the chain unit).
$\frac{dn}{dx}$ = refractive index increment
c = the weight of the solute in grams in 100 grams of solvent mixture

C = the amount of solute in moles of monomer units in 100 moles of solvent mixture

Then we have the following relations:

$$n_0 = \frac{m_s \cdot n_s + m_n \cdot n_n}{100}$$

$$C = M_0 \cdot \frac{c}{M}$$

$$M_0 = \frac{m_s \cdot M_s + m_n \cdot M_n}{100}$$

$$\Delta n = c \cdot \frac{dn}{dc}$$

$$(m_s - \Delta m + m_n)\ n_0' = (m_s - \Delta m)\ n_s + m_n \cdot n_n$$

hence

$$\Delta m = m_s - m_n \frac{n_n - n_0'}{n_0' - n_s} \tag{1}$$

and the solvation number is

$$S = \frac{\Delta m}{C} \tag{2}$$

being the number of solvent molecules bound to the solute per chain unit.

Of the values required in these calculations those for n_s, n_n, M_s, M_n, and often also that for M, may be found in the literature. Δn can be calculated if the refractive index increment of the solute in the pure solvent in question is known and $\Delta' n$ can be measured from the sedimentation picture (see later).

Thus, on the basis of the above, the conclusion can be drawn that the substances used in the experiments must fulfil the following conditions:

1. The solvent mixture consists of two components, component 1 being a good solvent with respect to the substance to be investigated on which substance component 2 is quite incapable of adsorption *. The components must be soluble in each other in the ratios used for the measurements.

* In the presence of a good solvent the danger of non-solvent adsorption on the solute molecules is evidently negligible.

2. The liquid components must possess as accurately as possible the same density and, in the solvent mixture used, about the same vapour pressure, but refractive indices which differ from each other as much as possible.

3. If the macromolecular substance to be investigated is polydispers, fractionated samples should be used, giving regular and well-defined sedimentation pictures for the calculation of the solvation numbers.

III. EXPERIMENTS

In the experiments carried out the solvation of cellulose nitrate in acetone, ethyl and butyl acetate was studied with benzene and xylene as non-solvents. The nitrogen content of the cellulose nitrate samples used varied from 11.7 to 13.2 % and all the samples were moderately polydisperse. Samples 682 and 708 were technical products of the Gyttorp factory and VF3 was studied by Jullander [7], while samples II 10 b and II 10 c were products of our own fractionation. The properties of the cellulose nitrate samples are evident from Table 1.

Table 1. The properties of the cellulose nitrate samples used.

Sample	N %	Degree of nitration	Basic molar weight	lim (η_{sp}/c)	lim s
II 10 b	13.19	2.63	280.5	2.3	11.4
II 10 c	13.19	2.63	280.5	1.2	
682	12.70	2.48	273.6		
708	11.75	2.18	260		
VF3	12.35	2.37	269	0.64	8.3

Table 2 shows the densities and refractive indices of the solvents and non-solvents used in the experiments and Table 3 the refractive index increments used in calculating the solvation numbers.

Table 2. The densities and refractive indices of the solvents and non-solvents used.

No.	Solvent	Density	Refractive index
A	Butyl acetate	0.882	1.39510
B	Ethyl acetate	0.901	1.37257
C	Acetone	0.792	1.35886
D	Benzene	0.894	1.50142
E	*m*-Xylene	0.868	1.49962

Table 3. The refractive index increments (dn/dc) of cellulose nitrate samples used in different solvents (concentration c = 1 g/100 g solution).

Sample	N %	Butyl acetate	Ethyl acetate	Acetone
II 10 b	13.19	74	(78)	84.5
II 10 c				
683	12.70	77		
708	11.75	80		

Of these increment values the one in acetone solution was measured by Jullander [7] and that in ethyl acetate is estimated on the basis of the increments in butyl acetate and amyl acetate.

Table 4 shows the results of the experiments. The composition of the solvent mixture is given in molar percents and the cellulose nitrate content of the solution in basic moles per 100 moles of solvent mixture.
The values in column $\Delta' n$ are generally mean values of the areas of the maxima of 3 to 4 sedimentation diagrams and the values in parentheses indicate the mean errors. In the two last columns the calculated solvation numbers per glucose unit and per nitrate group are given.

From Table 4 it is evident that the method gives reproducible results. The solvation numbers calculated from different parallel experiments differ not more then $\pm$ 10 per cent and often much less. The addition of nonsolvent does not seem to cause any association of the cellulose nitrate molecules; the sedimentation constants of the substances are the same both in pure solvent and solvent-nonsolvent mixtures (corrections for the change in density and viscosity must be introduced). Near the critical concentration association certainly occurs however. As the cellulose nitrate concentration and the proportion of nonsolvent added seem to have no effect on the solvation in the intervals used, the mean values of the solvation numbers S for each cellulose nitrate sample in the different solvent-nonsolvent pairs have been calculated, and collected in Table 5.

When calculating the values given in Table 5, experiment S 10, which gave a clearly erroneous result, and also experiment S 1, which was the only measurement with sample VF 3, were not taken into consideration. A mean value common for samples II 10 b and II 10 c has been calculated because these samples are subsequent fractions of a fractionation and apparently differ from each other only as to their degree of polymerization. The degree of nitration of these samples can differ from the value 2.63 used because this value has

Table 4. The data of solvation measurements.

Expt. no.	Solvent mixture	Cellulose nitrate	Degree of esterification	C	S	Δn	$\Delta' n$		Δm	S/gluc.	S/NO_3
S 3	42.74 A + 57.26 D	II 10 b	2.63	0.161	3.15	35.4	9.2	(1.1)	0.329	2.04	0.78
S 27	54.58 A + 45.42 D	II 10 b	2.63	0.110	3.34	23.0	11.2	(0.6)	0.225	2.05	0.78
S 28				0.0705	3.50	14.8	9.1	(0.2)	0.119	1.69	0.64
S 29				0.110	3.50	23.0	13.0	(1.3)	0.208	1.89	0.72
S 14		682	2.48	0.122	4.02	25.9	12.2		0.284	2.33	0.94
S 15				0.122	3.99	25.9	12.6	(2.1)	0.276	2.26	0.91
S 10				0.0986	4.67	21.0	7.4	(0.0)	0.282	(2.86)	(1.15)
S 16		708	2.18	0.0988	3.84	20.8	11.6	(0.2)	0.199	2.01	0.92
S 17				0.0657	4.95	13.8	7.4	(0.1)	0.133	2.02	0.93
S 18				0.0657	4.99	13.8	7.9	(1.0)	0.123	1.87	0.86
S 4	69.35 A + 30.65 D	II 10 b	2.63	0.222	2.58	44.2	22.4	(0.2)	0.604	2.72	1.03
S 5				0.151	2.99	30.0	18.5	(0.9)	0.351	2.32	0.88
S 6				0.0805	3.54	16.0	10.5	(0.5)	0.167	2.07	0.79
S 25	50.00 A + 50.00 E	682	2.48	0.100	4.11	18.9	3.7	(0.8)	0.290	2.90	1.17
S 26				0.100	4.07	18.9	4.0	(0.3)	0.284	2.84	1.15
S 23				0.0715	4.82	13.6	2.5	(0.3)	0.212	2.97	1.20
S 24				0.0715	4.86	13.6	1.2	(0.0)	0.237	3.31	1.33
S 19		708	2.18	0.0692	4.74	13.0	2.9	(0.8)	0.193	2.79	1.28
S 20				0.0692	4.53	13.0	3.2	(0.5)	0.189	2.73	1.25
S 21				0.0461	5.95	8.6	2.1	(0.4)	0.126	2.73	1.25
S 22				0.0461	5.74	8.6	2.4	(0.4)	0.120	2.60	1.19
S 1	46.99 B + 53.01 D	VF3	2.37	2.17	1.12	550	17.3	(2.0)	5.23	2.41	1.02
S 7	75.09 B + 24.91 D	II 10 c	2.63	0.121	4.14	30.8	17.8	(1.2)	0.405	3.35	1.27
S 8				0.0803	4.87	20.5	12.0	(0.0)	0.265	3.29	1.25
S 9				0.0601	5.04	15.4	10.7	(0.4)	0.148	2.46	0.94
S 33	70.00 C + 30.00 D	II 10 b	2.63	0.119	5.71	43.8	25.5	(2.5)	0.426	3.58	1.36
S 30				0.0736	6.72	27.1	13.0	(1.5)	0.329	4.47	1.70
S 32				0.0534	7.33	19.7	12.0	—	0.180	3.37	1.28
S 31				0.0445	7.56	16.4	7.6	(0.9)	0.205	4.61	1.75

Table 5. The mean values of the results of the solvation measurements.

Solvent mixture	Cellulose nitrate	Degree of nitration	S/gluc.	S/NO_3	Error
Butyl acetate	II	2.63	2.11	0.80	0.08
Benzene	682	2.48	2.30	0.92	0.01
	708	2.18	1.97	0.90	0.03
Butyl acetate	682	2.48	3.00	1.21	0.06
Xylene	708	2.18	2.71	1.24	0.02
Ethyl acetate	II	2.63	3.03	1.15	0.14
Benzene					
Acetone	II	2.63	4.02	1.52	0.20
Benzene					

been obtained for unfractionated cellulose nitrate, of which the samples II 10 b and II 10 c are almost the very lowest fractions. It is evident from the average errors (calculated as molecules of solvent per nitrate group) contained in the last column of the table that the data obtained from the measurements made in mixtures of butyl acetate and benzene as also of butyl acetate and xylene can be considered very reliable, while in other mixtures less accurate results have been obtained. This inaccuracy of the tests in the ethyl acetate-benzene mixture might possibly be due to the incorrect refractive index increment used, while that in the acetone-benzene mixture might be caused by the very different volatilities of the solvent components.

Summing up the solvation measurement tests given in Table 5, it is noted that the solvation number calculated as solvent molecules per glucose unit is dependent on the degree of esterification of cellulose nitrate. Above all, this is evident from the data obtained from the very successful test series made with samples 682 and 708. The solvation numbers of sample 708 calculated per glucose unit are 10—15 per cent lower than those for sample 682, but on calculating the solvation numbers per nitrate group this difference disappears. From this it may be concluded that solvation it apparently solely a phenomenon existing between the nitrate groups and the solvent molecules.

Moreover it is of interest to note that test S 1 has given a solvation number of the same magnitude as those of tests S 7—S 9, although the concentration of the former is about 7 per cent giving a gelatinous solution and those of the latter are only about 0.2—0.4 per cent. This shows that no difference exists in degree of solvation on transition from the gel-solution to the sol-solution.

It is evident that the solvation numbers given in Table 5 represent the minimum value of solvation, *i. e.* they report the smallest possible number of solvent molecules which on centrifuging are removed from the solution, together with the cellulose nitrate molecules to give the changes obtained in the refractive index measurements. If, on the other hand, the nonsolvent molecules also sediment with the solute molecules, the number of sedimenting solvent molecules must be greater than the values given in Table 4, and, indeed to an extent, which depends on the number of sedimenting nonsolvent molecules corresponding to the molar ratio of solvent mixture components.

On the basis of the data obtained from these tests the average solvation number of cellulose nitrate in butyl acetate is 0.85—0.91 when benzene is used as nonsolvent, but 1.23 in a solution containing xylene. It is difficult to explain the cause of this difference. Moll [3] has calculated, on the basis of the sorption investigation data given by McBain and collaborators [8], the solvation numbers for cellulose nitrate (the degree of nitration is not given) in different solvents and nonsolvents and reports that the solvation number in benzene is 0.33 molecules per glucose unit. Assuming that cellulose nitrate in butyl acetate solution also combines with this number of benzene molecules — which seems to be hardly probable — it would increase the solvation number only by 0.10—0.15 per nitrate group. Hence this does not explain the difference. The only possible explanation remaining is the suggestion that some nonsolvent molecules are involved in the primary solvate mantles mainly containing butyl acetate, and according to the results (Table 5) benzene molecules in a considerably greater quantity than xylene molecules. This can be an effect of association of solvent and nonsolvent molecules. If this is the case, both benzene and xylene should give too low solvation numbers and the actual solvation number for cellulose nitrate in butyl acetate would be at least 1.2 molecules per nitrate group.

Conclusions drawn from X-ray analysis infer that xylene, which as such is inert with regard to cellulose nitrate, associates with this if a small amount of acetone is present [9]. The X-ray diagram of this solvate is pronounced if the amount of acetone present is under 10 per cent but changes rapidly into a cellulose nitrate-acetone solvate diagram when the acetone concentration increases. On the basis of this it is obvious that the possibility of a nonsolvent action in the solvation of cellulose nitrate cannot be excluded. For a definite decision more experiments with other nonsolvents must be carried out.

Assuming Moll's report on the solvation number in butyl acetate conforms with the result, 1.23, obtained above, the degree of nitration of the cellulose nitrate used in McBain's investigations must be about 2. The solvation number 3.1 for cellulose nitrate in acetone solution reported by Moll then also

conforms with the number 1.55 in Table 5. On the contrary the solvation number in ethyl acetate obtained in this investigation does not seem to agree with Moll's value. This may be due to an incorrect refractive index increment used. It is true, that McBain's test does not include measurements in ethyl acetate, but on the basis of the data obtained in butyl, propyl, and methyl acetate it is not improbable that for the solvation number in ethyl acetate a value of 3.2—3.3 might have been obtained. This would give 1.6 molecules of ethyl acetate per nitrate group which is about 50 per cent higher than that obtained from ultracentrifuge measurements.

SUMMARY

1. A method is developed for determining the degree of solvation of substances sedimenting in the ultracentrifuge. This method is based on the fact that on dissolving a substance in a mixture of solvent and nonsolvent the solute molecules adsorb solvent molecules which form a primary solvate mantle.

2. The conditions which the components of the solvent mixture and the solute must fulfil in order that this method should give reliable data are discussed.

3. Using the method described the degree of solvation of some cellulose nitrate samples in four different solvent mixtures have been measured. Thus, the fact that the solvation number of cellulose nitrate in organic solvents is proportional to the degree of nitration is confirmed, wherefore it is likely that the hydroxyl groups do not take part in the solvation process of cellulose nitrate with organic solvents.

4. The obtained solvation numbers are minimum values of primary solvation.

5. The solvation numbers calculated from the measurements are for the great part in conformity with the data obtained by other methods. The solvation number in butyl acetate (possibly also in other solvents) seems to be dependent on the nonsolvent used.

The junior author wishes to express his indebtedness to the Finnish Chemical Central Association *(Kemian Keskusliitto)*, for a fellowship, making his stay at the Institute of Physical Chemistry in Uppsala possible.

LITERATURE

1. Eirich, F., and Mark, H. *Ergeb. Exakt. Naturw.* **15** (1936) 1.
2. Philippoff, W. *Viskosität der Kolloide* Dresden and Leipzig (1942).
3. Moll, W. L. H. *Kolloidchem. Beihefte* **49** (1939) 1.

4. Fikentscher, H., and Mark, H. *Kolloid-Z.* **49** (1929) 135.
5. Schulz, G. V. *Z. physik. Chem.* **A 184** (1939) 1.
6. Svedberg, The, and Pedersen, K. O. *The ultracentrifuge* Oxford (1940), *Die Ultrazentrifuge* Dresden and Leipzig (1940).
7. Jullander, I. Dissertation, Stockholm (1945).
8. McBain, J. W., Good, S. J., Bakr, A. M., Davies, D. P., Willevoys, H. J., and Buckingham, R. *Trans. Faraday Soc.* **29** (1933) 1086.
9. Tomonari, T., Trogus, C., and Hess, K. *Z. physik. Chem.* **B 17** (1932) 241.

Received May 28, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 486—500

Investigations in the Retene Field

I. Determination of the Structure of 4-Nitro-3-acetaminoretene

L. SIHLBOM

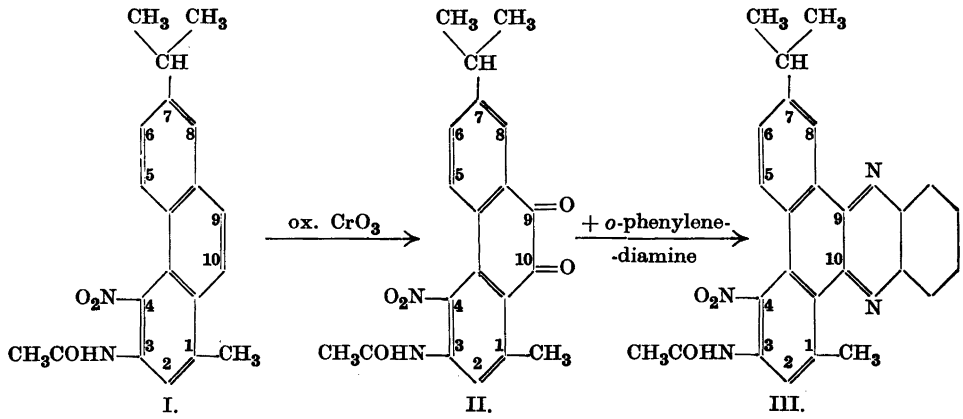
Institution of Organic Chemistry and Biochemistry, University of Stockholm, Sweden

In many earlier works on retene attempts have been described to obtain pure nitroretenes by means of direct nitration [1—3]. These experiments have not given satisfactory results, since only viscous impure products were obtained. Recently, however, some Danish workers have succeeded in performing more successful nitration. They thus obtained, on cautious nitration of retene, 9-nitroretene in a yield of approximately 5 per cent of the theoretical [2]. Since, however, the method requires chromatography and molecular distillation in order to obtain a pure product, it is not as yet particularly suitable for use in the production of nitroretene.

In order if possible to throw some light on the problem of the nitration of retene, it is my intention in this and subsequent investigations to prepare a series of nitroretenes and determine their structure. It may be of some value to obtain a more thorough knowledge of those products that can be formed by nitration of retene. In the methods of preparation described in the following, 3-acetaminoretene was used as the starting material, since this compound is easily nitrated and the amino group can be eliminated by diazotization [3]. The nitration of 3-acetaminoretene is described in earlier papers [3]. Under mild conditions of nitration, two mononitro derivatives are obtained simultaneously, *i. e.* 9-nitro-3-acetaminoretene and x-nitro-3-acetaminoretene. The derivatives were obtained in the approximate proportion of 3 : 2, the 9-derivative giving the largest yield. It is demonstrated in the present investigation that x-nitro-3-acetaminoretene, whose structure was hitherto unknown, has the nitro group in the 4-position. It can be mentioned in this connexion that among the previously produced derivatives of retene of known structure there is none that has any substituent on carbon 4.

In setting up the formulae for the structures it was assumed that the position of the nitro group in x-nitro-3-acetaminoretene was known. It was thus simpler to draw the formulae and the reaction series were more easy to survey. In order to obtain a simpler name, the designation »retenequinoxaline» is used instead, for example, of 3″-methyl-5′-iso-propyl-(dibenso-1′,2′: 1,2 ; 1″,2″ : 3,4-phenazine). The original position numbering of the retene molecule has been retained for derivatives of retenequinoxaline, thus indicating more satisfactorily their relations with the retenequinone derivatives from which they were produced.

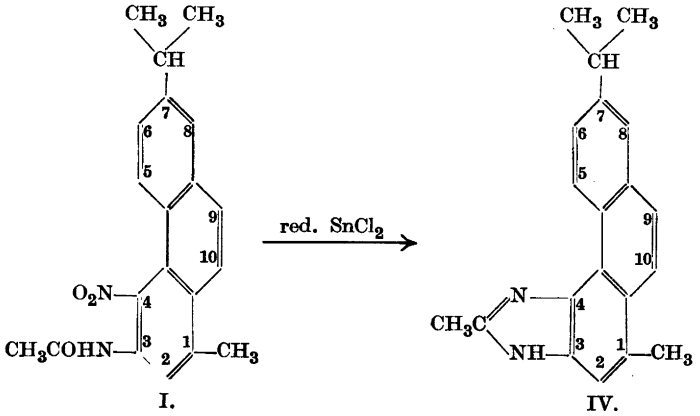
As pointed out in the foregoing, two mononitro derivatives, of which one has the nitro group in the 9-position, are obtained on mild nitration of 3-acetaminoretene. In a preliminary investigation it was established that the nitro group of x-nitro-3-acetaminoretene (I) was not in the 10-position. This was done by transferring the compound to quinone (II) by means of oxidation with chromic trioxide. Since the quinone also contained two nitrogen atoms, it could immediately be established that the nitro group must be bound to a carbon atom in one of the other two benzene nuclei. Had this not been the case, the nitro group would have been eliminated on oxidation. In order to check that quinone had been formed, x-nitro-3-acetaminoretenequinoxaline (III) was also prepared.



I. II. III.

From previous experiments[3] it was known that the acetyl group in 9-nitro-3-acetaminoretene is easily split off by boiling the compound in glacial acetic acid containing concentrated hydrochloric acid. We were not successful in producing x-nitro-3-retylamine hydrochloride in a similar manner. In attempts to split off the acetyl group in an alkaline solution, an intensely dark-red oil was formed which did not invite further treatment. This was, however, later identified as somewhat impure x-nitro-3-aminoretene.

In the hope of obtaining a compound more easy to work with, x-nitro-3-acetaminoretene was reduced with stannous chloride in a solution of glacial acetic acid containing a considerable quantity of concentrated hydrochloric acid. A white product was obtained that formed salts with acids and that could be readily acetylated with acetic anhydride. The values obtained on analysis and the fact that it was not possible to diazotize the compound indicated that something other than an amine was present. The analytical values are in good agreement with the theoretical values if we assume that an imidazole ring was formed (IV). This ring formation shows that the nitro group must be ortho to the acetylated amino group in the 3-position, thus either in the 2- or 4-position.



I. IV.

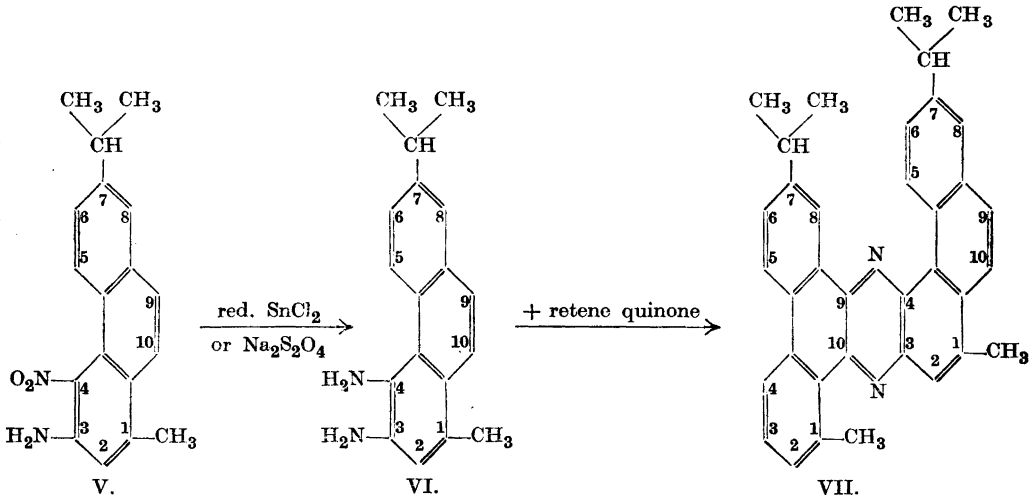
Analysis value:	Found	C 83.4	H 7.07	N 9.64
For the imidazole	Calc.	» 83.3	» 6.99	» 9.72
For x-amino-3-acetamino-retene	Calc.	» 78.4	» 7.24	» 9.15

In order to define (IV) more accurately, some simple derivatives were produced from it, such as the hydrochloride, the N-acetyl derivative and the picrate. Like other imidazoles of the same type, (IV) forms a very poorly soluble picrate.

Attempts were also made to reduce x-nitro-3-acetaminoretene in ethanol solution with sodium sulphide. This resulted in a product similar to that obtained in attempts to split off the acetyl group in alkaline solution (see foregoing). The dark-red oil was extracted in ether. On the introduction of hydrogen chloride into the dry ether solution, the hydrochloride of x-nitro-3-aminoretene was obtained, the yield being approximately 90 per cent of the theoretical. The nitroamine (V) could be diazotized, and yielded on acetyla-

tion the initial product (I). The sodium sulphide had thus not reduced the nitro group but had only acted as an alkali and had caused a splitting off of the acetyl group. As is clear from the description of the synthesis in the following, potassium hydroxide was subsequently used as an alkaliser instead of sodium sulphide. Under suitable conditions, the nitroamine was not obtained as an oily product but directly in crystallized form, analytically pure and with a yield of approximately 80 per cent of the theoretical.

The production of (IV) shows, as already pointed out, that the nitro group must be substituted in the ortho position to the amino group. In order to establish this more definitely, 3,x-diaminoretene (VI) was produced, which should give the reactions of an ortho-diamine. This was found to be the case. The diamine thus reacted with retenequinone by forming a quinoxaline (VII).



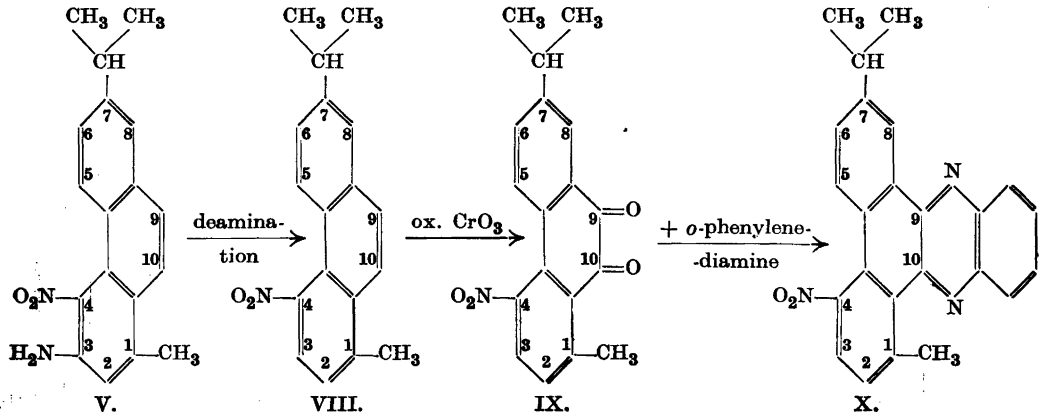
V. VI. VII.

As seen from the foregoing, the nitro group must occupy the 2- or 4-position. Two methods are available for determining which of these two possibilities was present. In both cases the nitroamine is deaminated and x-nitroretene (VIII) thus obtained. This compound can in turn either be transferred via x-aminoretene to x-hydroxyretene — which is compared with the known 2-hydroxyretene [4] — or can be oxidized to x-nitroretenequinone which is compared with 2-nitroretenequinone [5] recently obtained.

The first reaction series, which is not included in the description of the syntheses, was performed qualitatively by diazotization of the x-aminoretene and heating of the diazonium salt. A crystallized substance, too impure for analysis, was obtained, which was probably the x-hydroxyretene. This couples

with diazonium salts with formation of intensive colour. Since the 2-hydroxyretene does not couple, the hydroxyretene formed must therefore be a 4-derivative.

The other reaction series was performed under more accurate conditions. On oxidation of the x-nitroretene (VIII) with chromium trioxide a quinone (IX) was obtained which had its melting point at 185.0—185.5 °C. It crystallized as long yellow needles and reacted with o-phenylenediamine with the formation of a quinoxaline (X). 2-Nitroretenequinone melts at approximately 220 °C and crystallizes as flakes. Thus it can not be identical with x-nitroretenequinone which must, consequently, have the nitro group in the 4-position.



V. VIII. IX. X.

Since the structure of 4-nitro-3-acetaminoretene is known through the foregoing experiments, another structural problem is also solved at the same time. In an earlier investigation it was namely demonstrated that on stronger nitration of 9-nitro-3-acetaminoretene, a dinitro derivative is obtained that is identical with the dinitro-3-acetaminoretene formed when 4-nitro-3-acetaminoretene is exposed to further nitration. This dinitro derivative has thus one nitro group in common with 9-nitro-3-acetaminoretene and one with 4-nitro-3-acetaminoretene. Its formula must therefore be 4,9-dinitro-3-acetaminoretene.

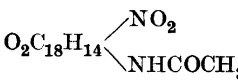
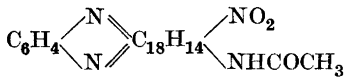
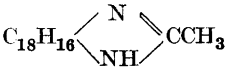
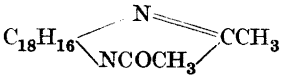
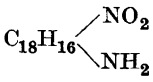
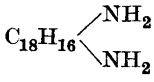
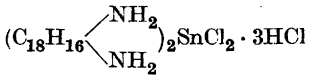
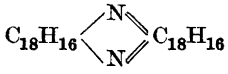
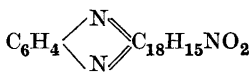
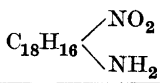
In addition to those substances that are essential to demonstrate the structure, others have been included in this investigation that are in close connexion with the proof of the structure as well as those which contribute towards characterizing the new compounds. Thus the hydrochloride and the picrate have been produced from the diaminoretene. A stannous complex with 3,4-diaminoretene was also isolated. On reduction of 4-nitro-3-aminoretene with stannous chloride in an ethanol solution containing hydrochloric

acid, the first product obtained was not the hydrochloride of the diamine but the complex of the hydrochloride with tin. This crystallized as long flat needles. Since a closer analysis of this complex is not within the scope of this investigation, I confined myself to analyzing all the elements comprising the complex and to calculating the empirical formula with the guidance of the analytic figures. The formula was found to be $\left(C_{18}H_{16}\begin{matrix}NH_2\\NH_2\end{matrix}\right)_2SnCl_2 \cdot 3HCl$. A reservation must be made for the number of hydrogen atoms, since a change of one or two such atoms has only a very slight effect on the percentage of hydrogen. The complex can also be produced directly from diamine and stannous chloride. Attempts to obtain it from stannic ion solution did not succeed. The tin atom in the complex must consequently be considered to be bivalent.

In the course of this investigation additional substances were also obtained, but it was not possible to determine the exact conditions of their synthesis and formulae. Thus 4-nitro- and 9-nitro-3-aminoretene at times crystallized in a dark red form instead of the reddish-yellow one usually obtained. It is probable, at any rate in the case of the 4-derivative, that the dark red form is a molecular combination with the solvent (ethanol or methanol). On reduction of 4-nitro-3-aminoretene, a product with m. p. 95 °C was isolated simultaneously with the 3,4-diaminoretene with m. p. 81 °C, the synthesis of which is described in the following. The former compound reacts with retenequinone to give a quinoxaline which has the same m. p. as the quinoxaline produced from the 81-degree diamine. No depression in the m. p. (300 °C) was achieved on mixing the two quinoxalines. Since the form with m. p. 95 °C also appears to be a diamine, and since the quinoxalines are presumably identical, it appears probable that it is only another form of 3,4-diaminoretene.

Finally, the previous assumption that 9-nitro-3-aminoretene is unstable in air [3] must be revised. The assumption is based on the following observation. If the hydrochloride of the nitroamine is suspended in ether and shaken with a solution of sodium carbonate, a yellow solution is obtained. If this is dropped on a filter paper, a yellow blot is formed which becomes intensely red when the ether has evaporated. The red colour was assumed to depend on the effect of the air. Closer investigation showed that the nitroamine, which is itself reddish-yellow, gives a pale yellow solution in ether. When the ether evaporates, the red-coloured nitroamine is once more obtained. The red colour is thus not caused by the nitroamine being discoloured in some way when it is exposed to air. That the red substance really is 9-nitro-3-aminoretene is demonstrated by the fact that on acetylation it yields 9-nitro-3-acetaminoretene. Moreover, the analytical values of both the nitroamine and its picrate show that this assumption is correct.

Table 1. Substances produced in the present investigation. (Picrates and hydrochlorides not included.)

No.	Name	Formula	M. p. °C *
2.	4-Nitro-3-acetaminoretene-quinone		187—188
3.	4-Nitro-3-acetaminoretene-quinoxaline		305 (decomp.)
4.	2-Methylretimidazole-(3′,4′)		199.0—199.5
5.	N-Acetyl-2-methylretimid-azole-(3′,4′)		202—203
6.	4-Nitro-3-aminoretene		124—125
7.	3,4-Diaminoretene		80—81
8.	3,4-Diaminoretene's tin complex		approx. 190
9.	Reto-3′,4′ : 2,3-reto-9″,10″ : 5,6-pyrazine		299—300
10.	4-Nitroretene	$C_{18}H_{17}NO_2$	136—137
11.	4-Nitroretenequinone	$O_2C_{18}H_{15}NO_2$	185.0—185.5
12.	4-Nitroretenequinoxaline		175—176
13.	9-Nitro-3-aminoretene		152—153

* The melting points are approximately corrected.

A modified method for the production of 4-nitro-3-acetaminoretene is described in the experimental part of this paper. The essential difference lies in the manner of separating 9-nitro-3-acetaminoretene from 4-nitro-3-acetaminoretene. The method is based on the fact that the 9-derivative is practically insoluble in benzene, whereas the 4-derivative is fairly soluble. The isomer mixture is therefore extracted with benzene and the derivative soluble in it is recrystallized in glacial acetic acid. In this way very pure 4-nitro-3-acetaminoretene is obtained. The compound insoluble in benzene consisted of practically pure 9-nitro-3-acetaminoretene.

EXPERIMENTAL PART

1. 4-Nitro-3-acetaminoretene

30 g of 3-acetaminoretene suspended in 225 ml of glacial acetic acid was mixed with 70 ml of propionic acid. (The propionic acid was added in order to prevent the glacial acetic acid from crystallizing when it was cooled to approximately 10° C). 180 ml of a mixture of concentrated nitric acid (d = 1.4) and 70 ml of glacial acetic acid was added in drops with vigorous stirring during approximately 20 minutes. The temperature was kept at 5—10° C. During nitration the reaction mixture was at first a thin fluid, then thickened considerably and once more became more thinly fluid. After the nitric acid had been added, nitration was allowed to continue for 35 minutes. The yellow reaction product was filtered off, thoroughly washed with water and dried. This crude product (30 g) was boiled with 250 ml of benzene and the mixture allowed to cool to 30—40 °C. The residue was filtered off and once more extracted with 150 ml of benzene. In the benzene solution practically only 4-nitro-3-acetaminoretene was present. The benzene was distilled off and the residue crystallized in 130 ml of glacial acetic acid. The crystals containing acetic acid thus obtained were dried at 125° C and the acetic acid thereby eliminated. The yield of the pure product was 12.5 g, m. p. 196—197° C.

The substance insoluble in benzene consisted of practically pure 9-nitro-3-acetaminoretene, m. p. 284—285 ° C. A recrystallization in glacial acetic acid gave an analytically pure product.

2. 4-Nitro-3-acetaminoretenequinone

10 g of chromium trioxide was added in portions to a suspension of 5.0 g of 4-nitro-3-acetaminoretene in 25 ml of glacial acetic acid. The heat of the reaction caused the temperature to rise to 100—110° C. Oxidation proceeded for 25 minutes. 200 ml of water was then added in drops. A yellow precipitate of the quinone then appeared. This was filtered off and washed carefully with water. The yield was 2.3 g. After recrystallization in glacial acetic acid (1.5 g in 5 ml) a pure 4-nitro-3-acetaminoretenequinone, m. p. 187—188° C, was obtained. It crystallized as flat yellow prisms.

$C_{20}H_{18}O_5N_2$	Calc.	C 65.6	H 4.95	N 7.65
	Found	» 65.4	» 4.92	» 7.57

3. 4-Nitro-3-acetaminoretenequinoxaline

0.4 g of 4-nitro-3-acetaminoretenequinone was dissolved in 40 ml of boiling absolute ethanol. A hot ethanol solution containing 0.4 g of o-phenylenediamine was added. Within half a minute the quinoxaline had crystallized out. It was filtered off and washed thoroughly with ethanol in which it is practically insoluble. A recrystallization of the crude product in 200 ml of glacial acetic acid gave a pale yellow product which crystallized as small needles, m. p. 305° C (decomp.). The yield was 0.3 g. The quinoxaline was coloured intensely reddish-brown by concentrated sulphuric acid.

$C_{26}H_{22}O_3N_4$	Calc.	C 71.2	H 5.06
	Found	» 71.1	» 5.08

4. 2-Methylretimidazole-(3′, 4′)

To a mixture consisting of 220 ml of glacial acetic acid and 30 ml of concentrated hydrochloric acid, 35 g of $SnCl_2$ $2H_2O$ and 10 g of 4-nitro-3-acetaminoretene were added. The temperature was raised to 80° C and a weak stream of hydrogen chloride was passed through. As the reduction progressed, an increasing amount of the nitro compound dissolved and the yellow colour shown by the reaction mixture at the beginning finally disappeared. When reduction had continued for 30 mins. and a clear solution was obtained, 45 ml of diluted hydrochloric acid was added. On cooling, short white needle-shaped crystals formed. These were filtered off, washed with diluted hydrochloric acid, water and finally with ether. The yield was 8.9 g. They were then suspended in ether mixed with alkaline (KOH) ethanol. Free imidazole was then obtained. The ether solution was washed with water, dried with sodium sulphate and the ether allowed to evaporate. The residue, which weighed 6.5 g (75 per cent yield) was recrystallized in 150 ml of benzene. The imidazole was obtained as very small, white needle-shaped crystals, m. p. 199.0—199.5° C.

$C_{20}H_{20}N_2$	Calc.	C 83.3	H 6.99	N 9.72
	Found	» 83.4	» 7.07	» 9.64

The foregoing reduction could also be carried out in a solution of ethanol containing hydrochloric acid, giving the same product.

The *picrate* was obtained when a few tenths of a gram of 2-methylretimidazole and 0.5 g of picric acid were boiled in 50 ml of propyl alcohol. A very voluminous yellow picrate was crystallized out of the clear solution. Melting point approx. 272° C (decomp.).

Calc.	C 60.3	H 4.48
Found	» 60.4	» 4.42

The *hydrochloride* crystallized as short needle-shaped crystals from a solution of 2-methylretimidazole in glacial acetic acid to which hydrochloric acid had been added. The hydrochloride was filtered off, washed with glacial acetic acid containing hydrochloric acid, and ether. Melting point > 300 °C. After drying at 130 °C for four hours it was titrated with 0.1 *N* sodium hydroxide.

Calc. Cl 10.92 Found Cl 10.95

5. N-Acetyl-2-methylretimidazole-(3′, 4′)

A few tenths of a gramme of methylretimidazole were boiled in 10 ml of acetic anhydride for 10 minutes and then heated on the water bath for 20 minutes. On cooling, the acetyl derivative crystallized as long needle-shaped crystals which, after recrystallization in acetic anhydride, had a melting point at 202—203 °C.

$C_{22}H_{22}ON_2$	Calc.	C 80.0	H 6.71	N 8.48
	Found	» 80.0	» 6.83	» 8.56

The *picrate* was obtained as small yellow, needle-shaped crystals when an alcoholic solution of picric acid had been added to the acetylretimidazole, dissolved in propyl alcohol. The solutions were mixed hot and boiled for about a minute. Sufficient propyl alcohol was then added to produce complete solution. The picrate is not readily soluble in propyl alcohol. Melting point approx. 269 °C (decomp.).

Calc.	C 60.1	H 4.50
Found	» 59.9	» 4.40

6. 4-Nitro-3-aminoretene

14.8 g of 4-nitro-3-acetaminoretene was suspended in 250 ml of ethanol. 8 g of potassium hydroxide dissolved in 100 ml of water was added and the reaction mixture was heated on the water bath for two hours. A clear dark-red solution was obtained from which long, needle-shaped yellowish-red crystals, m. p. 124—125 °C, crystallized out on cooling. Recrystallization did not raise the melting point. The nitroamine is thus obtained directly in pure form. The yield was 10.0 g, corresponding to 77 per cent of the theoretical.

$C_{18}H_{18}O_2N_2$	Calc.	C 73.5	H 6.16	N 9.52
	Found	» 73.5	» 6.19	» 9.61

The filtrate from the reaction mixture was diluted with water and extracted with ether. When the ether solution had been washed and dried, hydrogen chloride gas was passed through. 1 g of the hydrochloride of the nitroamine was then obtained. This brought the total yield of the nitroamine to 84 per cent.

The *picrate*. An excess of picric acid was added to a solution of 4-nitro-3-aminoretene in ethanol and the solution boiled for about one minute. On cooling, the picrate was obtained as needle-shaped reddish-brown crystals, m. p. 145 °C (decomp.).

Calc.	C 55.1	H 4.04
Found	» 55.3	» 3.98

The *hydrochloride*. Analytically pure 4-nitro-3-aminoretene was dissolved in ether. Hydrogen chloride was passed through and the hydrochloride then precipitated as a pale creamy-yellow product. This was filtered off and dried carefully. It decomposed at approximately 155 °C.

Calc.	C 65.4	H 5.79	Cl 10.72
Found	» 65.1	» 5.89	» 10.62

The chlorine was determined by titration of the hydrochloride with 0.1 *N* sodium hydroxide.

4-Nitro-3-acetaminoretene. 0.1 g of the nitroamine was dissolved in 5 ml of acetic anhydride and heated to boiling point during 10 minutes. A sufficient quantity of water was then cautiously added for the anhydride to be converted into acetic acid. The solution was then allowed to cool. Yellow needle-shaped crystals were obtained which, after recrystallization in glacial acetic acid, melted at 196—197 °C. No depression in the melting point was obtained by mixing with 4-nitro-3-acetaminoretene. The acetylation product is thus 4-nitro-3-acetaminoretene.

7. 3,4-Diaminoretene

1.0 g of 4-nitro-3-aminoretene and 3 g of $Na_2S_2O_4$ were suspended in 25 ml of methanol to which 5 ml of water had been added. The reaction mixture was heated to nearly boiling point during 10 minutes. Decolouration occurred and a practically colourless solution was obtained. A large quantity of the salt remained undissolved. Three times the volume of water was added and the reduction product was extracted with ether. The ether solution was washed with water and dried carefully with sodium sulphate and the ether then distilled off under vacuum. A viscous yellow residue was obtained and recrystallized in petroleum ether (b. p. 40—60 °C) containing 10 per cent benzene. On cooling, the diamine was obtained as needle-shaped crystals melting at 80—81 °C. Further recrystallization did not raise the melting point.

$C_{18}H_{20}N_2$	Calc.	C 81.8	H 7.63	N 10.60
	Found	» 81.8	» 7.65	» 10.52

The *picrate.* 3,4-Diaminoretene was dissolved in boiling ethanol containing 4 g of picric acid in 100 ml of ethanol. The picrate is sparingly soluble in ethanol and relatively large quantities of the solvent are therefore required. The picrate crystallizes as thin, feather-like crystals. It was, however, obtained in such a voluminous form that the contents of the vessel, after cooling, were an almost jelly-like mass. The picrate was filtered off and recrystallized in ethanol. Melting point approx. 165 °C (decomp.). The product was dried at 100 °C for three hours, during which it acquired a greenish colour.

Calc.	C 58.4	H 4.70
Found	» 57.4	» 4.80

The *hydrochloride.* 0.2 g of 3,4-diaminoretene was dissolved in 5 ml of ethanol. Concentrated hydrochloric acid was added in drops. The hydrochloride then crystallized out as flakes with a melting point at 242—244 °C. Titration showed that a dihydrochloride had been formed.

Calc. Cl 21.0 Found Cl 21.0

8. The complex of 3,4-diaminoretene with stannous chloride

0.5 g of 4-nitro-3-aminoretene was dissolved in 10 ml of ethanol. The solution was cooled to approximately 30° C and 2 ml of concentrated hydrochloric acid was added. The faintly creamy-yellow hydrochloride precipitated as a half-solid mass. A solution consisting of 3 g of $SnCl_2 \cdot 2H_2O$, 5 ml of ethanol and 2 ml of concentrated hydrochloric acid was then added. Hydrogen chloride gas was then passed through, the temperature rose to 80° C and a clear solution was formed. After the addition of 30 ml of water, the reaction mixture was allowed to cool. The stannous complex then crystallized as white needle-shaped prisms melting at approximately 190° C (the melting point varies somewhat depending on how long the sample remains in the melting point determination apparatus). The crystals were first washed with dilute hydrochloric acid and then with ether. The yield was 0.6 g, corresponding to 85 per cent.

$C_{36}H_{40}N_4SnCl_2 \cdot 3HCl$	Calc. C 52.3	H 5.23	N 6.77	Sn 14.3	Cl 21.4
	Found » 52.6	» 5.24	» 6.44	» 14.5	» 21.2

The chlorine was determined by titration with 0.1 *N* sodium hydroxide. For the tin determination the sample was first digested by boiling with concentrated sulphuric acid to which some concentrated nitric acid had been added. The tin was then precipitated as a sulphide which was ignited to SnO_2. Direct ignition with sulphuric acid gave considerably lower analytical values (Sn = 13.8 per cent).

This stannous complex could also be produced as follows. 0.1 g of 3,4-diaminoretene was dissolved in 3 ml of ethanol. A solution of 0.5 g of $SnCl_2 \cdot 2H_2O$, 2 ml of ethanol and 0.5 ml of concentrated hydrochloric acid was added and hydrogen chloride gas passed through for a few minutes. The temperature rose to 80° C. After 5 ml of water had been added, the clear solution was allowed to cool. A product similar in appearance to the stannous complex in the foregoing crystallized out. The crystals were filtered off, washed with dilute hydrochloric acid and ether. The melting point was approximately 190° C.

Calc.	C 52.3	H 5.23
Found	» 52.8	» 5.28

If the stannous complex is suspended in ether to which alkaline (KOH) ethanol has been added, the free diamine is formed and obtained according to the way described in the foregoing (v. under 3,4-diaminoretene).

9. Reto-3',4':2,3-reto-9'',10'':5,6-pyrazine

0.3 g of 3,4-diaminoretene was dissolved in 5 ml of ethanol. The solution was mixed with 0.25 g of retenequinone dissolved in 20 ml of absolute ethanol. A dark colouration of the reaction solution could be observed. It was boiled for a few minutes and then allowed to cool. The yellow quinoxaline then precipitated was filtered off and recrystallized twice in dioxane (solubility 1 g in 200 ml). It crystallized as canary-yellow needle-shaped prisms melting at 299—300° C. The yield of the pure product was 0.05 g. The quinoxaline was coloured intensely greenish-blue by concentrated sulphuric acid.

$C_{36}H_{32}N_2$	Calc.	C 87.8	H 6.55	N 5.69
	Found	» 87.3	» 6.44	» 5.61

10. 4-Nitroretene

A solution of 8.0 g of 4-nitro-3-aminoretene in 600 ml of glacial acetic acid was mixed with 185 ml of dilute sulphuric acid consisting of one volume of concentrated acid and three volumes of water. The clear red solution was cooled to + 5° C. The greater part of the sulphate of the nitroamine crystallized out. During 15 minutes a solution of 8 g of sodium nitrite in 35 ml of water was added drop-wise with vigorous stirring. A clear solution was gradually obtained. A reduction solution (see the following) containing hypophosphorous acid was added and the reaction mixture placed in the refrigerator for 20—30 hours. The nitroretene formed on reduction was obtained as a yellow, partly crystalline precipitation. It was filtered off and washed with large quantities of water. The yield was 6.5 g. The crude product was recrystallized in a mixture of 100 ml of absolute ethanol and 20 ml of propyl alcohol. The alcohol solution was boiled with 5 g of charcoal, filtered and allowed to cool. After a further recrystallization and boiling with charcoal, the pure 4-nitroretene was obtained as faintly yellow, long prism-shaped crystals melting at 136—137° C. The yield was 2.5 g. Calculated on the nitroamine this corresponds to a yield of approximately 30 per cent.

$C_{18}H_{17}O_2N$	Calc.	C 77.4	H 6.14	N 5.02
	Found	» 77.3	» 6.05	» 4.99

The 4-nitroretene is readily soluble in boiling glacial acetic acid; 4 g dissolves in less than 10 ml. The solubility in absolute ethanol at boiling point is approximately 1 g in 15 ml.

The reduction solution used in the foregoing was produced as follows. 100 g of dried and pulverised sodium hypophosphite was added in portions to 160 g of concentrated sulphuric acid. When the reaction mixture was left overnight at room temperature, it was diluted with water to a volume of 300 ml and mixed with the diazonium solution.

11. 4-Nitroretenequinone

0.5 g of 4-nitroretene was dissolved in 10 ml of glacial acetic acid. 1.0 g of chromium trioxide was added in portions. When reaction had started, the temperature rose to approximately 100° C on account of the heat of reaction. When oxidation was complete, 35 ml of water was added in drops and with stirring. The quinone was obtained as a flocculent yellow precipitate. It was filtered off and washed carefully with water. After recrystallization, pure quinone was obtained. It crystallized as long yellow needles melting at 185.0—185.5° C.

$C_{18}H_{15}O_4N$	Calc.	C 70.0	H 4.89	N 4.53
	Found	» 70.0	» 4.85	» 4.46

12. 4-Nitroretenequinoxaline

A solution of 0.3 g of 4-nitroretenequinone in 15 ml of ethanol was mixed with a solution of 0.2 g of o-phenylenediamine in 5 ml of ethanol. The mixture was boiled for a few minutes and then allowed to cool. The quinoxaline then crystallized out and was

recrystallized in ligroin (b. p. 65—90° C) containing a small percentage of toluene. It crystallized as faintly yellow, very short needles, melting at 175—176° C. It was coloured reddish-brown by treatment with concentrated sulphuric acid.

$C_{24}H_{19}O_2N_3$	Calc.	C 75.6	H 5.02
	Found	» 75.9	» 5.02

13. 9-Nitro-3-aminoretene

A few tenths of a gram of 9-nitro-3-retylamine hydrochloride were suspended in ether and shaken with a 20 per cent solution of sodium hydroxide. The free nitroamine then dissolved and a faintly yellow ether solution was obtained. This was washed, and dried with sodium sulphate. The ether was distilled off and the blood-red residue was recrystallized twice in ethanol. The nitroamine was obtained as reddish-yellow, needle-shaped crystals, melting at 152—153° C.

$C_{18}H_{18}O_2N_2$	Calc.	C 73.5	H 6.16	N 9.52
	Found	» 73.1	» 6.26	» 9.51

The *picrate* was produced from an ethanol solution in the usual way. 0.3 g of the 9-nitro-3-aminoretene was dissolved together with 0.7 g of picric acid in 50 ml of ethanol and the solution boiled for about one minute. On cooling, the picrate was obtained as thin yellow, needle-shaped crystals with the melting point at 182—183° C (decomp.).

Calc.	C 55.1	H 4.04
Found	» 55.1	» 3.97

For comparison it can be mentioned that the picrate of 4-nitro-3-aminoretene is intensely brown in colour.

9-Nitro-3-acetaminoretene was obtained when the nitroamine was boiled with an excess of acetic anhydride. A clear pale yellow solution was obtained, from which beautiful yellow crystals in the form of flakes were obtained on cooling. Their m. p. was 286—287° C. A mixture with a sample of 9-nitro-3-acetaminoretene [3] gave the same melting point. Since no depression of the melting point was observed, the acetylation product thus consists of 9-nitro-3-acetaminoretene.

SUMMARY

The present investigation is chiefly concerned with a determination of the structure of 4-nitro-3-acetaminoretene. At the same time a series of retene derivatives with some substituent in the 4-position was produced. No 4-derivative of retene has previously been obtained. Among the new retene derivatives special mention is made of 4-nitroretene. Only one nitro derivative of retene is hitherto known, *i. e.* 9-nitroretene. The investigation also in-

cluded the production of 9-nitro-3-aminoretene. This compound has hitherto been assumed to be unstable in air. This is not the case.

The present paper forms a part of a number of investigations with the object of producing a series of nitroretenes. It is thus hoped to throw some light on the problem of nitration of retene.

I wish to convey my sincere thanks to Professor Karl Myrbäck, who gave me the opportunity of carrying out these investigations and who has always shown great interest and willingness in discussing the problems that have arisen in connexion with the work.

I also thank Docent K.-J. Karrman, Lund, who kindly placed a sample of 2-nitroretenequinone at my disposal.

REFERENCES

1. Adelson, D. E., and Bogert, M. T. *Chem. Rev.* **24** (1939) 158.
2. Fredriksen, E., and Nielsen, E. J. *Acta Chem. Scand.* **1** (1947) 448.
3. Karrman, K.-J., and Sihlbom, L. *Svensk Kem. Tid.* **58** (1946) 189.
4. Komppa, G., and Wahlforss, E. *J. Am. Chem. Soc.* **52** (1930) 5009.
5. Karrman, K.-J. Personal communication.

Received May 29, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 501—517

The Crystal Structures of Mo_9O_{26} (β'-Molybdenum Oxide) and Mo_8O_{23} (β-Molybdenum Oxide)

ARNE MAGNÉLI

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

In a previous communication from this Institute [1], the existence of two molybdenum oxides of a composition close to $MoO_{2.90}$ was reported. The one formed at about 700° C was denominated the β'-oxide, the other obtained at a maximum temperature of about 650° C was called the β-oxide. A description of the appearance of the oxides and of the method of preparing them was given in [1].

The determination of the structure has been entirely based on Weissenberg photographs taken with Mo-K radiation. The estimation of the intensities was carried out visually, starting from 4 : 2 : 1 as relative values of the intensities of Mo-$K\alpha_1$, Mo-$K\alpha_2$, and Mo-$K\beta$ radiation. Relative values of the structure amplitude were obtained by dividing the intensity values by $(1 + \cos^2 2\Theta)/\sin^2 2\Theta$. The temperature factor was neglected.

The calculations of Patterson and electron density function projections and cuts were carried out by means of the electric machine for the summation of Fourier series, constructed by Hägg and Laurent [2].

DERIVATION OF THE STRUCTURE OF Mo_9O_{26} (β'-MOLYBDENUM OXIDE)

Unit cell and space-group

A crystal suitable for taking single crystal photographs was obtained from a preparation $MoO_{2.92}$, which had been heated at 700° C for 60 hours. Laue photographs showed the Laue symmetry to be C_{2h}—$2/m$. Rotation and Weissenberg photographs were taken around [0 1 0]. By using Mo-K radiation it was then possible to obtain layer lines with $k = 0$—5. Reflections 0 k l were

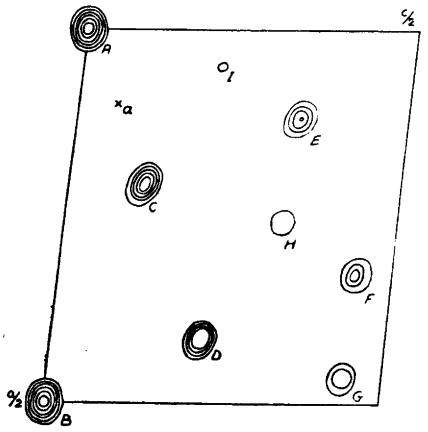


Fig. 1. Mo_9O_{26}. *Projection of the Patterson function on the ac plane, p (xz). Only maxima whose centres of gravity belong to the sketched area are indicated.*

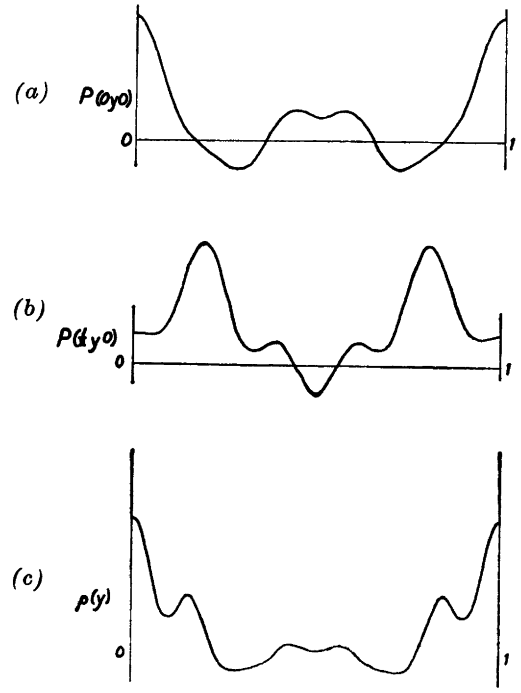


Fig. 2. Mo_9O_{26}. *The Patterson function along the lines 0,y,0 (a) and* $\frac{1}{2}$,*y*,0 (*b*). *Projection of the Patterson function on the b axis (c).*

registered on a Weissenberg photograph with [1 0 0] as axis of rotation. This made it possible to correlate the intensity scales of the former photographs.

The dimensions of the monoclinic unit cell proved to be:

$$a = 16.75 \text{ Å}, \; b = 4.03 \text{ Å}, \; c = 14.45 \text{ Å}, \; \beta = 96°$$

The volume of the unit cell is 970 Å^3. The density value, 4.26, obtained for a preparation evidently consisting of practically pure β'-molybdenum oxide ($MoO_{2.88}$ heated at 700° C for 60 hours)[1] corresponds to 17.5 $MoO_{2.88}$ units per cell. This makes it rather probable that the elementary cell contains two formula units Mo_9O_{26} (corresponding to 18 $MoO_{2.89}$). The density calculated under this assumption would be 4.38.

Reflections h 0 l only appear when h is even. Reflections 0 k 0 are present in all the orders. Thus one of the space-groups C_s^2—Pa and C_{2h}^4—$P2/a$ is probable. The investigation was started by examining whether the structure was consistent with the latter space-group, having the higher symmetry.

Molybdenum positions

From the F^2_{h0l} values the projection of the Patterson function on the *ac* plane, p(*xz*), was calculated (Fig. 1 and Table 1). The projection obtained shows a rather smooth background with discrete maxima rising from it. As the *b* axis of the unit cell is quite short it seems probable that the background corresponds to the p(*xz*) value zero. The height of a maximum above this lowest level may be looked upon as an approximate measure of its magnitude.

The shortness of the *b* axis excludes the possibility of two Mo atoms having the same *x* and *z* coordinates, which may also be inferred from the fact that the cut P(0 *y* 0) in the Patterson function (Fig. 2 a) only contains the single strong maximum at $y = 0$. The major peaks B—G of p(*xz*) are presumably chiefly due to Mo—Mo vectors and may all be interpreted as effected by 18 Mo atoms arranged according to one of the following alternatives *:

Alternative I.

2 Mo_1 in 2(*a*) or 2(*c*):	0 0 0 or 0 $\frac{1}{2}$ 0
4 Mo_2 in 4(*g*):	x_C y_2 z_C
4 Mo_3 in 4(*g*):	x_D y_3 z_D
4 Mo_4 in 4(*g*):	x_E y_4 z_E
4 Mo_5 in 4(*g*):	x_F y_5 z_F

Alternative II.

2 Mo_1 in 2(*e*):	$\frac{1}{4}$ y_1 0
4 Mo_2 in 4(*g*):	$(x_C + \frac{1}{4})$ y_2 z_C
4 Mo_3 in 4(*g*):	$(x_D - \frac{1}{4})$ y_3 z_D
4 Mo_4 in 4(*g*):	$(x_E + \frac{1}{4})$ y_4 z_E
4 Mo_5 in 4(*g*):	$(x_F - \frac{1}{4})$ y_5 z_F

The Mo positions of these two alternatives differ essentially with regard to the twofold axes. It is not possible to decide between them from reflections *h* 0 *l*.

The consistency of the Patterson function generated by the proposed Mo arrangements (Alternatives I and II) with the projection calculated from the F^2_{h0l} values, p(*xz*), appears from Table 1. The heights above the background level of the maxima of p(*xz*) agree rather well with those required by the metal atom lattices although they comprise Mo—O and O—O maxima. Two further maxima, H and I, required by the proposed structures and corresponding to 6 and 4 Mo—Mo vectors respectively, are found at the calculated positions. A remaining maximum due to 2 Mo—Mo vectors could not, however, be observed with certainty.

---

* Notations analogous to those of space-group C^4_{2h} — *P* 2/*c* in *International tables for the determination of crystal structures*. Berlin (1935).

Table 1. Mo_9O_{26}. Projection of the Patterson function on the ac plane, p(xz).

Maximum *Cf.* Fig. 1	Coordinates observed		Number of Mo—Mo vectors estimated from p(xz) (The influence of the O atoms being neglected.)	Number of Mo—Mo vectors required by the structures proposed.
	x	z		
A	0	0	18	18
B	$\frac{1}{2}$	0	18	18
C	0.208	0.113	14.8	16
D	0.413	0.225	12.5	14
E	0.123	0.337	12.0	12
F	0.327	0.448	10.2	10
G	0.466	0.440	8.8	8
H	0.257	0.328	7.5	6
I	0.050	0.209	6.3	4
—				
a (Mo—O vector)	0.10	0.058	—	—

F^2_{h0l} values calculated on the basis of the proposed structures are in good agreement with the observed values for reflections with moderately high indices. The $\varrho(xz)$ functions for the two alternative structures estimated from these F_{h0l} values contain 18 heavy peaks with only slight deviations from the assumed Mo positions. A repeated computation of the $\varrho(xz)$ functions with F_{h0l} values based on the coordinates thus obtained gives two sets of x and z parameters for Mo which bring about good agreement between observed and calculated F^2_{h0l}.

The two following alternatives of distributing the Mo atoms are thus possible:

Alternative I.

2 Mo_1 in 2(a) or 2(c):	0	0	0	or 0 $\frac{1}{2}$ 0
4 Mo_2 in 4(g):	0.206	y_2	0.113	
4 Mo_3 in 4(g).	0.412	y_3	0.221	
4 Mo_4 in 4(g):	0.119	y_4	0.334	
4 Mo_5 in 4(g):	0.332	y_5	0.450	

Alternative II.

2 Mo_1 in 2(e):	$\frac{1}{4}$	y_1	0
4 Mo_2 in 4(g):	0.456	y_2	0.113
4 Mo_3 in 4(g):	0.162	y_3	0.221
4 Mo_4 in 4(g):	0.369	y_4	0.334
4 Mo_5 in 4(g):	0.082	y_5	0.456

The error in the parameter values is estimated to be ± 0.002 corresponding to about ± 0.03 Å.

The vectors caused by Mo atoms mutually connected by means of the glide plane must correspond to maxima in the Patterson function on a line perpendicular to the *ac* plane and cutting this plane in $x = \frac{1}{2}$, $z = 0$. The appearance of P($\frac{1}{2}$ y 0) is given in Fig. 2 b. The major peaks at $y = 0.18$ and $y = 0.82$ are evidently due to Mo—Mo vectors. The corresponding Mo atoms must lie in planes normal to the *b* axis and 0.18*b* apart symmetrically to the glide planes, *i. e.* in $y = \pm 0.09$ or in $y = (\frac{1}{2} \pm 0.09)$. If there are Mo atoms in both of these sets of planes there must be a maximum in the projection of the Patterson function on the *b* axis, p(y), at $y \approx 0.32$. As such a maximum is totally lacking (Fig. 2 c) only one of the double planes ought to contain Mo atoms. The parameter values $y = 0.41$ and $y = 0.59$ are arbitrarily chosen for these atoms. The comparatively high value of P($\frac{1}{2}$ y 0) at $y = 0$ might possibly indicate that one of the Mo positions, corresponding to two or four Mo atoms, has the y parameter close to 0 or 0.5. This possibility cannot be excluded from the appearance of p(y). However, an arrangement with two or four Mo atoms near the plane $y = 0$ and the other ones equally distributed at $y = 0.41$ and 0.59 does not give calculated values of F^2_{0k0} compatible with the observed ones.

The establishment of the arrangement of the Mo atoms with respect to the remaining y parameter values (0.41, ~ 0.50, 0.59) may be carried out by examining reflections $h\,k\,l$ with h odd, as those with h even are not influenced by the distribution of the Mo atoms in the planes $y = 0.41$ and $y = 0.59$. In this way it is possible to decide between the proposed arrangements of the metal atoms, Alternatives I and II. The F^2_{hkl} values are computed for a number of reflections $h\,k\,l$ (h = odd) for the following alternatives:

Alternative I. The twofold Mo position has $y = \frac{1}{2}$ (corresponding to 2(*c*)); the other Mo positions are given the y parameters 0.41 and 0.59 in all arrangements.

Alternative II. a) All arrangements of one Mo position (two- or fourfold) at $y = 0.50$, the others at $y = 0.41$ and 0.59.

b) All arrangements of the Mo positions at $y = 0.41$ and 0.59.

Conformity between observed and calculated F^2_{hkl} is only obtainable for one of these arrangements (belonging to Alternative II b). The Mo atoms of the oxide thus have positions with the symmetry C^4_{2h}—$P2/a$, x and z coordinates equal to those given above for Alternative II and the following y parameters:

$$y(\mathrm{Mo}_1) = 0.59, \quad y(\mathrm{Mo}_2) = 0.41, \quad y(\mathrm{Mo}_3) = 0.41,$$
$$y(\mathrm{Mo}_4) = 0.59, \quad y(\mathrm{Mo}_5) = 0.41.$$

The error in the y parameters is probably less than $\pm$ 0.01 corresponding to about $\pm$ 0.04 Å.

The sign of most F_{hkl} values can be determined from this Mo arrangement as the influence of the Mo atoms in most cases dominates over the contribution of the O atoms.

By examining the electron density function (ϱ) along lines normal to the *ac* plane and cutting it in $x = x$(Mo) and $z = z$(Mo) it is possible to verify the y(Mo) parameters. This was done for Mo_1 and Mo_5. The curves show heavy maxima at the y values arrived at above. However it seems impossible to correct the y coordinates in this way owing to the low number of Fourier terms in the y direction, the maximum value of k being 5.

Oxygen positions

In the Patterson projection on the *ac* plane (Fig. 1) there are a number of minor peaks not interpretable as projections of Mo—Mo vectors and thus probably due to Mo—O interaction. The vector projection Aa, halving the projected Mo—Mo distance AC, may indicate O atoms situated between such adjacent Mo atoms of the structure. Thus it seems reasonable that a number of O atoms are located in or close to the plane $y = \frac{1}{2}$. From a computation of the electron density function in this plane, $\varrho(x\ \frac{1}{2}\ z)$, it appears that besides maxima caused by the proximity of the Mo atoms, there are minor maxima, evidently due to O atoms at the following points:

2 O_6	in 2(*c*):	0	$\frac{1}{2}$	0				
4 O_7	in 4(*g*):	0.355	0.50	0.06	4 O_{11}	in 4(*g*):	0.26	0.50	0.285
4 O_8	in 4(*g*):	0.21	0.50	0.115	4 O_{12}	in 4(*g*):	0.125	(<) 0.50	0.345
4 O_9	in 4(*g*):	0.06	0.50	0.18	4 O_{13}	in 4(*g*):	0.465	~0.50	0.42
4 O_{10}	in 4(*g*):	0.42	0.50	0.23	4 O_{14}	in 4(*g*):	0.32	~0.50	0.46

The y(O) values are arrived at in the following way. For space reasons it is evident that the O_6 atom at $x = 0$, $z = 0$ must occupy a twofold position and thus be situated in 2(*c*): $0\ \frac{1}{2}\ 0$. All the O maxima in $(x\ \frac{1}{2}\ z)$ are of the same magnitude, indicating that the atoms are located fairly close to the plane $y = \frac{1}{2}$. Similar to O_6 the O atoms indexed 7—11 seem to constitute the corners shared by two Mo—O polyhedra with the Mo atoms symmetrically displaced in relation to $y = \frac{1}{2}$. This makes it probable that the y parameters of these O atoms are equal to 0.50. O_{12} is situated between two Mo atoms with $y =$ 0.41, and $y(O_{12})$ might for that reason be somewhat less than 0.50. The y values of O_{13} and O_{14} cannot be easily corrected from similar considerations.

The Patterson function curves P(0 y 0) and P($\frac{1}{2}$ y 0) (Fig. 2 a and b) show minor peaks at $y \approx 0.4$ and $y \approx 0.6$ besides the main peaks due to Mo—Mo vectors. If these maxima are generated by Mo—O interaction, which seems probable, they would indicate O atoms having about the same x and z coordinates as a number of Mo atoms and located near the planes $y = 0.8$ or $y = 0$. The former possibility, however, is definitely excluded from considerations of space as it would give much too short O—O distances. The cut $\varrho(x\ 0\ z)$ in the electron density function contains maxima at points with the x and z coordinates arrived at for the Mo atoms. These peaks are higher than those corresponding to oxygen in $\varrho(x\ \frac{1}{2}\ z)$, evidently due to the influence of the Mo atoms. However, the maxima do not show any trace of distortion indicating a difference between the x and z coordinates of molybdenum and oxygen. Linear cuts through these points normal to the ac plane, for instance $\varrho(\frac{1}{4}\ y\ 0)$, are influenced by cutting off effects so that the correction of the y(O) coordinates has to be carried out from considerations of space.

The average distance between Mo atoms and neighbouring O atoms close to the plane $y = \frac{1}{2}$ amounts to 1.91 Å. If one assumes the shortest distance between Mo atoms and O atoms at $y \approx 0$ to have about the same value, these O atoms must have the y parameters equal to y(Mo) + 0.47, giving the shortest O—O distance a value of 2.5 Å. Thus the following O positions are obtained:

2 O_1 in 2(e):	$\frac{1}{4}$	0.06	0				
4 O_2 in 4(g):	0.455	0.94	0.115	4 O_4 in 4(g):	0.37	0.06	0.335
4 O_3 in 4(g):	0.16	0.94	0.22	4 O_5 in 4(g):	0.08	0.94	0.45

Final structure of Mo_9O_{26}

It is thus possible to describe the arrangement of the atoms of the oxide in the following manner:

Cell content: $2Mo_9O_{26}$.

Space group: $C_{2h}^4 - P\ 2/a$.

2 Mo_1	in 2(e):	$\frac{1}{4}$	0.59	0	4 O_4	in 4(g): 0.37	0.06	0.335
4 Mo_2	in 4(g):	0.456	0.41	0.113	4 O_5	in 4(g): 0.08	0.94	0.45
4 Mo_3	in 4(g):	0.162	0.41	0.221	2 O_6	in 2(c): 0	$\frac{1}{2}$	0
4 Mo_4	in 4(g):	0.369	0.59	0.334	4 O_7	in 4(g): 0.355	0.50	0.06
4 Mo_5	in 4(g):	0.082	0.41	0.450	4 O_8	in 4(g): 0.21	0.50	0.115
2 O_1	in 2(e):	$\frac{1}{4}$	0.06	0	4 O_9	in 4(g): 0.06	0.50	0.18
4 O_2	in 4(g):	0.455	0.94	0.155	4 O_{10}	in 4(g): 0.42	0.50	0.23
4 O_3	in 4(g):	0.16	0.94	0.22	4 O_{11}	in 4(g): 0.26	0.50	0.285
					4 O_{12}	in 4(g): 0.125	(<)0.50	0.345
					4 O_{13}	in 4(g): 0.465	~0.50	0.42
					4 O_{14}	in 4(g): 0.32	~0.50	0.46

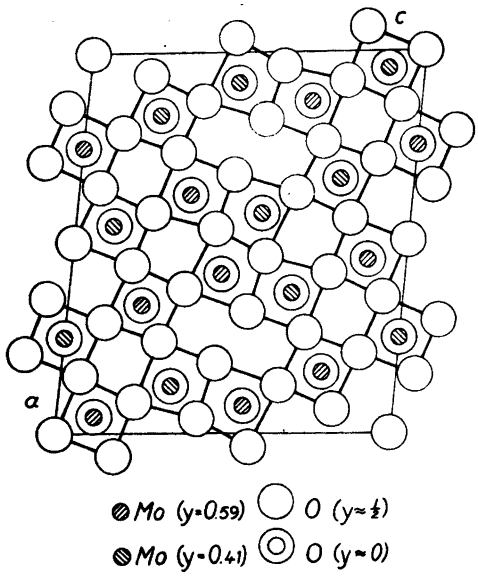


Fig. 3. Mo_9O_{26}. Projection of the structure parallel to [0 1 0].

A projection of the structure parallel to [0 1 0] is given in Fig. 3. The structure factors of a large number of reflections have been computed, some of them being given in Table 2. The agreement between observed and calculated F values is not perfect in every detail but may be looked upon as satisfactory considering the great number of parameters.

DERIVATION OF THE STRUCTURE OF Mo_8O_{23} (β-MOLYBDENUM OXIDE)

The method for the determination of the structure of Mo_8O_{23} has to some extent been analogous to the manner of solving the structure of Mo_9O_{26} and will therefore be reported briefly.

Unit cell and space-group

From a preparation $MoO_{2.90}$, which had been heated at 650° C for 24 hours, a minute needle-shaped single crystal was picked out. Laue photographs showed the Laue symmetry to be C_{2h}—$2/m$, with the digonal axis parallel to the needle axis. Rotation and Weissenberg photographs were taken with Mo-K radiation and rotation around this axis. In this way layer lines with $k = 0$—5 were registered. It was not possible to find a crystal suitable for taking photographs around another axis. Correlation between the intensity

Table 2. Mo_9O_{26}. Weissenberg photographs with Mo-K radiation. Comparison between observed and calculated $|F|$ values.

h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.
4 0 0	200	200	4 5 0	120	80	5 2 $\bar{8}$	130	120
6 0 0	—	60	5 5 0	—	20	5 2 $\bar{7}$	190	140
8 0 0	—	0	6 5 0	—	60	5 2 $\bar{6}$	120	100
10 0 0	300	300	7 5 0	—	40	5 2 $\bar{5}$	360	240
12 0 0	—	60	8 5 0	—	0	5 2 $\bar{4}$	350	230
14 0 0	—	10	9 5 0	—	20	5 2 $\bar{3}$	160	120
16 0 0	—	10	10 5 0	170	120	5 2 $\bar{2}$	—	40
18 0 0	—	80	0 0 4 —	—	<50	5 2 $\bar{1}$	—	40
4 1 0	160	100	— 0 0 8			5 2 1	130	140
5 1 0	—	60	0 0 9	470	590	5 2 2	200	140
6 1 0	—	50	0 0 10—	—	<70	5 2 3	130	120
7 1 0	180	130	— 0 0 17			5 2 4	230	230
8 1 0	—	0	0 0 18	230	340	5 2 5	230	230
9 1 0	—	70	0 1 0	450	680	5 2 6	140	130
10 1 0	260	180	0 1 1 —	—	<40	5 2 7	—	50
11 1 0	—	70	— 0 1 8			5 2 8	—	10
12 1 0	—	50	0 1 9	360	370	5 2 9	—	50
4 2 0	100	70	0 1 10—	—	<60	5 2 10	—	100
5 2 0	90	100	— 0 1 17			10 0 $\bar{10}$	330	310
6 2 0	—	30	0 1 18	210	240	10 0 $\bar{9}$	200	200
7 2 0	280	230	0 2 0	360	460	10 0 $\bar{8}$	—	30
8 2 0	—	0	0 2 1 —	—	<30	10 0 $\bar{7}$	—	60
9 2 0	140	150	— 0 2 8			10 0 $\bar{6}$	—	40
10 2 0	120	130	0 2 9	200	250	10 0 $\bar{5}$	—	50
11 2 0	100	120	0 2 10—	—	<40	10 0 $\bar{4}$	—	70
12 2 0	—	30	— 0 2 17			10 0 $\bar{3}$	—	80
4 3 0	—	20	0 2 18	120	150	10 0 $\bar{2}$	90	160
5 3 0	—	70	0 3 0 —	—	<40	10 0 $\bar{1}$	350	410
6 3 0	—	20	— 0 3 18			10 0 1	—	60
7 3 0	240	180	0 4 0	130	170	10 0 2	—	10
8 3 0	—	0	0 4 1 —	—	<30	10 0 3	—	30
9 3 0	120	120	— 0 4 8			10 0 4	—	20
10 3 0	—	10	0 4 9	150	190	10 0 5	—	30
4 4 0	—	60	0 5 0	260	250	10 0 6	—	90
5 4 0	—	60	0 5 1 —	—	<50	10 0 7	120	150
6 4 0	—	40	— 0 5 8			10 0 8	280	300
7 4 0	130	150	0 5 9	270	250	10 0 9	130	190
8 4 0	—	0	0 6 0	230	240	10 0 10	—	10
9 4 0	90	110	5 2 $\bar{10}$	—	60			
10 4 0	90	80	5 2 $\bar{9}$	110	100			

scales of the Weissenberg photographs had for that reason to be obtained from the estimated intensity values of reflections, belonging to the several layer lines, registered under similar conditions on the same film.

A rather small interval of oscillation was chosen, where good reflections had been observed in all the layer lines. Successive, equally exposed equi-inclination Weissenberg photographs were taken with oscillation within this interval, the film being moved a little between the exposures in order to get the resulting short Weissenberg photographs separated on the film. The influence of possible variations of the X-ray intensity from the ion tube used was reduced by repeating the sequence of exposures several times, the film being carefully adjusted to the exact position for each layer line.

The unit cell dimensions calculated from the single crystal photographs are:

$$a = 16.8 \text{ Å}, \quad b = 4.04 \text{ Å}, \quad c = 13.4 \text{ Å}, \quad \beta = 106° 5$$

The volume of the unit cell is 870 Å^3. For a preparation $MoO_{2.90}$, heated at 650° C for 24 hours, Hägg determined a density of 4.32 *. This corresponds to 15.9 $MoO_{2.9}$ units per cell. Thus a cell content of two formula units Mo_8O_{23} (= 16 $MoO_{2.875}$) is very probable, giving a calculated density of 4.33.

In the Weissenberg photographs all reflections $h\ 0\ l$ with h odd are missing. This is characteristic of the space-groups C_s^2—$P\,a$, C_{2h}^4—$P\,2/a$, and C_{2h}^5—$P\,2_1/a$. As reflections $0\ k\ 0$ were not registered it was impossible to use these reflections to decide between the last group and the other two. However, the existence of a twofold screw axis in the structure would cause a number of heavy maxima to appear in the Patterson cut $P(x\,\frac{1}{2}\,z)$, corresponding to vectors between Mo atoms, mutually connected by the axis. For a set of reflections $h\ k\ l$ with h and l constant the F^2 values vary regularly with k to give an approximately smooth curve with a minimum at $k = 3$ for h even and a maximum at $k = 3$ for h odd. Thus the amplitudes of the Fourier series representing $P(x\,\frac{1}{2}\,z)$, $\sum_k F_{hkl}^2 \cos 2\pi \frac{k}{2} = \sum_k (-1)^k F_{hkl}^2$, cannot reach any greater values. Heavy maxima will be lacking in this cut and the structure cannot contain twofold screw axes. The possibility of describing the structure by means of $C_{2h}^4 - P\,2/a$ having the higher symmetry of the two remaining space-groups will thus be examined.

Molybdenum positions

The appearance of the Patterson projection on the ac plane, $p(xz)$, is given in Fig. 4. As in Mo_9O_{26} the shortness of the b axis forbids more than one Mo atom having the same x and z coordinates. The heavy maxima of the projec-

* According to a private communication.

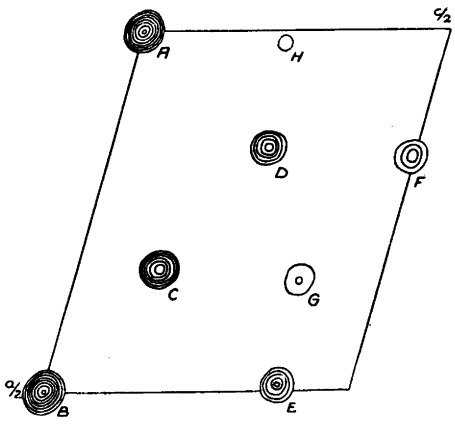


Fig. 4. Mo_8O_{23}. Projection of the Patterson function on the ac plane, p(xz). Only maxima whose centres of gravity belong to the sketched area are indicated.

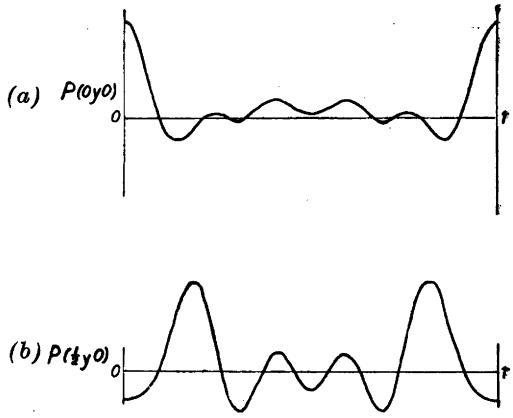


Fig. 5. Mo_8O_{23}. The Patterson function along the lines 0y0 (a) and ½y0 (b).

tion, B—F, are likely to depend chiefly on Mo—Mo vectors. It is possible to interpret them as caused by 16 Mo atoms arranged in two ways, which are different with regard to the distribution of the metal atoms around the digonal axes *.

Alternative I.

4 Mo_1 in 4(g):	$\frac{x_B + x_C}{2}$	y_1	$\frac{z_B + z_C}{2}$
4 Mo_2 in 4(g):	$\frac{x_C + x_D}{2}$	y_2	$\frac{z_C + z_D}{2}$
4 Mo_3 in 4(g):	$\frac{x_D + (x_E - \frac{1}{2})}{2}$	y_3	$\frac{z_D + z_E}{2}$
4 Mo_4 in 4(g):	$\frac{x_E + x_F}{2}$	y_4	$\frac{z_E + z_F}{2}$

Alternative II. Four fourfold Mo positions (g) with x parameters differing from those of Alternative I by ¼, while the z parameters are unchanged.

A comparison between the Patterson function required by the proposed alternative structures and the calculated projection p(xz) shows the conformity

* Notations analogous to those of space-group C_{2h}^4 — $P2/c$ in *International tables for the determination of crystal structures.* Berlin (1935).

to be as good as for Mo_9O_{26}. The weak maxima G and H correspond to 6 and 4 Mo—Mo distances whereas another maximum corresponding to 2 Mo—Mo vectors is not recognized with certainty. The agreement between calculated and observed F^2_{h0l} is also satisfactory for moderately high values of h and l. After two successive computations of the projection of the ϱ function on the ac plane, $\varrho(xz)$, the following corrected x and z parameters of the Mo positions are arrived at:

	Alternative I.		*Alternative II.*	
	x	z	x	z
Mo_1	0.415	0.063	0.165	0.063
Mo_2	0.246	0.188	0.496	0.188
Mo_3	0.077	0.316	0.327	0.316
Mo_4	0.404	0.445	0.154	0.445

The error in the parameter values may be ± 0.002 corresponding to about ± 0.03 Å.

The appearance of the linear Patterson cut $P(\frac{1}{2}\ y\ 0)$ is given in Fig. 5 b. There are high maxima at $y = 0.18$ and $y = 0.82$, evidently due to Mo—Mo interaction. The Mo atoms must therefore be arranged in planes normal to the b axis and 0.18 b apart, symmetrically to the glide planes, *i. e.* in $y = \pm 0.09$ and $y = (\frac{1}{2} \pm 0.09)$. However, an examination of a few reflections $0\ k\ l$ shows that only one of these double planes can be occupied by Mo atoms. In order to get conformity with Mo_9O_{26} the y parameters 0.41 and 0.59 are chosen. By arranging these coordinate values on the Mo positions of Alternatives I and II in all possible ways and comparing the corresponding calculated F^2_{hkl} values with the observed ones for a number of reflections with h odd it is found that only the following arrangement according to Alternative I, will give satisfactory agreement:

$$y(Mo_1) = 0.59,\quad y(Mo_2) = 0.41,\quad y(Mo_3) = 0.59,\quad y(Mo_4) = 0.41$$

The error in these parameters may hardly exceed ± 0.01 corresponding to ± 0.04 Å.

Oxygen positions

In the Patterson cuts $P(0\ y\ 0)$ and $P(\frac{1}{2}\ y\ 0)$, Fig. 5 a and b, minor maxima occur at $y \approx 0.4$ and $y \approx 0.6$, evidently analogous to the Mo — O maxima of the corresponding cuts of Mo_9O_{26}. The cuts in the electron density function

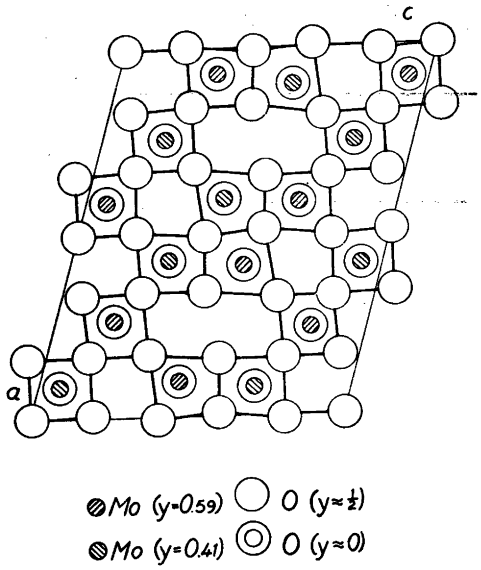


Fig. 6. Mo_8O_{23}. Projection of the structure parallel to [0 1 0].

$\varrho(x\ \frac{1}{2}\ z)$ and $\varrho(x\ 0\ z)$ actually show maxima which could be interpreted as due to the O atoms. The y(O) parameters could to some extent be adjusted from functional and space reasons as was done for Mo_9O_{26}.

Final structure of Mo_8O_{23}

The structure of the oxide may thus be described in the following way:

Cell content: 2 Mo_8O_{23}.

Space group: $C_{2h}^4 - P\ 2/a$.

4 Mo_1	in 4(g):	0.415	0.59	0.063
4 Mo_2	in 4(g):	0.246	0.41	0.188
4 Mo_3	in 4(g):	0.077	0.59	0.316
4 Mo_4	in 4(g):	0.404	0.41	0.445
4 O_1	in 4(g):	0.415	0.06	0.065
4 O_2	in 4(g):	0.245	0.94	0.19
4 O_3	in 4(g):	0.075	0.06	0.315
4 O_4	in 4(g):	0.405	0.94	0.445
2 O_5	in 2(c):	0	½	0
4 O_6	in 4(g):	0.165	0.50	0.065
4 O_7	in 4(g):	0.335	0.50	0.135
4 O_8	in 4(g):	0.495	0.50	0.195
4 O_9	in 4(g):	0.17	0.50	0.27
4 O_{10}	in 4(g):	0.325	(<) 0.50	0.325
4 O_{11}	in 4(g):	0.005	~0.50	0.405
4 O_{12}	in 4(g):	0.16	~0.50	0.46

A projection of the structure parallel to [0 1 0] is reproduced in Fig. 6. Part of the structure factor values computed for the structure are given in Table 3 together with the observed F values. Considering the great number of parameters the agreement may be looked upon as satisfactory.

Table 3. Mo_8O_{23}. *Weissenberg photographs with Mo-K radiation. Comparison between observed and calculated* $|F|$ *values.*

h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.
4 0 0	110	120	11 3 0	40	50	0 1 9 — 0 1 12	—	< 30
6 0 0	390	430	12 3 0	—	10			
8 0 0	—	0	13 3 0	80	100	0 2 4 — 0 2 7	—	< 20
10 0 0	110	90	14 3 0	—	0			
12 0 0	540	450	15 3 0	190	160	0 2 8	250	200
14 0 0	—	20	16 3 0	—	10	0 2 9 — 0 2 12	—	< 30
16 0 0	—	50	4 4 0	—	30			
4 1 0	60	60	5 4 0	—	0	0 3 4 — 0 3 7	—	< 20
5 1 0	—	0	6 4 0	270	230			
6 1 0	140	180	7 4 0	90	70	0 3 8	40	50
7 1 0	40	60	8 4 0	—	0	0 3 9 — 0 3 12	—	< 30
8 1 0	—	0	9 4 0	190	140			
9 1 0	90	110	10 4 0	40	40	0 4 4 — 0 4 7	—	< 30
10 1 0	—	50	11 4 0	—	40			
11 1 0	—	30	12 4 0	70	110	0 4 8	160	160
12 1 0	300	310	13 4 0	60	80	0 4 9 — 0 4 12	—	< 30
13 1 0	—	60	14 4 0	—	10			
14 1 0	—	10	15 4 0	160	140	0 5 4 — 0 5 7	—	< 30
15 1 0	70	100	16 4 0	—	20			
16 1 0	—	40	4 5 0	—	40	0 5 8	230	240
4 2 0	60	40	5 5 0	—	0	0 5 9 — 0 5 12	—	< 40
5 2 0	—	0	6 5 0	340	270			
6 2 0	120	140	7 5 0	—	20	9 2 $\bar{10}$	100	110
7 2 0	110	110	8 5 0	—	0	9 2 $\bar{9}$	80	80
8 2 0	—	0	9 5 0	60	30	9 2 $\bar{8}$	200	200
9 2 0	160	200	10 5 0	60	50	9 2 $\bar{7}$	110	160
10 2 0	—	30	11 5 0	—	10	9 2 $\bar{6}$	—	50
11 2 0	40	60	12 5 0	200	160	9 2 $\bar{5}$	—	40
12 2 0	210	220	13 5 0	—	20	9 2 $\bar{4}$	130	100
13 2 0	110	120	14 5 0	—	10	9 2 $\bar{3}$	110	140
14 2 0	—	10	15 5 0	—	30	9 2 $\bar{2}$	130	150
15 2 0	160	190	16 5 0	—	30	9 2 $\bar{1}$	110	130
16 2 0	—	20	0 0 4 — 0 0 7	—	< 40	9 2 1	140	180
4 3 0	—	10				9 2 2	70	80
5 3 0	—	0	0 0 8	480	490	9 2 3	—	10
6 3 0	160	110	0 0 9 — 0 0 12	—	< 30	9 2 4	—	30
7 3 0	110	90				9 2 5	130	110
8 3 0	—	0	0 1 4 — 0 1 7	—	< 20	9 2 6	120	140
9 3 0	180	170				9 2 7	120	120
10 3 0	—	10	0 1 8	270	310	9 2 8	140	160

Table 3 (contd.).

h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.	h k l	|F| obs.	|F| calc.
9 2 9	140	150	10 0 $\bar{4}$	60	60	10 0 4	60	90
9 2 10	60	80	10 0 $\bar{3}$	270	260	10 0 5	180	190
10 0 $\bar{10}$	300	420	10 0 $\bar{2}$	520	370	10 0 6	310	240
10 0 $\bar{9}$	180	120	10 0 $\bar{1}$	180	130	10 0 7	80	90
10 0 $\bar{8}$	80	60	10 0 1	80	60	10 0 8	50	70
10 0 $\bar{7}$	—	40	10 0 2	—	50	10 0 9	—	50
10 0 $\bar{6}$	—	40	10 0 3	40	50	10 0 10	—	40
10 0 $\bar{5}$	—	40						

DISCUSSION OF THE STRUCTURES

Mo_9O_{26} and Mo_8O_{23} are both built up of MoO_6 octahedra. The structures may formally be described as constructed of two-dimensional »boards», formed by MoO_6 octahedra sharing corners. (*Cf.* also the idealized structures in Fig. 7, which are further discussed later.) The boards are of infinite length parallel to the *b* axis but have an extension normal to the *b* axis of 9 octahedra for Mo_9O_{26} and 8 octahedra for Mo_8O_{23}. The two extreme octahedra of both ends of a board share edges with the corresponding octahedra of other boards. As to the rest, parallel boards are joined by octahedra sharing corners. In this way every unit cell at two localities will contain a characteristic group of four MoO_6 octahedra connected by sharing edges. (*Cf.* Fig. 7.) In the vana-

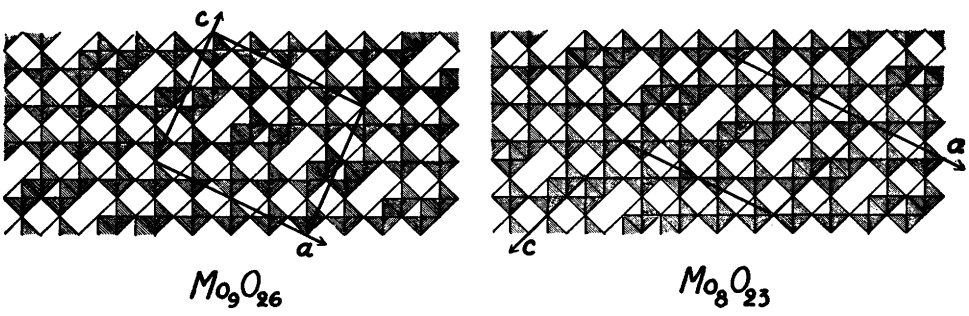


Fig. 7. Comparison between the structures of Mo_9O_{26} and Mo_8O_{23}. Idealized figures obtained by combining regular octahedra. (Mo_8O_{23} is here described on a right-handed coordinate system contrary to the other structure diagrams of this paper.)

dium oxide $V_{12}O_{26}$, investigated in this Institute by Aebi[3], a somewhat similar configuration is present.

The Mo layers parallel to (0 1 0) form a puckered network due to the Mo atoms being a little displaced mutually in the direction of the *b* axis (± 0.36 Å). The high value of the distances between adjacent Mo atoms in this direction being 4.03 Å for Mo_9O_{26} and 4.04 Å for Mo_8O_{23} is also worth mentioning. The regular alternation of the two *y* parameter values between adjacent Mo atoms is disturbed, as in the groups of four MoO_6 octahedra mentioned above the metal atoms of octahedra sharing corners have the same *y* coordinates. This arrangement is reminiscent of the distribution of the Mo atoms of the infinite zigzag shaped rows parallel to the *c* axis of MoO_3, which are also formed by MoO_6 octahedra sharing edges. In this case the mutual displacement of the Mo atoms in the direction of the *a* axis amounts to ± 0.35 Å (4) or ± 0.34 Å (5). The distance between adjacent metal atoms of different rows composing the double layers parallel to (0 1 0) is reported to be 3.954 Å (4), 3.92 Å (5), and 3.958 Å (1).

Within the network parallel to (0 1 0) the distances between Mo atoms of octahedra joined by corners for Mo_9O_{26} vary between 3.66 and 3.74 Å with an average value of 3.70 Å. For Mo_8O_{23} the corresponding limits are 3.74 and 3.82 Å and the average value 3.77 Å. The Mo atoms of octahedra joined by edges are 3.23 and 3.28 Å apart in Mo_9O_{26} and 3.23 and 3.25 Å in Mo_8O_{23}. This may be compared with the distances between Mo atoms of MoO_6 octahedra sharing edges in MoO_3, 3.42 Å (4) or 3.37 Å (5), and in MoO_2, 2.48 and 3.06 Å (6).

As the parameters of the O atoms are rather approximate the Mo—O and O—O distances of the structures are not very exact. In Mo_9O_{26} the Mo—O distances vary between 1.8 and 2.1 Å with an average value of 1.91 Å and the O—O distances between 2.5 and 3.0 Å. For Mo_8O_{23} the limits of the Mo—O distances are 1.8 and 2.2 Å with an average value of 1.94 Å while the O—O distances fall within 2.5—2.9 Å. In both oxides the lengths of O—O edges common to two MoO_6 octahedra are $\sim$ 2.5 Å.

The idealized structures, obtained by joining boards of regular octahedra in a manner corresponding to the way in which the two oxides are built up, are illustrated in Fig. 7, showing projections parallel to the direction of the infinite extension of the boards. The axial ratios of the actual structures based on 3.75 Å as unit length — which may be a probable value of the length of the space diagonal of a regular MoO_6 octahedron — are in harmony with the values calculated for the idealized lattices, apart from the *b* axes being extended. The agreement between idealized and actual values of the monoclinic angles is also good.

		$a:b:c$	β
Mo_9O_{26}	Idealized lattice	4.48 : 1 : 3.82	93°4
	Actual lattice	4.47 : 1.07 : 3.85	96°
Mo_8O_{23}	Idealized lattice	4.48 : 1 : 3.53	108°4
	Actual lattice	4.48 : 1.08 : 3.57	106°5

SUMMARY

Two molybdenum oxides of compositions close to $MoO_{2.90}$ (β' and β oxide) have been investigated by means of X-rays. Their formulae have been shown to be Mo_9O_{26} and Mo_8O_{23}. Complete determinations of the structure have been carried out, showing pronounced similarity between the two structures. Both oxides are built up of MoO_6 octahedra coupled together by sharing corners or edges. Formally, two-dimensional layers of MoO_6 octahedra joined by corners may be looked upon as larger elements of the lattices. These »boards» are of infinite length in one direction but of finite, characteristic extension in the other, *viz.* 9 octahedra for Mo_9O_{26} and 8 for Mo_8O_{23}.

The author wishes to thank Professor G. Hägg for his continued interest in this investigation and Miss Anna-Britta Sondell, Mrs Margareta Palme-Boman, Mr Bengt Lindqvist and Mr Bengt Tollander for their valuable assistance with the calculations. He is also indebted to the *Kungl. Svenska Vetenskapsakademien* for a grant from the Edlund fund. The *Statens Naturvetenskapliga Forskningsråd* has supported the research financially, which support is gratefully acknowledged.

REFERENCES

1. Hägg, G., and Magnéli, A. *Arkiv Kemi, Mineral. Geol.* **A 19** (1944) no. 2.
2. Hägg, G., and Laurent, T. *J. Sci. Instruments* **23** (1946) 155.
3. Aebi, F. *Helv. Chim. Acta* **31** (1948) 8.
4. Bräkken, H. *Z. Krist.* **78** (1931) 484.
5. Wooster, N. *Z. Krist.* **80** (1931) 504.
6. Magnéli, A. *Arkiv Kemi, Mineral. Geol.* **A 24** (1946) no. 2.

Received June 3, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 518—522

On the Determination of the Peptide-Amino-Nitrogen by the Copper Method

HEIKKI K. KERKKONEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Pope and Stevens [1] have developed a method for the determination of amino-nitrogen based on the formation of soluble copper compounds between the amino-acid or digested protein and the excess of copper present in the form of copper phosphate. In this laboratory we have used the same method, commonly called the copper method, since 1940, and found it to give correct figures with amino-acid-mixtures and with completely hydrolyzed proteins. In partially hydrolyzed proteins, on the other hand, the values are too high indicating that the soluble copper compounds of peptides formed in this reaction are not similar to those of the amino-acids as suggested by Pope and Stevens.

In order to investigate this question some determinations were carried out with synthetic peptides and with hydrolysates of zein and zein plastein. In the following, the results of these experiments are briefly given.

EXPERIMENTAL

Solutions of each synthetic peptide (Amino Acid Manufactures, University of California) were prepared containing about 10—20 mg total nitrogen per 25 ml. In each of these solutions the amino-nitrogen was determined by the copper method and by Van Slyke's volumetric nitrous acid method (5 min shaking). The total nitrogen was estimated by the micro method of Kjeldahl. Table 1 shows the results.

Table 1. Amino-nitrogen content of different synthetic peptides.

Peptide	Total N mg	Copper method		Van Slyke	
		N mg	N %	N mg	N %
Glycyl-*l*-leucine	12.19	11.85	97.2	6.15	50.5
	10.12	9.92	98.1	5.07	50.8
Glycyl-glycine	12.61	12.38	98.1	6.24	49.5
	16.44	16.14	98.2	8.17	49.7
l-Leucyl-*l*-tyrosine	12.29	12.10	98.3	6.10	49.6
	12.30	12.12	98.5	6.08	49.4
l-Leucyl-glycyl-glycine	17.75	11.82	66.6	6.00	33.8
	19.72	13.15	66.7	6.69	33.9

The mixture of the synthetic peptides mentioned in Table 1 dissolved in 25 ml water gave the values shown in Table 2.

Table 2. Amino-nitrogen content of the peptide mixture.

	Amino-N mg
Copper method	11.46
Van Slyke, 5 min. shaking	5.74
» » 30 » »	7.07
Free α-amino-N, calculated	5.75

The relation between the results obtained by the copper method and by Van Slyke's method in an enzymic digest appears from the following experiment. A sample of 200.6 mg purified zein (Corn Products Refining Company, U. S. A.) was suspended in 10 ml of 0.175 *N* hydrochloric acid in a Pyrex test tube, 9.8 mg crystalline pepsin (The Armour Laboratories, U. S. A.) and 0.5 ml toluene were added, the tube was sealed by melting up, and the ampul thus prepared was incubated at 37° C for seven days. Thereafter the hydrolysate was filtered off, and the ammonia nitrogen of the clear solution was determined according to Pucher *et al.*[2] The remaining solution was transferred with distilled water into a 25 ml volumetric flask, adjusted slightly acid and made up to the volume. Aliquots of this solution were then analyzed for total nitrogen (total soluble nitrogen was calculated by adding the value obtained in the estimation of ammonia nitrogen to that of total nitrogen),

Table 3. Amino-nitrogen content of the peptic digest of zein.

	Nitrogen	
	mg	% of total peptide-N
Total soluble N	24.82	—
Ammonia N	3.74	—
Free amino-acid N	0.52	—
Total peptide N	20.56	—
Van Slyke amino N	5.25	—
Peptide amino N	4.73	23.0
Copper amino N	9.86	—
Peptide amino N	9.34 (4.67) *	45.5

for amino-nitrogen according to Van Slyke and by the copper method, and for »ninhydrin nitrogen» (free amino-acid-N) according to Van Slyke *et al.*[3] The total peptide nitrogen can approximately be calculated by subtracting the »ninhydrin nitrogen» and the ammonia nitrogen from the total soluble nitrogen, and the peptide-amino-nitrogen by subtracting the »ninhydrin nitrogen» from the total amino-nitrogen. The results obtained are listed in Table 3.

Another experiment with an acid digest of zein plastein [4] gave the results shown in Table 4. 2 g of zein plastein were hydrolyzed with 0.175 *N* HCl

Table 4. Amino-nitrogen content of the acid digest of plastein.

	Nitrogen	
	mg	% of total peptide-N
Total soluble N	18.72	—
Ammonia N	0.297	—
Free amino-acid N	0.95	—
Total peptide N	17.47	—
Van Slyke amino N	4.82	—
Peptide amino N	3.87	22.1
Copper amino N	8.66	—
Peptide amino N	7.71 (3.86) *	44.2

* The number in parentheses — half of the value obtained by the Cu-method — represents the real NH_2-N in peptides.

for 43 days at 37° C, the clear hydrolysate was filtered off, and the same determinations were carried out as in the previous experiment.

In later experiments with pentaglycine ethyl ester the amino-nitrogen value obtained by the copper method was 3.82 mg (calculated 1.96 mg). When using the factor 0.14 instead of 0.28 the value 1.91 mg (97.5 %) should be obtained. Another determination with glycyl-tyrosine methyl ester gave by the copper method the value 4.16 mg (calculated 1.935 mg), by using the factor 0.14 2.08 mg (107.5 %).

DISCUSSION

The experiments described above show that the amino-nitrogen values obtained by the copper method (calculated in the same way as in the case of amino-acids) are twice as high as those obtained by Van Slyke's method. Woiwod [5], using a slightly modified copper reagent, has recently shown that the ratio α-amino-N/copper for a number of amino-acids and related compounds (hydroxyproline, aspartic acid, serine, threonine, valine, leucine, *iso*-leucine, glutamic acid, methionine, asparagine, glycine, tyrosine, arginine, phenylalanine, cystine, tryptophan, glutamine, alanine, proline) varies from 0.38 to 0.48, but the same ratio for the dipeptide alanyl-glycine is 0.22. For the formation of the complex A_2Cu the theoretical ratio should be 0.44 showing that alanyl-glycine binds twice as much copper as an amino-acid theoretically does. This finding is in good agreement with the results reported in this paper.

Unfortunately there was no possibility to determine the amino-nitrogen in synthetic peptides containing proline. It is well known that proline does not react in Van Slyke's method. We could decide this way whether proline occupies the end position in a peptide chain or not.

Pope and Stevens [1] have shown that in iodometrical titration of the copper compounds of amino-acids each ml of 0.01 *N* thiosulphate is equivalent to 0.28 mg amino-nitrogen. Now, when titrating the peptide-copper-compound in the same way, each ml of 0.01 *N* thiosulphate is equivalent to 0.14 mg amino-nitrogen. When calculating the results by using this factor instead of 0.28 correct values for the peptide-amino-nitrogen can be obtained.

SUMMARY

The copper method of Pope and Stevens has been applied to the determination of the free amino-nitrogen of the peptide chains. It has been found that the peptides bind twice as much copper as the amino-acids, consequently, in calculating the results the factor 0.14 instead of 0.28 should be used.

Grateful thanks are expressed to Prof. Dr. M. S. Dunn, University of California, U. S. A. for providing the samples of the synthetic peptides, to Sir Ian Heilbron and Dr. A. H. Cook, Imperial College of Science and Technology, London, England, for the samples of pentaglycine and glycyl-tyrosine, and to Corn Products Refining Company, Argo, Ill., U. S. A., for the samples of zein.

REFERENCES

1. Pope, C. G., and Stevens, M. F. *Biochem. J.* **33** (1939) 1070.
2. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S. *Ind. Eng. Chem., Anal. Ed.* **7** (1935) 152.
3. Van Slyke, D. D., Mac Fadyen, D. A., and Hamilton, P. *J. Biol. Chem.* **141** (1941) 671.
4. Virtanen, A. I., and Kerkkonen, H. K. *Nature* **161** (1948) 888.
5. Woiwod, A. J. *Biochem. J.* **42** (1948) xxviii.

Received June 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 523—526

Some Views on the Gelation of Aqueous Solutions of Ethyl Cellulose on Heating

SVEN SÖNNERSKOG

Mo and Domsjö AB, Örnsköldsvik, Sweden

Ethyl cellulose, containing 1.2—1.4 ethoxy groups per anhydroglucose residue, dissolves in cold water (below 20° C) to a clear viscous solution. Maximum clarity is obtained by cooling the solution until freezing. The viscosity increases with decreasing temperature, but a range of irregularity is observed just when the main part of the sample has passed into solution. The following diagram shows some measurements of the viscosity of a 2 per cent solution on cooling from 25 to 5° C.

The long horisontal part of the curve arises the question whether the dissolution involves some *special* kind of interaction between solvent and solute. Below this part the slope indicates no irregularities. Only the range between the two knees diverges from what could be considered as a standard.

If we compare the rapid increase in viscosity above the right knee-point with the comparatively small gradient at lower temperatures, we establish that some kind of immobilization of solvent molecules takes place simultaneously with a more regular association. The dissolution should therefore

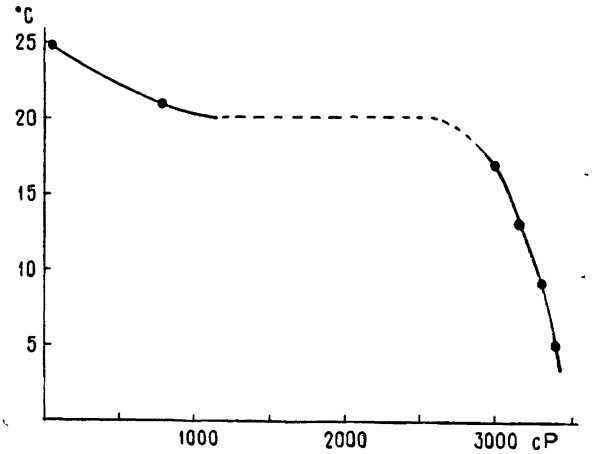
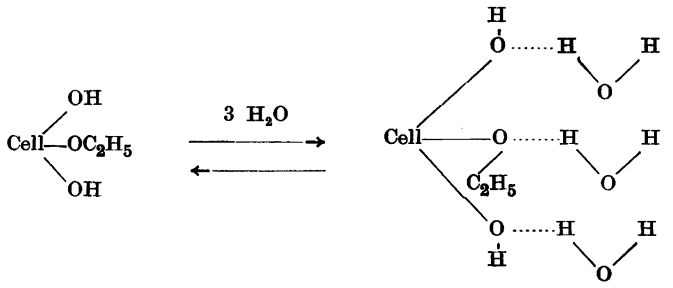


Fig. 1. The relation between temperature and viscosity for water soluble ethyl cellulose between 5 and 25° C.

be considered as a superposition of two processes, firstly a solvation of the hydroxyl groups by the water dipoles, secondly *an aggregate formation* between the layers of hydrated cellulose chains. The first phenomenon takes place at all temperatures, while the second mainly is localized to the neighbourhood of the range of irregularity.

What is stated above can be readily understood if we consider that dissolution of the cellulose ether implies an orientation by dipole forces between the water molecules and the hydroxyls or ethoxy radicals in the cellulose chains. The degree of orientation is depending on the temperature and increases with the ratio $1/T$[1]. (T = temp. in °K.) When the dipole forces are in equilibrium with the thermal movement energy of water molecules, a parallel or an antiparallel orientation might occur. The hydration may be symbolized as follows:



Such a molecule, however, would be able to contribute to an aggregate formation, for instance by further dipole interaction or simply a cross linkage formation by secondary valence forces. If two hydrated chains form an aggregate, the result is a network where the distance between adjacent chains roughly is determined by the sum of two hydrogen bridges (5.5 Å). Secondary valence lattices of noticeable stability do not show higher distances between their net planes than 4.5 Å [1]. The state represented by the formula must therefore be considered as a fairly supersaturated one which tends to contract, for instance by splitting off some immobilized water molecules. In fact, if a 3 % solution is heated in a test tube until complete gelation takes place, water is gradually deliberated until the solid gel is made free from the glass walls. This phenomenon should not be confused with the first period in the gelation process, where the volume increases [2, 3] thanks to the removal of highly compressed water molecules who have taken part in the dissolution but not in the aggregate formation.

It should be noticed that this immobilization of water molecules is analogous to the arrangement of crystal water in certain organic molecules, for instance in oxalic acid dihydrate [4]. The cohesion forces which are involved in aggregate formation there are the same as in the case of ethyl cellulose.

If we now proceed from the picture of the dissolved ethyl cellulose as being a framework of hydrated chains partly or totally immobilized by secondary valence forces between the hydroxyl or ethoxy groups, it is easy to understand that the irregularity in viscosity, shown in Fig. 1, as well as the gelation on heating, disappear, when an increasing part of the hydroxyls is displaced from the original position, for instance by introducing ethylene oxide in the ethyl cellulose. When a hydroxyl radical adds ethylene oxide catalytically, the displacement of the hydroxyl group from the original position corresponds to about 3 Å. The hydroxyl is no longer able to take part in the aggregate formation mentioned above and cross linkages with shorter bridging than 4.5 Å are very unlikely if we consider the large energy content which would be involved in a shortening of the linkages with 3 Å. The dehydration of glycol groups in cellulose can be estimated from the viscosity-temperature diagram of glycol cellulose. Fig. 2 shows some values measured on a 2 per cent solution. The curve is a hyperbola $VT = \text{const.}$, which is the same as $V = \text{const. } 1/T$, or in other words the *relation between temperature and the degree of orientation of rigid dipoles.*

If we now introduce various amounts of glycol groups into watersoluble ethyl cellulose, part of the hydroxyls become unsuitable for taking place in the aggregate formation which would imply a lower rigidity of the gel formed on heating a solution. The following diagram shows the gel hardness for a few glycol ethers of ethyl cellulose. The measurements are carried out on 2 per cent solutions at 80° C, using a test tube of stainless steel, fitted with an inlet for a metallic rod ending with a surface of 1 cm^2. The rod could be loaded with concentrical weights on the upper part.

The number of glycol groups are calculated from the total amount of ethylene and ethyl iodide formed by combustion with hydroiodic acid, supposing the ethoxy content known (DS = 1.2).

The similarity between the decrease of viscosity of glycol cellulose on heating and the weakening of the gel rigidity of ethyl cellulose by introducing glycol groups allows us to suppose that removal of purely oriented water molecules is the essential change that takes place on gelation. The glycol groups in the modified ethyl cellulose prevent part of the water molecules to join in the aggregate formation resulting in a corresponding decrease of gel hardness. Most of the water molecules fixed at the cellulose chains only by dipole cohesion are sloughed off on heating, causing a sudden increase of rigidity. In pure ethyl cellulose gels the remaining network is strong enough to keep the lattice structure, but when many glycol groups are present, the number of cross linkages is reduced and the lattice breaks down. This might be the reason why ethyl ethers of cellulose containing more than one glycol

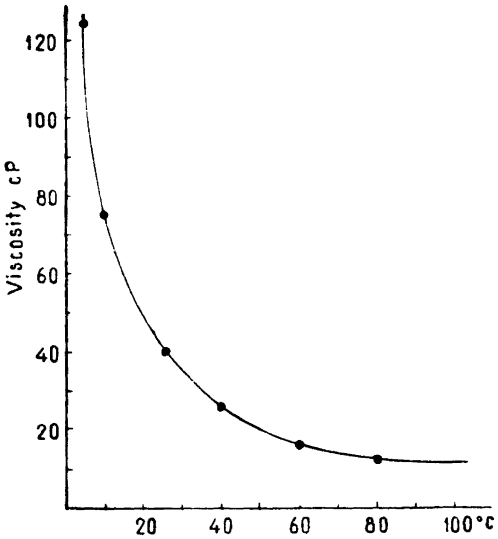


Fig. 2. Relation between temperature and viscosity for glycol cellulose between 5 and 80° C.

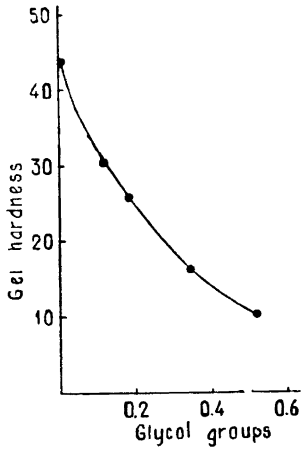


Fig. 3. Gel hardness of ethyl cellulose containing 0.11—0.52 glycol groups per anhydroglucose residue.

group hardly give any gel at all but flocculate very easy on heating to the gelation point.

SUMMARY

Ethyl ethers of cellulose, showing a degree of substitution between 1.2—1.4, dissolve in cold water to a clear, viscous solution. On cooling, a sudden increase in viscosity is observed just when the main part of the ether has passed into solution. The phenomenon is explained by an analysis of the possibilities of cross linking as well as simple orientation of the water dipoles with regard to the hydroxyls of the cellulose chains. The gelation on heating is shown to be essentially due to a removal of purely oriented water molecules.

REFERENCES

1. Mark, H. *High polymers* Vol. II (1940) pp. 91 and 129.
2. Heymann, E. *Trans. Faraday Soc.* **31** (1935) 846.
3. Heymann, E. *Trans. Faraday Soc.* **32** (1936) 462.
4. Hoffman, H., and Mark, H. *Z. Phys. Chem.* **111** (1924) 321.

Received June 6, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1947) 527—537

Short Communications

The Configuration of the α-Phloroglucitol Molecule

PER ANDERSEN and O. HASSEL

Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

The configurations of the two stereo-isomeric phloroglucitols [1,2] have not been determined and it has therefore not been known which of them corresponds to the 1 $\varkappa$, 3 $\varkappa$, 5 $\varkappa$ compound and which to the 1 $\varkappa$, 3 ε, 5 $\varkappa$ compound. Wislicenus [1] observed, however, that the phloroglucitol dihyrate crystallizes in rhombohedrons and we have carried out an X-ray analysis in order to ascertain whether the molecule itself has trigonal symmetry in these crystals or not.

The α-phloroglucitol examined was prepared from phloroglucinol using Wislicenus' original method [1]. The crystals (from aqueous solution) were indeed trigonal, and X-ray examinations based on Laue, rotation and Weissenberg photographs show that the unit cell is rhombohedral with $r = 8.71$ Å, $\alpha = 56° 10'$. The density of the crystals is 1.31 and the number of $C_6H_9(OH)_3 \cdot 2H_2O$ in the unit cell therefore 2. The Laue symmetry is D_{3d}—$\bar{3}m$. Besides the extinctions characteristic of a rhombohedral cell the only systematic extinction is the absence of *hhl* (rhomb.) where l is odd. Possible space groups are therefore C_{3v}^6—$R3c$ and D_{3d}^6—$R\bar{3}c$. Now according to observations by Mr. Erik Blomgren in Uppsala, the crystals exhibit strong piezoelectricity, and the correct space group is therefore C_{3v}^6—$R3c$. In this space group the coordinates of the centers of the phloroglucitol molecules may be chosen as 000 and $\frac{1}{2}\frac{1}{2}\frac{1}{2}$, and the molecules have trigonal symmetry. The coordinates of the four water molecules are: $u\,u\,u$; $u+\frac{1}{2} \cdot u+\frac{1}{2}$, $u+\frac{1}{2}$ and $v\,v\,v$; $v+\frac{1}{2}$, $v+\frac{1}{2}$, $v+\frac{1}{2}$. It is obvious therefore, that the α-phloroglucitol is the $\varkappa$, $\varkappa$, $\varkappa \rightleftharpoons \varepsilon$, ε, ε compound. The β-phloroglucitol must therefore be the $\varkappa$, ε, $\varkappa \rightleftharpoons \varepsilon$, $\varkappa$, ε compound.

We now assume the $\varkappa\varkappa\varkappa$ form to be present in the crystal, tetrahedral valency angles, and the C—C and C—O distances to be 1.54 and 1.42 Å respectively. Remembering that all the water molecules are situated on the trigonal axes, *one* parameter only has to be determined as far as the projection of the structure into the (111) plane is concerned. We choose as a parameter the angle φ between the line joining neighbouring molecular centers situated in the same (111) plane and the projection into that plane of the carbon-oxygen bond. Using the trial and error method it was found that this angle can only differ very little from zero.

This is far from surprising when we consider the positions of the water molecules. Throughout the structure two sets of equilateral triangles of alcoholic oxygen atoms will occur. The oxygen atoms of each triangle belong to three different phloroglucitol molecules, but they are all lying in the same (111) plane. If the angle φ is equal to zero the dimensions of the two sets of triangles are identical with a O—O distance of 4.67 Å.

On the [111] axis joining the centers of two neighbouring O_3-triangles *two* water molecules will have to be placed. The

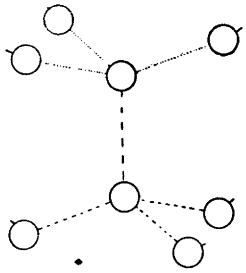


Fig. 1. The O_3—O—O—O_3 arrangement.

length of this axis is $\frac{21.96}{6}$ Å = 3.66 Å. The resulting 0—0 distances will then be shorter than should be expected in the absence of hydrogen bonds. The angle φ being zero or very nearly so, four hydrogen bonds from each water oxygen will be formed, one to the oxygen atom of the other water molecule and one to each of the three nearest alcoholic oxygens. The fact that the two O_3 triangles are identical or nearly identical in size suggests that the vertical distances between their planes and the nearest water oxygens will be nearly identical. The best agreement between calculated and estimated intensities was indeed reached if the O_3—O—O—O_3 group was assumed to be symmetrical and the distance between the two water oxygens equal to 2.76 Å. The distance from each of these oxygen atoms to the three alcoholic oxygen neighbours will then be 2.74 Å. These distances are in good agreement with hydrogen bond distances found in similar cases. It should be pointed out, however, that the oxygen tetrahedrons surrounding each of the water oxygens are not regular tetrahedrons. The shape of the O_3—O—O—O_3 groups linking the organic molecules together is illustrated in Fig. 1. In Fig. 2 a projection into the (111) plane of part of the structure is reproduced. As an illustration of the agreement between estimated intensities (chiefly from Weissenberg photographs) and calculated intensities Fig. 3 has been drawn. The figure contains calculated (joined by fully drawn lines) and estimated intensities (dotted lines) of 36 of the more important reflexions.

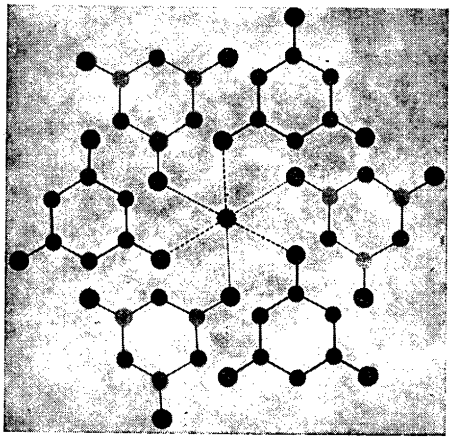


Fig. 2. Part of the structure projected along [111].

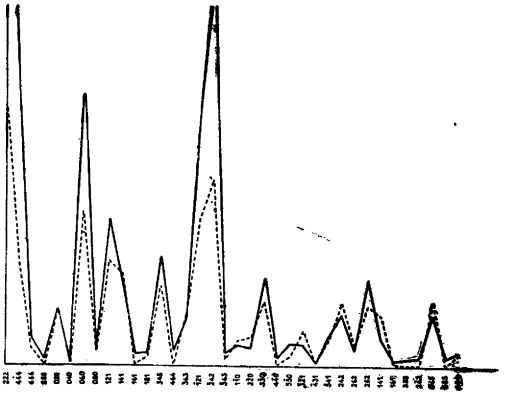


Fig. 3. Calculated (—) and estimated (- - -) intensities.

We are much indebted to cand. real. O. Bastiansen for valuable help.

1. Wislicenus, W. *Ber.* **27** (1894) 357.
2. Lindemann, H., and Baumann, H. *Ann.* **477** (1929) 78.

Received October 11, 1948.

In Vitro Inhibition of Alkaline Phosphatase by Estrogenic Hormones

BERTIL ALDMAN, EGON DICZFALUSY AND THOMAS ROSENBERG.

The Department of Women's Diseases, Karolinska Sjukhuset, Stockholm, Sweden, and the Physico-Chemical Institute, University of Copenhagen, Denmark

With regard to the mode of action of estrogenic substances it seemed to be of great interest to study the interaction between estrogenic hormones and alkaline phosphatase. Data available in the literature on diminished phosphatase activity in different organs [1] after the administration of sex hormones do not clear up the question whether this effect is due to a decreased concentration of alkaline phosphatase, or it is the result of a direct inhibition of the enzyme by the hormones. Therefore we have investigated the inhibition of crystalline kidney alkaline phosphatase and liver alkaline phosphatase by estrogenic hormones and some of their derivatives. In these experiments estradiol and estrone showed very slight or no inhibitory effect, while some of their phosphorylated derivatives were very strong inhibitors. These phosphorylated estrogens were not hydrolyzed by the phosphatase.

Experimental. Crystalline alkaline phosphatase from kidneys of juvenile rabbits was prepared by the method of van Thoai *et al* [2]. The final product with a nitrogen content of 13.5 per cent contained a small amount of amorphous material *. In 1 ml hydrolysis mixture, containing 1.2 μg dry enzyme, 0.0079 mmol disodium phenyl phosphate and 0.0008 mmol magnesium sulfate, 26 μg phenol was liberated within 24 minutes at pH 9.67 and 37° C. The enzyme was dissolved in distilled water and kept in a refrigerator. Its activity remained unchanged over a period of 60 days. The liver phosphatase was prepared from livers of juvenile rabbits. No attempts have been made to obtain the enzyme in crystalline form.

Estrone, estradiol and progesterone were dissolved in a solution of cetylpyridi-

* We are indebted to Ing. B. Högberg, AB. Leo, Hälsingborg, Sweden, for the nitrogen determinations.

Table 1. Inhibition of alkaline kidney phosphatase.

Inhibitor	Concentration	Per cent inhibition after min 4	8	16	24
Estrone	$5.7 \cdot 10^{-5}$ *M*	0	0	0	0
Estradiol	$5.7 \cdot 10^{-5}$ *M*	15	0	6	0
Estrone-3-phosphate	$5.0 \cdot 10^{-4}$ *M*	64	77	78	78
—»—	$8.0 \cdot 10^{-5}$ *M*	38	38	41	39
Estradiol-17-phosphate	$8.0 \cdot 10^{-5}$ *M*	8	12	6	3
Estradiol-3,17-diphosphate	$8.0 \cdot 10^{-5}$ *M*	47	47	52	57
Progesterone	$8.0 \cdot 10^{-3}$ *M*	30	30	35	
—»—	$8.0 \cdot 10^{-5}$ *M*	0	6	7	7

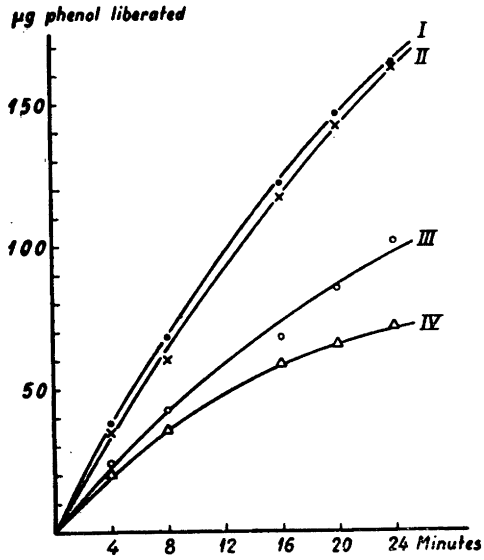


Fig. 1. Inhibition of alkaline kidney phosphatase by different phosphates of the estrogenic hormones.

I: without inhibitor
II: $8 \cdot 10^{-5}$ M estradiol-17-phosphate
III: $8 \cdot 10^{-5}$ M estrone-3-phosphate
IV: $8 \cdot 10^{-5}$ M estradiol-3,17-diphosphate

niumchloride, so that the final reaction mixture contained 80 µg cetylpyridiniumchloride. At this concentration cetylpyridiniumchloride has no inhibitory action on the phosphatase activity.

Estrone-3-phosphate, estradiol-17-phosphate and estradiol-3,17-diphosphate were kindly supplied by Ing. B. Högberg, AB. Leo, Hälsingborg.

For the determination of the phosphatase activity the method of King and Armstrong [3] has been used, in the modification described by Buch and Buch [4]. The amount of phenol liberated was determined after 4, 8, 16 and 24 minutes.

Results. The results of the experiments with the kidney phosphatase are presented in Fig. 1. and Table 1.

Furthermore the inhibitory effect of $1.6 \cdot 10^{-4}M$ estradiol-3,17-diphosphate on alkaline liver phosphatase was investigated. After 4, 8, 16 and 24 minutes the inhibition was 46, 64, 68 and 69 per cent.

From the results presented above it is seen that among the compounds investigated, those phosphorylated in the 3-position have the strongest inhibitory effect on the phosphatase activity. Nothing is known at present about the occurrence of estrogenic hormones as phosphates in living organisms. A discussion of the significance of the present findings together with further experiments will be published later.

We wish to express our sincere thanks to AB. Leo, Hälsingborg, whose financial support has made possible this investigation.

1. Kochakian, Ch. D. *Recent progress in hormone research* Vol. I (1947) 177.
2. van Thoai, N., Roche, J., and Sartori, L. *Comp. rend. soc. biol.* **138** (1944) 47.
3. King, E. J., and Armstrong, A. R. *Can. Med. Assoc. J.* **31** (1934) 376.
4. Buch, I., and Buch, H. *Acta Med. Scand.* **101** (1939) 211.

Received September 13, 1948.

On the Structure of Salt Monohydrates

INGVAR LINDQVIST

The Chemical Institute, University of Uppsala, Uppsala, Sweden

The crystal chemistry of salt hydrates is a vast field in Inorganic Chemistry, which has not yet been definitely clarified. Recently, A. Tovborg Jensen [1] has written an excellent, critical review on these substances. He has also given some rules for the neighbourhood of the water molecules in crystals of this type.

Tovborg Jensen states that a water molecule has always negative neighbours

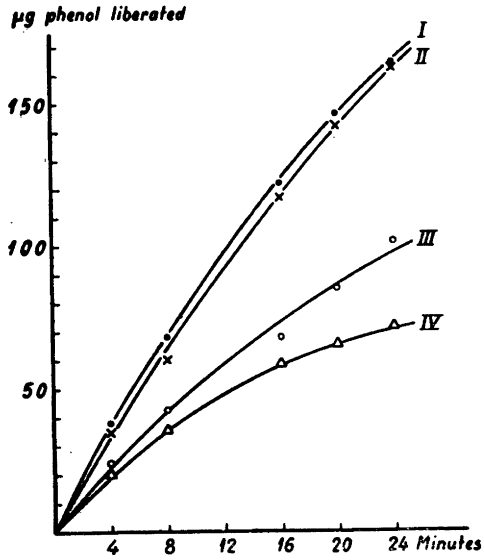


Fig. 1. Inhibition of alkaline kidney phosphatase by different phosphates of the estrogenic hormones.

I: without inhibitor
II: $8 \cdot 10^{-5}$ M estradiol-17-phosphate
III: $8 \cdot 10^{-5}$ M estrone-3-phosphate
IV: $8 \cdot 10^{-5}$ M estradiol-3,17-diphosphate

niumchloride, so that the final reaction mixture contained 80 µg cetylpyridiniumchloride. At this concentration cetylpyridiniumchloride has no inhibitory action on the phosphatase activity.

Estrone-3-phosphate, estradiol-17-phosphate and estradiol-3,17-diphosphate were kindly supplied by Ing. B. Högberg, AB. Leo, Hälsingborg.

For the determination of the phosphatase activity the method of King and Armstrong [3] has been used, in the modification described by Buch and Buch [4]. The amount of phenol liberated was determined after 4, 8, 16 and 24 minutes.

Results. The results of the experiments with the kidney phosphatase are presented in Fig. 1. and Table 1.

Furthermore the inhibitory effect of $1.6 \cdot 10^{-4}M$ estradiol-3,17-diphosphate on alkaline liver phosphatase was investigated. After 4, 8, 16 and 24 minutes the inhibition was 46, 64, 68 and 69 per cent.

From the results presented above it is seen that among the compounds investigated, those phosphorylated in the 3-position have the strongest inhibitory effect on the phosphatase activity. Nothing is known at present about the occurrence of estrogenic hormones as phosphates in living organisms. A discussion of the significance of the present findings together with further experiments will be published later.

We wish to express our sincere thanks to AB. Leo, Hälsingborg, whose financial support has made possible this investigation.

1. Kochakian, Ch. D. *Recent progress in hormone research* Vol. I (1947) 177.
2. van Thoai, N., Roche, J., and Sartori, L. *Comp. rend. soc. biol.* **138** (1944) 47.
3. King, E. J., and Armstrong, A. R. *Can. Med. Assoc. J.* **31** (1934) 376.
4. Buch, I., and Buch, H. *Acta Med. Scand.* **101** (1939) 211.

Received September 13, 1948.

On the Structure of Salt Monohydrates

INGVAR LINDQVIST

The Chemical Institute, University of Uppsala, Uppsala, Sweden

The crystal chemistry of salt hydrates is a vast field in Inorganic Chemistry, which has not yet been definitely clarified. Recently, A. Tovborg Jensen [1] has written an excellent, critical review on these substances. He has also given some rules for the neighbourhood of the water molecules in crystals of this type.

Tovborg Jensen states that a water molecule has always negative neighbours

on one side, positive neighbours on the opposite side, and furthermore that a water molecule touchs one or two positive neighbours on one side and almost always two negative neighbours on the opposite side. As a conclusion of these rules he makes the statement that structural suggestions, where the surroundings of the water molecule do not show its positive and negative side, may be discarded as false. Structural suggestions, where the water molecule has more than two positive neighbours may be considered as very improbable. Suggestions which imply more than two negative neighbours must be very firmly founded in order to be accepted. One may expect, that hydrates with such a structure have rare properties, in which they depart from most salt hydrates.

A year ago the author published a crystal structure [2] determination of $(NH_4)_2FeCl_5H_2O$ *, which has not been considered by Tovborg Jensen. The lattice was found to be built up by NH_4^+ ions and distorted octahedral ions $[FeCl_5H_2O]^{2-}$. The complete structure does not indicate one positive and one negative side of the water molecule, which furthermore has more than two positive and two negative neighbours. The crystals give sharp X-ray reflections, a fact which has also been confirmed by Tovborg Jensen. This situation made it seem advisable to examine the structure once more. In view of this examination it does not appear that there is anything to be changed in the structure except possibly small variations of the parameter values, especially those of H_2O and NH_4^+. These cannot, however, remove the contradictions to the rules quoted. The formula of the salt also makes the obtained structure most probable.

* In this paper the cell dimensions were by mistake given in the first approximation from the rotation photographs The more accurate values obtained from powder photographs are $a = 13.68$ Å; $b = 9.88$ Å; $c = 7.02$ Å.

The conclusions drawn by Tovborg Jensen on the other hand are very well founded on the crystal structures of the salt hydrates considered. An examination of the list of these structures shows that there is not more than one salt of a trivalent positive ion among them, namely the hexahydrate $AlCl_3.6\ H_2O$. It seems probable that the contradictions mentioned above are due to too far reaching generalisations made by Tovborg Jensen. In a monohydrate of a salt with a trivalent positive ion, it seems as if the possibilities for the water molecule of getting its ideal surroundings are small compared with the tendency of Fe^{3+} to form the monohydrated octahedral ion. And every ion $[Me\ X_5\ H_2O]^{2-}$ in a crystal structure does of course disagree with the conditions stated by Tovborg Jensen. Although Tovborg Jensen's rules seem to be of a great importance in the salt hydrates which were studied by him, his conclusions seem to have been drawn too far. In any case, it remains for the theory to explain the structure of $(NH_4)_2FeCl_5 \cdot H_2O$, which now establishs the existence of monohydrated octahedral ions.

In order to obtain further confirmation of the proposed structure I intend to carry out a complete structure determination on $K_2FeCl_5\ H_2O$, which is isomorphous with $(NH_4)_2FeCl_5\ H_2O$. This may also lead to a more accurate determination of the parameter values.

According to a recent private communication by Mr. M. Atoji at the Osaka Imperial University $Cs_2(TlCl_5H_2O)$ seems to be isomorphous with $(NH_4)_2FeCl_5H_2O$ and then must give good possibilities to determine the parameter values of the monovalent positive ions.

Quite recently a structure determination of $(NH_4)_2InCl_5H_2O$, which is isomorphous with $(NH_4)_2FeCl_5H_2O$, is reported [3]. The parameter values obtained by Fourier methods agree very well with those obtained for $(NH_4)_2FeCl_5H_2O$.

Some further Remarks on the Structure of Salt Monohydrates

AKSEL TOVBORG JENSEN

Chemical Laboratory, Royal Agricultural and Veterinary College, Copenhagen, Denmark

The empirical rules governing water molecules in salt hydrates which I attempted to find were derived from the study of the 25 presumably correct structures then published. These 25 structures I took to be representative for all salt hydrate types. As pointed out by Lindqvist the list contains not many salts of trivalent cations. Apart from $AlCl_3,6H_2O$ mentioned by Lindqvist and the isomorphous $CrCl_3$, $6H_2O$ the list contains only the alum structure in which however a large number of different trivalent cations may be accomodated. An obvious flaw of the list is that it contains no salts of mononuclear acido-aquo-anions, which ought not to have been overlooked. It might — even without knowledge of Lindqvist's structure determination for $(NH_4)_2FeCl_5,H_2O$ — have been inferred that acido-aquo-anions only with difficulty could be packed in a crystal in such a fashion that a water molecule will have »negative neighbours» on the opposite side of that touching the cntral atom of the complex anion. In the case of Lindqvist's salt it is almost a physical impossibility.

Even if the rule that a water molecule in a crystal shall have positive neighbours on one side, negative neighbours * on the opposite side is not without exceptions, the number of exceptions will tend to be small for the following reason: Imagine the full mathematical expression for the lattice energy of a salt in which this rule is not obeyed. The terms in this expression which pertain to the attraction between a water molecule and its immediate neighbours will be small compared with the corresponding terms in the lattice energy expression for a salt hydrate in which a water molecule has positive and negative neighbours on opposite sides. Consequently the total lattice energy can reach a reasonable size only if the lattice energy expression contains *other* terms which are *extraordinarily* large, and only in that *exceptional* case can the salt be stable.

Because the lattice energy expression of such salts contain terms which as pointed out are extraordinarily small one should expect such salts to be very soluble rather than insoluble in water.

Now the question arises: in which compounds can the de-stabilizing effect of these small lattice energy terms be counterbalanced to such an extent that a stable crystal (even if a very soluble one) results? One may perhaps conjecture: in some crystals in which shared electron forces are found, crystal which contain complex ions known as definite chemical entities from solutions.

But not necessarily in all such crystals. $CrCl_3,6H_2O$ and a number of alums contain aquo-lattice-complexes which dissolve as such. Nevertheless all water molecules in these crystals obey the rules formulated by me, which is another reason why I think that exceptions from the rules tend to be rare.

A hydrate like K_2FeCl_5,H_2O contains a water molecule in a state of strain as compared with the state of a water molecule in one of the 25 hydrates from which the rules were derived, and the forthcoming accurate parameter determination will be of more than usual interest.

* Position and negative *per se* or by polarization.

1. Tovborg Jensen, A. *Krystallinske Salt hydrater* Copenhagen (1948). With a summary in English.
2. Lindqvist, I. *Arkiv Kemi, Mineral. Geol.* **A 24** (1946) no. 1.
3. Klug, H., Kummer, E., and Alexander, L. *J. Am. Chem. Soc.* **70** (1948) 3064.

Received September 25, 1948.

Received November 3, 1948.

The Influence of Nitrate and Ammonia Nitrogen on the Formation of Different Nitrogen Fractions in *Torula utilis*

ARTTURI I. VIRTANEN, TIHAMÉR Z. CSÁKY* AND NIILO RAUTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Low-nitrogen *Torula* suspended in nitrate solution under strong aeration takes rapidly up nitrate nitrogen. The uptake of ammonia nitrogen occurs at a still higher speed in corresponding experiment from ammonium sulphate suspension. The fate of the nitrogen taken up is in both cases somewhat different. This appears clearly from Fig. 1.

The nitrogen fraction first formed from ammonia nitrogen in the cells is almost entirely soluble in trichloracetic acid (soluble N-fraction). During the first half an hour the difference between the increase of total nitrogen in the cells and that of the soluble nitrogen is still very small. Not until after about an hour does the protein synthesis intensively begin. The results are thus in agreement with the findings by Roine [1]. In the nitrate solution, on the other hand, the accumulation of soluble nitrogen in the cells is relatively small, while the protein synthesis is intensive right from the start. During 75 minutes nitrate nitrogen produced nearly twice as much protein nitrogen as soluble nitrogen (100 mg or 62 % protein N of the total uptake 162 mg N) while ammonia nitrogen produced nearly three times as much soluble nitrogen as protein nitrogen (91 mg or 29 % protein N of the total uptake 344 mg N). In spite of the much smaller uptake of nitrate nitrogen in comparison to ammonia nitrogen the formation of protein was in both cases practically equal.

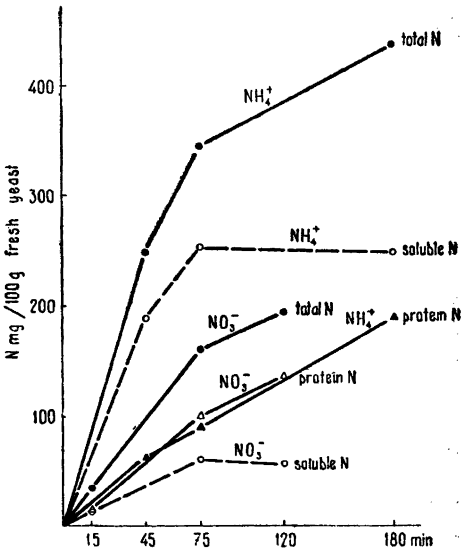


Fig. 1. Low-nitrogen T o r u l a suspended in ammonia and nitrate solutions. Different N-fractions during 180 minutes.

The soluble N-fraction formed in the cells of low-nitrogen *Torula* both in ammonium and nitrate solutions has to a great extent similar composition in spite of its different amount. The amino dicarboxylic acids, in the first place glutamic acid, their amides, alanine and some ammonia as well as a very small quantity of oxime nitrogen [2] constitute in the nitrate experiment during experimental times of 15 and 30 minutes practically the entire soluble N-fraction. In the ammonia experiment no oxime-N is formed but otherwise the composition of the soluble N-fraction is similar to that found in the nitrate experiment.

A detailed report on this work with discussion will be published in *Biochimica et Biophysica Acta.*

1. Roine, P. *Ann. Acad. Sci. Fennicae* Ser. A. II. Chem. (1947) no. 26.
2. Virtanen, A. I., and Csáky, T. Z. *Nature* 144 (1948) 597.

Received November 18, 1948.

* Visiting scientist from the Hungarian Biological Research Institute, Tihany, Hungary.

Action of Strong Acids on Acetylated Glycosides

II. Synthesis of Tetraacetyl-α-tertiary Butylglucoside

BENGT LINDBERG

Organisk-Kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In a previous communication [1] it was reported that attempts to prepare tetraacetyl-α-tertiary butylglucoside had been started and that the β-compound yielded acetochloroglucose by action of titanium tetrachloride.

Tetraacetyl-β-tertiary butylglucoside was treated with boron trifluoride in absolute benzene at room temperature. From the reactivity series: Isopropyl > ethyl > methyl, it was evident that the reaction time necessary would be still shorter than for the isopropyl derivative, which needed 30 minutes. For that reason 10 minutes was chosen. When the mixture was worked up, however, an oil of $[\alpha]_D^{20}$ + 80° was obtained, which refused to crystallize. The experimental conditions were varied but the result was always the same. The oil was analysed with respect to alkoxyl, acetyl and hydroxyl groups. The analyses were consistent with a mixture of about 60 % tertiary butylglucoside and 40 % 2,3,4,6-tetraacetylglucose. The glucoside ought to be insoluble in water and soluble in ether. 2,3,4,6-Tetraacetylglucose is known to be rather soluble in water and only slightly soluble in ether. It should therefore be possible to concentrate the glucoside by partition between ether and water. Thus, with a synthetic mixture of tetraacetyl-α-isopropylglucoside and β-2,3,4,6-tetraacetylglucose 1 : 1, the glucoside was isolated in an almost pure state and a yield of 80 % by partition between ether + petrol ether and water. When the oil was worked up in the same way, a crystalline compound was isolated from the ether phase. It was purified by recrystallization from methanol and petrol ether and analysis showed it to be tetraacetyl-α-tertiary butylglucoside. M. p. 69—70°. $[\alpha]_D^{20}$ + 113° *.

Table 1. Molecular rotations of some alkyl glucosides.

Tetraacetyl-glucoside	M	2A	2B
α-methyl	+ 47400	+ 54200	+ 40600
β-methyl	— 6800		
α-ethyl	+ 49700	+ 58200	+ 41200
β-ethyl	— 8500		
α-isopropyl ..	+ 55800	+ 65300	+ 46300
β-isopropyl ..	— 9500		
α-tert. butyl..	+ 45700	50400	+ 41000
β-tert. butyl..	— 4700		

This seems to be the first α-glucoside of a tertiary alcohol that has been prepared. It is of interest to notice its small molecular rotation. The Hudson 2A value increases in the series: Methyl > ethyl > isopropyl, but for tertiary butyl it is even smaller than for methyl. The 2B value varies in the same way, but here the differences are smaller.

Tetraacetyl-β-tertiary butylglucoside. Tetraacetyl-β-tertiary butylglucoside, previously prepared by Veibel [2], was prepared here by a modification of the method of Zemplén for alkyl cellobiosides [3].

Acetobromoglucose (22 g) was dissolved in absolute benzene (100 ml). Tertiary butylalcohol (40 ml) and mercury acetate

* All melting points uncorrected. All specific rotations in chloroform, about 2 %.

(7.8 g) were added and the solution was boiled for 15 minutes on the steam bath. When cold the solution was washed with water, dried over calcium chloride and concentrated under reduced pressure. The residue was recrystallized from ethanol. Yield 6.2 g. M. p. 141—143°. After further recrystallizations from ethanol the m. p. was raised to 144—144°.5. $[\alpha]_D^{20}$ −11°.

Tetraacetyl-β-tertiary butylglucoside and titanium tetrachloride. Tetraacetyl-β-tertiary butylglucoside (2 g) was dissolved in absolute chloroform (20 ml) and titanium tetrachloride (1.3 g) in absolute chloroform (12 ml) was added. The clear, yellow solution soon grew turbid and both white and yellow particles appeared in the resulting precipitate. The solution was heated for 40 minutes on the steam bath. When cold it was shaken with ice water, the chloroform phase washed with water and dried over calcium chloride. The brown solution was filtered through a column of aluminium oxide (Brockmann). After filtration it was yellow. The chloroform was evaporated under reduced pressure and the residue dissolved in ethanol and diluted with water. Pale yellow crystals (0.4 g) precipitated. M. p. 72—73°. $[\alpha]_D^{20}$ + 165°. By recrystallization from ether-petrol ether the m. p. was raised to 74—75° and the specific rotation to + 166°. As the substance prooved to contain chlorine and the data are consistent with those of acetochloroglucose, it must evidently be that substance.

Tetraacetyl-α-tertiary butylglucoside. Tetraacetyl-β-tertiary butylglucoside (3 g) was dissolved in absolute benzene (75 ml), saturated with boron trifluoride and allowed to stand at room temperature for 10 minutes. The solution was then neutralized with absolute pyridine (10 ml) and shaken with cold bicarbonate solution. The benzene solution was washed with water, dried over sodium sulfate and concentrated under reduced pressure. The residual oil was dissolved in ether (40 ml) and extracted with water (5 × 100 ml). After each extraction the ether volume was made up to 40 ml again. Now petrol ether was added (20 ml) and the extraction with water continued (5 × 100 ml). The ether phase was dried over sodium sulfate and concentrated under reduced pressure. The residual oil was dissolved in ethanol and poured into water. An oil separated which soon solidified to white crystals of m. p. 65—66°. Yield 1.1 g. After recrystallization, first from methanol and then from petrol ether, the substance was obtained in a pure state. M. p. 69—70°. $[\alpha]_D^{20}$ + 113°.

The acetyl content was determined according to Clark.

$C_{10}H_{16}O_6(OCCH_3)_4$ (404.2)
Calc. Acetyl 42.6 Found Acetyl 42.5

A grant from *Statens Naturvetenskapliga Forskningsråd* is gratefully acknowledged.

1. Lindberg, B. *Acta Chem. Scand.* **2** (1948) 426.
2. Veibel, S., and Nielsen, E. *C. R.* **203** (1936) 126.
3. Zemplén, G., and Gerecs, A. *Ber.* **63** (1930) 2720.

Received November 5, 1948.

Aromatic Keto- and Hydroxy-polyethers as Lignin Models. I

HOLGER ERDTMAN AND BENGT LEOPOLD

Organisk-Kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

According to modern views regarding the structure of lignin, this material is built up from simple building stones containing an aromatic nucleus with a three carbon atom side chain. This idea is supported by a multitude of experimental results *e. g.* the fact that lignin and several lignin derivatives give a comparatively

(7.8 g) were added and the solution was boiled for 15 minutes on the steam bath. When cold the solution was washed with water, dried over calcium chloride and concentrated under reduced pressure. The residue was recrystallized from ethanol. Yield 6.2 g. M. p. 141—143°. After further recrystallizations from ethanol the m. p. was raised to 144—144°.5. $[\alpha]_D^{20}$ −11°.

Tetraacetyl-β-tertiary butylglucoside and titanium tetrachloride. Tetraacetyl-β-tertiary butylglucoside (2 g) was dissolved in absolute chloroform (20 ml) and titanium tetrachloride (1.3 g) in absolute chloroform (12 ml) was added. The clear, yellow solution soon grew turbid and both white and yellow particles appeared in the resulting precipitate. The solution was heated for 40 minutes on the steam bath. When cold it was shaken with ice water, the chloroform phase washed with water and dried over calcium chloride. The brown solution was filtered through a column of aluminium oxide (Brockmann). After filtration it was yellow. The chloroform was evaporated under reduced pressure and the residue dissolved in ethanol and diluted with water. Pale yellow crystals (0.4 g) precipitated. M. p. 72—73°. $[\alpha]_D^{20}$ + 165°. By recrystallization from ether-petrol ether the m. p. was raised to 74—75° and the specific rotation to + 166°. As the substance prooved to contain chlorine and the data are consistent with those of acetochloroglucose, it must evidently be that substance.

Tetraacetyl-α-tertiary butylglucoside. Tetraacetyl-β-tertiary butylglucoside (3 g) was dissolved in absolute benzene (75 ml), saturated with boron trifluoride and allowed to stand at room temperature for 10 minutes. The solution was then neutralized with absolute pyridine (10 ml) and shaken with cold bicarbonate solution. The benzene solution was washed with water, dried over sodium sulfate and concentrated under reduced pressure. The residual oil was dissolved in ether (40 ml) and extracted with water (5 × 100 ml). After each extraction the ether volume was made up to 40 ml again. Now petrol ether was added (20 ml) and the extraction with water continued (5 × 100 ml). The ether phase was dried over sodium sulfate and concentrated under reduced pressure. The residual oil was dissolved in ethanol and poured into water. An oil separated which soon solidified to white crystals of m. p. 65—66°. Yield 1.1 g. After recrystallization, first from methanol and then from petrol ether, the substance was obtained in a pure state. M. p. 69—70°. $[\alpha]_D^{20}$ + 113°.

The acetyl content was determined according to Clark.

$C_{10}H_{16}O_6(OCCH_3)_4$ (404.2)
Calc. Acetyl 42.6 Found Acetyl 42.5

A grant from *Statens Naturvetenskapliga Forskningsråd* is gratefully acknowledged.

1. Lindberg, B. *Acta Chem. Scand.* **2** (1948) 426.
2. Veibel, S., and Nielsen, E. *C. R.* **203** (1936) 126.
3. Zemplén, G., and Gerecs, A. *Ber.* **63** (1930) 2720.

Received November 5, 1948.

Aromatic Keto- and Hydroxy-polyethers as Lignin Models. I

HOLGER ERDTMAN AND BENGT LEOPOLD

Organisk-Kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

According to modern views regarding the structure of lignin, this material is built up from simple building stones containing an aromatic nucleus with a three carbon atom side chain. This idea is supported by a multitude of experimental results *e. g.* the fact that lignin and several lignin derivatives give a comparatively

high yield of vanillin on oxidation with aromatic nitro compounds (Freudenberg [1]).

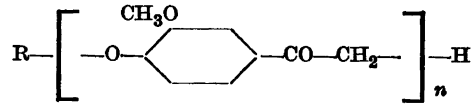
Biogenetic considerations and the results of dehydrogenation experiments with *iso*-eugenol [2] and ferulic acid [3] appear to show that the β-carbon atom of the side chain may be involved in the connection of the phenyl propane building stones of many binuclear or polynuclear natural products of phenyl propane type.

Lignin contains reactive groups responsible for its sulphonation by sulphite cooking acid. Holmberg found that α-phenetylalcohol reacted with bisulphite with the formation of α-phenethylsulphonic acid [4]. Thus the hydroxyl group was substituted by a sulphonic acid group. In his extensive analytical studies on lignin and the products obtained by the reaction between lignin and various SH-containing reagents he arrived of the conclusion that a substitution of a hydroxyl group in lignin was a more probable course of reaction than for example a fission of an oxide ring [5].

Lindgren [6] recently found that suitably substituted benzyl alcohols or phenethyl alcohols constitute excellent lignin models, reacting by substitution of hydroxyl groups by sulphonic acid groups.

In unpublished experiments it has been directly shown that during the sulphonation of lignin, hydroxyl groups disappear when sulphonic acid groups are introduced.

Consequently we were interested in synthesising compounds containing several building stones similar to those assumed to occur in lignin and connected in various ways, which would furnish vanillin on oxidation and sulphonic acids when heated with sulphite cooking acid. The two following series of ethers were first synthesised:



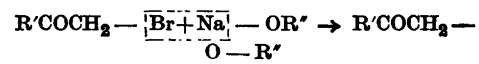
R=H

n=2, m.p. 140—142°. n=3, m.p. 181—183°. n=4, m.p. 201—202°.

R=CH_3

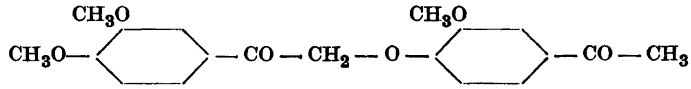
n=2, m.p. 138—139°. n=3, m.p. 145—146°. n=4, m.p. 146—147°.

The syntheses were accomplished in the following way:

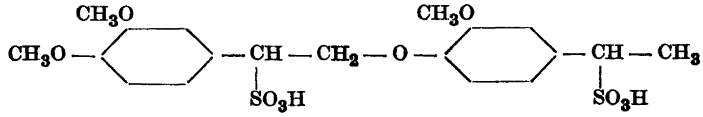


These ketonic compounds did not react with sulphite cooking acid but, of course, yielded vanillin on oxidation with nitrobenzene and alkali. If the keto groups were reduced to hydroxyl groups, compounds capable of sulphonation should be obtained.

In our first experiments we have subjected the following compound to reduction with aluminium isopropoxide in boiling isopropyl alcohol [7].



An alcohol was obtained in excellent yield. This readily reacted with sulphite cooking acid to yield the corresponding sulphonic acid: (isolated as the barium salt)



	$C_{19}H_{22}O_{10}S_2Ba$	
Calc.	Ba 22.5	OCH_3 15.2
Found	» 22.6	» 15.1

A more complete communication on the reactions of the abovementioned and related compounds will follow shortly.

1. Freudenberg, K., Lautsch, W., and Engler, K. *Ber.* **73** (1940) 167.
2. Erdtman, H. *Ann.* **503** (1933) 283.
3. Erdtman, H. *Svensk Kem. Tid.* **47** (1935) 223.
Cartwright, N. J., and Haworth, R. D. *J. Chem. Soc.* (1944) 535.
4. Hedén, S., and Holmberg, B. *Svensk Kem. Tid.* **48** (1936) 207.
5. Holmberg, B., and Gralén, N. *IVA:s handlingar* no. 162.
6. Lindgren, B. *Acta Chem. Scand.* **1** (1947), 779.
7. Wilds, A. L. *Organic reactions* Vol. II, New York (1946) p. 178.

Received November 6, 1948.

New Books

Jacob Berzelius. *Reiseerinnerungen aus Deutschland.* Verlag Chemie, Weinheim, 1948. 72 pp. DM 2.—

Verlag Chemie has edited a volume in order to celebrate the 100th anniversary of Berzelius' death. The intention of the book is not to enter into the details of Berzelius' scientific work, which is only indicated now and then, but rather to give a picture of his personality, from his own words and from the impression he made on others.

The first part contains extracts from Berzelius' letters and from his diary notes from his seven journeys to and through Germany between the years 1819 and 1845. The second part is taken from Wöhler's *Jugenderinnerungen eines Chemikers;* it is the well-known description of Wöhler's visit to Berzelius in 1823—1824.

The text has been ably compiled, translated and commented on by Dr Gisbert Klingelmann. A few Swedish names, like Uddevalia and Nykyrba, appear in unusual forms, which are easily recognized by a Swedish reader and do not disturb a foreign eye. The book, which has by the way a very handy size, can be recommended to anyone who would like to know something about Jöns Jacob Berzelius as a charming personality, and not only as a famous scientist.

Lars Gunnar Sillén

Otto Hahn. *Künstliche neue Elemente. Vom Unwägbaren zum Wägbaren.* Verlag Chemie, Weinheim, 1948. 51 pp. DM 2.50

For a long time German scientists have had no, or very limited, access to the scientific literature of the Western countries. When Otto Hahn, on May 9th, 1947, gave a lecture on artificial new elements to the *Gesellschaft deutscher Chemiker* in Mühlheim, he could assume that his audience had a good scientific background dating from before 1939 but no previous knowledge of the details of recent Western scientific work on the subject. Just for this reason the present book, which is founded on this lecture, may also prove of considerable value to scientists other than Germans who want a short, intelligent, and thorough account, without too much of the sugar and whipped cream of popularisation.

The lecture begins with a few pages on nuclear reactions, followed by the history of uranium fission and the principles of the U reactors and the A bombs. Then for each of the new elements 43 Tc, 61 Pm, 85 At, 87 Fr, 93 Np, 94 Pu, 95 Am, and 96 Cm there follows a list of the different ways of preparation, the chemical and radioactive properties, and a discussion of the amounts available. (But should not technetium be spelt with a *t* ?) German scientific work from the forties has naturally also been included. Even the radioactive »$4n + 1$» (Np)-series is recorded, which had scarcely been released for publication at the date of the lecture.

Lars Gunnar Sillén

ACTA CHEMICA SCANDINAVICA 2 (1948) 539—544

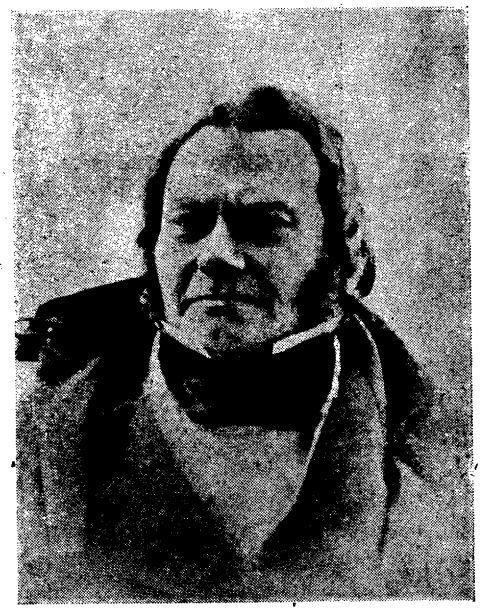
Jöns Jacob Berzelius

A Centenary

August 7th 1948 was the 100th anniversary of the death of Berzelius, the famous Swedish chemist. In his own country, he was a pioneer scientist whose concrete achievements, in a number of different spheres, were very considerable — and it can safely be said that few Swedish scientific workers have achieved a degree of international celebrity equal to his.

On September 20th 1948, some of the institutions to which he once devoted his energies arranged a commemorative meeting in Stockholm Town Hall. Invitations were sent out by *Kungliga Vetenskapsakademien* (The Royal Academy of Science), *Kungliga Karolinska Institutet* (The Royal Caroline Medico-Surgical Institute), *Kungliga Lantbruksakademien* (The Royal Academy of Agriculture) and *Svenska Läkaresällskapet* (The Swedish Medical Association). The opening address was given by Professor Gunnar Holmgren, the President of the Royal Academy of Science, and then followed speeches by Professor Arne Fredga, Professor Hilding Bergstrand, Professor Olof Svanberg and Professor Hugo Theorell, who acted as spokesmen for the organisations mentioned above. A number of scientific societies abroad — and in particular, those to which Berzelius himself had belonged — also sent representatives, who conveyed the greetings of their respective organisations.

This paper has been written with the object of sketching the main features of Berzelius' life and work — with perhaps a stress on certain achievements which were particularly remembered during the Centenary celebrations.



Jöns Jacob Berzelius.
From a daguerreotype taken in Berlin in 1845.

Like many another Swedish scientific worker who achieved fame, Berzelius came from a family of clergymen, originally of peasant stock. The name Berzelius was derived from a farmstead namely Bergsäter in the parish of Motala, where the last of his peasant ancestors lived and laboured. His father, Samuel Berzelius, was a schoolmaster at the *gymnasium* in Linköping. The family sometimes spent a holiday at Väversunda Sörgård in Väversunda Parish, the home of Mrs. Berzelius' parents, and it was here that Jöns Jacob Berzelius was born on

The yard of 'Tyska Bagarns Hus' ('The German Baker's House') in the corner of Nybrogatan and Riddaregatan, Stockholm. During the important period 1809—1818, Berzelius' residence and laboratory were on the first floor of the building on the right.

August 20th 1779. His childhood was however not particularly happy, for at an early age he lost both parents, and he was brought up in the homes of various relatives, under constantly changing and at times unpleasant circumstances. But there is plenty of evidence that at an early stage in his development, Berzelius showed clear signs of a strong-willed and independent character. At the *gymnasium* in Linköping, he became interested in scientific matters and at length decided to break with the family tradition and become a doctor. But his single-minded pursuit of his own special interests led quite naturally to the neglect of certain other studies which seemed to him of less importance — the Hebrew language, for instance. His final certificate, which has often been quoted, mentions his good natural gifts, his bad manners and doubtful future prospects!

In 1786, Berzelius commenced his medical studies at Uppsala University. At first, he derived very little stimulation from the existing text-books on chemistry, but as soon as he had the opportunity of participating in experiments, he 'felt something he had never felt before'. He at once realised that he was irrevocably predestined to work in this field. With great industry and enthusiasm, he went ahead with experiments of various kinds — generally in his own lodgings, but sometimes in the University laboratory, to which he was admitted a century and a half ago: in the autumn of 1798.

Berzelius was an orphan, and had to contend with very great economic difficulties. At the University — just as at the *gymnasium* — he was periodically forced to interrupt his studies in order to earn something as a private tutor. During the summer months, he worked as the doc-

tor's assistant at the Medevi mineral wells, and his very first printed paper was in fact a dissertation *pro exercitio* dealing with an analysis of the water at these wells. This early interest in mineral wells was something which lasted throughout the whole of his life.

At the conclusion of his studies, Berzelius moved to Stockholm with the intention of earning a livelihood as a doctor. All his spare time, however, was devoted to chemical research. He had not been very long in Stockholm before he was engaged as a lecturer at the *Kirurgiska Undervisningsverket* (The Institute of Surgical Education), but it was an appointment which unfortunately carried no salary with it. But it did at any rate lead to Berzelius becoming acquainted with several influential men who soon became his friends — amongst them the erudite ironmaster Wilhelm Hisinger, and Johan Gottlieb Gahn, a member of the Board of Mines. In collaboration with Hisinger, Berzelius founded a kind of review entitled *Afhandlingar i Fysik, Kemi och Mineralogi* (Transactions in Physics, Chemistry and Mineralogy). These papers were published in six volumes during 1807—1818, and while Berzelius was the author of the greater part of the contents, it was Hisinger who — at least at the beginning — paid most of the expenses.

In 1805, Berzelius was passed over in connection with the appointment of a professor at the Institute of Surgical Education, but two years later, when the post again became vacant, he obtained it without difficulty. The following year he was elected a member of the Royal Academy of Science, and in 1818 he was made secretary for life. The time had now come when he did not need a review of his own in order to get his works printed. In time, however, he found his duties as secretary so arduous that he took leave of absence from his professorship, and gave it up altogether in 1832. He remained until his death in the service of the Academy.

Berzelius' first great work dealt with the electrical decomposition of salts — at that time a completely new field of research. He had collaborated with Hisinger in this, and the results were destined to greatly influence Berzelius' theoretical outlook. He had also begun to take an interest in mineralogy, and during his analytical studies in this field, he discovered a new element, namely *cerium*. This was no mere chance, for the mineral in question had already been analysed by Scheele, who however had not found anything worthy of special note. At this time, Berzelius was also very much occupied with the subject now known as physiological chemistry, and it was while working in this field that he discovered sarcolactic acid. He also published two volumes of *Föreläsningar i Djurkemien* (Lectures on Animal Chemistry).

Soon however he became occupied with a problem which absorbed most of the energy of his best years, and which contributed very largely to his future fame, namely his researches into the multiple proportions. At this period in history, the knowledge of chemical compounds was too uncertain to allow of definite conclusions as to the general laws for their composition. This deficiency in human knowledge, arising from the undeveloped state of quantitative analysis, had already been pointed out by Lavoisier. And for some considerable time past, Berthollet and Proust had been discussing the question wether the composition of a compound had to be looked upon as fundamentally constant, or variable. Berzelius, above all things a lover of order and clarity, found this state of affairs rather unsatisfactory — not least from the angle of teaching. He therefore decided to go into the whole complex problem, and at length state as exactly as possible the proportions in which elements unite with each other.

This naturally meant nothing less than the quantatitive analysis of thousands of compounds, with constant and strict control of the methods, which usually called for improvement, and sometimes had to be rejected for quite new ones.

The task was gigantic, and took more than ten years: from 1807 to 1818. But the result was that the laws of definite and multiple proportions became settled once for all. It was this work — which found its theoretical background in Dalton's contemporary atomic theory — which provided a firm basis for the further development of chemistry. In the matter of accuracy, Berzelius had moved far in advance of his predecessors, and it has been said that it was he who made chemistry an exact science. Because of his unhesitating and masterly knowledge of facts, Berzelius was able to utter the decisive word in many of the burning controversies of his time, and in consequence he came to be regarded as the greatest living authority in the field of chemistry. It was a position which he held for more than two decades.

During these years he also created our language of chemical symbols. Of course, even before his time, there had been a system of signs and symbols, and they were both venerated and antiquated — an inheritance from the alchemists of the Middle Ages. Berzelius, with his firm grasp of realities, saw that if a system, whatever it might be, was to be of practical use in writing and printing, it had to be founded upon common numbers and letters. Having come to that conclusion, he made a complete break with ancient and hoary traditions. We now know that Berzelius' system of alphabetical symbols proved to be capable of development and adaptation in the general evolution of science, and in our own time it is the indispensable means of formal expression in chemistry.

In the field of mineralogy, the application of his new-won knowledge of the laws of chemical compounds enabled Berzelius to create a quite new system — one which contrasted very strongly with the earlier way of looking at these matters, which was natural-historical. Berzelius' system became the norm in later development. During this period of his life, he also performed a number of other tasks in certain special fields — one of them led to his discovery of the element *selenium*.

Journeys to England in 1812, and to England, France and Germany during 1818—19 were of great value to Berzelius. In these countries, he established connections with leading scientists, and both gave and received many fresh impulses. Later still, he often travelled abroad, but by that time the visits had acquired another character. He was now a celebrity, and much of his time was taken up by curious visitors and by all kinds of social functions.

After 1818, when Berzelius was appointed permanent secretary of the Royal Academy of Science, a new period of work began. The affairs of the Academy absorbed much time, but so also did his labours on his great text-book, which at length amounted to ten volumes. There was hardly ever a time when he was not busy with a new edition or translation, or with negotiations with foreign editors. Quite often, the latter had very different ideas to his on questions of precision and accuracy, and they were often made to feel his displeasure. From 1821 onwards he also wrote annual reports on the progress of chemistry, and these became the prototypes of later scientific reference works. Originally, these reports were written only to be read at the annual celebrations of the Academy of Science, but they soon developed into volumes containing 400—700 pages. They were translated into several foreign languages, and were perused by the chemists of every civilised country. Quite naturally, Berzelius' reputation thus became still further enhanced, and it has

been said that he came to be regarded as a kind of 'Supreme Court of Chemistry'.

But in spite of all these far-reaching activities, Berzelius continued with practical experiments until far into the 1840's. He made very important investigations in the realm of sulpho and seleno salts and fluoro and chloro salts, and in the chemistry of tellurium and the platin metals. He also investigated various groups of organic compounds. For instance: he discovered pyruvic acid and stated that racemic acid and tartaric acid were of identical composition. But in many instances, his work in organic chemistry had rather the character of a preliminary survey of the field. He also continued with mineral analyses, and in doing so he discovered the element *thorium*. He also revised and corrected earlier determinations of atomic weights and he carried out numerous controlling investigations in connection with his annual reports, or for the purpose of checking and supplementing the work of others.

Berzelius received both Swedish and foreign disciples into his laboratory — only a few at a time, however. It was there that Arfwedson discovered *lithium* and Sefström discovered *vanadium*. But in both cases, it is probable that Berzelius was the actual discoverer, though he allowed his disciples to take the credit. Amongst the foreigners who worked with him in this way we note, first and foremost, the Germans Mitscherlich, Rose, Magnus and Wöhler — and it was the last-named whose personal relations with Berzelius were closest.

We may say that Berzelius sought to survey, put in order and systematize the whole of his field of science. As a young man, he had penetrated very deeply into Lavoisier's way of thinking, and his subsequent development was determined by two important experiences, namely the definite proportions and the electrolytic phenomena. The former constituted a foundation that could be built upon — the latter did not. The primitive electrical science of the early 19th century could not solve the problem of chemical affinity. From his comparatively simple starting points, Berzelius could not in spite of much ingenious toil master the tangled puzzles of organic chemistry, and the general development of science travelled along other paths. Hence it happened that towards the end of his life, he found himself strongly opposed to certain of the younger scientists who, searching for new ways and viewpoints, suffered from the pressure of his authority. But it is only in our own time that the electrical nature of chemical affinity has become more clear.

Berzelius' outstanding characteristic was a respect for facts, and he was averse to all speculation with no solid base. It is certain that this personal quality contributed in no small measure to his ultimate success, for he began his chemical researches at a time when that science sorely needed, above all things, established facts and reliable data. But later in Berzelius' life, all this hardened into a somewhat conservative outlook, and he became very reluctant to alter an opinion unless he had very certain proofs that it was inaccurate. Thus it was that rather late in the day, he agreed to recognise the halogenes as elements. His criticisms, however, were very rarely no more than negative, and as soon as he had convinced himself of the accuracy of an observation, he was eager to give it a place in his system, to generalise upon it, and to search for a suitable terminology. And in this way he coined the terms *isomerism* and *catalysis*.

We ourselves can see Berzelius' personal character revealed in a very clear light, for his was a most open and frank nature. He wrote a clear, easy and fluent style, and his correspondence is very extensive. Further, we have much information from people who were closely acquainted with him. It is said that in his younger days,

he was inclined to be a little 'choleric', and given to 'holding on to his rights', but in later life he is described as having an equable temperament: amiable, benevolent and with a great sense of humour. It was a sometimes rather drastic humour, and his letters reveal a rich fund of it. In his personal relations — as for instance in any home circle — he made a most profound impression on everybody, and it was very easy for him to acquire both friends and influence.

Additional activities were in the realm of public affairs — especially those relating to higher education and health. It is clear that he was a man of very great practical ability — a faculty that revealed itself not least in his energetic and far-seeing management of the affairs of the Academy. But he had no flair for money-making by means of the practical application of his scientific knowledge, and his attempts in that direction brought him little else but trouble. His discovery of *selenium* was however the outcome of an industrial commitment. As a professor, he worked energetically for the development of the Institute of Surgical Education (after 1810 known as *Kungliga Karolinska Institutet*) into a complete medical school, and in doing so he met with strong opposition from the old universities — often the objects of his biting sarcasm.

From the time of its foundation, Berzelius was a member of the Royal Academy of Agriculture, and for several decades he was president of the Scientific Department of that institution. He was also one of the founders of the Swedish Medical Association, and as a member of it played an important rôle.

Because of his absorption in his numerous tasks, Berzelius had very little time even to think of marriage and family life, but at the age of 56, however, he married the daughter of one of his closest friends. And on his wedding day, King Charles XIV. John conferred upon him the title of baron. As the years passed, Berzelius acquired still further honours, and he was made a member of more than 80 academies and scientific societies in different parts of the world.

As one becomes more familiar with Berzelius' character, his most imposing features are perhaps his encyclopaedic mind and his enormous capacity for work. Undaunted, he undertook tasks which might well have been thought above the capacity of any one man. But with never-failing persistence, he tackled them and solved them. And although he was very careful about correctness of detail, it was always the main outline of a problem which aroused his interest. He did not care for special hypotheses of limited range, and he never allowed himself to move within the narrow groove of one special and particular study. As a man and as a scientist, Berzelius had the qualities of solidity and honesty, and his scientific motto was in fact: 'Clarity and Truth'.

The chief work on Berzelius (published only in Swedish) is H. G. Söderbaum's *Jac. Berzelius I—III* (Uppsala 1929—31). His 'Autobiographical notes' have been published in Swedish (Stockholm 1901), English (Baltimore 1934) and German (Leipzig 1903). Six volumes of letters have been published by H. G. Söderbaum (Uppsala 1912—32). Berzelius' correspondence with Liebig, Wöhler and Magnus was published in Germany.

Arne Fredga

ACTA CHEMICA SCANDINAVICA 2 (1948) 545—549

The Diagnostic Value of the Titration-curves of the *p*-Carboxyphenylhydrazones of Hydroxy-substituted Aromatic Aldehydes and Ketones

STIG VEIBEL and HANS WILLUMSEN SCHMIDT

Department of Organic Chemistry, Technical University of Denmark, Copenhagen, Denmark

In a previous paper [1] one of us mentioned that from the potentiometrical titration-curves of the *p*-carboxyphenylhydrazones of hydroxy-substituted aromatic aldehydes and ketones conclusions may be drawn with regard to the number of phenolic groups present in the carbonyl compound examined. We have now continued the investigation in order to see if, in addition to the number of phenolic groups, also the place of these groups may be determined or discerned from the position of the titration-curve compared with the titration-curve of the *p*-carboxyphenylhydrazone of the unsubstituted carbonyl compound. To some extent this seems to be the case.

The investigation has covered the determination of the titration-curves of alcoholic solutions of benzaldehyde, *o*-, *m*- and *p*-hydroxybenzaldehyde, acetophenone, *o*-, *m*- and *p*-hydroxyacetophenone, 2,4- and 2,5-dihydroxyacetophenone (resacetophenone and chinacetophenone). In addition we have examined the *p*-carboxyphenylhydrazones of 3 aminosubstituted carbonyl compounds, *viz.* *p*-dimethylaminobenzaldehyde and *m*- and *p*-aminoacetophenone, in order to see if the amino-groups have an effect opposite to the effect of the hydroxy-groups.

From Figs. 1—2 it is seen that on the acidic side of the neutral-point a hydroxy-group in *o*-position to the carbonyl group makes the carboxygroup slightly more acid than in the unsubstituted carbonyl compound, whereas hydroxyl groups in *m*- or *p*-positions have practically no effect or, if an effect is discernable, it tends to go in the opposite direction.

Fig. 3 shows that another hydroxy group in position 4 diminishes the effect of the hydroxy group in position 2, whereas the effect is enhanced if the second hydroxy group is introduced in position 5.

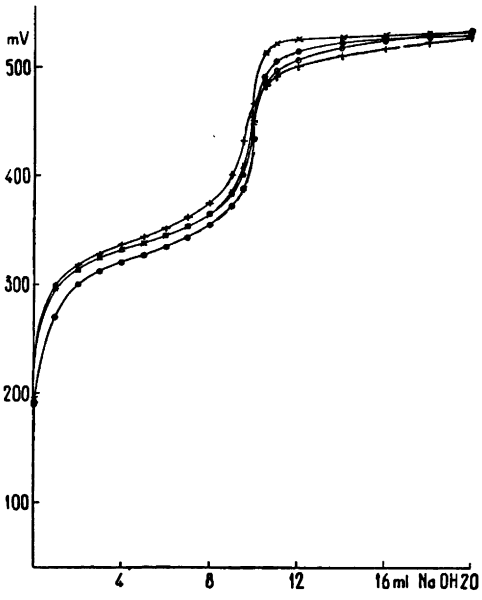


Fig. 1. Titration curves of p-carboxyphenylhydrazones of hydroxy-substituted benzaldehydes.

×——× *Benzaldehyde*
●——● *o-Hydroxybenzaldehyde*
○——○ *m-Hydroxybenzaldehyde*
+——+ *p-Hydroxybenzaldehyde*

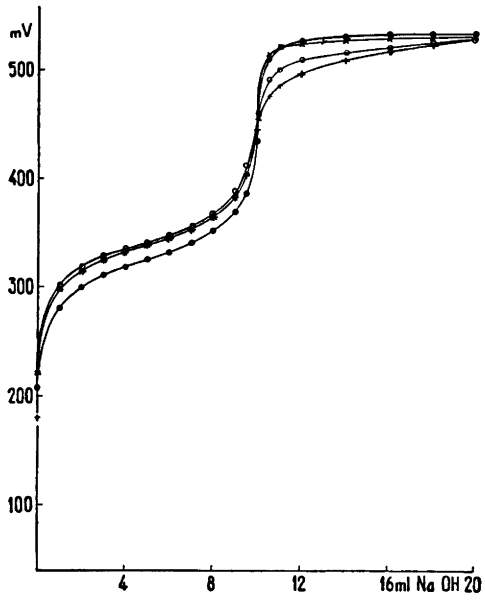


Fig. 2. Titration curves of p-carboxyphenylhydrazones of hydroxy-substituted acetophenones.

×——× *Acetophenone*
●——● *o-Hydroxyacetophenone*
○——○ *m-Hydroxyacetophenone*
+——+ *p-Hydroxyacetophenone*

Figs. 4—5 show that an amino group in *m*- or *p*-position to the carbonyl group is without any significant effect (acetophenone) or makes the carboxy group slightly less acidic (benzaldehyde) than in the unsubstituted carbonyl compound.

These results agree tolerably well with the previously known effect of hydroxy substitution on the dissociation constant of benzoic acid:

Table 1. Dissociation constants of hydroxy- and amino-substituted benzoic acids.

	k (25°)	k_B (25°)
Benzoic acid	$6.86 \cdot 10^{-5}$	
o-Hydroxybenzoic acid	$1.06 \cdot 10^{-3}$	
m-Hydroxybenzoic acid	$8.3 \cdot 10^{-5}$	
p-Hydroxybenzoic acid	$2.85 \cdot 10^{-5}$	
2,4-Dihydroxybenzoic acid	$5.5 \cdot 10^{-4}$	
2,5-Dihydroxybenzoic acid	$1.08 \cdot 10^{-3}$	
m-Aminobenzoic acid	$1.67 \cdot 10^{-5}$	$1.22 \cdot 10^{-11}$
p-Aminobenzoic acid	$1.21 \cdot 10^{-5}$	$2.33 \cdot 10^{-11}$

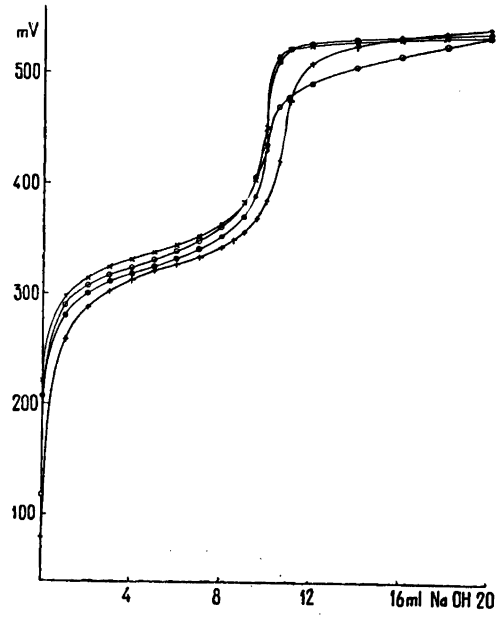


Fig. 3. Titration curves of p-carboxyphenylhydrazones of dihydroxysubstituted acetophenones.

×——× *Acetophenone*
●——● *o-Hydroxyacetophenone*
○——○ *2,4-Dihydroxyacetophenone*
+——+ *2,5-Dihydroxyacetophenone*

When the titration is continued beyond the neutralisation of the carboxyl group the effect becomes more differentiated. In *o*-position the hydroxy group is practically without effect. A hydroxy group in *m*-position displaces the titration curve towards lower potentials and in *p* position the hydroxy group has a still greater effect, *i. e.* the phenolic group in *m*- or *p*-position acts as a very weak acid whereas the acidic character of the hydroxy group in *o*-position is negligible. Here too the effect is in accordance with facts known from the hydroxysubstituted benzoic acids, *e. g.* that salicylic acid may be titrated with phenolphthalein as indicator, whereas the indicator used in the titration of *p*-hydroxybenzoic acid is methyl red.

When two hydroxy groups are present the effect is greater for 2,4-dihydroxyacetophenone than for 2,5-dihydroxyacetophenone, in accordance with the greater effect of a hydroxyl group in *p*-position than in *m*-position.

The effect of amino groups is a displacement of the titration curve towards higher potentials than for the unsubstituted *p*-carboxyphenylhydrazone, *i. e.* the salts of the aminosubstituted *p*-carboxyphenylhydrazones have a somewhat more alkaline reaction than the salts of the correspondant unsubstituted acids (compare the k_B-values of the aminobenzoic acids indicated above).

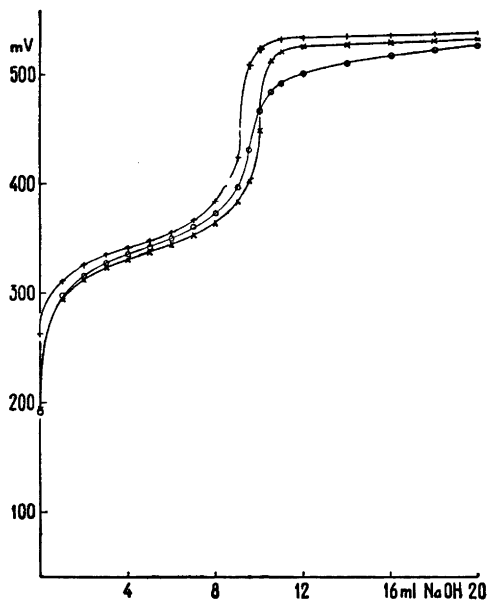


Fig. 4. Titration curves of p-carboxyphenylhydrazones of amino-substituted benzaldehydes.

×———× *Benzaldehyde*
○———○ *p-Hydroxybenzaldehyde*
+———+ *p-Dimethylaminobenzaldehyde*

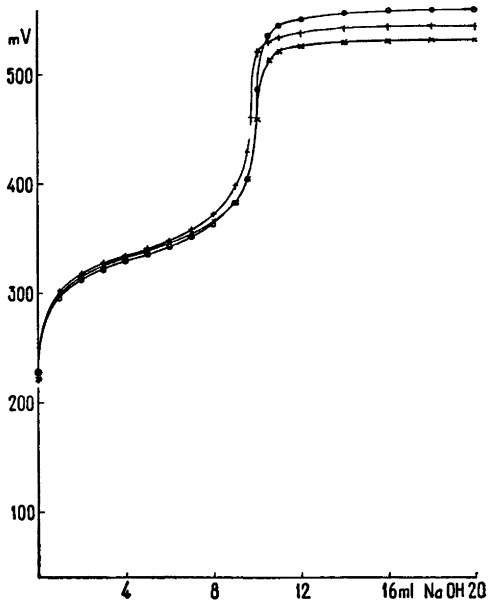


Fig. 5. Titration curves of p-carboxyphenylhydrazones of amino-substituted acetophenones.

×———× *Acetophenone*
●———● *m-Aminoacetophenone*
+———+ *p-Aminoacetophenone*

EXPERIMENTAL PART

The *p*-carboxyphenylhydrazones of benzaldehyde, *o*-hydroxy- and *p*-dimethylaminobenzaldehyde and of acetophenone, *o*-hydroxy- and *p*-aminoacetophenone have been described previously [1].

m-Hydroxybenzaldehyde was prepared from *m*-nitrobenzaldehyde according to the indications of Woodword [2]. The *p*-carboxyphenylhydrazone of the compound was prepared as usually [1]. It contains 1 mol of water which is lost during the determination of the melting point, *i. e.* the m. p. indicated below is that of the anhydrous substance.

The water is lost, too, by heating the substance for 18 hours to 78° in vacuum over P_2O_5. The anhydrous substance regains its water of crystallisation if it is left at room temperature by ordinary degree of humidity for some days.

m-Aminoacetophenone was prepared from *m*-nitroacetophenone by reduction with sodium dithionite [3] and exchange of the amino group for a hydroxygroup by current methods (diazotation).

Resacetophenone and chinacetophenone were prepared by current methods. In the preparation of the *p*-carboxyphenylhydrazones the usual methodique was employed. In case of the aminosubstituted carbonyl compounds it must be remembered that one

equivalent of sodium hydroxyde has to be added to the reaction mixture in order to avoid contamination of the *p*-carboxyphenylhydrazone with its hydrochloride. The *p*-carboxyphenylhydrazones of the dihydroxysubstituted acetophenones are more soluble in water than usual for *p*-carboxyphenylhydrazones.

Table 2. Melting points of p-carboxyphenylhydrazones not previously described.

Carbonyl compound	M. p.	Carbonyl compound	M. p.
m-Hydroxybenzaldehyde	227—228°	*m*-Aminoacetophenone	251—52°
p-Hydroxybenzaldehyde	264—266°	2,4-Dihydroxyacetophenone	304—306°
m-Hydroxyacetophenone	247—248°	2,5-Dihydroxyacetophenone	287—288°
p-Hydroxyacetophenone	288—291°		

SUMMARY

Titration curves of the *p*-carboxyphenylhydrazones of hydroxy- and amino-substituted benzaldehydes and acetophenones have been compared with the titration curves of the *p*-carboxyphenylhydrazones of benzaldehyde and acetophenone. The displacements of the curves, caused by the substituents, seem to agree tolerably well with those predicted from the dissociation constants of the hydroxy- and amino-substituted benzoic acids.

REFERENCES

1. Veibel, S. *Acta Chem. Scand.* **1** (1947) 54.
2. Woodword, R. B. *Org. Synth.* **25** (1945) 55.
3. Comp. D. R. P. 218364 (1910), Friedländer 10 (1913) 162.

Received June 6, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 550—556

Investigations in Serum Copper

II. Isolation of the Copper Containing Protein, and a Description of some of its Properties

CARL G. HOLMBERG and C.-B. LAURELL

Laboratory of Clinical Chemistry, University of Lund, Lund, Sweden

When pigs serum is precipitated with ammonium sulphate, the fraction obtained between 40 and 50 % saturation, has a distinctly bluish-green color and contains most of the copper present in this serum.

We have attempted to purify the blue protein contained in this fraction by alcohol precipitation in the cold. In this way it is possible to get rid of a considerable amount of colorless or yellow proteins, and to obtain a preparation, giving solutions of a deep blue color. The copper content of the best preparations thus obtained is about 0.03 %. When these preparations have been investigated with electrophoresis they show a big main peak with the mobility of an α_1-globulin and two smaller peaks, one on each side. It has, however, not been possible with this technique to obtain an electrophoretically homogeneous preparation. Some of the best preparations have been subjected to ultracentrifugation by Dr. K. Pedersen in Upsala. He found that the main component had a sedimentation constant, $S_{20} = 17.8$, two other smaller components had sedimentation constants $S_{20} = 7.2$ and 4.2 respectively. Preparative centrifugation at high speed revealed that the main component was colorless. It seemed obvious that the difficulties in preparing the blue protein originated from its tendency to be adsorbed on other proteins when these were precipitated.

As it seemed very probable to us that the hemocuprein of Mann and Keilin [1] had some connection with this blue protein we tried to treat our preparations with alcohol and chloroform at pH 6.5 expecting to obtain in this manner a preparation of pure hemocuprein. With such treatment one obtains a bulky precipitate mainly consisting of denaturated protein and a practically colorless

supernatant. By washing the precipitate several times with 0.9 % NaCl a blue substance is brought in solution. A solution of the blue substance is dialyzed and precipitated with ammonium sulphate.

When the blue protein thus obtained was further investigated it was at once clear that it differed in at least one property from hemocuprein. Its reduction by hydrosulphite ($Na_2S_2O_4$) was completely reversible, whereas Keilin and Mann state that hemocuprein, which has been decolorized with hydrosulphite, has definitely lost its blue color. It also retained the property which Holmberg [2] found for the impure preparation of catalyzing the oxidation of paraphenylenediamine.

We have now worked out a method which permits a preparation of this blue protein in a fairly pure state. The principles of this method are as follows:

PREPARATION

Principles of preparation

I. Salt precipitation of the blue protein together with the bulk of the globulins.

II. Precipitations of some euglobulins at pH 6.2 and 5.5, followed by precipitation of the blue component together with the main part of the globulins at pH 5.5 with alcohol (15 %) at low ionic strength. The hemoglobin and the main part of the albumins remain in the supernatant.

III. Denaturation of all proteins except the blue component in two steps with chloroform-alcohol at pH 6.5 and 5.5 respectively at low ionic strength and room temperature.

Preparation from pigs serum

Pigs serum is precipitated with ammonium sulphate. The fraction obtained between 36 % and 55 % saturation is dissolved in water and dialyzed for at least 24 hours against running tap water.

After dialyzing, the pH is adjusted with dilute acetic acid to 6.2. A yellow precipitate is centrifuged off and discarded. Now the pH is adjusted to 5.5. The solution is cooled down to 0°. The precipitate which is formed is centrifuged off and discarded. Alcohol is now added to a concentration of 15 % at a temperature between 0 and — 5°. The strongly blue precipitate is centrifuged down at low temperature and dissolved in water containing some NaCl. The solution is dialyzed over night.

After the pH has been adjusted to 6.5 this solution is precipitated at 0° with an equal volume of chloroform-alcohol (9 parts 90 % alcohol, and 1 part

chloroform). The temperature of the solution is allowed to rise, and the precipitate is centrifuged down at room temperature. The colorless supernatant is discarded *.

The faintly blue precipitate is extracted on the centrifuge with about equal volume of 0.9 % NaCl, until no more blue color is obtained in the supernatant. The pooled extracts are dialyzed over night. Next day the dialyzed solution is adjusted to pH 5.5, and again treated with chloroform-alcohol in exactly the same way as the day before. If the supernatant, after the treatment with chloroform-alcohol, has some blue color this solution is cooled down to — 15° and filtered at this temperature. The precipitate is dissolved in water and added to the pooled NaCl-extracts and the whole quantity dialyzed first against running tap water and after that against distilled water. A small precipitate, which appears during the dialysis, is centrifuged down and discarded. If the solution, which now contains practically pure blue protein, is too dilute for further work, it can easily be concentrated by precipitating with ammonium sulphate to 65 % saturation, redissolving the precipitate in a small volume of distilled water, and again dialyzing.

Preparation from human serum

The corresponding blue protein can easily be prepared from human pregnancy serum by applying the same method as previously described. Here, however, the blue protein begins to precipitate when the pH is adjusted to 5.5 before adding alcohol. Therefore, this precipitate is not discarded. Alcohol is added to a concentration of 10 % instead of 15 % used with pigs serum. Otherwise the procedure is exactly the same.

CHEMICAL PROPERTIES OF THE COPPER CONTAINING PROTEIN

Solubility

Impure preparations precipitate at pH 5.4 after prolonged dialysis. Pure preparations do not precipitate even *after prolonged* dialysis against distilled water, but *the protein comes down on acidification to the isoelectric point* (4.4). Even very small impurities greatly influence all solubility tests, as the *blue protein is adsorbed* on *most precipitating proteins in neutral and slightly acid solutions*, which might depend upon the very acid i. p. of the blue protein.

* The supernatant is colorless, if the ionic strength has been low enough (specific resistance $> 1000\ \Omega$).

On salt precipitation it comes down, when the ammonium sulphate concentration is raised *over 48* % saturation. From the preparation method it is evident that the blue protein has an alcohol solubility like most albumins but *is less sensitive to room temperature in alcohol-water mixtures than most albumins.*

Electrophoretic mobility

On electrophoresis in Tiselius-Svensson apparatus the blue protein moves as a practically homogeneous substance over a wide pH range (8—3).

Table 1. Electrophoretic measurements on blue protein from pig.

pH	Mobility u. 10^5 cm^2/volt sec		
	Anode	Cathode	
7.40	ascending 2.7	descending 2.7	
6.32	» 2.1	» 2.0	Phosphate
5.75	» 1.7	» 1.6	buffers
5.10	» 1.4	» 1.3	
4.87	» 0.76	» 0.73	
4.48	» 0.27	» 0.19	Acetate
4.33	descending 0.16	ascending 0.16	buffers
3.70	» 1.4	» 1.6	

We have determined its mobility in phosphate and acetate buffers. The total ionic strength was maintained at 0.2 with 0.18 derived from NaCl and 0.02 from the buffer. The protein concentration was about 0.2 %. It is evident from Fig. 1. that *the isoelectric point* when determined *in the presence of acetate ions is about 4.4.* At this pH the protein is partially decolorized.

Blue protein from pig and man have the same isoelectric points.

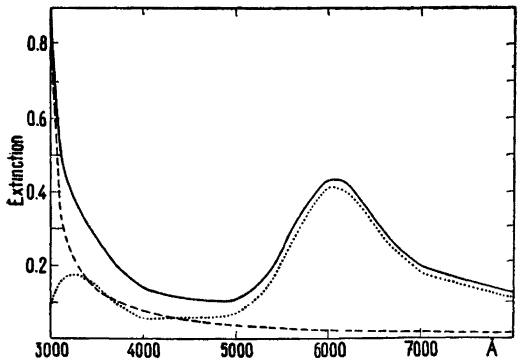


Fig. 1. Absorption spectrum.
——— *Blue protein (pH 6.5).*
- - - - *Blue protein decolorized with ascorbic acid.*
. . . . . *Spectrum of chromatic groups by subtracting 2 from 1.*

Molecular weight

Dr. Pedersen in Upsala has made an investigation into some of the physical constants of the blue protein. His results will be published separately in a special paper in this series. His value for the *molecular weight is about 151 000.* Identical for pig and man.

Copper content

The copper content has been determined in 6 different preparations, 3 from pig and 3 from man. The results are to be found in Table 2. Assuming the molecular weight of Pedersen to be correct, and taking into consideration the small amount of impurities present, we find *a copper content of eight atoms per molecule.* The hemocuprein of Keilin and Mann contains two atoms of copper in a molecule weighing about 35000. Therefore, it seems possible that our protein might contain four units of hemocuprein.

Absorption spectrum

The absorption spectrum at neutral pH is shown in curve 1, in Fig. 1. It shows *a broad absorption band* with a maximum of about *6050* Å. In the ultraviolet there is a maximum at about 2800 Å corresponding to the ordinary protein absorption maximum in this region.

When the blue protein is reduced with ascorbic acid in an inert gas atmosphere (curve 2) the *maximum at 6050 Å disappears and a progressive absorption between 3000 and 4000 Å is decreased. Upon reoxidation with oxygen the spectrum reverts* to the one pictured in curve 1.

Table 2. Light absorption, copper and protein contents in some preparations from pig and man.*

	Protein %	*E* (6050 Å 1 cm cuvette)	Cu mg/100 ml	Cu content of protein in %	Atoms Cu per protein molecule
Pig I.	0.37	0.238	1.26	0.34	8.1
II.	0.59	0.395	1.92	0.33	7.9
III.	1.28	0.778	4.14	0.32	7.6
Man I.	0.96	0.570	2.99	0.31	7.4
II.	1.2	0.750	3.85	0.32	7.6
III.	0.62	0.428	1.96	0.32	7.6

The spectrum of the chromatic group (curve 3) is obtained by subtracting curve 2 from curve 1. This spectrum contains two maxima, one at 6050 Å, and another at 3250 Å.

The *spectrum given for the blue protein is the same in a pH interval between 5.5 and 8.*

On *acidification to pH 3 the same spectrum is obtained as after reduction.* At this pH the copper becomes completely dialyzable. *At pH 12.5 the maximum at 6050 Å also disappears.* What happens to the maximum at 3250 Å is not so easy to determine as the protein maximum is shifted to a longer wavelength.

There exists a direct proportionality between copper content and extinction at 6050 Å.

$E \cdot 5 =$ Cu (mg/100 ml) when the extinction is measured in an 1 cm cuvette.

DISCUSSION

A blue protein has been isolated from serum of pig and man. It has a great similarity to the hemocuprein of Mann and Keilin but is not identical with this substance. The molecular weight is about 151000 and it contains eight atoms

* Biuret method.

of copper. It can be reduced to a colorless compound with reducing agencies such as sodium hydrosulphite, ascorbic acid, hydroxylamine and thioglycolic acid. It should be emphasized, however, that we have tried reduction with purified hydrogen gas in the presence of catalysts, such as palladium black and Raney nickel, without success. The reduction is completely reversible in the presence of oxygen, but attempts to oxidize with potassium ferricyanide were not successful. The absorption spectrum is similar to that of the hemocyanines. The question, therefore, seems pertinent whether we have here to do with a real oxidation and reduction, or with a reversible oxigenation. All that can be said at the present moment is that attempts to decolorize solutions of this protein by evacuation or saturation with an inert gas have been unsuccessful. The problem is being further investigated.

As has been pointed in the first paper of this series [3], it is probable that most if not all serum copper is present in the form of this blue compound. We propose that the new protein is named *cæruloplasmin.*

SUMMARY

A new copper containing protein has been isolated from mammalian plasma. A description is given of the method of the preparation and of some of the properties of this protein.

Our thanks are due to Professor Alf Sjövall, chief of the Obstetrical Clinic in Lund, and to Dr. Olle Dahl at Scan's cooperative butchery for providing us with material.

REFERENCES

1. Mann, T., and Keilin, D. *Proc. Roy. Soc. London* **B 126** (1938) 303.
2. Holmberg, C. G. *Acta Physiol. Scand.* **8** (1944) 227.
3. Holmberg, C. G., and Laurell, C.-B. *Acta Chem. Scand.* **1** (1947) 847.

Received June 14, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 557—560

The Stability of Dried Cytochrome c

KARL-GUSTAV PAUL

Institute of Biochemistry, Medical Nobel Institute, Stockholm, Sweden

Cytochrome c is known to be stable even under conditions which may destroy many other enzymes. Thus it does not change at extreme pH values [1], at high temperatures in salt free solutions [2] nor upon drying. Since this last property is of great practical importance, we found it to be of interest to investigate some properties of a sample of cytochrome c, which had been kept in the dry state for 12 years. We believe this preparation to be one of the oldest of a fairly pure cytochrome c available as yet.

Patch, Morrison, Ciminera and Beyer [3] found no changes in the absorption spectrum and catalytic properties of cytochrome c which had been lyophilized and stored for four months. Drabkin [4] has found that the activity of acetone-dried cytochrome c is not completely retained when the preparation is stored at room temperature for some time. Recently Keilin and Wang [5] published an investigation of the stability of hemoglobin and certain endoerythrocytic enzymes from various animals. Their samples had been stored as solutions in ampoules and kept in darkness for 26—47 years. If the samples were taken under sterile conditions and without addition of chloroform no inactivation of the proteins occurred. These authors also give a review of some other investigations on the stability of enzymes upon storage.

Our sample of cytochrome had been prepared by Theorell in 1936 according to a method worked out by him [6], using adsorption on cellophane as an essential step. According to data from that time, the preparation was of 93 % purity, which, since pure cytochrome c at that time was believed to contain 0.34 % iron, corresponds to 0.316 % iron. It had been dialyzed against 0.2 % ammonia, evaporated to dryness in a desiccator at room temperature and then kept in a glass tube, sealed with a rubber stopper and protected against light. The tube had been standing at room temperature and humidity.

The sample was easily and almost completely soluble in redistilled water. Some insoluble particles were centrifuged down. The clear, red solution

Table 1. Analysis of old and fresh cytochrome c.

	Dry weight mg/ml	Iron content μg/ml	Iron %
Old cytochrome c	10.00	32.36	0.324
Fresh cytochrome c	4.84	18.56	0.383

showed the same absorption bands both in the reduced and oxidized states as a sample, freshly prepared according to Keilin and Hartree [7].

The slight increase in the iron content of the old cytochrome c as compared to the analysis 12 years ago indicates that at least a part of the insoluble particles were impurities.

Table 2. Optical densities at the tops of the absorption bands of old and fresh cytochrome c. To give suitable density values the old cytochrome was diluted 0.100 ml to 5.20 ml and the fresh cytochrome 0.300 ml to 8.30 ml, both with M/15 sodium phosphate buffer of pH 6.8. The values in the table are for these diluted solutions.

Wave-length in mμ	Old c	Fresh c	Density of fresh c / Density of old c
	oxidized		
278	0.613	0.322	0.53
408	1.17	1.31	1.12
528	0.117	0.129	1.10
600	0.018	0.020	1.11
690	0.008	0.009	(1.12)
	reduced		
416	1.44	1.65	1.15
520	0.168	0.195	1.16
549	0.276	0.341	1.23
650	0.003	0.006	(2.00)

The light absorption curves (Table 2) for this old cytochrome and for the fresh preparation were determined with a Beckman spectrophotometer. As neither preparation was completely pure, it is of no value to give the whole curves, and only the densities at the tops of the bands are listed. The maximal absorptions of the different bands had the same wave-lengths for the two preparations. The figures in the right column indicate that there is no considerable spectrophotometrically observable change in the old cytochrome. The low ratio at wave-length 278 mμ suggests that the impurities in the old preparation were of a protein nature.

Table 3. Catalytic activity of old and fresh cytochrome upon oxygen uptake in the oxidation of succinic acid. Reaction time 30 min. Temperature 37° C. Dry weight of kidney preparation 27.0 mg/ml. »Density» means the value calculated from tables 2 and 3 of the α-band density of added cytochrome in a final volume of 3.45 ml if it had been completely reduced. Thus for vessel 7 »density» = 0.276 × 5.2 × 0.0464/0.10 × 3.45 = 0.193.

Vessel no.	1	2	3	4	5	6	7
Well:							
0.20 ml 2 *M* KOH	→						
Main chamber:							
2.00 ml phosph. buff. *M*/15 pH 7.3	→						
0.50 ml kidney prep.	→						
ml fresh cytochrome	0	0.0162	0.0377	0.0808	0	0	0
ml old cytochrome	0	0	0	0	0.093	0.0216	0.0464
μg of Fe from added cytochrome	0.0	0.3	0.7	1.5	0.3	0.7	1.5
ml water	0.65	0.63	0.61	0.57	0.64	0.63	0.60
Side bulb:							
0.30 ml 10 % Nasuccinate pH 7.3							
0_2 uptake in μl	80.5	150.5	199	242	142	186.5	231
»Density»	0.000	0.044	0.103	0.221	0.039	0.090	0.193

For the activity determinations (Table 3) we used a modification of a method by Keilin and Hartree [8] which we have used for some other activity determinations of cytochrome c [1].

If one plots oxygen uptake *versus* micrograms of iron in the cytochrome c added to the Warburg vessels, the old cytochrome shows a slightly decreased activity. However, if one calculates the concentrations of added cytochrome c in the manometer vessels from the densities of the α-bands for the reduced cytochrome and plots the oxygen uptake against these densities, it is obvious that the old cytochrome sample has the same catalytic activity as the fresh one (Fig. 1). This again means that there must be some foreign iron in the old cytochrome.

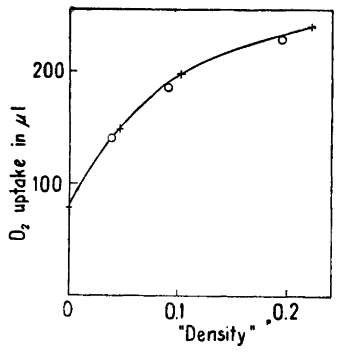


Fig. 1. Oxygen uptake plotted against concentrations of cytochrome, expressed as densities. + fresh, O old cytochrome.

SUMMARY

Cytochrome c, which had been kept in the dry state for 12 years, had the same properties as a fresh preparation with regard to solubility in water, light absorption and catalytic activity in oxidation of succinic acid.

REFERENCES

1. Paul, K.-G. *Acta Chem. Scand.* **2** (1948) 430.
2. Theorell, H. Personal communication.
3. Patch, E., Morrison, H., Ciminera, J., and Beyer, K. *Fed. Proc.* **3** (1944) 35.
4. Drabkin, D. L. *J. Biol. Chem.* **171** (1947) 409.
5. Keilin, D., and Wang, Y. L. *Biochem. J.* **41** (1947) 491.
6. Theorell, H. Unpublished experiments.
7. Keilin, D., and Hartree, E. F. *Biochem. J.* **39** (1945) 289.
8. Keilin, D., and Hartree, E. F. *Proc. Roy. Soc. London* **B 129** (1940) 277.

Received May 12, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 561—573

On the Hematin and the Bile Pigments in Catalase

ROGER K. BONNICHSEN

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The protein part of blood and liver catalase from horse has previously been found to be identical [1], but there is a great divergence of opinion about the relation between the hematin and the bile pigments in liver catalase in the literature.

Sumner, Dounce and Frampton [2] found that the activity and the protohematin iron content varied much in their liver catalase preparations.

Lemberg and Legge [3] also found a great variation in the ratio bile pigment iron/total iron, from 0.3—0.5. They were of the opinion that this large amount of bile pigment could not be formed during the preparation and considered it more likely that bile pigment is preformed *in vivo* in liver catalases.

In a previous publication we reported bile pigment corresponding to only 10 % of the total hematin iron in our liver catalase preparations [4].

The object of this work was to study this discrepancy in the ratio bile pigment/total hematin.

Ox blood catalase [5], and horse blood catalase [6] contain only protohematins, so bile pigments are not a constituent of these catalases.

In our experiments, the properties of blood catalase, which contains 4 protohematins/molecule, were compared with those of liver catalase, which has a varying number of protohematins/molecule. In each case our improved method of determining the activity was used [4].

As a result of these experiments, the conclusion is drawn that the bile pigment in liver catalase preparations is an artifact, or in any case only small amounts can be present in the liver catalase *in vivo*.

Additional investigations of blood and liver catalases from different species of animals have been made. Besides information about the bile pigments in liver catalases other conclusions have been drawn in the discussion from the results obtained.

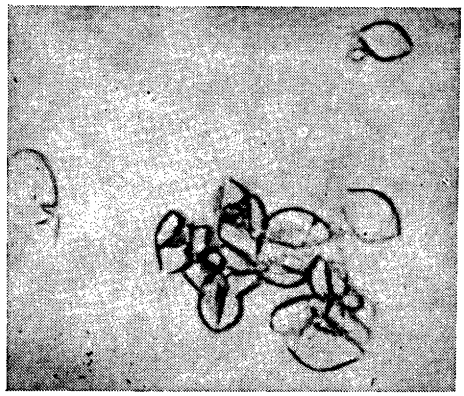


Fig. 1. Crystalline guinea pig liver catalase, crystallized from ammonium sulphate (× 200).

EXPERIMENTAL PART

Preparations

The catalases referred to later as pure are prepared according to earlier descriptions [1]; they were crystallized from ammonium sulphate solution.

The guinea pig liver catalase was prepared by a simplified method. Other liver catalases can be prepared in this way and the method allows a very rapid preparation. One hour is enough to achieve a preparation with a ratio of the extinction at 280 mμ to 405 mμ, 2 : 1; then the dialysis takes some time corresponding to a percity of 50 %.

1. The livers are ground in a »Turmix» with a double amount of water for a couple of minutes.

2. Immediately afterwards the extract is shaken with one tenth of its volume of an alcohol-chloroform mixture 5 : 1 and centrifuged.

3. The alcohol concentration is then increased to 70 vol %. The enzyme then precipitated together with some inactive protein.

4. The enzyme is dissolved in distilled water and dialyzed, whereby some inactive protein precipitates and is discarded. Crystallisation from ammonium sulphate can now be carried out as usual [1]. Other liver catalases at this step contain large amounts of ferritin, that can be removed using acetate buffer and alcohol as previously described [1].

In Table 1 are shown some figures from two guinea pig liver catalase preparations. No. 1 is prepared from healthy animals. No. 2 from animals used for experiments with infective material.

The yield is 700—800 mg enzyme from 500 g of liver. Fig. 1 shows the crystalline enzyme. Guinea pig livers weighing from 12—20 g contain about 50 mg catalase, and it is possible to prepare the pure enzyme from a single liver.

Certain precautions should be taken in all liver catalase preparations as will be shown later:

1. The livers should be used as soon after the animal is killed as possible, and the extraction time only last a couple of minutes.

2. The alcohol-chloroform added should be just enough to denature the hemoglobin.

3. The enzyme must never be left standing in the alcohol-chloroform solution, but immediately either be precipitated with alcohol or the alcohol-chloroform removed by evaporation *in vacuo*.

Bile pigment determination

The bile pigment was determined in some cases after the method of Lemberg, Lockwood and Legge (Addition of glacial acetic acid and extraction with ether)[7]. By this method Lemberg and Legge [3] recovered 50—70 % of the protohematin; the rest was bound to the denatured protein. In the determination of bile pigment they assumed the same loss as found in the protohematin determinations. Evidence given below indicates that this calculation gives too high a figure for the bile pigment in our preparations.

In our experiments the method of Lemberg and Legge [3] also gave about the same loss. When the enzyme was split in HCl-acetone we found that the loss of hematins was much less from 10—30 %, depending upon the amount of HCl used, but the recovery of bile pigment was found to be constant and independent of the strength of the acid.

Even small amounts of HCl (*e. g.* 0.2 ml *N*/1 HCl to 20 ml acetone) split off all the bile pigments, in agreement with earlier determinations by Agner [8].

As a control the denatured protein from catalase that was split in either acetic acid or in HCl-acetone was dissolved in water, and strong HCl was added. If the protein did not dissolve in water the HCl acetone was added with stirring. After this treatment only a trace of bile pigment was found in the aqueous phase. While almost all the protohematin was recovered.

When a blood catalase is split either in strong acetic acid or in HCl-acetone (*e. g.* 0.05 ml *N*/1 HCl to 20 ml acetone) the hematins are completely split off leaving a white protein which does not give any pyridine hemochromogen spectrum.

If the hematins are determined in the blood catalase before it is split by the standard method of Lemberg and Legge or by Agner [8], and the amount of recovered hematins determined, there is always a constant loss in hematins ranging from about 7 % when the enzyme is split in HCl-acetone to 10—12 % when split in acetic acid.

Both these methods require many manipulations, and the mentioned loss is probably due to minor losses adding up during the procedure in the standard method to the figures above. When calculating the loss of bile pigment during the splitting of liver catalase with the same methods, these same losses were assumed.

Some figures from bile pigment determinations in different liver catalases are given in Table 1.

Table 1. Analytic data for different catalase preparations.

Catalase	$\beta \times 10^7 \cdot cm^2 \cdot mole^{-1}$ CN⁻			k_1^4 22°C	Bile pigment % of total hematin	Protohematin % of dry weight	Ratio $^{630}/_{570}$ mμ of the hemochromogen spectrum	Iron % of dry weight	Calc. ratio bile pigm. / total hematin
	280 mμ	405 mμ	425 mμ						
Human blood	70	86	73	3.5	—	1.08	0.12	0.09	—
Horse blood	68	86	73	3.5	—	1.08	0.11	0.098	—
Horse liver 1	69	76	62.9	3.0	14	0.92	0.23	0.097	0.10
Horse kidney	78*	84	—	3.4	—	1.01	0.20	0.100	0.15
Guinea pig liver 1	68	84	70	3.4	—	1.01	0.15	0.089	—
Guinea pig liver 2	70	65	53	2.4	22	0.80	0.52	0.100	0.31
Horse liver 2	68	50	40.3	1.1	40	0.62	0.74	0.098	0.45
Horse liver 3	68	63	47	1.4	29	0.76	0.59	0.092	0.35

Hemochromogen spectra

Lemberg and Wyndham [9] found that their liver catalase preparations possessed a hemochromogen with an absorption band in red at 651 mμ. They assumed this was due to another hemochromogen besides protohemochromogen. The ratio of the absorption at 651 and 557 mμ was found to be 0.25.

Fig. 2 shows the hemochromogen spectrum of some of the catalases from Table 1. Horse blood has very little and horse liver catalase no. 1 has some absorption in the red, the ratio 650/557 mμ being about 0.1.

Horse liver nos. 2 and 3 have a much larger absorption in the red, the ratio being 0.36 and 0.25. A definite band can be seen at 651 mμ, which also was visible in a hand spectroscope.

Lemberg and Legge [3] have described the CO-hemochromogen spectra of liver catalase. They found an absorption band at 630 mμ, thus confirming Lemberg and Wyndham's assumption that another hemochromogen besides protohemochromogen was present in their liver catalase preparations. The ratio between the absorptions at 630 and 570 mμ was 0.50 in most cases. Assuming that the catalase CO-hemochromogen band at 630 mμ had the same molar extinction as CO-cholehemochromogen they could calculate the ratio bile pigment/total hematin from the formula $[\varepsilon(630/\varepsilon 570)—0.047] / [(0.55\ \varepsilon 630/\varepsilon 570) + 0.96]$ ($\varepsilon = \log Io/I$) and found agreement with the results obtained from the direct determination of the bile pigment.

* Some cloudiness in the solution.

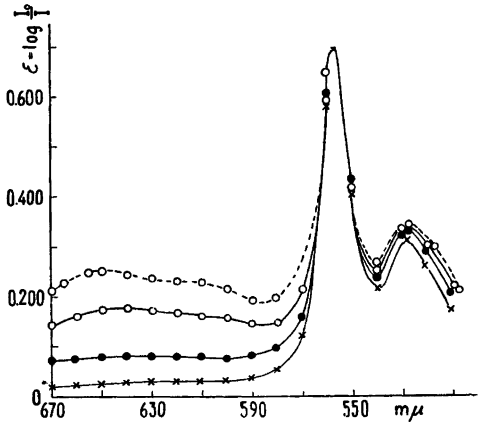


Fig. 2. Hemochromogen spectra of different catalases, extinction corrected to the same value at 557 mμ. ×——× *horse blood cat.,* ●——● *horse liver cat. no. 1,* O——O *horse liver cat. no. 3,* O — — O *horse liver cat. no.2.*

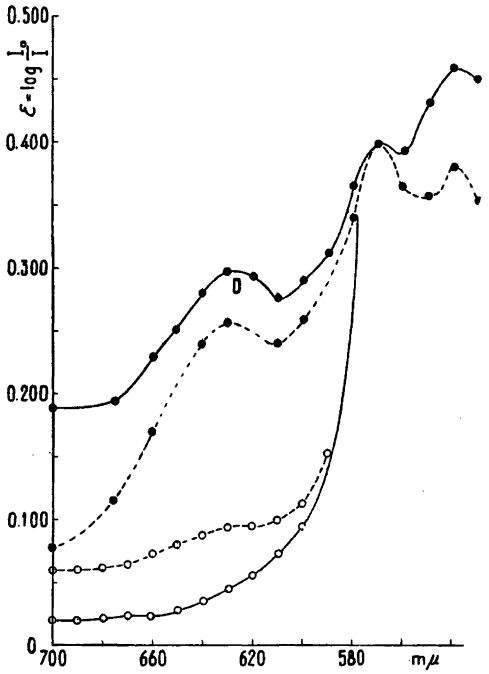


Fig. 3. CO-hemochromogen spectra of catalases with varying number of hematins. Extinction corrected to the same value at 570 mμ. O——O *horse blood cat.,* O — — O *horse liver cat. no. 1,* ●— — ● *horse liver cat. no. 3,* ●——● *horse liver cat. no. 2.*

Fig. 3 shows the CO-hemochromogen spectrum of some of the catalases from Table 1. In the table the bile pigment has also been calculated from the above mentioned formula, but the blood catalase absorption at 630 mμ has first been subtracted. With this modification we also found agreement between the determination of bile pigment from the above mentioned formula and the direct determination.

Analyses of liver catalase in the different steps of the preparation

A large number of experiments have been carried out on different liver catalase preparations in order to find the cause of the disagreement between our results and those of earlier authors concerning the bile pigments.

The reasons could be that:

1. Catalase where more than one hematin is converted to verdohematin might be more susceptible to denaturation by the reagents used by us in the preparation — thus the final preparation would consist of a more resistant catalase fraction with less bile pigment.

2. The bile pigment content could vary from horse to horse.

3. The methods of determining activity and bile pigment might give different results in the hands of different authors.

4. If hematin disintegrating enzymes are found in the liver, the way in which it is extracted and the time elapsed since the animal was killed could influence the bile pigment in the liver catalase.

The bile pigment content and the activity have been followed through the different steps in horse and guinea pig liver preparations. Some results are seen below. The activity had to be measured as a relative activity. This was done by determining the absorption at 405 mμ and using a β of $86 \cdot 10^7 \text{cm}^2 \cdot \text{mole}^{-1}$ in the calculation.

	Bile pigment % of total hematin	Ratio 630/570 mμ	k_1^4 mole^{-1} · seconds^{-1}
Before acetone fractioning	10.5 %	0.15	3.4
After acetone fractioning	12.8 %	0.12	3.3
After alcohol fractioning and crystallization	14.0 %	0.20	3.3

We have found that alcohol and chloroform used in a high concentration denatures the enzyme, but even the usual alcohol-chloroform amount slowly inactivates the catalase. Inactive hematins have a Soret band and gives the same pyridine hemochromogen as protohematin, but they have no activity and do not combine with CN^-. The figures below show a catalase before and after standing four days in chloroform-alcohol solution.

	Bile pigment % of total hematin	Ratio 630/570 mμ	k_1^4 mole^{-1} · seconds^{-1}
Immediately after adding alcohol-chloroform	8.5 %	0.12	—
After 4 days in the alcohol-chloroform	22 %	0.62	1.7

The same catalase after purification and crystallization is referred to as H. L. no. 3 in Table 1. It was not possible to obtain any fraction with more or less bile pigment.

In one single case in a horse liver containing numerous small and large parasitic granuloma, bile pigment in much greater proportion than usual was found in the catalase. The ratio bile pigment/total hematin kept nearly constant during the preparation though increasing a little, and even repeated fractionation with both alcohol and acetone did not denature any part of the enzyme, and no fraction with more or less bile pigment could be obtained. This catalase after crystallization is no. 2 in Table 1.

Catalase with about 2.3 hematins/molecule seems to be very resistant towards the reagent used for the preparation.

These two catalases with a low hematin content could not be crystallized from ammonium sulphate alone, but using dioxane as described by Sumner *et al.*[10] the enzyme could easily be crystallized. The crystals were green and not black as those of usual catalase.

The hematin content of intact catalase

Some control experiments have been carried out by Chance in this institute on the hematin content of the above mentioned catalases with a low hematin content. The discovery of the primary intermediate compounds of catalase with hydrogen peroxide and with alkyl hydro-peroxides bound to respectively one and all intact hematins in the catalase molecyle permits a determination of the number of intact hematins by a comparison of the spectral shifts on formation of these complexes [11].

Also the velocity of oxidation of alcohols by the primary alkyl hydro-peroxide complexes depends not only upon the catalase concentration but also upon the number of intact hematins in the catalase and thus enables us to determine the number of hematins in relation to blood catalase, which contains 4 hematins/molecule [11].

The density change at 405 mμ on the formation of catalase-hydrogen peroxide is 11.5 scale divisions and therefore four hematins bound to peroxide would give 46 scale divisions. But this liver catalase preparation gave only 26 scale divisions on reaction with methyl hydro-peroxide, thus there are $\frac{26}{46} \cdot 4 = 2.3$ hematins.

The velocity constant for the oxidation of alcohols is 590 mole^{-1} · seconds^{-1} for this preparation whereas blood catalase (of the same density at 405 mμ)

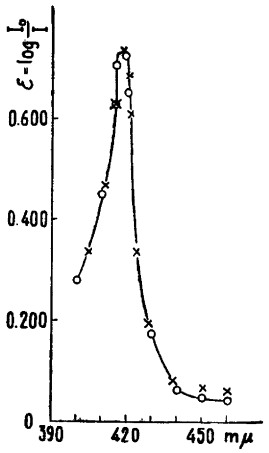


Fig. 4. CO-pyridine hemochromogen spectra of horse liver and blood catalase. 0.3 ml 1 N NaOH, 0.5 ml pyridine and 10.4 γ hematin, made up to 4 ml with water. × blood catalase, O liver catalase.

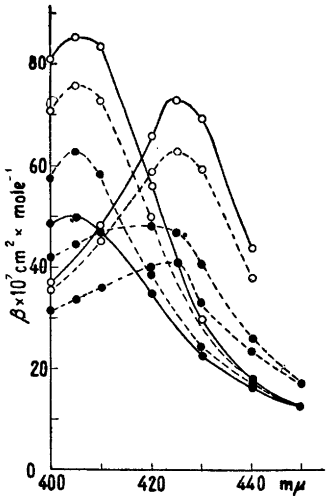


Fig. 5. The Soret band of different catalases from Table 1, and their CN^- compound.
O——O *horse blood catalase*
O— —O *horse liver catalase no. 1*
●——● *horse liver catalase no. 2*
●— —● *horse liver catalase no. 3*

gives a velocity constant of 1000 mole^{-1} · seconds^{-1}. The relative hematin content is $\frac{590}{1000} \cdot 4 = 2.4$ hematins.

Similar experiments with H. L. no. 2 gave 2.5 active hematins/molecule.

Post mortem changes of catalase in liver tissue and in liver extract.

No measureable increase in the bile pigment was found in the liver catalase prepared from liver tissue that was stored for two days in the ice-chest, but 14 days storing gave 20.7 % bile pigment.

In water extracts of liver tissues with 10 % ammonium sulphate kept at 20° C the catalatic activity is diminishing in a few days. The solution turns green due partly to conversion of hemoglobin into choleglobin. The catalase is destroyed if the solution is shaken with alcohol-chloroform, and fractionation with ammonium sulphate does not allow a complete separation of hemoglobin and choleglobin from the catalase.

The method of Bonnichsen, Chance and Theorell [4] used in this paper is more accurate than older methods and gives somewhat higher activity values

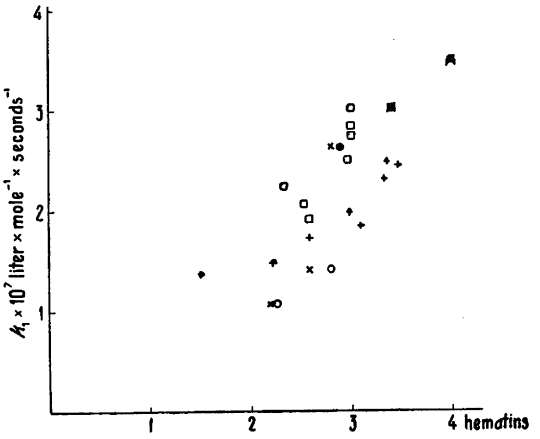


Fig. 6. k_1 plotted against the number of hematins determined from pyridine hemochromogen determinations and from the CN^- compound. △ horse blood cat., ● guinea pig liver cat., ○ horse liver cat., hematins determined from pyridine hemochromogen, × the CN^- compound of the same catalases. + values from Sumner et al.[2], □ *values of Lemberg and Legge*[3] *see text. 22° C.*

of the catalase preparations. The inaccuracy of older methods alone, however, cannot be the only cause of the great variation in the activity values of the different preparations prepared by other authors.

The bile pigments have been determined spectrophotometrically using the extinction coefficient used by Lemberg and Legge [3] and the values given in this paper are comparable to those of Lemberg and Legge.

Absorption spectra

Table 1 gives the extinction coefficient for the Soret band and the maximum at 280 mμ for several catalases in phosphate buffer pH 6.8. The purity of these preparations can be judged from the absorption at 280 mμ [1].

The human blood catalase since it was first described (1), has been further purified using acetate buffer as for the blood catalase from horse.

The horse blood and the human blood catalase have the same spectral properties. Horse kidney and one of the guinea pig liver catalases are almost identical to blood catalases. The extinction coefficient at 280 mμ is seen to be about the same for catalases from different animals.

The table shows further that in agreement with the results of Holden and Lemberg [12] on isolated verdohematin the bile pigment group in the liver cannot have any light absorption comparable to that of protohematin in the region of 405 mμ.

As the group of the liver catalases that yields bile pigment might interfere with the pyridine-hemochromogen determination, the hematin content was

also determined from the peak of the Soret band at 418 mμ of the CO-pyridine hemochromogen, as neither bile pigment nor verdohematin have any Soret band [12]. The result agreed as well as could be expected with the direct determination of hematins from the pyridine hemochromogen band at 557 mμ, thus the absorption of the verdohematin or the bile pigment is low at these two wavelengths compared to the strong band at 557 mμ and the still stronger band at 418 mμ.

Fig. 4 shows the Soret band of CO-pyridine hemochromogen at 418 mμ of blood and liver catalases from horse. There is no sign of any Soret band different from that of protohematin in this region.

The extinction coefficient for the peak of the cyanide compound at 425 mμ in the table seems only to be influenced by the number of active hematins in the molecule. Fig. 5 shows the Soret band of the different catalases in phosphate buffer and in cyanide. The band from horse liver no. 3 though seems to have its maximum shifted towards ultraviolet, but the absorption at 425 mμ was used to calculate the numbers of active hematins. This catalase as mentioned contained some inactive hematins.

In Fig. 6 the activity k_1 [4] is plotted against the number of hematins calculated from the pyridine hemochromogen determination and also from the cyanide compound $\beta \cdot 73 \cdot 10^7$ cm^2 · mole^{-1} from the blood catalase used as standard for 4 hematins per molecule.

DISCUSSION

Guinea pig liver catalase has been prepared without bile pigment so this cannot be a normal constituent of this catalase. Horse liver catalase has been prepared with about 8 % bile pigment in the first steps of the preparation increasing to about 14 % in the crystalline enzyme.

The conclusion is drawn that under normal conditions *in vivo* there is probably no bile pigment present in liver catalase. Several factors have been found to increase the bile pigment content of the liver catalase: post mortem changes, infections in the animals, the use of too much alcohol-chloroform or the standing for some time in the alcohol-chloroform solution.

No reliable method can be given to obtain horse liver catalase entirely free from bile pigment. Even if the greatest care is taken variable amounts of bile pigments or inactive hematins are found to be present.

In our usual preparations the number of active hematins are 3—3.5/ molecule. A rather drastic treatment of the catalase or the use of abnormal livers was necessary to prepare catalase with 2.3—2.5 hematins per molecule.

The former agrees with both Agners determination of protohematin in liver catalases [13, 8], and with Theorell and Agners [14] magnetic titration of liver catalase as well as with the data of Agner and Theorell [15] for titration with floride. These data show a variation of active hematins from 3 to about 3.5.

In blood catalases bile pigment has never been found in any preparation. On standing for some time in the alcohol-chloroform they are inactivated without any formation of bile pigment [16]. The only difference found in the behaviour of the protohematin of blood and liver catalase is the difficulty with which part of the liver hematins is split off from the protein, and this difficulty seems to vary from preparation to preparation.

Lemberg and Legge [3] increased the bile pigment content of their liver catalases by adding large amounts of hydrogen peroxide to an enzyme solution containing ascorbic acid. Some preliminary results have shown that if this experiment is repeated without ascorbic acid at pH 7 on a blood catalase it results in the hematins becoming more difficult to split off from the protein. At the same time the activity decreases somewhat. No bile pigment is formed. Hydrogen peroxide plus ascorbic acid oxidize hemoglobin [17] and probably the partly inactivated hematins of a catalase are also affected by ascorbic acid and hydrogen peroxide.

Nothing is yet known of the function of blood catalase but liver catalase may oxidize alcohols by coupled oxidations [18], or directly by the means of the catalase-peroxide compound [11]. Thus catalase may function as a peroxidase with hydrogen or alkyl hydroperoxides in the liver metabolism. If the function in the blood is similar the higher metabolic rate of the liver could explain the fragility of the liver enzyme.

More than one hematin/molecule seems to be necessary for the catalatic activity. This would speak in favor of the »internal» reaction type for the splitting of hydrogen-peroxide by catalase in agreement with the theory put forward by Theorell [19].

Sumner, Dounce and Frampton [2] have given some figures about the variation of activity with the bile pigment content in their catalase preparations. 4 hematins should have a Kat. f. of 60,000, 3 hematins 45,000 etc. The points of the curve published by them do not go through the origin, however, as already pointed out by Lemberg and Legge [3], and figures can also be taken in favor of one hematin/molecule being catalatically inactive. See Fig. 6 where the figures of Summer *et al.* have been plotted.

Lemberg and Legge [3] have also plotted bile pigment/total hematin against activity. They found the activity proportional to the number of active hematins. Assuming that the bile pigment value determined by them is too high,

this might change their curve in favor of our data. In figure 6 the data of Lemberg and Legge [3] are plotted having been recalculated on the base that the loss in bile pigment is constant and about 10 %; corrections have also been made for the Kat. F. They assumed that Kat. F. for a four hematin catalase was 52,000, whereas we found 61,000 [4]. None of their data would then speak against one hematin being catalatically inactive.

Since the catalase Soret band is due to the conjugated double bonds of the tetra pyrrol ring and this ring has been opened in the verdohematin [12], it could thus be expected, that the »verdohematin» in catalase does not have a noticable light absorption in region of the Soret band. The experimental results confirm this.

CN- is bound with covalent bonds to the hematin iron [14] and probably to the same place as is the hydrogen-peroxyde [11], to the hydroxyl group found by Agner and Theorell [15].

That peroxides do not combine with verdohematin has been shown by Chance [11]. The cyanide absorption band has also been found to be inversely proportional to the activity, and the peak of the cyanide band at 425 mμ seems to be a reliable value for the determination of the number of active hematins in the catalase molecule.

The content of hematins in catalase preparations can be determined if iron impurities can be excluded. The data in this paper indicate that pyridine-hemochromogen or CO-hemochromogen determinations give reliable values for the protohematins in liver catalases, the absorption of bile pigment in this region being negligible. Ferritin can be completely removed using acetate buffer in the preparation and by dialysis the rest of foreign iron can be removed. The difference between the total iron and the protohematin iron gives the bile pigment iron/molecule. The absorption coefficient for catalase from different animals at 280 mμ was found to be the same, and the hematins equal in catalatic activity in spite of the immunologic differences of the protein part. This indicates that only the heme-linked groups of the protein, as suggested by Theorell [20], take part in the catalatic activity, and that they are the same in the different animal catalases.

SUMMARY

A comparison has been made between different blood and liver catalases. Crystalline guinea pig liver catalase has been prepared without any demonstrable bile pigment. In horse liver catalase preparations bile pigment was found in an amount of 10 to 14 % of the protohematin content.

Chloroform-alcohol treatment especially increases the amount of bile pigments or of inactive hematins in the liver catalase molecule. A further increase in bile pigments was found in a preparation from an infected liver from horse.

The conclusion has been drawn that under normal conditions in vivo there is probably no bile pigment present in liver catalases. The small amounts invariably found in horse liver catalase can be explained as resulting from the preparation.

Part of the hematins in liver catalases, from 10 to 25 %, is much more difficult to split off from the protein than in blood catalases.

The catalatic activity has been found to be identical for catalases with the same hematin content but from different animals and with immunologically different proteins.

Catalase with little more than 2 active hematins/molecule was found to have so little catalatic activity that more than one hematin seems to be necessary for the catalatic activity.

CN^- was found to combine with active hematins only. The absorption band at 418 mμ of the CO-hemochromogen spectrum or at 557 mμ in the pyridine hemochromogen spectrum was found to be a reliable measure for the hematin content of liver catalases.

REFERENCES

1. Bonnichsen, R. K. *Arch. Bioch.* **12** (1947) 83.
2. Sumner, J. B., Dounce, A. L., and Frampton, V. L. *J. Biol. Chem.* **136** (1940) 343.
3. Lemberg, R., and Legge, J. W. *Biochem. J.* **37** (1943) 117.
4. Bonnichsen, R., Chance, B., and Theorell, H. *Acta Chem. Scand.* **1** (1947) 685.
5. Laskowski, M., and Sumner, J. B. *Science* **94** (1941) 615.
6. Agner, K. *Arkiv Kemi, Mineral. Geol.* **B 17** (1943) no. 9.
7. Lemberg, R., Lockwood, W. H., and Legge, J. W. *Biochem. J.* **35** (1941) 363.
8. Agner, K., *Arkiv Kemi, Mineral. Geol.* **A 16** (1942) no. 6.
9. Lemberg, R., and Wyndham, R. A. *Proc. Roy. Soc. N. S. W.* **70** (1937) 343.
10. Sumner, J. B., and Dounce, A. L. *Science* **85** (1937) 366.
11. Chance, B. *Acta Chem. Scand.* **1** (1947) 236.
12. Holden, H. F., and Lemberg, R. *Austral. J. Exptl. Biol. Med. Sci* **17** (1939) 133.
13. Agner, K. *Naturwissenschaften* **27** (1939) 418.
14. Theorell, H., and Agner, K. *Arkiv Kemi, Mineral. Geol.* **A 16** (1942) no. 7.
15. Agner, K., and Theorell, H. *Arch. Bioch.* **10** (1946) 321.
16. Bonnichsen, R. K. Unpublished experiments.
17. Lemberg, R., Legge, J. W., and Lockwood, W. H. *Biochem. J.* **35** (1941) 328.
18. Keilin, D., and Hartree, E. F. *Biochem. J.* **39** (1945) 293.
19. Theorell, H. *Experientia* **4** (1948) 100.
20. Theorell, H. *Advances in Enzymol.* **7** (1947) 265.

Received May 3, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 574—582

Some Technical Improvements in Adsorption Analysis

LENNART HAGDAHL

Institutes of Biochemistry and Physical Chemistry, Uppsala, Sweden

The interferometer for adsorption analysis constructed by Tiselius and Claesson [1] has shown itself to be a useful instrument which is easy to operate and which, by virtue of its accuracy and sensitivity, has given valuable results [2].

In order that the interferometer work well, it is necessary that the solution which passes through the cuvette be thoroughly mixed. This, unfortunately, is not always the case. If the filter used does not give even and horizontal fronts, the solution in the cuvette consequently becomes layered.

THE USE OF THE MIXER

In the large number of experiments performed it has been evident that the fringes, as a rule, become blurred preceding each component which is detected. The fringes become inclined and their upper portions progressively disappear until the entire fringes are obliterated. By screwing the compensator plates one can find the fringes again after several milliliters have passed. In most cases it is the upper portions of the fringes which first reappear. The fringes grow forth and become more clear until they are as clear as they were before the boundary came into the cuvette. Occasionally it happens that fringes can be seen at two different levels.

In an attempt to remedy this unsatisfactory condition, a mixer was made through which the solution passes before it enters the cuvette. The mixer consists of a metal rod, 7.5 cm long, through which a 1 mm capillary tube passes. The construction is indicated in Fig. 1. This mixer is placed between the lowest filter and the cuvette, and it is equipped with threads to fit these. The capillary is filled with quartz sand which is held in place by a little wad of cotton at each end. The volume of liquid in the capillary filled with sand is less than 0.05 ml. Because the mixer is made of metal the sample is easily brought to even temperature.

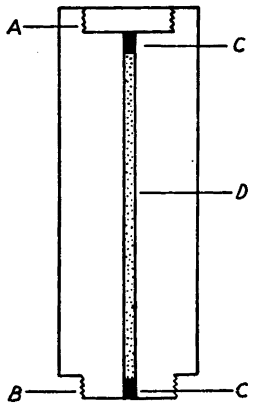


Fig. 1. Mixer.

A. Threads which fit the filter.
B. Threads which fit the cuvette.
C. Wad of cotton.
D. Capillary filled with quartz sand.

When the mixer is coupled into the system, the fringes are straight and clear as long as the solution's index of refraction remains constant. With an increase in refractive index, at least if the solution traverses the cuvette somewhat fast, the fringes bend sharply in one direction and with a decrease in refractive index they bend in the other direction. This is shown schematically in Fig. 2 where the center fringes' approximate appearance is shown throughout the experiment, placed directly over the corresponding points. The bending effect is caused by the solution streaming faster in the center of the cuvette capillary. By using the mixer one can, in most cases, follow the fringes during the entire experiment. It is only in extraordinary cases that the change in refractive index is so sudden that the fringes momentarily disappear.

When the mixer was used, the transitions between components were not found to be as sharp as expected. To ascertain the cause, several frontal analyses were performed in which the mixer was omitted but in which the eluate

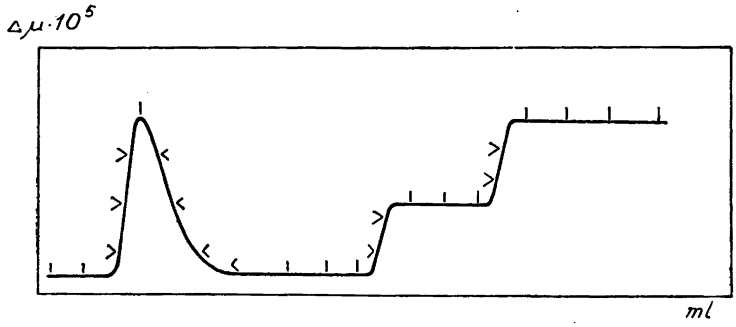


Fig. 2. The approximate appearance of the center fringes throughout the experiment. Drawn schematically.

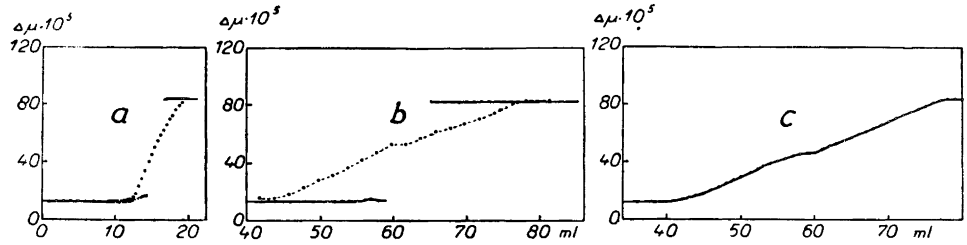


Fig. 3. Frontal analysis of 0.5 % sucrose in water.
Adsorbent: Carboraffin Supra.
Filter: a) 500 π, b) 2000 π, c) 2000 π + mixer.
a) and b) The interrupted lines show the same experiment according to the fractions taken during the experiment.

was divided into small fractions corresponding to the interferometric readings. At the close of the experiment the refractive index was measured on the fractions in the same cuvette, using the same filling of the comparison tube as was used during the experiment itself. The results are shown in Fig. 3a and 3b. The solid lines show the direct readings in the interferometer. The broken lines show the same experiment according to the fractions.

The results of the experiment shown in Fig. 3b are surprising, for the change of refractive index has proceeded for more than 15 ml, without movement of the fringes. They merely become blurred and obscured. The upper portions progressively disappeared, to be sure, but the rest of the fringes did not advance.

An experiment was performed with the same filter and the same solution as in Fig. 3b but with the difference that a mixer was placed between the filter and the cuvette. The curve for this experiment, shown in Fig. 3c, corresponds closely with the brokenlined curve in Fig. 3b, showing the course of the concentration change in the solution which actually comes out of the apparatus.

The horizontal portions on the broken lines in Fig. 3b and 3c (between 60—62 and 58—60 ml respectively) were caused by turning off the pressure approximately half a minute for replenishing the solution. It is then clearly important that the stream velocity be the same throughout the course of the experiment if the best possible results are to be obtained, and that turning off the pressure temporarily is disastrous, since one runs the risk of getting steps and irregularities which are artifacts.

The cause for the unsharp transition zones between the different components is the unevenness of the fronts in the column or the fact that these fronts often become inclined (not horizontal). This is due to the difficulty in

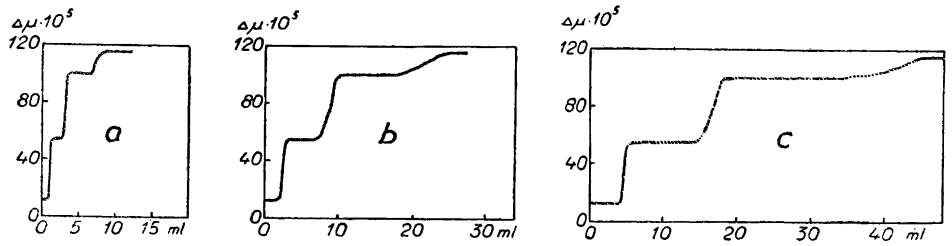


Fig. 4. Frontal analysis of (0.25 % glucose + 0.25 % sucrose + 0.25 % raffinose). Adsorbent: Carboraffin Supra. Filter: a) 100 π + mixer, b) 250 π + mixer, c) 500 π + mixer.

packing the adsorbent evenly — the direct obstacle in all chromatographic work, which is particularly disturbing when one wants to investigate adjacent zones closely, as in frontal and displacement analysis. The uneven adsorption front in the column nevertheless is usually quite sharp. When using the interferometer without mixer one tends to follow a system of fringes which corresponds only to one of the simultaneously streaming solutions in the channel.

The effect is most marked with the broad filters (*e. g.* 2000 π mm³, height 20 mm, diameter 20 mm) and is less pronounced with narrow filters where the fronts are relatively more even (see further below).

THE USE OF COUPLED FILTERS

Since it is impossible to pack an adsorption filter homogeneously, the solution's front always becomes more or less irregular. These irregularities increase with the size of the filters, but even such small filters as 500 π mm³ give quite poor fronts with large retention volumes. In Figs. 4a, b, and c some frontal analyses with three components are shown. It can be seen there that the lack of sharpness in the fronts increased partly with the size of the filter and partly with the increased retention volumes. The blurring of the fronts which increases with the retention volume cannot merely be due to poorer fronts in the filter itself. It must be due to the relationship of the components' slower movement and the consequently increased volume between the first appearance of the front at the filter's lower side and the saturation of the entire filter.

In an adsorption filter a substance in low concentration wanders more slowly than it does in high concentration. If one, therefore, presses a solution into a filter and afterward introduces a higher concentration of the same substance, the weaker front is overtaken by the stronger, if the filter is suffi-

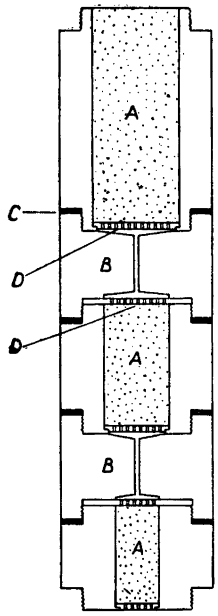


Fig. 5. Coupled filter.

A. Filters with adsorbent.
B. Constricted couplings.
C. Packing.
D. Perforated disks.

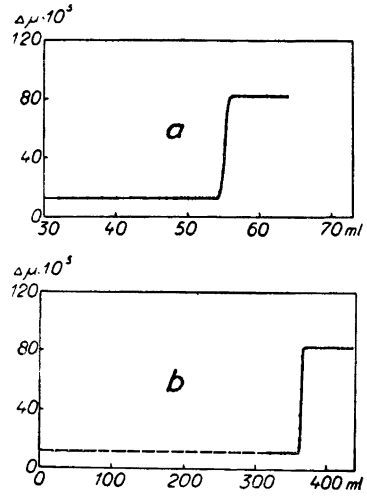


Fig. 6. Frontal analysis of 0.5 % sucrose. Adsorbent: Carboraffin Supra.
Filter: a) 875 π + 500 π + 250 π + 175 π + 100 π + mixer (total 1900 π).
b) 8000 π + 4000 π + 1250 π + mixer (total 13250 π).

ciently large. Then a single front is formed having the same concentration as the stronger solution's front.

When a frontal analysis is performed on a substance, it happens that solvent and solution emerge simultaneously when the uneven front emerges from the filter. By letting the emerging mixture pass through a capillary where it is mixed, and then by allowing the diluted solution to start on a new filter, a new front is formed. Because this corresponds to a weaker concentration and consequently moves more slowly, the chief front eventually overtakes the first and thus only one front is obtained. If the filter is smaller, and in addition has suitable proportions between height and diameter, a better front is formed. This can be repeated until one comes down to a filter which gives a satisfactory front.

An arrangement of this kind was introduced by Claesson [3] particularly for use with large amounts of adsorbent. The present author, independently,

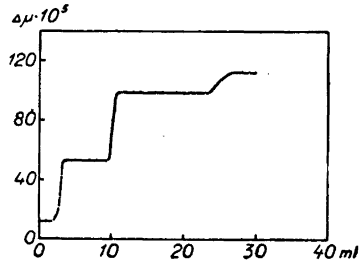


Fig. 7. Frontal analysis of (0.25 % glucose + 0.25 % sucrose + 0.25 % raffinose). Adsorbent: Carboraffin Supra. Filter: 175 π + 100 π + mixer.

observed the favorable influence on the evenness of adsorption fronts by introducing constricted couplings between successive filters with different adsorbents, used in group separations of amino acids [4]. It was also found that this type of filter coupling is very valuable for straightening out fronts in adsorption analysis with quantities commonly used for analytical separations.

In Fig. 5 is given an example of a coupled filter which has been found suitable. The important feature to be noted is the special type of coupling. This consists simply of metal rods with threads and depressions in the form of two flat cones whose points are joined by a small canal. (The depression volume in the coupling pieces ought to be as small as possible.)

Any number of filters can be coupled together without harming the fronts if one only takes care that the filters do not increase in size faster than the chief front can overtake the weaker front in each filter it traverses. If it is found advantageous, filters of the same size can be coupled together under each other. Therefore one is not bound to certain filter sizes but can choose those which seem most suitable.

In Figs. 6a and 6b two examples are shown of frontal analysis with one component, where such coupled filters were used. If, instead, all these filters had been coupled together to one continuous filter, a front only 2 ml broad would have been obtained in a retention volume of about 415 ml. But then a considerable increase in the duration of the experiment should be expected as the final filter, because of too small a diameter, would limit the rate of flow.

Frontal analysis with several components gives similar satisfactory results (compare Fig. 7 with Fig. 4b). Even with displacement analysis coupled filters give the same excellent results with several components as with one. (Fig. 8, a, b, c.) In Fig. 8c the free component's front was so sharp that the fringes temporarily disappeared. However, the falling concentration on the free component's back side could be read.

In Fig. 8d is shown an experiment performed on a 500 π mm³ filter with the same solution and displacer as in the experiment 8c. To permit a strict comparison of the two experiments even the same stream velocity was used.

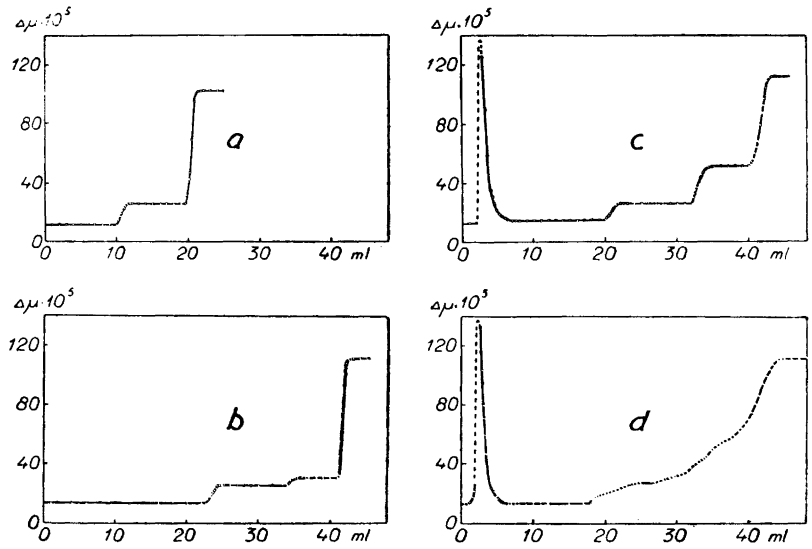


Fig. 8. Displacement development of some sugars.
Displaced substance:
a) 1.5 ml 0.5 % sucrose.
b) 2 ml (0.5 % sucrose + 0.5 % maltose).
c) and d) 1 ml (1 % glucose + 1 % sucrose + 3 % raffinose).
Developer: 0.5 % ephedrine.
Adsorbent: Carboraffin Supra.
Filter: a) 175 π + 100 π + mixer.
b) and c) 250 π + 175 π + 100 π + mixer.
d) 500 π + mixer.

The free component in this case is also very sharp, but the two other components and the displacer have such poor fronts that in the diagram it is seen that they practically form a single slowly rising front.

In elution analysis sharper fronts are obtained than previously, but the components back sides may be somewhat heightened. Since most components displace each other to a certain extent, the separation in elution experiments

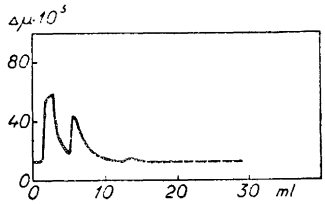


Fig. 9. Elution analysis of 2 ml (1/3 % alanine + 1/3 % valine + 1/3 % leucine) Eluent: Water. Adsorbent: Carboraffin Supra. Filter: 500 π + 250 π + 100 π + 50 π + mixer.

may be better than judged from the diagrams. Also in this case the sharpening effect of the coupled filters improves the separation.

In all the experiments described here the filters have been packed with wet charcoal. The charcoal is mixed with water and the charcoal suspension transferred to the filter by a broad-tipped pipette. During the filling of the filter excess water is sucked away by vacuum from below the filter. During the entire procedure one must be careful that the charcoal is never sucked dry so that the charcoal forms cracks. Enough charcoal is added so that a small heap is formed on top of the filter. This is then pressed into the filter with a spatula until it is filled with charcoal of a sufficiently stiff consistency. Then the excess charcoal is scraped away and the surface is made as even as possible with a spatula. This pressing of the charcoal filling is done so that it will not sink during the experiment. In experiments using coupled filters pockets must be avoided as much as possible for these can bring about unnecessarily poor results.

Through this wet packing better fronts are obtained and air is avoided in the filter. All the filters can be screwed together and washed at the same time. With dry packing each filter must be washed separately and until all air disappears before it may be screwed to another filter, for otherwise air may remain in the couplings between the various filters. These must be completely filled with liquid for best performance.

Filters with the following internal dimensions have been used.

Diam. (mm)	20	20	20	10	10	10	7.1	7.1	5	3.6
Height (mm)	80	40	20	50	35	20	20	14	16	15.5
Volume (π mm^3)	8000	4000	2000	1250	875	500	250	175	100	50

The above filters should not be considered as optimum ones for experimental work. They merely represent those models which were in use here at the time this work was under way. One of them, the 2000 π filter, is actually very poor. If possible it is better to use longer and narrower filters, but when coupled filters are used this is not so important.

SUMMARY

The advantages of using the mixer described for interferometric experiments are that the fringes can be followed during the entire experiment and that consequently the results obtained are more truly consistent than those formerly obtainable.

Through the use of coupled filters with constriction pieces, a considerable improvement of fronts in adsorption analysis is obtained, both with small and large amounts of adsorbents. The coupled filters are of advantage in all types of adsorption analysis — frontal analysis, elution analysis, and displacement development.

I wish to express my gratitude to Professor Arne Tiselius for the encouraging interest he has taken in this work, and for his helpful discussions concerning this material.

Also, I sincerely wish to thank Dr Ralph Holman for the English translation of this article.

This work is part of a research program on adsorption analysis which is financially supported by a grant to the Institute of Biochemistry from *Naturvetenskapliga forskningsrådet.*

LITERATURE

1. Tiselius, A., and Claesson, S. *Arkiv Kemi, Mineral. Geol.* **B 15** (1942) no. 18.
2. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **A 23** (1946) no. 1.
3. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **A 24** (1947) no. 16.
4. Tiselius, A., Drake, B., and Hagdahl, L. *Experientia* III/1, 651 (1947).

Received July 7, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 583—591

Potentiometric Titration in Two-phase Systems

Determination of Bitter Acids in Hops

INGVAR HEDLUND and MAJ STENINGER

Forskningslaboratoriet LKB, Äppelviken, and AB Stockholms Bryggerier, Stockholm, Sweden

If an aqueous buffer solution is shaken with a water immescible solvent, which is able to extract the free acid, the pH of the solution increases. This increase, determined with a glass electrode, is shown in the following table for some buffer solutions of acids, neutralized to 50 % with sodium hydroxide.

The buffer solution, 20 ml, was shaken with 5 ml of organic solvent, the pH was taken before the procedure and after the separation of the solvent. The separation was not always complete, a light fog could sometimes be observed in the water solution. The glass electrode gives less reliable results in emulsions, hence the small increases, some hundredths in pH, with acetic and propionic acid.

Table 1. Increases in pH by the extraction of buffer solutions.

Acid	Solvent						
	1	2	3	4	5	6	7
0.02 *M* Acetic	0.04	0.04	0.20	0.02	0.06	0.02	0.02
0.02 *M* Propionic	0.05	0.00	0.27	0.00	0.00	0.00	0.00
0.02 *M* *iso*-Valeric	0.04	0.03	0.81	0.10	0.30	0.11	0.03
0.01 *M* Caprylic	1.25	—	2.40	1.61	1.86	1.49	1.35

The solvents are: 1 — n-C_6H_{14}, 2 — Petroleum ether b. p. 45—55° C, 3 — Ether, 4 — C_6H_6, 5 — $CHCl_3$, 6 — CCl_4, 7 — CS_2.

The procedure is a suitable expedient for selecting solvents for extraction, but it can also be used for analytical purposes. Observation of the changes in pH during the extraction of an acid might furthermore be very helpful in

preparative and manufacturing practise in order to get the best possible separation as will be shown in regard to bitter acids in hops. (Quoted by Lundin [1]).

THEORY

The theory for the distribution of a substance between immiscible liquids has long been common knowledge [2]. Below follows an application of the theory to a case where an acid is successively neutralized in a two-phase water-oil system with continous determination of the pH.

In the water solution of a partially neutralized acid the pH is determined by the relation

$$\mathrm{pH} = \mathrm{p}k + \log C_{\mathrm{salt}} - \log C_{\mathrm{acid}}$$

where C_{salt} and C_{acid} represent the concentrations of salt and acid respectively. If such a water solution is shaken with a solvent wherein the acid but not the salt is soluble, the factor C_{acid} decreases to a degree determined by the distribution coefficient of the acid and the ratio between the volumes of the water and oil phases. This decrease in the concentration of free acid causes a raise in the pH.

Assuming that the distribution coefficient f is independent of the concentration — a condition seldom valid for acids, but might be accepted as practical in routine work — and if C_{oil} and C_{water} designate concentrations of free acid in an oil phase of volume o, and in a water phase of volume v respectively, and if equilibrium has been established, this equation holds:

$$C_{\mathrm{oil}} = f \cdot C_{\mathrm{water}} \text{ and } v \cdot C_{\mathrm{acid}} = v \cdot C_{\mathrm{water}} + o \cdot C_{\mathrm{oil}}$$

$$\text{or } v \cdot C_{\mathrm{acid}} = (v + o \cdot f)\ C_{\mathrm{water}}$$

From this latter relation and the simultaneously valid equation:

$$\mathrm{pH} = \mathrm{p}k + \log C_{\mathrm{salt}} - \log C_{\mathrm{water}} \tag{1}$$

we get

$$\mathrm{pH} = \mathrm{p}k + \log C_{\mathrm{salt}} - \log C_{\mathrm{acid}} + \log \left(\frac{o}{v} \cdot f + 1\right) \tag{2}$$

In this formula applied to a two-phase system, C_{acid} thus denotes the concentration which the free acid should have had if the oil phase had not been present.

The formula implies that the titration curve in a two-phase system is constantly being displaced towards a higher pH by a factor that depends partly on the volume ratio of the phases, and partly on the value of f, a coefficient characteristic of the acid.

For acids, that mainly go into the oil phase, the last term in equation (2) can without major error be written:

$$\log \left(\frac{o}{v} \cdot f + 1\right) = \log \frac{o}{v} + \log f$$

If we introduce m for the total amount of non-neutralized acid, *i. e.* $v \cdot C_{\text{acid}}$, the equation becomes:

$$\text{pH} = \text{p}k + \log f + \log C_{\text{salt}} - \log m + \log o \qquad (3)$$

But as a rule the coefficient f is unfortunately not independent of the concentration. In organic solvent acids are often associated into double molecules. The equilibrium between oil and water phases can then approximately be represented by the relation:

$$C_{\text{oil}} = \psi^2 \cdot (C_{\text{water}})^2 \text{ where } \psi \text{ is a constant.} \qquad (4)$$

For the total amount of non-neutralized acid we get the expression:

$$v \cdot C_{\text{acid}} = o \cdot \psi^2 \cdot (C_{\text{water}})^2 + v \cdot C_{\text{water}}$$

When the oil-solubility of the acid prevails strongly the last term in this analogy may be omitted and from equation (1) we obtain:

$$\text{pH} = \text{p}k + \log \psi + \log C_{\text{salt}} - \tfrac{1}{2} \log m + \tfrac{1}{2} \log o \qquad (5)$$

The pH curve of a two-phase titration in such a case becomes somewhat flatter than without association into double molecules, but it still is on a higher level than the corresponding curve for neutralization in pure water. With increasing volume of the oil phase the pH of the buffer increases to a smaller degree for associating than for non-associating molecules.

The values of $\log f$ or $\log \psi$ can be calculated from the pH increase on the addition of an oil phase to a partially neutralized solution of acids, whose dissociation constants in water are known. But it is more convenient to merge $\text{p}k$ and $\log f$, $\text{p}k$ and $\log \psi$ respectively into a new term, especially as the distri-

bution coefficients often are unknown. For nonassociating acids the term »apparent pk» is suggested, denoted ap$k$. From equation (3) we then derive:

$$\mathrm{pH} = \mathrm{ap}k + \log C_{\mathrm{salt}} - \log m + \log o \qquad (6)$$

It is easy to see that ap$k$ = the pH of a semineutralized solution when water and oil phases are of equal volume.

This simple definition cannot be used for associating acids, because in that case the pH of a semineutralized solution depends on the absolute concentrations as well. We introduce the term »normal pk» = np$k$.

According to equation (5) we then get:

$$\mathrm{pH} = \mathrm{np}k + \log C_{\mathrm{salt}} - \tfrac{1}{2} \log m + \tfrac{1}{2} \log o \qquad (7)$$

Here np$k$ denotes the pH in a system where the water phase is 1 N as regards salt, and the oil phase is 1 N as regards acid. Deviations caused by activity coefficints have not been taken into consideration.

EXPERIMENTAL TECHNIQUE

The two-phase titration can be used for analytical characterization of an acid. It is then sufficient to study the change in the pH when an oil phase is added to a partially neutralized water solution. Mixed acids can be separated even if they have identical dissociation constants in water under the premise that their distribution coefficients differ rather widely.

Two different techniques can be used for the titration: either the volume of the water phase is kept constant by titrating with rather concentrated alkali, which gives a curve, entirely (equation 6) or closely (equation 7) resembling ordinary titration curves, or otherwise a standard alkaline solution is added to *e. g.* a petroleum ether solution of one acid or a mixture of acids. If only one acid is present C_{salt} when using the second method, becomes constant and a very flat curve results. The only variable is then log m. With either method ap$k$ and np$k$ can easily be determined.

When using the second technique for a mixture of acids, it is advisable to draw away the water solution before the next portion of alkali is added. If the portions are collected separately when the pH tends to change rapidly, a separation of the acids is attained.

Fig. 1 is a sketch of the apparatus used for the two-phase titration.

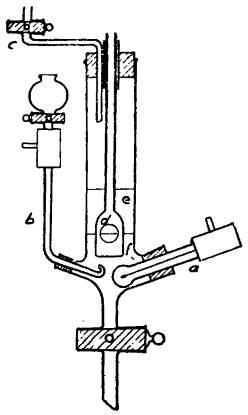


Fig. 1. Apparatus for two-phase titration. a = glass electrode, b = reference electrode, c = burette, d = centrifugal stirrer, e = oil phase, f = water phase.

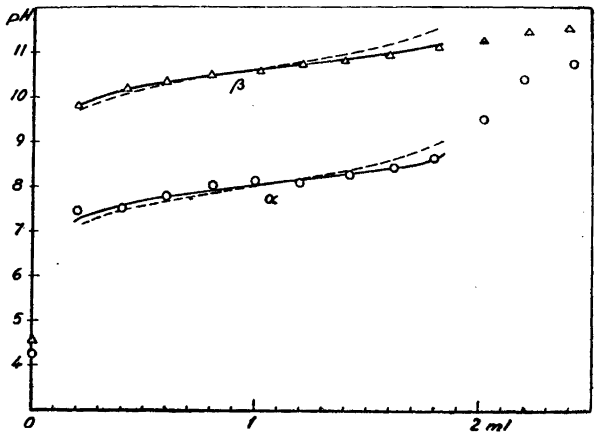


Fig. 2. Two-phase titration in water — petroleum ether on 1 millimole of α-resp. β-acid, with 0.49 N NaOH.

EXPERIMENTS WITH BITTER ACIDS IN HOPS

Hops contain several bittering substances or bitter acids, two of them, humulon and lupulon, also called α-acid and β-acid, have known chemical structures [3, 4] and are soluble in hexane. As possible degradation products from these, hops also contains so-called soft resins and hard resins. The former are soluble in hexane, the latter are not. Humulon is easily determined as an insoluble lead salt [5–7], for lupulon, on the contrary, no specific reagent is known and it is usually determined together with the soft resin.

In methanol solution humulon and lupulon have practically the same acid strength ($pk = 5.5$). The solubility in water is very low. If a solution of sodium salts of humulon or lupulon is titrated with hydrochloric acid an emulsion is formed. Windisch, Kolbach and Winter [8] found that such emulsions give neutralization curves, which are the higher situated in the pH scale the higher the concentration. The pH values were higher with lupulon than with humulon. A mixture of these two bitter acids gave a neutralization curve permitting at best a very rough estimate of the two components.

The performance of neutralization in two-phase systems gives more accurate results.

As a rule the titrations have been performed in the following way: 10 ml of a petroleum ether solution of the bitter substance is introduced into the titration vessel. About 9 ml CO_2-free water is added, and the phases are brought to equilibrium through vivid stirring for 2 min. The stirrer is stopped and the pH of the water layer is measured with the glass electrode. Small portions of 0.50 *N* alkali are added, every portion followed by 2 min stirring and the pH is measured when the phases have separated. Now and then emulsions appear, especially at higher pH; they often need up to 30 minutes to separate. With pure bitter-acids however the titrations can usually be completed in about 2 hours. The amount of bitter-acid was chosen to give a consumption of about 2 ml of titration liquid, which gave petroleum ether- and water-phases of equal volume when the acid was half-neutralized.

Fig. 2 shows the result of a two-phase titration on 1 millimole α-acid and 1 millimole β-acid respectively with 0.49 *N* NaOH, both systems consisting of 10 ml petroleum ether and about 10 ml water.

The points mark the results of the measurements. The lined curves have been calculated according to equation (7) with $npk = 8.70$ for α-acid and 11.27 for β-acid. The dashed curves have been calculated according to equation (6) with $apk = 8.03$ for α-acid and 10.64 for β-acid. There is no great difference between the curves, but the points fit closely to the lined curve. It is obvious that equation (7) is valid for this two phase system, which means that the bitter-acids are associated to double molecules in the petroleum ether phase. The attention is drawn to the great difference between the two acids, which must mainly depend on different distribution constants. In alcohol *e. g.* the acids are of practically the same strength. As is shown by the bottom curve it is easy to determine the end point of the α-acid titration but more difficult to do so for the β-acid, where the pH is very high. At such a high pH an excess of alkali causes but a slight change of the pH. Besides, the glass electrode exhibits a certain inertia at these higher values of pH.

The association to double molecules appears also in another experiment. One millimole of α-acid, neutralized to 50 % in 10 ml water + 10 ml petroleum ether had a pH = 8.03. A fivefold increase of the petroleum ether phase should — according to equation (7) — increase the pH by 0.35 units ($\frac{1}{2}$ log 5), while for simple molecules — according to equation (6) — the increase should be 0.70 units. Experimentally an increase in the pH-value of 0.28 units was measured. On 27-fold dilution of the petroleum ether the increase was 0.75 units, as compared with 0.72 units which had been calculated for double molecules. A fourfold dilution of the water phase resulted for the sodium salt of the α-acid in a decrease of 0.54 pH-units (0.60 calc.); thus the salt shows a tolerably classical behaviour. Corresponding experiments with β-acid gave

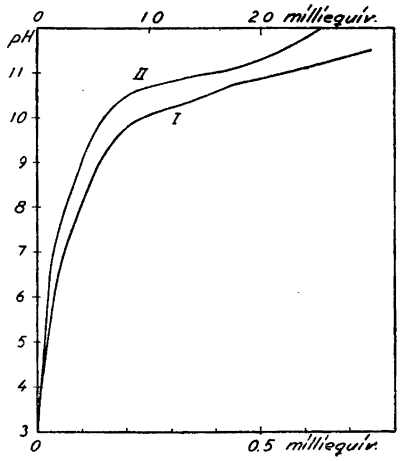


Fig. 3. Two-phase titration (diagram I) and fractionated alkaline extraction (diagram II) of the β-fraction of lupuline in petroleum ether solution.

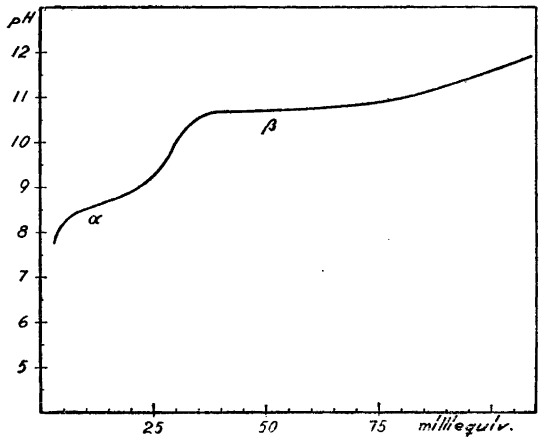


Fig. 4. Two-phase titation of a β-fraction contaminated by some α-acid.

somewhat uncertain results, which is explainable considering the inertia of the glass electrode at higher pH values. But everything indicates that even in this case an association to double molecules occurs.

The two diagrams in Fig. 3 refer to the same preparation of lupulon from lupuline. After precipitation of the α-acid from methanol solution with lead acetate in the usual manner, the solution was diluted with water, acidified with hydrochloric acid and extracted with petroleum ether. We titrated 10 ml of the petroleum ether solution, containing 0.26 g of bitter substances, with 0.50 *N* NaOH in a two-phase system containing 10 ml water, and the diagram I in fig. 3 was obtained. Diagram II shows the results of pH measurements on repeated extractions with 10 ml portions of 0.1 *N* NaOH of 400 ml of the same petroleum ether solution. The scales of the abscisses have been chosen in accordance with the total contents of bitter-acids in the solutions and are given at the bottom resp. at the top of the figure as added milliequivalents of alkali. The deviation in pH between the two diagrams corresponds to the differing concentrations and volumes in the two experiments. Each water phase of 10 ml in the experiment referred to in diagram II was acidified and extracted after the pH-measurement. The extracts were evaporated and dried in vacuum, and the residues of bitter-acids were compared. From the alkaline extracts within the pH-range 10.6—11.8 well crystallized β-acid was obtained. At higher and at lower pH-values we mainly obtained a noncrystalline product

besides a small amount of crystallized β-acid. Up to pH 9 mostly soft resins were extracted, possibly contaminated with such α-acid as had been incompletely precipitated by lead acetate. In accordance with the non-crystallization of the products extracted at a lower pH-value, the two-phase titration of the oxidation products from pure α- and β-acids gave values of pH corresponding to the ap$k$:s of the resins from 4 up to 7 or 8. In these cases most curves had no distinct platforms, proving that the products were not homogenous.

An approximate estimation of soft resins in such a β-fraction as is obtained with hitherto prevalent methods of analysis should be rendered possible by a two-phase titration in water-petroleum ether. The alkali needed to reach a pH value of about 8.5 would then correspond to the amount of soft resins. Gravimetrically the analysis could be performed in a simpler manner: by extraction with a buffer of about pH 8.5 which is then acidified and extracted with ether. For comparatively fresh lupuline this latter method seems to give rather reliable results. But as soon as the bitter acids have been exposed to more serious destruction, the values become less reliable.

In preparations of β-acid from lupuline the extraction with small portions of alkali under continuous control of the pH value has been very useful. In the pH-range 10.5—11.7 we have got a comparatively pure β-bitter acid. The acid has been further purified by recrystallization, first from petroleum ether and then from 90 % methanol.

Another example of how the fractionation of an alkaline extract can be surveyed by continuous measurement of the pH value is given in Fig. 4. The diagram represents a preparation from lupuline from which the α-acid, because of incomplete precipitation by lead acetate, had not been entirely removed. This α-acid is revealed by the step at pH 8.5.

This fractionation can be regarded as a development of the method of Windisch, Kolbach and Winter [9], where the acid is purified by successive extractions with ammonium carbonate, sodium carbonate and potassium hydroxide.

How solvents, other than petroleum ether, influence the pH value in a two-phase system, has only been examined in some preliminary experiments. For β-acid we got the following ap$k$-values: in hexane 10.4, in carbon tetrachloride 10.3, in ether 11.1 and in chloroform 12.3. The ap$k$-value of 10.6 in petroleum ether (b. p. 45—55° C) has already been mentioned. Chloroform thus extracts β-acid a hundred times better than do both hexane and petroleum ether.

SUMMARY

Acids, slightly soluble in water, can be titrated with an alkali and characteristic titration curves obtained, provided that the titration is performed in a two-phase system consisting of water and an organic solvent able to dissolve the acids. The distribution equilibrium of the acids must be attained before the determination of the pH.

The composition of lupuline from hops is studied by this method and it is shown that the analytical determination of lupulon is possible by titration in a water-petroleum ether system.

In the extraction of acids from an organic solvent with small successive portions of an alkaline water solution different acids can sometimes be separated. In this way a fairly good separation of the bitter acids and soft resins in hops can be achieved.

This work was performed in 1945. We wish to express our thanks to Professor H. Lundin, to Mr. E. Sandegren at *AB Stockholms Bryggerier* (Stockholm's Brewery Company) and to Dr. S. Brohult, head of *Forskningslaboratoriet LKB* (LKB Research Laboratory) for their stimulating interest.

REFERENCES

1. Lundin, H. *European brewery convention* (1947) p. 49.
2. A comprehensive study is to be found *e. g.* in Taylor, Hugh, S. *A treatise of physical chemistry* New York (1930). See also: Smith, H. W., and White, T. A. *J. Phys. Chem.* **33** (1929) 1953; Moelwyn-Hughes, E. A. *J. Chem. Soc.* (1940) 850.
3. Wöllmer, W. *Ber.* **49** (1916) 780, **58** (1925) 672.
4. Wieland, H. *Ber.* **58** (1925) 102, 2012, **59** (1926) 2352.
5. Siller, R., *Untersuch. Nahr. u. Genussm.* **18** (1909) 241.
6. Wöllmer, W. *Woch.schr. Brau* **46** (1929) 121.
7. Windisch, W., Kolbach, P., and Winter, M. *Ibid.* **46** (1929) 101.
8. Windisch, W., Kolbach, P., and Winter, M. *Ibid.* **46** (1929) 124.
9. Windisch, W., Kolbach, P., and Winter, M. *Ibid.* **46** (1929) 102.

Received July 9, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 592—596

A Technique for Partition Chromatography on Starch

PEHR EDMAN

Department of Physiological Chemistry, University of Lund, Lund, Sweden

In the last years the principle of counter current liquid-liquid distribution has gained interest because of the realisation of its potential high capacity in the resolution of mixtures of substances with differing distribution coefficients in a pair of immiscible solvents. The principle has been employed methodically along two somewhat different lines. In the method of Craig [1, 2] the resolution of the mixture is achieved through systematic, consecutive distributions of the substances to be resolved between two immiscible solvents. The essential feature in the method of Martin, Synge [3] and collaborators is that one of the solvent phases is finely dispersed on a supporting medium and in that way immobilized whereas the other phase flows in intimate contact over the immobile phase. The immobile phase is usually water and as a support silica gel, cellulose or starch have been used. The partition chromatography, as the latter method has been called, has an exceedingly high resolving capacity. It can be performed in a column essentially in the same way as an ordinary chromatographic procedure.

Potato starch has proved to be a superior support for the immobile phase [4, 5]. A difficulty, however, arises because of the slow rate of flow of liquid through a potato starch column with the consequence that one fractionation may take several days. This makes an automatic collector of the effluents almost indispensable. In this paper is described a technique for starch chromatography which has been used successfully for a long time.

EXPERIMENTAL

Pretreatment of the starch. Commercial potato starch contains contaminants which must be removed before the starch can be used for a chromatogram. Unless this is done disturbances will appear in the development of the chromatogram. The nature of these impurities is unknown except that they are

water soluble and can be removed by means of thorough washings of the starch with water. The practice has been to stir up the starch in five volumes of water, let it settle and decant the water. This process is repeated four times with tap water and finally two times with distilled water. The starch is sucked as dry as possible on a Buchner funnel and then spread and dried in air. The lumps of dry starch are broken up and the powder passed through a sieve, mesh 20/cm. For many purposes this treatment is sufficient. In certain instances, however, it was found desirable to defat the starch and to remove material absorbing in the ultraviolet. This was accomplished through extraction of the starch in a Soxhlet apparatus for 24 hours with dioxane or methyl alcohol containing 20 % water. Afterwards the organic solvent was removed from the starch by means of repeated washings with water and the starch then treated in the way already described. It is advisable to work up as large a batch as possible since there may be individual variations in properties between different batches. When the starch is stored in closed glass containers no changes in properties have been observed. One batch has been used with reproducible results for more than two years.

Preparation of the column. A suitable tube for the column can be made from a Buchner funnel with a sintered glass filter to which is sealed a glass tubing of the same diameter as the funnel so that the height of the funnel will be about 40 cm from the filter to the upper rim. A funnel made of American Pyrex glass with a plane filter of porositles C or M has been found most suitable.

The starch is dispersed in two volumes of the water-saturated solvent which will later be used for the development of the chromatogram and immediately transferred to the tube. The tube is inverted carefully several times to ensure an even dispersion of the starch in the solvent. Care should be taken that no air bubbles are introduced into the slurry. The tube is then placed in exactly the vertical position and the starch left to settle. Fresh solvent is constantly siphoned into the tube by means of the arrangement seen in Fig. 1. After 4—5 hours the starch column will have attained its final height and should present as mooth and rather firm upper surface. It is essential that this period is not shortened since the starch requires time to attain equilibrium with the water-saturated solvent. The period given has been found minimal when *n*-butanol is used as a solvent.

The chromatographic procedure. The solvent above the starch is now left to drain. At the moment when the solvent meniscus disappears into the starch the solution of the sample to be analyzed is introduced on the top of the column, care being taken not to disturb the surface of the starch. The sample should preferably be dissolved in the smallest possible volume of the water-

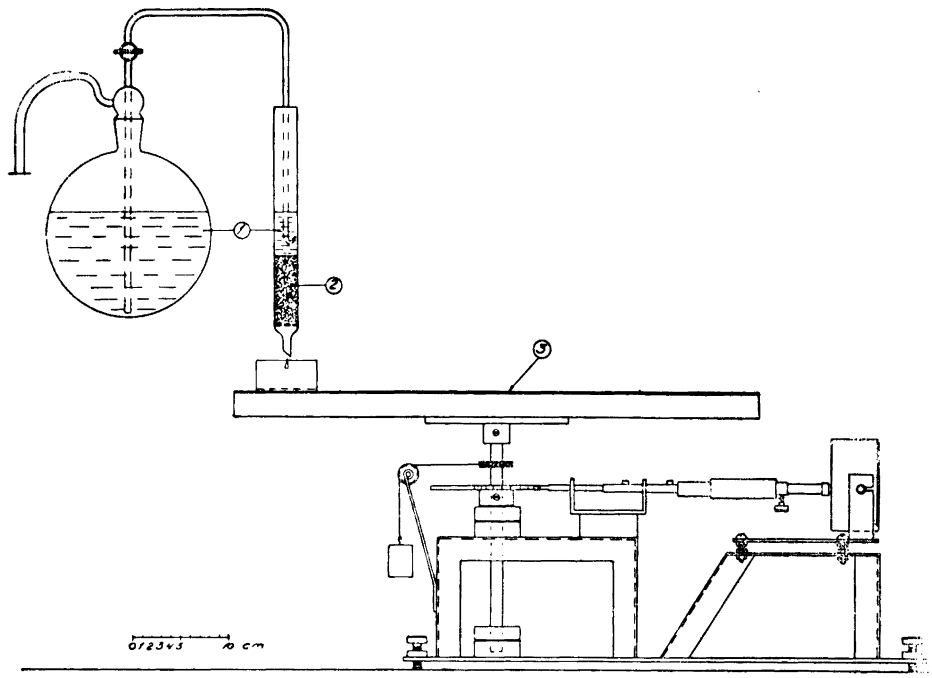


Fig. 1. Assembled chromatographic setup. 1. Solvent. 2. Potato starch. 3. Disk supporting the vessels.

saturated solvent since a small initial zone width is desirable. After the sample has drained into the column it is followed by an equal volume of the same solvent which is also left to drain into the column. Thereafter solvent is siphoned from the large storage bottle (Fig. 1) to the column. The rate of flow through the column is adjusted by raising or lowering the level of the liquid above the starch. By means of this arrangement it is easy to keep the flow of solvent at a fairly constant rate throughout a whole fractionation.

The rate of flow of the solvent requires some further consideration. In several cases investigated, the zones have been found to move at a rate which is directly proportional to the flowrate of the solvent. This relationship held true up to the highest rate of flow employed namely 4 ml per hour per cm^2. The sharpness of the zones were not appreciably impaired at this rate of flow. It is conceivable, however, that the maximal rate of flow in other instances may be lower.

In principle the same solvents as those used in paper chromatography can be employed in starch chromatography although for various reasons some of them are less suitable. Their use, however, in starch chromatography requires

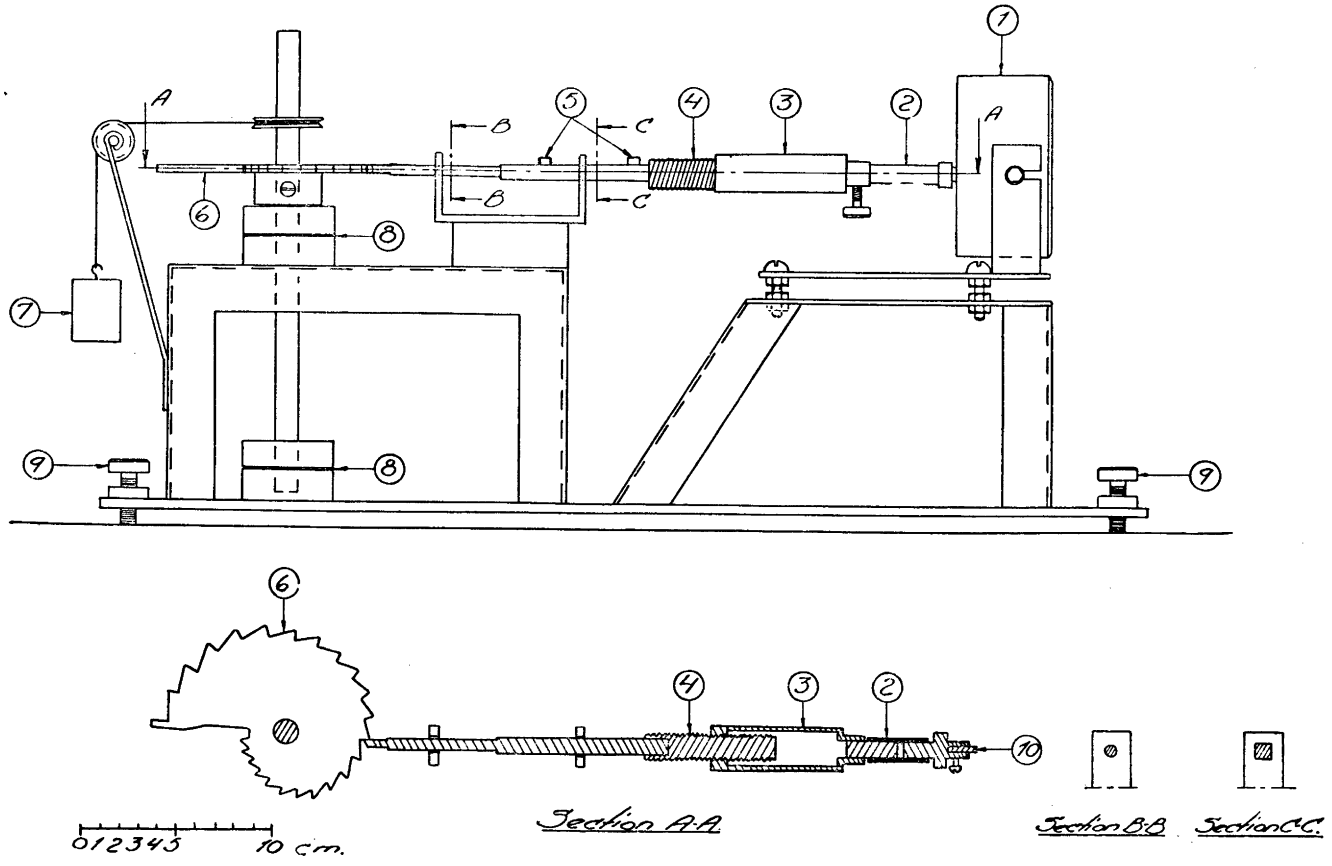


Fig. 2. Automatic collector (except the disk). 1. Clock work. 2. Rubber tubing. 3. Nut. 4. Screws. 5. Stop screws. 6. Notched wheel. 7. Weight. 8. Ball bearing. 9. Levelling screw 10. Axis of the clock.

a more rigorous purification. It is preferable not to saturate the organic solvent completely with water in order to avoid the formation of emulsions.

The automatic collector. This machine (Figs. 1 and 2) shifts the receiving vessel at the regular time interval of one hour and it is designed to collect 24 samples *. The operation of the machine is regulated by a clock work (1 in Fig. 2). To the axis of the clock work is attached a nut (3). The nut turns on a screw (4) connected to a rod. In this way the rod is made to retract from the notches of a spiralformed wheel (6). Every time a notch is released the disk (3 in Fig. 1) supporting the receiving vessels will move 1/24 of a complete revolution bringing a new receiver under the column. The disk should be made from a material which will not warp and should be covered with a rubber mat to prevent the receivers from sliding. It is essential that the levelling screws (9 in Fig. 2) are so aligned that the disk is exactly in the horisontal plane. The position of the clock work should be so aligned that its axis (10) will coincide as close as possible with the axis of the rod although the soft rubber connection (2) will take care of any strain resulting from a small deviation. All moving parts should be freely greased. The apparatus, once started, requires no attention for 24 hours.

As receiving vessels low beakers have been found very convenient. When a fractionation is finished the beakers with their contents are transferred to a vacuum oven and the solvent evaporated at a temperature of around 40° C. For subsequent analyses the dry residues are dissolved in a suitable medium.

The technique described here has been employed successfully for the separation of peptides [6], purines [7], and nucleosides [8].

SUMMARY

A technique is given for the performance of partition chromatography on starch. The pretreatment of the starch, the preparation of the column, the chromatographic procedure and an automatic collector of the effluents are described.

LITERATURE

1. Craig, L. C. *J. Biol. Chem.* **155** (1944) 519.
2. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E. *J. Biol. Chem.* **161** (1945) 321.
3. Martin, A. J. P., and Synge, R. L. M. *Biochem. J.* **35** (1941) 1358.
4. Elsden, S. R. and Synge, R. L. M. *Biochem. J.* **38** (1944) ix.
5. Synge, R. L. M. *Biochem. J.* **38** (1944) 285.
6. Edman, P. *Acta Chem. Scand.* **1** (1947) 684.
7. Edman, P., Hammarsten, E., Löw, B., and Reichard, P. *J. Biol. Chem.* In press.
8. Reichard, P. *Nature* **162** (1948) 662.

Received July 21, 1948.

* The apparatus is manufactured by Lindmark & Holm, Nybrogatan 76, Stockholm.

ACTA CHEMICA SCANDINAVICA 2 (1948) 597—610

Über eine Totalsynthese der Camphersäure resp. des Camphers

N. J. TOIVONEN, SALLI NIININEN (TOMMILA), SALLI ESKOLA, SYLVI LÄNG (LOUKAMO), ELLI TURUNEN und AARNE TUHKANEN

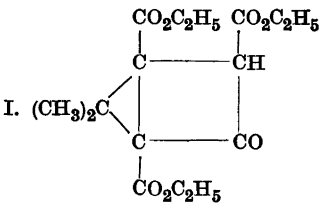
Chemisches Institut der Universität Helsinki, Finnland

Über die hier zu beschreibende Camphersäuresynthese ist schon vor längerer Zeit in einem anderen Zusammenhang [1] ganz kurz mitgeteilt worden. Vor einer näheren Beschreibung war es aber verlockend, über die Konfiguration der in die Synthese eingehenden 5-Oxycamphersäuren auch in Bezug auf die sterische Stellung der Hydroxylgruppe ins Klare zu kommen. Dies erwies sich indessen als eine ziemlich komplizierte Aufgabe, weshalb das Veröffentlichen der Untersuchung aufgeschoben wurde. Da wir kürzlich [2, 3] einige von diesen Oxysäuren auf einem ganz anderen Wege dargestellt haben — und zwar mit der ausdrücklichen Absicht, ihre Konfigurationen sowie dadurch auch diejenigen des Borneols und Isoborneols aufzuklären — soll diesmal nur der eigentlich synthetische Teil der früheren Untersuchungen wiedergegeben werden.

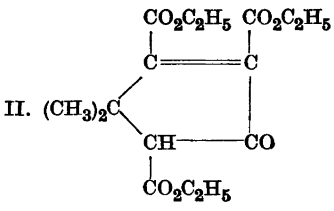
Um ein Bild über den ganzen Verlauf der Synthese zu geben, muss man zuerst die Darstellung und Konstitution einiger von Perkin jun., Thorpe und Walker [4] synthetisierten, von ihnen als Bicyclopentanderivate angesehenen Verbindungen kurz beschreiben.

Die genannten Forscher behandelten den α,α'-Dibrom-β,β-dimethylglutarsäurediäthylester mit überschüssigem Natriummalonsäureester und Natriumäthylat in Alkohollösung und erhielten in guter Ausbeute das gelbe Natriumsalz eines Ketotricarbonsäureesters (A), dem sie die Formel I gaben.

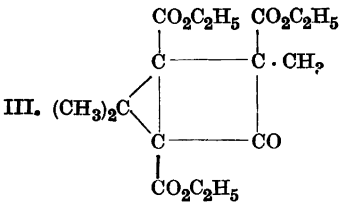
A. I.

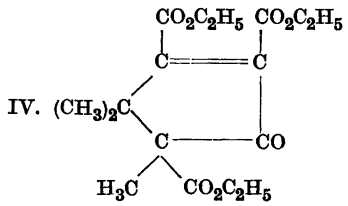


II.



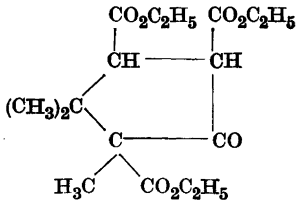
Durch Behandeln dieses Natriumsalzes mit Methyljodid erhielten dieselben Autoren ein Methylierungsprodukt (B), dem sie, sowie auch einige spätere Forscher [5], die bicyclische Formel III zuschrieben.

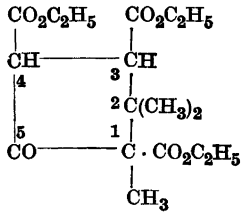
B. III. 

IV. 

Dass dieses Methylierungsprodukt jedoch mindestens zum allergrössten Teil aus der monocyclisch gebauten Verbindung IV besteht — was seinerseits auch für die monocyclische Formel II des dem gelben Natriumsalz entsprechenden Esters spricht — wurde von uns in der schon oben erwähnten Abhandlung [1] auf mehrere Weisen bewiesen. Einer dieser Beweise bestand eben darin, dass man das Methylierungsprodukt durch die im folgenden näher zu beschreibenden Operationen in Camphersäure überführen kann.

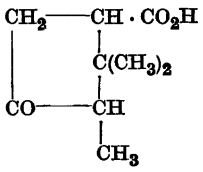
Wie schon früher [6] gefunden worden war, werden alle Glieder der obigen Verbindungsreihe durch Zinkstaub und Eisessig glatt zu gesättigten Cyclopentanoncarbonsäuren bzw. deren Ester reduziert, so auch das Methylierungsprodukt B (IV) zur Verbindung V. Die nahe Beziehung dieser Verbindung zur Camphersäure tritt durch die anders (rechts) geschriebene Formel, wonach man sie als *4-Carbäthoxy-5-ketocamphersäurediäthylester* bezeichnen kann, klar zutage.

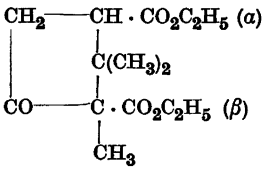
V. 

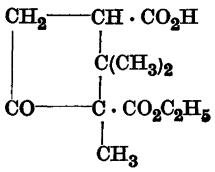
= 

Um zur Camphersäure zu gelangen, galt es zunächst, aus der Verbindung V die 4-ständige Carbäthoxygruppe zu beseitigen.

Wenn man den Ester V mit Säuren oder Alkalien verseift, so wird auch das 1-ständige Carboxyl abgespalten, und man gelangt zu der Ketomonocarbonsäure VI, die in erster Stelle zu der Konstitutionsbestimmung dieser Verbindungen, insbesondere zum Ermitteln der Stellung der eingeführten Methylgruppe, benutzt wurde [1].

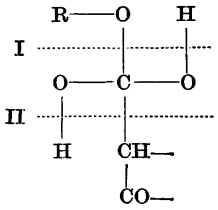
VI. 

VII. 

VIII. 

Dagegen gelang die alleinige Abspaltung der 4-ständigen Carbonestergruppe und damit die Bildung des *5-Ketocamphersäurediäthylesters (VII)* durch Erhitzen der Verbindung V in wasserhaltigem Glycerin. Diese Methode zur Abspaltung von Carboxalkyl aus β-Keto- und β-Dicarbonsäureestern sowie auch ihre Anwendung zu reaktionskinetischen Zwecken wurde vor kurzem näher beschrieben [7]. Dort ist auch die Darstellung der Verbindung VII aus der Verbindung V näher beschrieben worden.

Der Umstand (vgl. den experimentellen Teil, S. 602), dass der β-Ketosäureester (hier V) sich gerade im Beginn der Reaktion in dem wasserhaltigen Glycerin löst, deutet darauf hin, dass er dabei ein leicht lösliches Hydrat, etwa



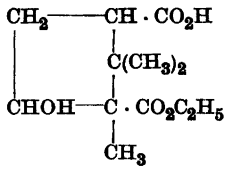
bildet. Dieses Hydrat könnte sich zersetzen entweder nach I: Verseifung, danach Abspaltung des Carboxyls unter Bildung des Ketons (hier VII), oder nach II: Spaltung zu Keton (hier VII) und Kohlensäuremonoester, der sofort in Kohlendioxyd und Alkohol zerfällt. — Weitere Versuche über diesen Gegenstand sind im Gange.

Die direkte Reduktion des Ketocamphersäureesters (VII) zum Camphersäureester nach den Methoden von Clemmensen und Wolff-Kishner gelang nicht. Um die Reduktion der Oxo- in die Oxygruppe z. B. durch Behandlung mit Natriumamalgam in wässriger, alkalischer Lösung zu ermöglichen, dabei aber die Abspaltung des 1-ständigen Carboxyls zu verhüten, wurde der Diäthylester VII partiell verseift. Dies gelang in sehr glatter Weise sowohl mit sauren als auch mit alkalischen Mitteln. So wurden zwei verschiedene *5-Ketocamphersäure-β-äthylester (VIII)* erhalten, von denen sich durch weiter unten zu beschreibende Versuche die eine, schwerer lösliche und höher (bei 150°) schmelzende als die *trans*-, die andere, leichter lösliche und niedriger (bei 106°) schmelzende sich als die *cis*-Form erwies. Über die relativen Mengen beider Formen und deren Bedingtheit s. S. 603. Dass sie beide die Formel VIII

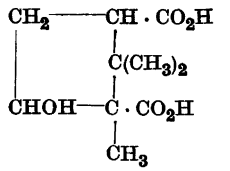
besitzen und dass in ihnen also das 1-ständige Carboxyl noch esterifiziert ist, war schon von vornherein, wegen der sehr geschützten Lage dieses Carboxyls zu erwarten. Ausserdem sind beide als solche beständig; erst nach Verseifung der noch vorhandenen Estergruppe wird aus beiden auch das zugehörige Carboxyl leicht abgespalten und die Ketomonocarbonsäure VI gebildet.

Die Reduktion dieser Estersäuren (VIII) mit Natriumamalgam findet aber äusserst langsam statt, was wohl auf die Nachbarschaft eines quartären Kohlenstoffatoms zur Ketogruppe zurückzuführen ist. Zur Reduktion der Estersäuren wurde deshalb die von Bredt [8] zur Reduktion der Camphononsäure ausgearbeitete elektrolytische Reduktion benutzt. Bei Anwendung von Kaliumamalgam als Kathode werden die beiden Ketocamphersäuremonoester in Kaliumcarbonatlösung ziemlich schnell und vollständig zu den entsprechenden *5-Oxycamphersäure-β-äthylestern (IX)* reduziert.

IX.



X.

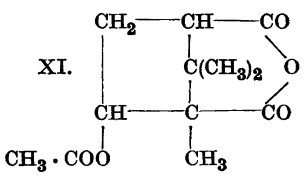


Hierbei reagiert die niedriger schmelzende (*cis*-) Ketoestersäure bedeutend schneller. Die aus ihr gebildete Oxyestersäure wurde nur zum Teil (bis nahe zur Hälfte) in kristallisierter, der Rest in flüssiger Form erhalten, während die aus der höher schmelzenden (*trans*-) Ketoestersäure gebildete Oxyestersäure, obwohl nicht ganz einheitlich, vollständig kristallisiert war.

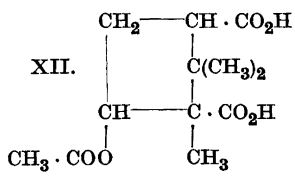
Durch Kochen in überschüssiger Natriumhydroxydlösung oder auch in verdünnter Salzsäure werden die Oxyestersäuren (IX) zu den entsprechenden *5-Oxycamphersäuren (X)* verseift.

Diese Oxysäuren verhalten sich zu Acetylchlorid verschieden, je nachdem, ob sie der niedriger oder der höher schmelzenden 5-Ketocampherestersäure (VIII) entstammen. Die ersteren reagieren mit dem Acetylchlorid ziemlich schnell und werden dabei in neutrale *5-Acetoxycamphersäureanhydride (XI)* überführt.

XI.



XII.

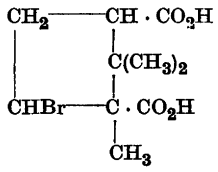


Die aus der höher schmelzenden Ketocampherestersäure gebildeten Oxysäuren reagieren dagegen mit Acetylchlorid nur sehr langsam und bilden dabei nur *5-Acetoxycamphersäuren (XII)*. Die niedriger schmelzende 5-Ketocampherestersäure und die aus ihr durch Reduktion und Hydrolyse gebildeten Verbindungen müssen also, in bezug auf die sterische Stellung der Carboxyle, der *cis*-Camphersäurereihe, die höher schmelzende Ketocampherestersäure bzw. die aus ihr gebildeten Verbindungen der *trans*-Camphersäurereihe angehören.

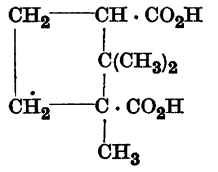
Die 5-Oxyderivate beider Reihen sollten wieder, wie ersichtlich, in bezug auf die sterische Stellung der Hydroxylgruppe, zweierlei Art sein können. In der Tat haben wir *zwei verschiedene 5-Oxy-cis-camphersäuren (X)* isoliert. Die eine, höher (207—208°, Zers.) schmelzende Form wurde durch Verseifung des auskristallisierten, schwerer löslichen Teils der Oxy-*cis*-campherestersäure (vgl. oben) gewonnen. Aus dem leichter löslichen, ölig ausfallenden Teil der Oxy-*cis*-estersäure wurde dagegen durch Verseifung und nach mehreren Umkristallisationen eine etwas niedriger (194°, Zers.) schmelzende Oxysäure erhalten. — Auf Grund des etwas variierenden Schmelzpunktes der jeweils erhaltenen 5-Oxy-*trans*-camphersäure ist auch hier das Vorkommen zweier Diastereomeren wahrscheinlich; bisher ist hier nur die eine Form isoliert worden.

Um diese 5-Oxycamphersäuren zu Camphersäure zu reduzieren, wurden sie zuerst, durch Behandlung mit Bromwasserstoff-Eisessig bei etwa 115°, in *5-Bromcamphersäuren (XIII)* übergeführt.

XIII.



XIV.



Aus diesen Bromcamphersäuren, die natürlich, ganz wie die 5-Oxycamphersäuren, im ganzen in vier verschiedenen diastereomeren Formen auftreten können, wurde bisher nur eine einzige, vermutlich die am schwersten lösliche, in reinem Zustande isoliert. Die leicht erfolgende Anhydridbildung zeigt, dass sie zu der *cis*-Camphersäurereihe gehört.

Das erhaltene Gemisch von Bromcamphersäuren wurde mit Zinkstaub in Eisessiglösung behandelt. Dabei entstand endlich die *Camphersäure (XIV)*.

Aus der 5-Oxy-*cis*-camphersäure werden durch den obigen Prozess sowohl *cis*- wie *trans*-Camphersäure gebildet; ganz gleich verhält sich die 5-Oxy-*trans*-camphersäure. Mit Bromwasserstoff-Eisessig findet also bei 110—120°

dieselbe sterische Umlagerung der *cis*- und *trans*-Formen statt, die gewöhnlich mit Salzsäure-Eisessiggemisch bei 180° bewirkt wird [9]. — Bezüglich der Isolierung und Identification der durch die Synthese gebildeten *cis*- und *trans*-Camphersäuren vgl. den experimentellen Teil.

EXPERIMENTELLER TEIL

Verbindungen A, B und V (S. 597, 598)

Die Darstellung und einige Eigenschaften dieser Verbindungen sind schon früher [4,6,5,1] beschrieben worden. Vorteilhafter als in Äthylalkohol wird die Methylierung der Verbindung A aber in kochender Aceton- oder am besten in kochender Benzollösung ausgeführt; ihr Natriumsalz ist nämlich auch in heissem Benzol ziemlich leicht löslich.

5-Ketocamphersäurediäthylester (VII)

Die Darstellung dieser Verbindung durch Erhitzen der Verbindung V mit wasserhaltigem Glycerin ist im Zusammenhang mit der Beschreibung dieser Ketonspaltungsmethode [7] schon geschildert worden.

In einer neuen Operation wurden 120 g der Verbindung V mit soviel (390 g) bei 161° siedendem (5 % Wasser enthaltendem) Glycerin erhitzt, dass die Menge des Wassers 3 Mol (statt früher 1,5 Mol) auf 1 Mol Ester betrug. Jetzt begann eine kräftige Reaktion schon bei 151° (statt früher bei 170°) und die bis dahin ungelöst gebliebene obere Schicht (Verb. V) löste sich schnell und vollkommen im Glycerin. (In wasserfreiem Glycerin löst sich der Ester V erst bei etwa 240° und trennt sich daraus beim Erkalten nahezu unverändert wieder ab.) Die Temperatur sank bis 146°, offenbar zufolge der gewaltigen Kohlendioxydentwicklung und des Abdestillierens des Äthanols. Nach einigen Minuten trennten sich die Schichten wieder und die Reaktion lief nur sehr träge fort, obwohl die Temperatur gesteigert wurde. Bei 174° war Kohlendioxydentwicklung nicht mehr wahrnehmbar und die durch zugesetzte Ferrichloridspuren ursprünglich kräftig rot gefärbte Esterschicht (V) ganz farblos (VII) geworden. Die Erhitzung wurde eingestellt, das Reaktionsprodukt wie früher weiter behandelt und i. V. destilliert. Es war sehr einheitlich:

Frakt.	g	Siedep. 6 mm	d_4^{20}	n_D^{20}	M_D ber.	M_D gef.	Ber.		Gef.	
							C	H	C	H
I	23,7	162—164°	1,0945	1,46235	68,01	67,91	62,17	8,21	62,25	7,86
II	64,4	164—166°	1,0962	1,46275	68,01	67,85	62,17	8,21	62,07	8,00
III	8,5	166—169°	1,0979	1,46325	68,01	67,81	62,17	8,21	60,69	7,90

Durch mehrtägige Einwirkung von Semicarbazidhydrochlorid und Kaliumacetat in Alkohollösung bei Zimmertemperatur wurde aus dem Ester ein Gemisch von *Semicarbazonen* gebildet. Das durch mehrmaliges Umkristallisieren aus Essigester und Ligroin erhaltene Produkt — kleine flache Nadeln — schmolz bei 147—150°.

$C_{15}H_{25}O_5N_3$(327,2)	Ber.	C 55,01	H 7,70	N 12,84
	Gef.	» 55,19	» 7,49	» 13,04

Durch Kochen in Eisessig-Salzsäure (vgl. unten) wurde das obige Semicarbazon in ein Gemisch der höher und der niedriger schmelzenden 5-Ketocamphersäure-β-äthylester (VIII) hydrolysiert.

Partielle Verseifung des 5-Ketocamphersäurediäthylesters (VII): Die β-Äthylester (VIII) der *cis*- und *trans*-5-Ketocamphersäuren

A. Saure Verseifung. 100 g Ester (VII) und je 250 ml Eisessig und 20 %iger Salzsäure wurden eine Stunde unter Rückfluss gekocht und die Lösung auf dem Wasserbad eingedampft. Der Rückstand wurde mit Sodalösung alkalisch gemacht und der neutrale Ester (7 g) mit Äther entfernt. Aus der wässrigen, gut umgerührten Lösung (ca. 300 ml) wurden die Estersäuren durch Zutröpfeln von 20 %iger Salzsäure fraktioniert ausgefällt, wobei die Kristallform der ausgeschiedenen Kristalle mikroskopisch verfolgt wurde. So wurden folgende Kristallfraktionen erhalten: I, hauptsächlich spulförmige Kristalle, Schmp. 140—142°, 18,1 g; II, hauptsächlich undeutliche Prismen, Schmp. 81—84°, 19,2 g; III, fast ausschliesslich Prismen, Schmp. 85—88°, 20,8 g; IV, undeutliche Prismen, Schmp. 85—87°, 10,7 g. Der Rest fiel ölig aus und wurde in Äther aufgenommen.

B. Alkalische Verseifung. I. *Mit 2 N Natriumhydroxyd* (1,5 Mol NaOH) *bei Zimmertemperatur* geschüttelt (zur Einleitung der Reaktion wurde ein wenig Alkohol zugesetzt) ging der Ester während einiger Stunden fast völlig in Lösung. Nach Entfernung des neutralen Esters mit Äther wurden die Estersäuren wie oben ausgefällt.

II. *In wässrig-alkoholischer Kaliumhydroxydlösung bei Zimmertemperatur.* Die klare Lösung (450 ml) enthielt: 81,05 g (0,3 Mol) Ketocamphersäureester, 16,83 g (0,3 Mol) Kaliumhydroxyd, 150 ml Wasser und ca. 220 ml Alkohol. Das Fortschreiten der Hydrolyse wurde titrimetrisch verfolgt. Schon nach 10 Minuten waren von dem Alkali ca. 15 %, nach 4 Stunden ca. 80 %, nach 21 Stunden ca. 94 % verbraucht worden. Nach Abdestillieren des Alkohols i. V. wurde der neutrale Ester und, nach Zusatz von verd. Schwefelsäure, das Hydrolysat in Äther aufgenommen. Nach Trocknen und Eindampfen der Ätherlösungen wurden 8,3 g des neutralen und 63,8 g des sauren Esters gewonnen. Der letztere wurde durch Kristallisieren in verschiedene Kristallfraktionen aufgeteilt.

Die ersten dieser Fraktionen wurden aus stark alkoholhaltigem, die späteren aus essigsäurehaltigem Wasser durch allmähliches Abdunsten des Lösungsmittel erhalten. Die Fraktionen I—III, zusammen 11,2 g, bestanden hauptsächlich aus rhombischen Blättern und schmolzen alle zwischen 147 und 149,5°, die Fraktionen IV—VIII, zusammen 50,4 g, hauptsächlich Prismen, schmolzen zwischen 92 und 100°.

Wie ersichtlich, bilden die durch partielle Verseifung erhaltenen Kristallfraktionen deutlich zwei Gruppen, die höher bzw. niedriger schmelzen. Durch weitere Reinigung werden aus ihnen zwei verschiedene Ketoestersäuren gewonnen (s. unten). Wie aus den weiter unten zu beschreibenden Versuchen erhellt (vgl. auch schon S. 599), stehen die Carboxyle der höher schmelzenden Estersäure in *trans*-, die der niedriger schmelzenden in *cis*-Stellung zu einander. Das Verhältnis zwischen den Mengen beider Formen, das wohl in der Hauptsache schon bei der Reduktion der Verbindung IV mit Zinkstaub und Eis-

essig bestimmt wird, dazu aber auch durch die Verseifungsbedingungen der Verbindung VII beeinflusst werden kann, ist etwas wechselnd, liegt aber entschieden zugunsten der *cis*-Form.

5-Keto-*cis*-camphersäure-β-äthylester (VIII). Diese niedriger schmelzende Ketoestersäure (vgl. oben) erhält man ziemlich rein durch Umkristallisieren der später abgeschiedenen, leichter löslichen Kristallfraktionen aus verdünntem Alkohol oder Eisessig. Die so erhaltenen Kristalle (Prismen) sehen sehr rein und wohlgebildet aus, Schmp. 98—100°. Den höchsten Schmelzpunkt erreicht man, allerdings unter bedeutendem Verlust an Substanz, wenn man die Estersäure einige Zeit in kalter, konzentrierter methylalkoholischer Kalilauge stehen lässt und dann mit Mineralsäure allmählich ausfällt. Schöne, flächenreiche Prismen mit stumpfen Enden, Schmp. 105—106°.

0,1766 g Sbst. verbrauchten 7,30 ml 0,1000 *N* NaOH.

$C_{12}H_{18}O_5$	Ber.	C 59,48	H 7,49	Äquiv.-Gew.	242,1
	Gef.	» 59,52	» 7,34	» »	242,0

Semicarbazon der Estersäure. Das Natriumsalz der Estersäure in konzentrierter wässriger Lösung wurde mit einer konzentrierten Lösung von Semicarbazidhydrochlorid und Kaliumacetat (je 1,5 Mol pro 1 Mol Estersäure) versetzt und über Nacht bei Zimmertemperatur stehen gelassen. Das durch Zusatz von verdünnter Salzsäure ausgeschiedene Semicarbazon wurde aus verdünntem Methanol umgelöst. Nädelchen, Schmp. 228° (Zers.). Leicht löslich in Natriumbicarbonatlösung; wird durch Zusatz von Salzsäure unverändert ausgefällt.

$C_{13}H_{21}O_5N_3$(299,2)	Ber.	C 52,14	H 7,08	N 14,05	
	Gef.	» 51,46	» 6,95	» 14,27,	13,96

5-Keto-*trans*-camphersäure-β-äthylester (VIII). Die höher schmelzenden Ketoestersäurefraktionen (vgl. oben) wurden aus verdünntem Alkohol umkristallisiert. Rhombische Blätter, Schmp. 150°.

0,1369 g Sbst. verbrauchten 5,62 ml 0,1000 *N* NaOH.

$C_{12}H_{18}O_5$	Ber.	C 59,48	H 7,49	Äquiv.-Gew.	242,1
	Gef.	» 59,55	» 7,36	» »	243,6

Das *Semicarbazon* wurde wie oben dargestellt; nur wird es viel langsamer gebildet. Kristallisiert aus verdünntem Methanol in kleinen Blättern, Schmp. 226° (Zers.). Mischschmelzpunkt mit dem Semicarbazon der niedriger schmelzenden Ketoestersäure: starke Depression.

$C_{13}H_{21}O_5N_3$(299,2) Ber. N 14,05 Gef. N 14,12

Elektroreduktion der β-Äthylester (VIII) der 5-Keto-*cis*- und *trans*-camphersäuren

Je 10 bis 35 g der Ketoestersäuren wurden unter Zusatz von etwas Kaliumcarbonat in etwa 600 bis 1000 ml Wasser gelöst und diese Lösung in den Kathodenraum des in der Hauptsache nach Bredt [8] konstruierten Apparats gebracht. Als Kathode diente festes, körniges Kaliumamalgam, das aus 1 kg reinem Quecksilber durch Elektrolyse einer konzentrierten Kaliumcarbonatlösung hergestellt worden war. Die Anode, ein Platinblech von etwa 10 × 20 cm, befand sich in einer mit konzentrierter Kaliumcarbonatlösung gefüllten porösen Tonzelle. Kühlen der Lösungen, Einleiten von Kohlendioxyd in die Kathodenflüssigkeit und Rühren derselben geschah wie bei Bredt.

Während der Reduktion wurde durch die Lösung ein Strom von 4—5 Amp. und 10—18 Volt geleitet. Bei Anwendung stärkerer Stromdichte scheint auch die Carbäthoxylgruppe teilweise reduziert zu werden, ebenso, wenn die Elektrolyse zu lange dauert.

Bei den angegebenen Stromstärken und Substanzmengen sind etwa 2 bis 6 Stunden genügend.

Bei der Reduktion der *cis*-Modifikation wird Wasserstoffentwicklung an der Kathode erst nach etwa 30—40 Minuten bemerkbar, während sie bei der Reduktion der *trans*-Modifikation schon von Anfang an wahrzunehmen ist. Die *cis*-Modifikation wird auch bedeutend schneller als die *trans*-Modifikation reduziert; sie wird sogar durch längeres Einwirken des elektrolytisch hergestellten Kaliumamalgams ohne den elektrischen Strom reduziert.

Nach der Reduktion wurde die vom Kaliumamalgam abgetrennte Kathodenflüssigkeit mit Salzsäure mineralsauer gemacht, die aus beiden Ketoestersäureformen gebildete, sofort ausfallende Menge einer sehr schwer löslichen, hochschmelzenden Verbindung nach ein paar Stunden abfiltriert und das Filtrat in den Abzug gestellt, um die leichter löslichen Oxyestersäuren allmählich auskristallisieren zu lassen.

5-Oxy-*cis*-camphersäure-β-äthylester (IX)

35,0 g 5-Keto-*cis*-camphersäure-β-äthylester (VIII) gaben nach dem obigen Reduktionsverfahren:

A. Schwerlösliches Reduktionprodukt,	Schmp.	ca. 260°,	1,65 g
B. Kristallisierte Oxyestersäure	»	» 96—102°,	13,6 »
C. Grösstenteils ölige	»	ca.	18 »

Die Fraktion B wurde aus 50 %iger Essigsäure umkristallisiert. Flache Nädelchen und Prismen mit spitzen Enden, Schmp. 111—112°.

0,1432 g Sbst. verbrauchten 5,93 ml 0,1000 *N* NaOH.

$C_{12}H_{20}O_5$	Ber.	C 58,98		H 8,25		Äquiv.-Gew.	244,2
	Gef.	» 58,74,	58,72	» 8,36,	8,27	» »	241,5

Über die Behandlung der Fraktion C s. weiter unten (S. 606). — Die Fraktion A ist nicht genügend genau untersucht worden.

5-Oxy-*cis*-camphersäuren (X)

Höher schmelzende 5-Oxy-*cis*-camphersäure. 10 g der obigen Oxyestersäure wurden durch vierstündiges Kochen in 100 ml 10 %iger Natriumhydroxydlösung verseift. Die Lösung wurde mit Salzsäure mineralsauer gemacht und in den Abzug gestellt. Die allmählich gebildeten Kristalle, zusammen 7,5 g, wurden aus etwa 10 %iger Essigsäure umgelöst. Schöne Prismen mit spitzen Enden, Schmp. 207—208° (Zers.).

0,0519 g Sbst. verbrauchten 4,75 ml 0,1000 *N* NaOH.

$C_{10}H_{16}O_5$	Ber.	C 55,53	H 7,46	Äquiv.-Gew.	108,1
	Gef.	» 55,37	» 7,74	» »	109,3

Acetoxy-anhydrid (XI) der höher schmelzenden 5-Oxy-cis-camphersäure. Die Oxysäure wurde mit einem grossen Überschuss von Acetylchlorid übergossen, wobei die Säure allmählich in Lösung ging. Nach zweitägiger Einwirkung bei Zimmertemperatur wurde das Acetylchlorid abdunsten gelassen, der neutral reagierende Rückstand in Äther gelöst, die Lösung mit Soda gewaschen, getrocknet und der Äther abdestilliert. Aus Benzol schöne Nadeln und Prismen mit stumpfen Enden, Schmp. 115—116°.

$C_{12}H_{16}O_5$(240,1)	Ber.	C 59,97		H 6,72	
	Gef.	» 59,48,	59,91	» 6,85,	6,46

Durch Verseifung wird aus dem Acetoxyanhydrid die ursprüngliche Oxysäure wiedergewonnen.

Niedriger schmelzende 5-Oxy-*cis*-camphersäure. Die flüssige Oxyestersäure (Frakt. C, S. 605) wurde in Äther aufgenommen und die nach dem Abdestillieren des Äthers sehr langsam kristallisierende Substanz wie oben mit Natriumhydroxyd verseift. Die allmählich kristallisierte Säure wurde mehrere Male aus verdünnter Essigsäure umgelöst. Zuletzt schöne Rhomboide, meistens als Zwillinge, Schmp. 194° (Zers.). — Mischschmp. mit der höher schmelzenden *cis*-Oxy-camphersäure: starke Depression des Schmelzpunktes.

0,0460 g Sbst. verbrauchten 4,29 ml 0,1000 *N* NaOH.

$C_{10}H_{16}O_5$	Ber.	C 55,53	H 7,46	Äquiv.-Gew.	108,1
	Gef.	» 55,44	» 7,23	» »	107,2

Das *Acetoxy-anhydrid (XI) der niedriger schmelzenden 5-Oxy-cis-camphersäure* wurde ganz wie das der höher schmelzenden Oxysäure (vgl. oben) hergestellt. Aus Benzol glänzende, dünne, leicht zerbrechliche Blätter, Schmp. 124°.

$C_{12}H_{16}O_5$(240,1)	Ber.	C 59,97	H 6,72
	Gef.	» 59,80	» 6,46

β-Äthylester (IX) der 5-Oxy-*trans*-camphersäure

20 g des β-Äthylesters (VIII) der 5-Keto-*trans*-camphersäure, Schmp. 148—149°, wurden in obenbeschriebener Weise (s. S. 605) elektrolytisch reduziert. Einwirkungsdauer 4 Stunden bei 4 Amp. und 18 Volt Elektrodenspannung.

Die nach dem Ansäuern der Kathodenlösung erhaltenen Produkte (vgl. S. 605) waren:

A. Schwerlösliches Reduktionsprodukt, Schmp. etwa 200° (Zers.), 0,36 g
B. Kristallisierte Oxyestersäure, » 115—115,5° 8,3 »
C. » » » 102—109° 7,5 »

Die Fraktionen B und C wurden aus verdünnter Essigsäure umgelöst. Lange, blättrige Nadeln, Schmp. 116°.

0,1002 g Sbst. verbrauchten 4,15 ml 0,1000 *N* NaOH.

$C_{12}H_{20}O_5$	Ber.	C 58,98	H 8,25	Äquiv.-Gew.	244,2
	Gef.	» 59,11	» 8,25	» »	241,4

5-Oxy-*trans*-camphersäure (X)

Diese Säure wurde durch die Verseifung der obenbeschriebenen 5-Oxy-*trans*-campher-säure-β-äthylester in Natriumhydroxydlösung erhalten. Nach Umlösen aus 10 %iger Essigsäure dicke Prismen, Schmp. 240° (Zers.).

0,2667 g Sbst. verbrauchten 24,48 ml 0,1000 *N* NaOH.

$C_{10}H_{16}O_5$	Ber.	C 55,53	H 7,46	Äquiv.-Gew.	108,1
	Gef.	» 55,55	» 7,46	» »	108,9

Acetoxyverbindung (XII) der obigen 5-Oxy-trans-campher-säure. Die Oxysäure löste sich in einem grossen Ueberschuss von Acetylchlorid bei Zimmertemperatur nur sehr langsam, in etwa 2 Wochen. Der beim Abdunstenlassen des Acetylchlorids gebildete kristalline Rückstand war völlig löslich in Natriumbicarbonatlösung. Aus heissem Essigester schöne, scharfkantige Prismen, Schmp. 233°.

0,0492 g Sbst. verbrauchten 3,845 ml 0,1000 *N* NaOH

$C_{12}H_{18}O_6$	Ber.	C 55,80	H 7,03	Äquiv.-Gew.	129,1
	Gef.	» 55,64	» 7,00	» »	128,0

Bei alkalischer Verseifung wird die Acetoxyverbindung in die ursprüngliche 5-Oxy-*trans*-campher-säure zurückverwandelt.

5-Brom-*cis*-campher-säure (XIII)

2 g 5-Oxy-*cis*-campher-säure (hauptsächlich der höher schmelzenden Form) wurden im Einschmelzrohr mit 15 ml Bromwasserstoff-Eisessig (bei 0° gesättigt) 12 Stunden bei 115° erhitzt. Der Bromwasserstoff und zuletzt auch der Eisessig wurden i. V. bei möglichst niedriger Temperatur verjagt. Der aus zwei solchen Versuchen gewonnene, dunkle, zum grössten Teil kristallisierte Rückstand wurde auf dem Tonteller getrocknet, in

ätherischer Lösung mit Aktivkohle entfärbt und aus Äther umkristallisiert. So wurden folgende Kristallfraktionen erhalten: I. 0,20 g, Schmp. 225—229°, II. 0,32 g, Schmp. 224—228°, III. 0,65 g, Schmp. 216—222°, IV. 0,40 g, Schmp. 210—216°. Die höchstschmelzenden Fraktionen aus mehreren Operationen wurden noch aus Essigester umkristallisiert, Schmp. 238—239° (Zers.).

0,1085 g Sbst. verbrauchten in der Kälte 7,70 ml 0,1000 *N* NaOH.

$C_{10}H_{15}BrO_4$	Ber.	C 43,00	H 5,42	Br 28,64	Äquiv.-Gew. 139,5
	Gef.	» 42,78	» 5,46	» 28,53	» » 140,9

Bei der Aufbewahrung wird diese Bromcamphersäure unter Bromwasserstoffabspaltung allmählich zersetzt.

Das *Anhydrid der obigen 5-Bromcamphersäure* wurde durch Auflösen von 1,75 g derselben in 50 ml Acetylchlorid und Stehenlassen über Nacht dargestellt. Nach Abdunstenlassen des Acetylchlorids wurde der kristalline Rückstand in Äther gelöst, die Lösung mit Natriumbicarbonatlösung gewaschen, getrocknet und der Äther abdestilliert. Rückstand 0,83 g. Dreimal aus Äther umkristallisiert schmolz das Anhydrid bei 199—201°. Grosse, monokline, dicke Blätter. Leicht löslich in Essigester, Benzol und Dioxan, schwerer in Äther.

$C_{10}H_{13}BrO_3$ (261,0) Ber. Br 30,63 Gef. Br 30,85

Reduktion der 5-Bromcamphersäuren (XIII) zu Camphersäure

Die auf obenbeschriebene Weise aus den 5-Oxysäuren sowohl der *cis*-(A) wie der *trans*-Camphersäurereihe (B) dargestellten Bromcamphersäuren wurden zur Reduktion zu Camphersäure benutzt, ohne sie durch Umkristallisation zu einheitlichen Verbindungen wie oben zu reinigen. Die eingeengten, dunkelgefärbten Eisessiglösungen wurden unter Rühren zuerst in Eiskälte, dann bei Zimmertemperatur und zuletzt auf dem Wasserbad allmählich mit Zinkstaub (fünfmal die berechnete Menge) versetzt. Der Zinkstaub wurde abgesaugt und mit heissem Wasser und Alkohol gewaschen. Das Filtrat mit den Waschflüssigkeiten wurde mit einer kleinen, nach der verbrauchten Menge des Zinkstaubs berechneten Menge verdünnter Schwefelsäure versetzt und, um die Essigsäure zu verjagen, mehrere Male unter wiederholtem Zusatz von Wasser auf dem Wasserbad eingedampft. Nach Zusatz von Wasser und verdünnter Schwefelsäure wurde die Lösung mit Äther extrahiert und die ätherische Lösung mit Aktivkohle entfärbt. Die aus dem Äther in Sodalösung aufgenommenen Säuren wurden hierin mit Kaliumpermanganat behandelt, die von Braunstein befreite Lösung nach dem Einengen mit Schwefelsäure angesäuert, mit Äther extrahiert, die ätherische Lösung getrocknet und der Äther abdestilliert. Der Rückstand wurde in Acetylchlorid gelöst, das Acetylchlorid am folgenden Tage i. V. verjagt und der kristalline Rückstand in Äther gelöst. Zur Isolierung der *trans*-Camphersäure (»Isocamphersäure») wurde die Ätherlösung mit verdünnter Sodalösung gewaschen, mit Natriumsulfat getrocknet und so nach Abdestillieren des Äthers das Anhydrid der *cis*-Camphersäure erhalten. Aus der Sodalösung wurde ihrerseits die Isocamphersäure abgeschieden.

A. Aus der 5-Oxy-*cis*-camphersäure (höher schmelzende Form) wurden auf die obenbeschriebene Weise die folgenden Verbindungen erhalten:

I. *Anhydrid der cis-r-Camphersäure.* Rohprodukt 1,41 g aus 4 g 5-Oxy-*cis*-camphersäure. Aus Alkohol umkristallisiert schmolz das Anhydrid bei 225°*. Mit dem aus Naturprodukten dargestellten *cis-d,l*-Camphersäureanhydrid (Schmp. 226°) gemischt war keine Depression des Schmelzpunktes wahrzunehmen. Auch die Kristalle des durch die hier beschriebene Synthese gebildeten Camphersäureanhydrids (Rhomboeder) zeigen gerade die sehr charakteristischen Merkmale (»Briefkuvert» form) des gewöhnlichen *r*-Camphersäureanhydrids.

$C_{10}H_{14}O_3$ (182,1)	Ber.	C 65,89	H 7,75
	Gef.	» 65,61	» 7,66

II. Die *r*-Camphersäure wurde aus dem Anhydrid durch Erwärmen desselben in Natriumhydroxydlösung und Ansäuern mit Salzsäure gewonnen. Nach Umkristallisieren aus essigsäurehaltigem Wasser flache Prismen, die denen der gewöhnlichen *r*-Camphersäure völlig glichen; Schmp. 202—203°. Mischschmp. mit der letztgenannten Säure (Schmp. 203—204°): keine Depression.

0,2180 g Sbst. verbrauchten 21,70 ml 0,1000 *N* NaOH.

$C_{10}H_{16}O_4$	Ber.	C 59,97	H 8,06	Äquiv.-Gew. 100,1
	Gef.	» 60,07, 60,22	» 7,67, 7,95	» » 100,5

III. Die *trans-r*-Camphersäure wurde aus der zum Waschen des Camphersäureanhydrids (vgl. S. 608) benutzten Sodalösung durch Zusatz von Salzsäure und Extrahieren mit Äther in geringer Menge isoliert. Aus essigsäurehaltigem Wasser umkristallisiert bildet sie mikroskopische flache Prismen, Schmp. 192—192,5°. Mit der gewöhnlichen *r*-Isocamphersäure, Schmp. 193—194°, gemischt keine Depression des Schmelzpunktes.

0,0351 g Sbst. verbrauchten 3,48 ml 0,1000 *N* NaOH.

$C_{10}H_{16}O_4$	Ber.	C 59,97	H 8,06	Äquiv.-Gew. 100,1
	Gef.	» 60,01	» 8,04	» » 100,9

B. Aus der 5-Oxy-*trans*-camphersäure wurden auf die schon beschriebene Weise dieselben Verbindungen gewonnen.

I. *Anhydrid der r-Camphersäure.* Rohprodukt 1,0 g aus 2 g 5-Oxy-*trans*-camphersäure. Aus Alkohol umkristallisiert Schmp. 226°. Kristallform und Mischschmp. mit dem gewöhnlichen *r*-Camphersäureanhydrid ganz wie oben (A.I.).

$C_{10}H_{14}O_3$ (182,1)	Ber.	C 65,89	H 7,75
	Gef.	» 65,92	» 7,50

* Roths Apparat wie immer in dieser Arbeit.

II. *r*-Camphersäure aus dem obigen Anhydrid. Darstellung und Identitätsnachweis ganz wie bei A.II.

III. *r*-Isocamphersäure. Auch diese Säure wurde hier in vorzüglicher Reinheit wie bei A.III. erhalten.

ZUSAMMENFASSUNG

Eine Totalsynthese der Camphersäure resp. des Camphers, deren Hauptzüge schon früher [1] kurz mitgeteilt worden sind, wird ausführlich beschrieben. Den Ausgangspunkt bilden einige von Perkin jun., Thorpe und Walker [4] schon vor etwa 50 Jahren synthetisierte Verbindungen, die lange Zeit als Abkömmlinge des Bicyclopentansystems angesehen wurden. Ausser den vorherigen Untersuchungsergebnissen zeigt auch die Bildung der Camphersäure aus diesen sowohl synthetisch wie auch valenztheoretisch wichtigen Verbindungen, dass ihnen nicht der Bicyclopentan- sondern der Cyclopentenkern zugrunde liegt. Eine von den Hauptstufen der Synthese besteht in einer neuen, bequemen Methode zur Abspaltung der Carbonestergruppe aus β-Ketocarbonsäureestern, wodurch man zu 5-Ketosäureestern und daraus weiter über 5-Oxy- und 5-Bromcamphersäuren zu *cis*- und *trans*-Camphersäuren gelangt.

LITERATUR

1. Toivonen, N. J., mit Nieminen, S., und Eskola, S. *Ann. Acad. Sci. Fennicae* **A29**, No. 20 (1927) 8.
2. Toivonen, N. J., und Halonen, A. *Suomen Kemistilehti* **B 19** (1946) 1.
3. Toivonen, N. J. *Berättelse och föredrag 6. Nordiska Kemistmöte* Lund (1947) S. 276.
4. Perkin, W. H. (jun.), Thorpe, J. F., und Walker, C. *J. Chem. Soc.* **79** (1901) 729.
5. Grimwood, R. Ch., Ingold, Ch. K., und Thorpe, J. F. *J. Chem. Soc.* **123** (1923) 3303.
6. Toivonen, N. J. *Soc. Sci. Fennica, Commentationes Phys.-Math.* I. **26** (1922) 1.
7. Toivonen, N. J., mit Niininen (Tommila) S., Eskola, S., Laakso, P. V., und Laukkanen, P. *Acta Chem. Scand.* **1** (1947) 133.
8. Bredt, J. *J. prakt. Chem. N. F.* **84** (1911) 786.
9. Vgl. Aschan, O. *Chemie der alicyklischen Verbindungen* Braunschweig (1905) S. 384.

Eingegangen am 16. Juli 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 611—619

The Utilization of Peptide Bound Amino Acids by Lactic Acid Bacteria. II

GUNNAR ÅGREN

Department of Medical Chemistry, University of Uppsala, Sweden

Some of the reasons for studying the peptide utilization of lactic acid bacteria were given in a previous paper [1]. Briefly, it could be demonstrated that some lactic acid bacteria were capable of using the essential amino acids leucine and valine supplied to the basal medium in the form of synthetic peptides only when these amino acids were incorporated in certain orders in the peptides. It was also suggested that these investigations further developed could be useful in the identification of the order in which the amino acids of natural peptides are linked together. Similar results have subsequently been obtained by Krehl and Fruton [2]. These authors also suggested that differences in the effectiveness of several leucine peptides in replacing the free amino acid may be taken to reflect differences in the rate of cleavage of the peptides by bacterial peptidases. A direct comparison of the results obtained by Krehl and Fruton with those obtained by Ågren [1] were in some cases possible when the same bacteria, *Streptococcus faecalis* (9790) and the same peptides, leucyl-glycine and glycyl-leucine, were tested. According to Krehl and Fruton only 70 to 80 per cent of the bound leucine was available in these peptides while we found a 100 per cent utilization. These quantitative differences could possibly be explained by differences in the composition of the synthetic media used by the investigators. However, very little is known about the nutritional requirements of lactic acid bacteria with regard to essential amino acids supplied in peptide form to different basal media. In order to extend the basis for comparison the peptides previously studied [1] on the basal media of Stokes *et al.*[3] were now supplemented in the same way by to the recently described medium of Henderson and Snell [4]. For some of the lactic acid bacteria the medium was used in 0.5 times the concentration of all ingredients of the original Henderson and Snell medium with the exception of Salt C which was kept at the original concentration (*cf.* Ågren [5]).

EXPERIMENTAL

The microorganisms used were *Streptococcus faecalis* (9790),* *Lactobacillus delbrückii* LD5 (9595), *Leuconostoc mesenteroides* P 60 (8042), *Streptococcus faecalis* R (8043) *Lactobacillus casei* (7469), *Lactobacillus citrovorum* (8081), *Lactobacillus citrovorum* (8082), *Lactobacillus dextranicum* (8086), *Lactobacillus arabinosus* 17—5 (8014) and *Lactobacillus fermenti* 36 (9595). The assay technique of Henderson and Snell [4] was followed. Separate solutions of basal medium, standard amino acid, peptides and inocula were delivered to 6 inch test tubes. The final volume in each case was 6 ml. The standard and peptides were each run at three levels and from five to six tubes were employed at each level. The peptides described in the previous communication [1] were used and in

Table 1. Lactic acid production of Streptococcus faecalis (9790) and Streptococcus faecalis R (8043) in the medium of Henderson and Snell supplemented with leucine, tryptophane or tyrosine containing peptides. Incubation time 48 hours.

Peptides tested	*Streptococcus faecalis* (9790)				*Streptococcus faecalis* R (8043)					
	Amount of free or bound essential amino acid** per tube in γ									
	0	15	30	60	0	3	6	15	30	60
	ml***	ml	ml	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.6	5.0	8.4	10.8	0.5	—	—	3.3	5.4	8.8
DL-Alanyl-DL-leucyl-glycine	1.5	4.8	6.2	8.9	0.5	—	—	3.3	5.4	8.5
Glycyl-DL-leucyl-DL-alanine	1.8	2.1	2.2	2.1	0.6	—	—	0.8	0.8	0.8
Glycyl-DL-leucylglycine	1.6	4.9	7.8	9.9	0.5	—	—	3.4	5.3	8.9
DL-Leucylglycylglycine	1.5	5.0	7.6	9.9	0.5	—	—	3.1	5.7	8.6
DL-Alanyl-DL-leucine	1.5	3.6	5.8	8.6	0.5	—	—	2.5	4.1	6.7
DL-Leucyl-DL-alanine	1.6	3.6	5.1	6.9	0.6	—	—	1.5	1.6	3.7
Glycyl-DL-leucine	1.7	4.8	7.5	10.1	0.6	—	—	3.0	4.6	8.0
DL-Leucylglycine					0.7	—	—	3.2	5.3	8.5
L-Tryptophane					0	4.5	7.7	12.5	13.4	—
Glycyl-L-tryptophane					0	4.7	6.0	13.5	14.0	—
L-Tyrosine					5.8	—	—	7.9	9.4	12.5
Glycyl-L-tyrosine					5.8	—	—	8.9	10.2	11.1
	Complete medium 14.4 ml				Complete medium 14.0 m					

* All bacteria from the American type culture collection, Georgetown University, Washington, D. C.

** Peptides were added in amounts calculated to give the same levels of L-leucine, L-tryptophane or L-tyrosine as used in the corresponding standard curves.

*** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

addition DL-leucyl-glycyl-glycine, glycyl-L-tryptopane and glycyl-L-tyrosine (Hoffmann-La Roche) were investigated. The standard amino acids were Merck preparations. The concentration levels of the tested peptides were as in the previous communication such that the amount of the essential amino acid of natural configuration they could yield fell within the range of the corresponding free amino acid amounts used in the standard curve.

RESULTS

The data obtained with *Streptococcus faecalis* (9790) and *Streptococcus faecalis* R (8043) are presented in Table 1. It is obvious that L-leucine in the tripeptide glycyl-DL-leucyl-DL-alanine was not available for any one of these two microorganisms and the L-leucine equivalent of the dipeptides DL-leucyl-DL-alanine and DL-alanyl-DL-leucine only partially. In the other DL-leucine peptides the L-leucine was completely available. These results are in agreement with those previously obtained [1], on the medium of Stokes *et al.* Tryptophane in the dipeptide glycyl-L-tryptophane and tyrosine in the dipeptide glycyl-L-tyrosine were also completely utilized. The high blank values in the

Table 2. Lactic acid production of Lactobacillus delbrückii LD5 and Lactobacillus casei in medium supplemented with leucine peptides. Incubation time 72 hours. The medium of Henderson and Snell used in 0.5 times the concentration as given by these authors.

Peptides tested	*Lactobacillus delbrückii* LD5				*Lactobacillus casei*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.5	5.2	7.1	8.9	1.5	5.2	7.1	10.2
DL-Alanyl-DL-leucylglycine	1.5	4.9	7.0	9.1	1.5	5.4	8.2	9.7
Glycyl-DL-leucyl-DL-alanine	1.6	5.5	6.5	9.5	1.9	5.6	7.6	9.7
Glycyl-DL-leucylglycine	1.6	5.6	8.3	10.2	1.8	5.4	8.5	10.3
DL-Leucylglycylglycine	1.4	4.9	7.7	9.9	1.6	5.1	7.9	10.0
DL-Alanyl-DL-leucine	1.5	4.2	7.0	9.7	1.5	5.8	8.0	10.4
DL-Leucyl-DL-alanine	1.8	5.1	6.1	9.8	1.8	5.7	7.6	9.7
Glycyl-DL-leucine	1.6	5.7	7.8	10.8	1.5	4.7	6.9	9.7
DL-Leucylglycine	1.5	5.2	7.0	8.9	1.6	5.0	6.8	10.0
	Complete medium 9.8				Complete medium 10.8			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

two tyrosine series are interesting. They seem to demonstrate that on the medium of Henderson and Snell this microorganism can at least partially synthezise tyrosine but better growth is obtained when tyrosine is added to the medium. While Snell [6] previously has reported that this microorganism is less suitable for microbiological determinations of tyrosine, Dunn *et al.*[7] indicate that the amino acid is indispensable for *Streptococcus faecalis* R (8043). The series of leucine peptides assayed with *Lactobacillus delbrückii* LD5 and *Lactobacillus casei* (Table 2) showed that these two microorganisms completely utilized all of the tested peptides. This result is especially interesting when compared with those obtained on the medium of Stokes *et al.*[1]. There is no difference with regard to *Lactobacillus casei* but on the medium of Stokes *et al.* the L-leucine of the tripeptide glycyl-DL-leucyl-glycine was not at all available and the L-leucine of the dipeptide DL-leucyl-glycine only partially available for *Lactobacillus delbrückii* LD5.

Next the leucine peptides were tested on *Leuconostoc mesenteroides* P-60 and *Lactobacillus dextranicum*. The results are given in Table 3. In the series with *Leuconostoc mesenteroides* P-60 there was only two peptides, glycyl-DL-leucyl-glycine and DL-leucyl-glycine where the peptide bound L-leucine had

Table 3. Lactic acid production of Leuconostoc mesenteroides and Lactobacillus dextranicum in medium supplemented with leucine peptides. Incubation time 72 hours. Lactobacillus dextranicum was grown on the medium 0.5 times the concentration as given by Henderson and Snell.

Substances tested	*Leuconostoc mesenteroides*				*Lactobacillus dextranicum*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.1	4.8	6.7	12.2	0	1.7	3.2	5.9
DL-Alanyl-DL-leucylglycine	1.3	3.3	3.8	5.5				
Glycyl-DL-leucyl-DL-alanine	1.3	1.3	1.2	1.1				
Glycyl-DL-leucylglycine	1.3	4.2	6.7	11.4				
DL-Leucylglycylglycine	1.2	3.4	5.5	8.7				
DL-Alanyl-DL-leucine	1.0	3.4	4.7	7.0				
DL-Leucyl-DL-alanine	1.2	2.6	4.2	7.0				
Glycyl-DL-leucine	1.1	3.6	5.8	9.1				
DL-Leucylglycine	1.1	4.5	6.0	11.5	0	0.9	1.0	3.4
	Complete medium 13.0 ml				Complete medium 6.0 ml			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

the same activity as the corresponding free amino acid. L-leucine in the tripeptide glycyl-DL-leucyl-DL-alanine was not utilized at all and of the dipeptides the two containing leucine and alanine were clearly less active than the two glycine-leucine peptides. These results are the most convincing of all obtained in the present investigation to indicate that the growth-promoting activity of the tested leucine peptides depends upon the position of the leucine residue with respect to the other amino acid residues, as well as upon the nature of the other amino acid residues. The data obtained with *Lactobacillus dextranicum* are few but none the less of interest. Dunn *et al.*[7] characterize leucine as a dispensable amino acid for this microorganism. However, on the medium of Henderson and Snell it has been found in repeated experiments that leucine has the character of an indispensable amino acid for this microorganism. It is also of interest that DL-leucyl-glycine is so incompletely utilized for growth by this microorganism in contrast to the other lactobacilli so far investigated on the medium of Henderson and Snell.

When the leucine peptides were assayed on *Lactobacillus citrovorum* (8081) and *Lactobacillus citrovorum* (8082) (Table 4) it was found that in both cases

Table 4. Lactic acid production of Lactobacillus citrovorum (8081) and Lactobacillus citrovorum (8082) in medium supplemented with leucine peptides. Incubation time 72 hours. The medium of Henderson and Snell was used in 0.5 times the concentration as given by these authors.

Substances tested	*Lactobacillus citrovorum* (8081)				*Lactobacillus citrovorum* (8082)			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	0.6	3.4	5.2	5.6	0.4	2.8	4.1	6.1
DL-Alanyl-DL-leucylglycine	0.6	4.5	5.9	6.4	0.4	3.5	5.0	6.4
Glycyl-DL-leucyl-DL-alanine	0.5	0.9	1.2	1.2	0.4	2.4	3.5	5.0
Glycyl-DL-leucylglycine	0.6	4.6	5.2	5.2	0.5	2.5	4.4	6.1
DL-Leucylglycylglycine	0.5	5.0	5.5	6.3	0.3	2.9	4.2	6.2
DL-Alanyl-DL-leucine	0.5	2.3	3.8	4.4	0.3	3.3	4.3	5.7
DL-Leucyl-DL-alanine	0.5	1.4	2.4	4.3	0.1	2.2	4.0	5.9
Glycyl-DL-leucine	0.4	4.9	5.5	6.0	0.4	3.3	4.9	6.3
DL-Leucylglycine	0.5	4.9	5.5	6.0	0.5	3.0	4.2	6.0
	Complete medium 5.8 ml				Complete medium 6.8 ml			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

the utilization of L-leucine in glycyl-DL-leucyl-DL-alanine was less than that expected if all of the peptide bound L-leucine was available for growth. The leucine equivalents of alanyl-leucine and leucyl-alanine were completely available only for *Lactobacillus citrovorum* (8082). Finally, the series of leucine peptides assayed on *Lactobacillus arabinosus* 17—5 and *Lactobacillus fermenti* 36 (Table 5) showed that the L-leucine content of the tripeptide DL-alanyl-DL-

Table 5. Lactic acid production of Lactobacillus arabinosus 17—5 and Lactobacillus fermenti 36 in the medium of Henderson and Snell supplemented with leucine peptides. Incubation time 72 hours.

Substances tested	*Lactobacillus arabinosus*				*Lactobacillus fermenti*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml*	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.8	5.7	8.6	12.7	4.0	7.5	10.8	15.1
DL-Alanyl-DL-leucylglycine	2.3	4.9	6.1	8.0	3.9	3.1	4.5	4.5
Glycyl-DL-leucyl-DL-alanine	2.0	4.8	7.3	11.7	3.7	6.4	8.4	13.0
Glycyl-DL-leucylglycine	1.8	6.2	8.5	13.4				
DL-Leucylglycylglycine	2.2	5.8	10.2	15.9				
DL-Alanyl-DL-leucine	2.0	5.6	9.8	12.5				
DL-Leucyl-DL-alanine	2.0	5.1	7.8	13.1				
Glycyl-DL-leucine	2.0	5.5	8.6	12.0				
DL-Leucylglycine	1.8	5.6	8.5	11.5				
	Complete medium 21.1 ml				Complete medium 20.5 ml			

leucyl-glycine was only partially available for *Lactobacillus arabinosus* 17—5 and was not utilized for growth at all by *Lactobacillus fermenti* 36.

DISCUSSION

The present investigation in agreement with the previous results of Ågren [1] and Krehl and Fruton [2] confirmed the assumption that the capability of some lactic acid bacteria to use leucine of leucine containing peptides supplemented to the basal medium instead of the free amino acid depends upon the position of leucine with respect to the other amino acid residues and upon the nature of the other amino acids. In order to facilitate a comparison semiquantitative

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

Table 6. Qualitative results of assays of leucine peptides with ten lactic acid bacteria.

++, + and 0 signify the same growth, approximately half the growth respectively no growth effect of peptide bound L-leucine when compared with the effect of the corresponding amounts of free L-leucine.

Micro-organism	Leucine peptides							
	Ala-Leu Gly *	Gly-Leu Ala	Gly-Leu Gly	Leu-Gly Gly	Ala-Leu	Leu-Ala	Gly-Leu	Leu-Gly
(8086)	—	—	—	—	—	—	—	—
(9595)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(7469)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(8082)	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +
(8014)	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(8043)	+ +	0	+ +	+ +	+	+	+ +	—
(9790)	+ +	0	+ +	+ +	+	+	+ +	+ +
(8081)	+ +	0	+ +	+ +	+	+	+ +	+ +
(9338)	0	+ +	—	—	—	—	—	+ +
(8042)	+	0	+ +	+	+	+	+	+ +

data of the present investigation are collected in Table 6. Comparing the data in the Table for the ten bacteria it is clear that L-leucine of the tripeptide glycyl-DL-leucyl-DL-alanine is less available for growth than in any of the other tripeptides. DL-alanyl-DL-leucyl-glycine ranked as second in this respect. Leucine of the corresponding dipeptides DL-alanyl DL-leucine and DL-leucyl-DL-alanine was less available than leucine in combination with glycine. The suggestion has been made by Simmonds *et al.*[9] that in the case of many peptides, utilization for growth is preceded by enzymatic hydrolysis to yield essential amino acids. If that is true the combination of leucine and alanine in di- and tripeptides must be considered as rather resistant against the action of lactic acid bacterial peptidases.

Further on, if the suggestion of Simmonds *et al.*[9] is accepted it may be of interest to compare some of the results of the present investigation with the corresponding results previously obtained [1] from this point of view. In both investigations the same strains of bacteria and the same leucine peptides were used. The only difference between the two sets of data was the media, the medium of Henderson and Snell used in the present investigation and that of Stokes *et al.* in the previous work. The use of the two media did not influence the complete utilization of leucine of the eight leucine peptides by *Lactobacillus*

* The abbreviations are those proposed by Brand and Edsall [8].

casei. The results obtained with *Streptococcus faecalis* (9790) were also in agreement. The same leucine peptides stimulated a maximal growth, the same were partially stimulating and in both media there was no growth when leucine was added in the form of glycyl-leucyl-alanine. If an enzymatic hydrolysis of the leucine peptides preceded the utilization, the process seemed not to be influenced by the comparatively small differences in the composition of the two basal media.

Comparing the results obtained on the two media with *Lactobacillus delbrückii* LD5 another picture is obtained. On the medium of Henderson and Snell a complete utilization of leucine peptides was obtained. On the medium of Stokes *et al.* the L-leucine of glycyl-DL-leucyl-glycine was not used at all and the L-leucine of DL-leucyl-glycine only partially. There is no obvious reason why the suggested enzymatic hydrolysis just in this case would be totally or partially blocked on the medium of Stokes *et al.* and completely active on the medium of Henderson and Snell. Here it may only briefly be reminded of the present discussion of the relationship between gene activity and enzyme activity. Small changes in gene activity may be reflected in changes in enzyme activity. The adaptability of bacterial enzymatic processes in certain microorganisms to changes in the hydrogen ion concentration of the surrounding media is well known. On the other hand the medium of Henderson and Snell contains considerably higher concentrations of mangan and magnesium ions, both known as activators of bacterial peptidases. The variability of results obtained when analysing the same synthetic peptides by the same microorganism on two slightly different media may also occur when studying a biological active peptide on different media [5].

The present investigation also demonstrated the difficulties in characterizing an amino acid as dispensable or indispensable. According to Dunn *et al.* leucine is a dispensable amino acid for *Lactobacillus dextranicum* on the medium specified by these authors. In the present investigation it was shown that on the medium of Henderson and Snell leucine is an indispensable amino acid for this microorganism. According to Dunn *et al.* tyrosine is an indispensable amino acid for *Streptococcus faecalis* R (8043) while on the medium of Henderson and Snell the microorganism is able to grow when this amino acid is not present in the medium. Finally, it may again be stressed that the type of analysis illustrated in Table 6 may be useful for the identification of the order in which amino acids are linked together. The peptides analyzed in this work did not show strepogenin activity.

SUMMARY

Analysis of the utilization of various peptides especially leucine peptides by ten lactic acid bacteria have shown that the growth-promoting activity of such peptides depends on the position of leucine in the peptide and on the nature of the surrounding amino acids. This type of analysis may also be useful for the establishment of the order in which the amino acids in peptides are linked together. It is also demonstrated that the utilization of a peptide bound essential amino acid may be changed by cultivation on slightly different media. Changes in dispensability and indispensability of amino acids for lactobacilli cultivated on different media were also observed.

Parts of the expenses for this investigation were defrayed by a grant from *Statens naturvetenskapliga forskningsråd.*

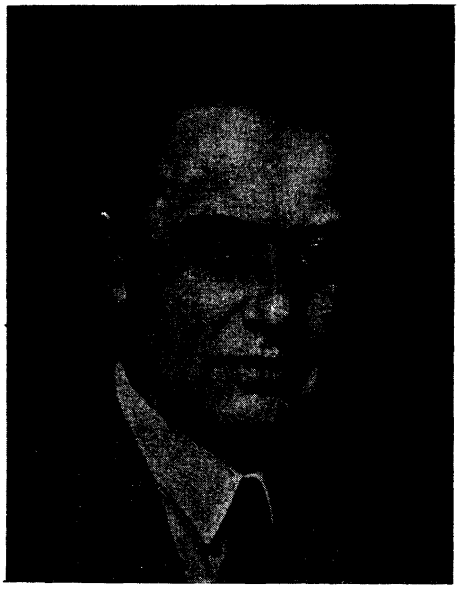
REFERENCES

1. Ågren, G. *Acta Physiol. Scand.* **13** (1947) 347.
2. Krehl, W. E., and Fruton, J. S. *J. Biol. Chem.* **173** (1948) 179.
3. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C. *J. Biol. Chem.* **160** (1945) 35.
4. Henderson, L. M., and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
5. Ågren, G. In press.
6. Snell, E. E. *Ann. N. Y. Acad. Sci.* **47** (1946).
7. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H. *J. Biol. Chem.* **168** (1947) 1.
8. Brand, E., and Edsall, J. T. *Ann. Rev. Biochem.* **16** (1947) 223.
9. Simmonds, S., Tatum, E. L., and Fruton, J. S. *J. Biol. Chem.* **170** (1947) 483.

Received August 30, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 620—624

Arne Tiselius



On November 4, 1948, it was announced that the Royal Swedish Academy of Science had awarded Professor Arne Tiselius, Upsala, the Nobel Prize for 1948 in chemistry for his work on electrophoresis and adsorption analysis and in particular for his discovery of the complex nature of the serum proteins.

Arne Wilhelm Kaurin Tiselius was born in Stockholm, on August 10, 1902 as son of the Swedish mathematician Dr. Hans Tiselius and his Norwegian wife Rosa Kaurin.

At school Tiselius had a very inspiring teacher in chemistry and biology who profoundly aroused his interest for chemistry. When the teacher discovered Tiselius' ability in chemistry, he gave him a private key to the laboratory of the highschool, so that it was possible for Tiselius to carry out his own chemical studies when the lessons were over in the afternoon. In these years his intense love for science and especially chemistry was born. It soon became evident to him that he desired to go to Upsala to study under The Svedberg.

In 1921 he matriculated at the University of Upsala, where he took his first degree (fil. mag.) in chemistry, physics and mathematics in 1924. In 1925 he became research assistant in physical chemistry under The Svedberg and his scientific life started.

His first publication, in 1926, was together with Svedberg on a new method for determination of the mobility of proteins [1]. In this preliminary paper the light absorption method, already successfully used by Svedberg for following the rate of sedimentation in the ultracentrifuge, was introduced for the first time in the moving boundary method for studying the electrophoretic migration of proteins. In the next years Tiselius investigated very carefully the possible sources of errors in connection with the moving boundary method both from a theoretical and from an experimental point of view. He devoted special attention to all the effects that were found to cause »boundary anomalies» and he indicated how the method could be used in the study of the purity of proteins. Some experiments were also carried out on mixtures, but it was evident that if the method should be used for the analysis of protein mixtures the resolving power had to be improved considerably. He showed, however, that serum globulin prepared according to the classical methods

was definitely non-uniform in electrophoresis. His investigations were published as a thesis for the degree of doctor of Science at the University of Upsala in 1930 [2] and gave him an assistant professorship in chemistry at this university.

By this time Tiselius had a family and had to think of the future. There was then in Upsala only one chair in general and inorganic chemistry, one in organic chemistry and a personal chair for The Svedberg in physical chemistry. At the other universities in Sweden the number of permanent positions in chemistry was not greater. Since the chair in general and inorganic chemistry was to become vacant in 1935, according to the rules, and as it could not be foreseen that other chairs in chemistry would become vacant in Sweden for quite a period he felt it his duty to try to get this chair.

After he had finished his work on the thesis it was quite natural for him to want to get some change in his studies. The electrophoresis problems were, therefore, put aside for several years, and he attacked new problems that gave him experience in other fields. The question of adsorption and diffusion in solid matter attracted him very much and he saw clearly that if progress was to be gained in this field one had to work with well defined and transparent crystals. Among the minerals belonging to the zeolite family he found valuable test material. As, however, it was difficult to find sufficient samples of selected material suitable for the experiments in the mineralogical collections in Sweden, he made a long trip to some of the most lonely islets among the isolated Faroe Islands in the Atlantic Ocean in the summer of 1932. He returned to Upsala with a beautiful collection of selected zeolite crystals.

When Tiselius started his work on the diffusion of water into a zeolite crystal, it was known that the optical properties changed when the dried crystal became rehydrated. Up to that date no quantitative study of the phenomenon had been made. Tiselius, however, saw the possibilities of this accidental observation, found the governing factors and developed a very elegant and accurate method for the quantitative measurement of the diffusion of water vapour and other gases into zeolite crystals. The work was extended by kinetic studies of the diffusion and adsorption processes occurring in the unhydrated crystal in the presence of various gases [3]. The later part of the work was carried out at the Frick Chemical Laboratory at Princeton in 1934—35, when Tiselius had a Rockefeller fellowship for studying under Hugh S. Taylor.

Shortly after his return from the States, the chair he had aimed at was given to Gunnar Hägg, a choice that Tiselius also considered the most correct. The committee investigating the candidates' qualifications, however, emphasised Tiselius' great experimental and theoretical qualifications and the high standard of his publications. There was no question that he was very well qualified for a chair in chemistry but there was only one vacant and he had to continue as assistant professor. One may question the wisdom of the Swedish system with the very few permanent university positions, where it is rather accidental whether the universities could keep the right people on their permanent staffs. If a young assistant professor does not get a chair in his subject before his seven year period has passed, the university has very few possibilities to keep him. Even in the case of Tiselius there was undoubtedly a risk that he could be forced to leave the university when his term as assistant professor was over.

Before Tiselius went to Princeton he admitted that if he was completely independent and free to do whatever he liked, he would concentrate on biochemistry, where he thought some of the most fascinating problems of modern science were still waiting for their solution. During

his stay in Princeton, he had much contact with the Rockefeller Institute situated nearby. He became good friend with John H. Northrop and M. L. Anson, and these scientists evidently further stimulated his interest in biochemical problems.

After his return to Upsala, he resumed his electrophoretical work this time with serum globulin which he earlier had found to be non-uniform in electrophoresis [4]. He soon found that if real progress should be made in solving the problem of the nature of the electrophoretical inhomogeneity of this protein, a complete reconstruction of the apparatus had to be made and a new experimental technique had to be introduced. From the work on his doctoral thesis he was familiar with all the factors that were important in electrophoretical experiments. On the basis of this knowledge a new apparatus for electrophoretical analyses was constructed in 1936 [5]. In this instrument the movement of the boundaries could be followed optically and the U-tube could be divided into well defined sections after the conclusion of the experiments allowing samples to be taken out from different parts of the U-tube for chemical analysis and for tests of biological activity. While the U-tube up to that date had always a circular cross section, the new Tiselius apparatus was equipped with cells with long and narrow rectangular cross sections. This gives for a given cross section an increased optical sensitivity and a better removal of the heat generated by the electric current, thus minimizing the risk of heat-convection currents. This severe source of error is furthermore much reduced by carrying out the experiments at a temperature of 0° to 4° C, where the buffer solutions applied have their density maxima. The result is that the new Tiselius apparatus can be safely used with a potential gradient in the U-tube at least ten fold higher than in any of the earlier constructions. It also means, that a much higher resolving power for protein mixtures is obtained. The first experiments with horse serum immediately demonstrated the advantages of the new instrument. The schlieren pattern showed five bands moving with different speed through the electrophoresis cell. The fastest band corresponded to the serum albumin boundary. The four other boundaries were tentatively by Tiselius called the α-, β-, γ-, and δ-boundaries, respectively. He also showed that the α-, β-, and γ-boundaries corresponded to three different groups of globulins, and that the antibodies usually were found in the γ-globulin or between the β- and γ-globulins. It was also discovered that the δ-boundary was a salt boundary depending upon the protein concentration and the nature of the buffer salts. By suitable choice of the buffer salts and by using protein concentrations below 1 per cent the δ-boundary could be practically eliminated.

In the following years the method was tested by Tiselius and by his coworkers in all possible ways and various minor changes were made. A considerable improvement in the observation of the boundary was made when the Philpot-Svensson method was introduced [6].

In the meantime Svedberg had been active trying to get a permanent position for Tiselius at the University of Upsala. At the end of 1937 he succeeded in finding a generous patron who made an endowment of half a million Swedish crowns to the University of Upsala for a permanent chair in biochemistry. A few months later Tiselius was called to be the first Karin and Herbert Jacobsson professor of biochemistry in Upsala.

Shortly after the publication of the new Tiselius apparatus his electrophoresis technique was brought into use in laboratories outside Upsala. The introduction of the Philpot-Svensson and its American modification, the Longsworth schlieren scanning method improved the usefulness of the apparatus and hundreds of them are at

present in use all over the world. For the scientist working with biocolloids, especially proteins, Tiselius' method has been of immense importance. It has given the biochemist a tool by which he can visually determine whether a substance under investigation is electrophoretically pure or consists of several components. In a purification process it gives valuable information about the progress of the fractionation. It is now generally considered that one of the necessary criteria of purity for a protein is that it is homogeneous in electrophoresis over a wide pH-range. It is, however, by no means a sufficient criterion.

Tiselius has also used his apparatus for preparative purposes and he has shown how the separation between electrochemically closely related substances may be improved by superimposing a constant movement of the solution in the U-tube over the movement of the individual boundaries. This procedure was used by Tiselius *et al.* for the isolation of the α-, β- and γ-globulins preceeding their chemical analyses. It was hereby found that the chemical properties of the three fractions were different [7]. Tiselius suspected early that each of these three main globulin components might consist of several individual proteins that by chance had similar mobilities. This point of view has recently been verified particularly by the results from the large scale fractionation of blood plasma carried out in the United States during and after the last war.

The work of Tiselius and his coworkers on serum and serum proteins greatly stimulated new research on this important group of substances. The method is being used more and more in other kinds of biochemical investigations and the number of publications in which Tiselius' method has been used is sharply increasing from year to year. At present it is already in the hundreds. Short surveys on electrophoresis have been given by Tiselius [8].

Tiselius himself had hoped that his new electrophoresis technique, besides the above mentioned applications, might also have been useful to him in the solution of a problem which interested him deeply, *viz.*, the question of the identification and the isolation of large fragments and polypeptides obtained by a mild break down of protein molecules. In this respect the method was, however, a disappointment. He therefore started to look for other methods that could possibly serve his purpose and he thought that he had found it in adsorption analysis.

In preparative organic and biological chemistry adsorption methods had been used to some extent. Willstätter and his school purified their enzymes by adsorption to colloidal aluminium hydroxide, separation of the adsorbed complex from the mother liquor and subsequent elution of the enzyme from the colloidal hydroxide. The chromatographic method originally developed by Tswett had been extensively used for the separation, fractionation and purification of a number of natural pigments. Tiselius saw the possibility of this method, investigated it thoroughly and developed the adsorption method to an exact quantitative method for analysis of mixtures of closely related substances such as for instance amino acids, carbohydrates and fatty acids [9,10].

In the preliminary experiments Tiselius made use of the same optical arrangements as in electrophoresis. However, because the accuracy obtained was not quite adequate and because sometimes organic solvents were used, it was advisable to introduce other methods for following the experiments. In 1941 successive interferometric observation of the filtrate was introduced [11] and this has since been the standard method. A survey on adsorption analysis has been given by Tiselius [12] and a more detailed description of the various methods used may be found in Claesson's thesis [13]. Quite recently a very interesting

and promising new application of the adsorption method has been made by Claesson, who has shown how molecular weight frequency curves may be obtained from the adsorption analysis diagrams from high polymers[14]. With other high molecular weight substances, such as for instance the proteins, this kind of adsorption method has not been as successful, and since Tiselius himself has been especially interested in this group of substances, he has been looking for a modification of the technique which could make the method useful in this field, too. In the search for this, very encouraging results have been achieved by the introduction of »salting out adsorption»[15]. It is based on his observation that »proteins and other substances which are precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly already in salt solutions of lower concentration than is required for their precipitation, and that some adsorbents which in salt free solutions show no or only slight affinity for proteins, at moderately high salt concentrations become excellent adsorbents».

Tiselius is at present working on the development of this method along different lines and it seems as if it may offer a number of new possibilities in the fractionation of mixtures of proteins and high molecular peptides.

Beside his scientific activity Tiselius has also been engaged in very important committee work. In 1944 he became member of a governmental committee for proposing ways and means for promoting and intensifying pure scientific research in Sweden. He has been one of the most active members of that committee and his point of view has evidently played an important role in the editing of the report to the government. The majority of the proposals made by the committee were accepted by the government and the parliament, and became effective on July 1, 1946. It has really meant a great improvement in the conditions for pure science in Sweden. A number of new positions for assistants and technicians were given to the universities, they received more money for their scientific institutes and last but not least a Research Council for Natural Science (Statens Naturvetenskapliga Forskningsråd) was created, which could spend 1 million Swedish crowns a year on pure research mainly at the university institutes. Tiselius was chosen as its first chairman and he is as such playing an important role in the organization of Swedish science.

1. Svedberg, T., and Tiselius, A. *J. Am. Chem. Soc.* **48** (1926) 2272.
2. Tiselius, A. *Nova Acta Regiae Soc. Sci. Upsaliensis IV* **7** (1930) no. 4.
3. Tiselius, A. *Z. phys. Chem.* **A 169** (1934) 425; **A 174** (1935) 401; *J. phys. Chem.* **40** (1936) 223.
4. Tiselius, A. *Biochem. J.* **31** (1937) 313, 1464.
5. Tiselius, A. *Trans. Faraday Soc.* **33** (1937) 524.
6. Svensson, H. *Kolloid-Z.* **87** (1939) 181; **90** (1940) 141; *Arkiv Kemi, Mineral. Geol.* **A 22** (1946) no. 10.
7. Blix, G., Tiselius, A., and Svensson, H. *J. Biol. Chem.* **137** (1941) 485.
8. Tiselius, A. *Harvey Lectures* **35** (1939—40), 37; *XI Intern. Congr. Pure Applied Chem. (London)* (1947, Suppl. to *Chemistry & Industry* (1948) 9; *Les Prix Nobel en 1948* Stockholm (1949). To be published.
9. Tiselius, A. *Arkiv Kemi, Mineral. Geol.* **B 14** (1940) no. 22; *B* **14** (1941) no. 32; **B 15** (1941) no. 6; **A 16** (1943) no. 18.
10. Tiselius, A. In *The Svedberg 1884 30/8 1944* Upsala (1944) 370.
11. Tiselius, A., and Claesson, S. *Arkiv Kemi, Mineral. Geol.* **B 15** (1942) no. 18.
12. Tiselius, A. *Advances in Protein Chem.* **3** (1947) 67.
13. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **A 23** (1946) no. 1.
14. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **A 26** (1948) no. 24.
15. Tiselius, A. *Arkiv Kemi, Mineral. Geol.* **B 26** (1948) no. 1.

Kai O. Pedersen.

ACTA CHEMICA SCANDINAVICA 2 (1948) 625—638

Antibiotic Substances from the Heart Wood of *Thuja plicata* D. Don

II.* The Constitution of γ-Thujaplicin

HOLGER ERDTMAN and JARL GRIPENBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In a series of investigations relating to coniferous resin components and heart wood constituents we have hitherto only been concerned with trees belonging to the family *Pinaceæ*. Apart from ordinary terpenes and resin acids the resins and heart woods of these trees generally contain components of a phenolic nature.

Some of these, *e. g.* the pinosylvin of the heart wood of *Pinus silvestris* appear to be characteristic constituents of a whole genus. Other pines, however, in addition to pinosylvin contain a characteristic pattern of other heart wood constituents including flavones, flavanones, pinitol and arabinose [1]. Ultimately it appears to be possible to construct a chemical taxonomy for the genus *Pinus* on the basis of these findings. This should support and amplify that based on purely botanical evidence.

The amount or distribution of a component, *e. g.* a heart wood constituent, may sometimes differ widely from one individual to another probably through racial causes.

Some of these extraneous components occur in several genera of the *Pinaceæ* family either as such or in a structurally slightly modified state. Examples are conidendrin in *Picea*, *Tsuga* (and in the *Podocarpaceæ* family in *Podocarpus*), pinoresinol in *Picea* and *Pinus* and the closely related lariciresinol in *Larix*. This and other evidence indicate phylogenetic relations within the group.

Other large groups of conifers include the families *Taxodiaceæ* and *Cupressaceæ*. Their chemistry has been less closely investigated. However, there

* Part I, *Nature* **161** (1948) 719.

appears to be a marked difference between *Pinaceæ* and these families, although there are a few examples of common constituents. Redwood (*Sequoia sempervirens* Endl., *Taxodiaceæ*), *e. g.* contains in its heart wood pinitol, and *Chamæcyparis obtusa* Sieb. et Zucc. (*Cupressaceæ*) among other heart wood constituents hinokinin, a lignan closely related to the matairesinol of *Podocarpus spicatus*, conidendrin, pino- and lariciresinol.

On the whole, in the *Cupressaceæ* there appear to be less phenolic extraneous constituents, the majority being related to terpenes and sometimes possessing unusual structures. Examples are dehydrogeranic acid [2] (*Callitropsis araucarioides* Compt.), rhodinic acid [3] (*Chamæcyparis obtusa* Sieb. et Zucc., *Thujopsis dolabrata* Sieb. et Zucc.), citronellic acid [4] (*Callitris glauca* R. Br.), »dehydroperillic acid» [5] (*Thuja plicata* D. Don) and shonanic acid [6] (*Libocedrus formosana* Florin). When phenols are present they usually exhibit close relations to the terpene group *e. g.* carvacrol and hydrothymoquinone [7] (*Callitris quadrivalvis* Vent.). Hence, also from a chemical point of view these families appear to be separated from the *Pinaceæ*.

The present series of investigations deals with the elucidation of the unusual structure of some components of the heart wood of western red cedar (*Thuja plicata* D. Don, *Cupressaceæ*) which were previously unknown or regarded as being phenols.

Thuja plicata grows abundantly in the north western part of America between Alaska and California. It has been introduced frequently into Europe and in Sweden there is a plantation at Böda, island Öland in the Baltic.

Western red cedar possesses a very light non-resinous wood which, nevertheless, is very resistant to decay and is extensively used as timber. When in contact with unprotected iron the wood develops a black stain and the metal corrodes.

In 1907 Blasdale [8] isolated from the heart wood of *Thuja plicata* a compound $C_{10}H_{12}O_2$ m.p. 80°. In 1933 Anderson and Sherrard [5] described the isolation of an acid termed »dehydroperillic acid» m. p. 88° having the same elementary composition. In addition these authors reported the isolation of a »phenolic» isomeride m. p. 82° which was highly toxic towards *Fomes annosus*.

Anderson and Sherrard recovered their substances by steam distillation of the wood. In the course of their investigations they also obtained a liquid material possessing the composition $C_{10}H_{12}O_2$ (unpublished observations). Dr. Anderson has been kind enough to provide us with this product, which in the meantime had partly crystallised. From this material a new isomeride m. p. 52—52.5° was easily obtained. From reasons which will become obvious we term this compound *β-thujaplicin* and the elucidation of its structure will be the object of a joint communication with dr. Anderson (Part IV).

The present authors have extracted the heart wood of Swedish *Thuja plicata* with ether. It was found that in this way an almost negligible amount of the above mentioned compounds could be isolated. When the wood was extracted with acetone, however, a dark coloured material was obtained which was partly soluble in ether. The ether insoluble portion resembled Brauns' »native lignin» and was partly dissolved by hot bisulphite cooking acid.

This material or part of it apparently forms ether insoluble membranes in the wood within which the ether soluble constituents are deposited exactly as the pinosylvin of pines and conidendrin of the spruce [9].

»Dehydroperillic acid» was removed from the ether solution by sodium hydrogen carbonate. Less acidic components were extracted with sodium hydroxide. Acidification of the latter followed by steam distillation afforded an oil which was dissolved in alkali. Through a fortunate observation by one of us (J. G.) it was found that only part of the material was easily precipitated by carbon dioxide, forming an oil which readily crystallised in the ice chest.

This substance melts at 34° and constitutes still another isomeride $C_{10}H_{12}O_2$. It has been termed *α-thujaplicin* for reasons given in Part III. Prolonged treatment with carbon dioxide resulted in the precipitation of a more acidic fraction which crystallised directly proving to be identical with Anderson and Sherrards »phenol» m. p. 82° and for which we suggest the term *γ-thujaplicin.*

Thus, no less than four isomerides $C_{10}H_{12}O_2$ have been isolated from *Thuja plicata;* from American trees »dehydroperillic acid», γ- and β-thujaplicin but no α-thujaplicin; from Swedish grown trees »dehydroperillic acid», α- and γ-thujaplicin but no β-thujaplicin.

All the thujaplicins are stable compounds and no transformation of *e. g.* α-thujaplicin into β-thujaplicin has been observed. It remains to be investigated whether this difference between American and Swedish grown trees is due to racial causes.

α-, β- and γ-thujaplicin exhibit one and the same green ferric reaction. With copper acetate green copper salts are obtained which are readily soluble in chloroform. This is a characteristic and sensitive test for the presence of thujaplicins.

Their U. V. absorption spectra (Fig. 1) are very similar but differ greatly from that of »dehydroperillic acid». Hence the thujaplicins are evidently structurally closely related.

On catalytic hydrogenation γ-thujaplicin absorbs four molecules of hydrogen yielding a crystalline product $C_{10}H_{20}O_2$ (II) together with an oil which probably is a mixture of stereoisomeric octahydro-γ-thujaplicins.

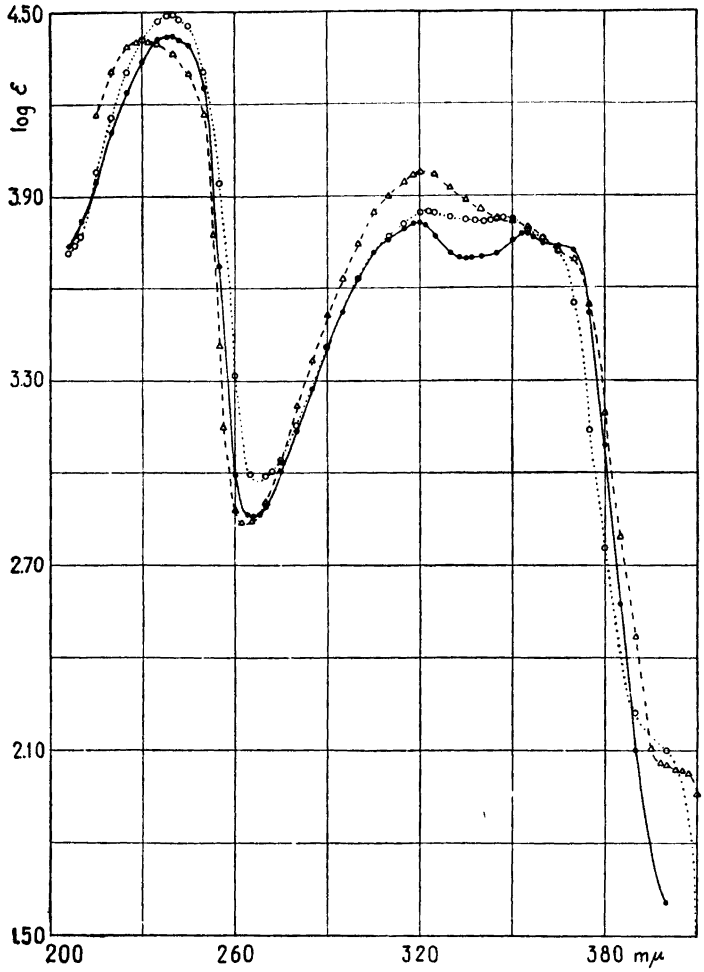


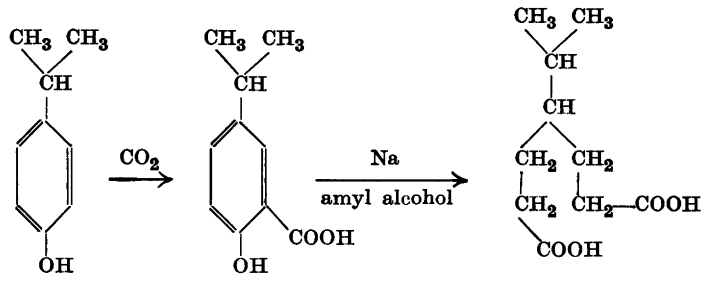
Fig. 1. Ultraviolet absorption curves: ●—● *α-thujaplicin,* ○···○ *β-thujaplicin and* △--△ *γ-thujaplicin in alcohol.*

On oxidation with periodate the compound $C_{10}H_{20}O_2$ affords a dialdehyde containing all ten carbon atoms of the original substance (III). The dialdehyde was characterised as its crystalline 2,4-dinitrophenylhydrazone. When oxidised with potassium permanganate both the crystalline and the liquid hydrogenation products of γ-thujaplicin yielded a dicarboxylic acid $C_{10}H_{18}O_4$ m. p. 65.5—66.5° (IV). These reactions characterise the hydrogenation products as being cyclic 1,2-diols.

Careful oxidation of γ-thujaplicin with chromic acid resulted in the formation of *iso*butyric acid, indicating the presence of an *iso*propyl group. Estimation of methyl attached to carbon yielded 0.49 moles of acetic acid. This is a normal yield from compounds containing *iso*propyl groups and excludes the presence of additional C-methyl groups.

On dry distillation of the barium salt of the dicarboxylic acid m. p. 65.5—66.5° an oil was obtained showing ketonic properties (V). This oil yielded a 2,4-dinitrophenylhydrazone and a semicarbazone which proved to be identical with the corresponding derivatives of 4-*iso*propyl*cyclo*hexanone. Furthermore, the oil, on oxidation with nitric acid, afforded β-*iso*propyladipic acid m. p. 76—77° (VI) identical with an authentic specimen.

It follows that the acid m. p. 65.5—66.5° must be γ-*iso*propylpimelic acid (IV). This acid which previously has not been described was prepared from *p*-*iso*propylphenol according to the following route:

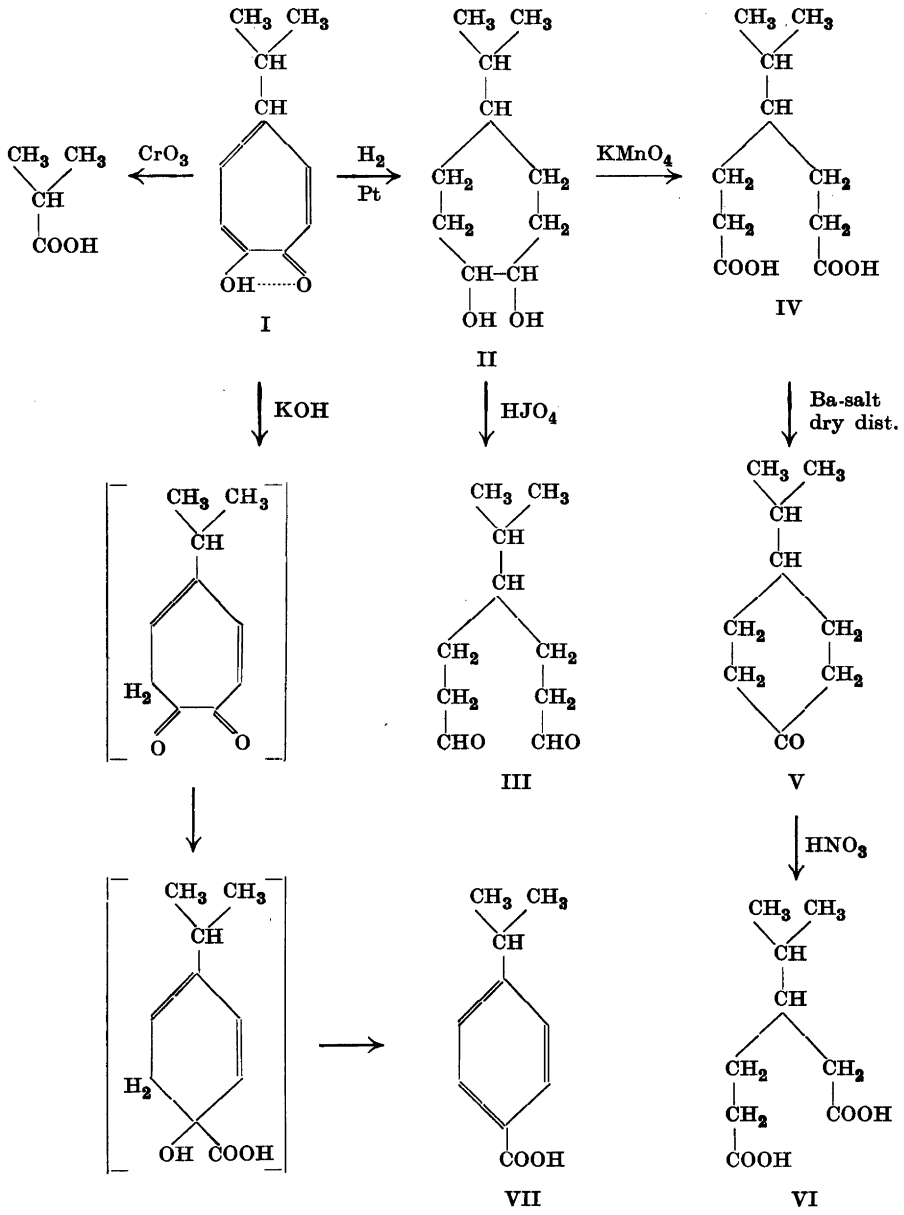


IV.

With diazomethan γ-thujaplicin yields an oily methyl derivative. It does not give any colour reaction with ferric chloride and therefore is obviously an O—CH_3 derivative. γ-Thujaplicin does not react with carbonyl reagents but reacts with bromine with formation of hydrogen bromide and a crystalline bromo derivative. It couples with diazotised amines giving red products. These reactions and the general stability of the compound recall the »aromatic nature» of benzene and its derivatives.

When heated with very strong alkali γ-thujaplicin is transformed into cuminic acid (*p*-*iso*propylbenzoic acid) (VII).

All these experimental results are readily explained on the basis of formula I for γ-thujaplicin and they are summarised in the following scheme:



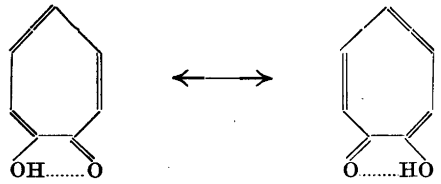
The name γ-thujaplicin refers to the fact that the *iso*propyl group occupies the γ-position relative to the oxygen atoms in the cycloheptatrien system. It is worth noting that γ-thujaplicin does not obey the isoprene rule although its close relation to the terpenes appears unmistakeable.

The formula I is in complete harmony with the properties of γ-thujaplicin. Since the structures of α- and β-thujaplicin are closely related to γ-thujaplicin (compare the two following papers) the discussion following below does also apply to them.

The acidic nature of the thujaplicins is to be expected by analogy with the carboxylic acid group, the hydroxy quinones and β-diketones. O = C — OH *viz.* O = C — C = C — OH. In the thujaplicins there are three double bonds interposed between the carbonyl group and the hydroxyl group. The acidity is due to the resonance:

$$(O{=}C{-}C{=}C{-}C{=}C{-}C{=}C{-}\overset{-}{O})\overset{+}{H} \longleftrightarrow \overset{-}{(O}{-}C{=}C{-}C{=}C{-}C{=}C{-}C{=}O)\overset{+}{H}$$

and this also explains the unreactive nature of the carbonyl group and the yellow colour of alkaline solutions of the thujaplicins. The formation of metal complexes soluble in chloroform probably arises through chelation. As a matter of fact the hydrogen atom of the hydroxyl group may be regarded as being shared between both oxygen atoms (hydrogen bond resonance VIII



VIII IX

—IX) although there appears to be some objection to this arising from the somewhat too long O—O-distance which can be calculated and which would not permit an ordinary hydrogen bond [10]. This is not a point of major structural concern but the peculiar properties of this novel structure merit further physico-chemical investigation.

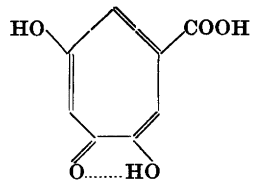
From the heart wood of the Japanese »hinoki» (*Chamaecyparis obtusa* Sieb. et Zucc.) *l*-rhodinic acid and a liquid compound $C_{10}H_{12}O_2$ termed »hinokitiol» have been isolated [3]. With ferric acetate a red crystalline complex was obtained which could be sublimed in a high vacuum (»hinokitin»). Several other metal complexes have been described [3, 11]. Hinokitiol was thought at first to be a cyclic β-diketone containing a five membered ring. Later the compound has obviously been regarded as containing a seven membered ring. In the *Chemical Abstracts* of 1947 a paper by Hiroshi Iinuma on »Coordination compounds of hinokitiol, *o*-diketone with seven membered ring» has been abstracted [11]. We have been unable, however, to find any article substantiating this statement.

Hinokitiol appears to be closely related to the thujaplicins. As it appears not to have been obtained in crystalline form it may perhaps be a mixture of different thujaplicins.

The unusual *cyclo*heptatrien ring structure of the thujaplicins appears not to be unique in the vegetable kingdom. It resembles, of course, the azulenes and could be regarded as an azulene in which the five membered ring has been removed by oxidation.

Some years ago Birkinshaw, Chambers and Raistrick [12] described a metabolite of the mould *Penicillium stipitatum* called stipitatic acid.

Dewar [13] interpreted their experimental results and advanced the formula X for this acid:

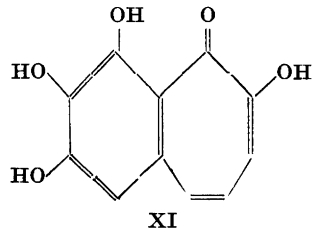


X

It exhibits a red ferric reaction and is converted into 5-hydroxy*iso*phthalic acid on heating with alkali.

Dewar [14] also suggested that colchicine might possess a similar *cyclo*heptatrienolone (»tropolone») structure and this idea has recently been confirmed by Arnstein, Tarbell, Huang and Scott [15]. With dilute acid colchicine is cleaved into methyl alcohol and colchiceine which is a true *cyclo*heptatrienolone. The latter compound shows a green ferric colour indistinguishable from that of the thujaplicins. It also gives a green copper complex soluble in chloroform. E. Rennerfelt has been kind enough to test the toxicity of colchiceine and its O-methyl derivative, colchicine, to fungi and reports that colchiceine is highly toxic whilst colchicine only possesses a low toxicity.

Quite recently Barltrop and Nicholson [16] advanced a new formula for purpurogallin (XI) the well known dehydrogenation product of pyrogallol, a glucoside of which is said to be a constituent of different galls (dryophantin) [17, 18].



XI

The tetramethyl ether of this compound on oxidation with permanganate, affords 3,4,5-trimethoxyphthalic acid, not formed, however, from the trimethyl ether which consequently must contain methoxyl groups distributed in both nuclei *.

When warmed with 2 *N* hydrochloric acid purpurogallin tetramethyl ether affords purpurogallin trimethyl ether identical with that obtained on methylation of purpurogallin with diazomethane. Thus the methoxyl group adjacent to the carbonyl group of the seven membered ring is not hydrolysed but one of the methoxyls of the benzene ring, probably the neighbouring one.

Similar to stipitatic acid purpurogallin [19] readily undergoes a benzilic acid change with formation of a six membered ring when subjected to the action of strong alkali.

All thujaplicins are highly toxic towards a great variety of wood destroying fungi and are obviously responsible for the great durability of the wood. A comprehensive study of this mycological aspect will be published elsewhere by Docent Rennerfelt, Statens Skogsforskningsinstitut, Stockholm. Thujaplicin appears to be a fungicidic substance rather than a fungistatic. When spores of *Pullularia pullulans* were treated with a 0.02 % solution of γ-thujaplicin all were alive after one hour. After three hours treatment 11 % were alive and after 6 and 24 hours all spores were killed. Quite recently the antibiotic character of the crude aqueous extract of the heart wood of *Thuja plicata* has been emphasized by Southam [20].

The toxicity of β-thujaplicin (compare part IV) to fish has been studied. *Lebistes reticulatus* died in a 0.02 % solution within 10 minutes and within 3 hours in a 0.002 % solution of this substance.

It appears to be more toxic than pinosylvin (Erdtman [9] 1939).

EXPERIMENTAL

Extraction of the wood

Batches of about 2 kg of finely powdered heart wood of *Thuja plicata* were extracted for 48 hours with acetone in a continuous extractor. From the combined extracts of several such batches the acetone was distilled off on a water bath. The remaining thick,

* This agrees with earlier unpublished results by one of us (H. E.) in University College, London, 1929. It was found that the trimethyl ether did not yield O-trimethylgallic acid as would be expected from the old Willstätter-Heiss formula. On oxidation with permanganate in the presence of magnesium sulphate a brown red crystalline compound (m. p. 163—165°) of hydroxyquinone character was obtained from the manganese sludge. From the tetramethyl ether 3,4,5-trimethoxyphthalic acid was obtained, isolated as the corresponding phthalimid and identified with an authentic specimen. On this occasion XI and alternatives with different distribution of the ionisable hydrogen atoms was discussed.

almost black oil was poured into a large volume of ether when a sticky resinous precipitate was formed. The ether was decanted and the precipitate washed thoroughly with ether. The combined ethereal solutions were extracted with a saturated solution of sodium hydrogen carbonate in order to remove »dehydroperillic acid». This was followed by extractions with 2 *N* sodium hydroxide solution. The very dark alkaline solution was acidified with sulphuric acid and extracted with ether. The ether was distilled off and the residue distilled with steam, until the distillate no longer showed any green colour in the ferric test. The distillate was saturated with sodium chloride and extracted with ether. The ether was extracted first with sodium hydrogen carbonate solution then with 2 *N* sodium hydroxide. The alkaline solution was agitated with a mechanical stirrer and carbon dioxide passed in. At pH ~ 9 an oil began to separate. The introduction of carbon dioxide was continued to pH ~ 8. The precipitated red oil was extracted with ether (Extract A). Carbon dioxide was passed again when a further precipitate appeared. When the solution had been saturated with carbon dioxide the precipitate was filtered and the solution extracted with ether (Extract B). The slightly brown crystalline precipitate consists of γ-thujaplicin.

Extract A was evaporated to dryness and the oily residue kept in a refrigerator overnight when it partly crystallised. The crystals were α-thujaplicin.

On evaporation extract B yielded an oil from which a small amount of γ-thujaplicin slowly crystallised. The combined mother liquors of extract A and B were dissolved in ether, the ethereal solution extracted with sodium hydroxide and the stepwise precipitation of α- and γ-thujaplicin with carbon dioxide repeated. In this way further amounts of α- and γ-thujaplicin were obtained. The yield varies considerably ranging from about 0.05 per cent down to almost nothing.

The γ-thujaplicin was distilled in a high vacuum and recrystallised from light petroleum. It formed long colourless needles, m. p. 82°.

$C_{10}H_{12}O_2$(164.1)	Calc.	C 73.13	H 7.37	$-CCH_3$ 9.15
	Found	» 72.97	» 7.37	» 4.0

With ferric chloride the alcoholic solution gives a green colour. By shaking its chloroform solution with a cupric acetate solution the chloroform is coloured intensely green. Evaporation of the chloroform gives a green crystalline copper complex m. p. 259—260°, easily soluble in the common organic solvents except light petroleum.

Oxidation of γ-thujaplicin

γ-Thujaplicin (0.2 g) was dissolved in dilute sulphuric acid (1 : 1, 20 ml). Sodium bichromate (1.5 g) dissolved in a small amount of water was then added in small portions. By external cooling the temperature was kept below 20°. After standing overnight the solution was distilled with steam. In the distillate the acid was determined by titration with standard alkali. This showed the presence of 0.53 millimoles of volatile acid (44 % of theoretical amount). The acid was converted to its p-bromophenacyl ester in the usual manner. After recrystallisation from dilute methanol it had m. p. 76—77°, undepressed in admixture with authentic *iso*butyric acid p-bromophenacyl ester.

Catalytic hydrogenation of γ-thujaplicin

γ-Thujaplicin (2.5 g) was dissolved in absolute ethanol and hydrogenated with Adams' platinum oxide catalyst. 1400 ml of hydrogen were consumed (calculated for 4 moles: 1360 ml). The catalyst was removed by filtration and the alcohol evaporated in a vacuum. On cooling the remaining oil partly crystallised. The crystals (1.3 g) were recrystallised from ligroin, m. p. 87—88°.

$C_{10}H_{20}O_2$ (172.2)	Calc.	C 69.69	H 11.73
	Found	» 69.60	» 11.85

A solution of this glycol, octahydro-γ-thujaplicin, in water consumes 1 mole of periodic acid. On addition of 2,4-dinitrophenylhydrazine dissolved in 2 *N* hydrochloric acid to the solution of the oxidised glycol a yellow precipitate was slowly formed. This was recrystallised from ethyl acetate, m. p. 194—195°.

$C_{22}H_{26}O_8N_8$ (530.3)	Calc.	N 21.14
	Found	» 21.24

Oxidation of octahydro-γ-thujaplicin

Octahydro-γ-thujaplicin (0.95 g) was suspended in 100 ml of water containing a small amount of sodium carbonate and 1.8 g potassium permanganate was then added in small portions at room temperature. When the permanganate colour had disappeared, dilute sulphuric acid was added and the manganese dioxide dissolved with sulphur dioxide. The solution was extracted with ether, the ethereal solution extracted with sodium hydrogen carbonate, the acid liberated by sulphuric acid and recovered with ether. After evaporation of the ether, the remaining oil (0.8 g) soon crystallised. On recrystallisation from a mixture of ether and light petroleum it had m. p. 51—52.5°, but after a few days the melting point increased to 65.5—66.5°. Subsequently only the higher melting point was observed.

$C_{10}H_{18}O_4$ (202.2)	Calc.	C 59.35	H 9.00
	Found	» 59.43	» 8.95

The oily portion of the hydrogenated γ-thujaplicin (0.47 g) on oxidation in a similar manner gave 0.4 g of the same acid.

4-*Iso*propyl*cyclo*hexanone

The above acid was converted into its barium salt. Dry distillation of this afforded an oil with a menthone-like smell. The oil was divided into two parts and these treated with 2,4-dinitrophenylhydrazine and semicarbazide respectively. After recrystallisation from methanol the 2,4-dinitrophenylhydrazone was obtained as orange red needles m. p. 118—119°.

$C_{15}H_{20}O_4N_4$ (320.2)	Calc.	N 17.50
	Found	» 17.62

The semicarbazone was recrystallised from dilute alcohol, m. p. 188—189°.

$C_{10}H_{19}ON_3$ (197.2)	Calc.	N 21.30
	Found	» 21.01

Neither substance showed any depression of melting point when mixed with the corresponding derivative of synthetic 4-*iso*propyl*cyclo*hexanone prepared according to Vavon and Callier [21] from *p-iso*propylphenol.

Oxidation of 4-*iso*propyl*cyclo*hexanone to *β-iso*propyladipic acid

The crude 4-*iso*propyl*cyclo*hexanone (0.05 g) was treated with conc. nitric acid. When the evolution of nitric oxides had ceased and the oil had disappeared the solution was neutralised and then made acid to congo red. After extraction with ether and evaporation of the ethereal extract an oil remained which partly crystallised. The crystals were dried on a porous plate and recrystallised from ether—light petroleum. A few milligrams of a substance m. p. 77—78° were obtained. The melting point was not depressed in admixture with an authentic sample of *β-iso*propyladipic acid, m. p. 78—80°.

Rearrangement of γ-thujaplicin to cuminic acid

γ-Thujaplicin (0.1 g) was added to potassium hydroxide (1 g) and a few drops of water and the mixture heated to about 230° in a nickel crucible. After 30 min. at this temperature the top layer was almost black. The melt was dissolved in water, a small amount of unchanged γ-thujaplicin precipitated with carbon dioxide and dilute sulphuric acid added. A precipitate (0.01 g) was formed. This was sublimed and recrystallised from dilute acetic acid, m. p. 115—116°, undepressed by an authentic sample of cuminic acid, m. p. 116—117°.

Synthesis of γ-*iso*propylpimelic acid

2,2-Di-(*p*-hydroxyphenyl)-propane (20 g) was mixed with about 1 g of a 15 % palladium charcoal catalyst and distilled very slowly (2—3 hours). The distillate was fractionated through a short column to remove phenol. When the temperature had reached 220° the distillation was interrupted and the residue distilled in a vacuum from an ordinary Claisen flask, b. p. 109—110°/10 mm. The distillate (5 g) immediately solidified and had m. p. 61°. The yield obtained by this process, which does not require any high pressure apparatus, is quite comparable with that reported by Cook, Philip and Somerville [22].

The 4-*iso*propylphenol was converted into 2-hydroxy-5-*iso*propylbenzoic acid as described by Paternò and Mazzara [23].

This acid (0.55 g) was dissolved in amyl alcohol (50 ml) and to the boiling solution was added small pieces of sodium (3 g). After the sodium had dissolved the amyl alcohol was removed by steam distillation. The residue was acidified and extracted with ether. The ether was evaporated and the remaining oil distilled with steam. The water solution

containing the nonvolatile acid was evaporated on a water bath. An oil which readily crystallised was obtained. After recrystallisation from ether—light petroleum the melting point was 65—66°.

$C_{10}H_{18}O_4$ (202.2)	Calc.	C 59.30	H 9.00
	Found	» 59.26	» 8.95

When mixed with the acid m. p. 65.5—66.5° obtained by oxidation of octahydro-γ-thujaplicin no depression of the melting point was observed.

Hydrolysis of O-tetramethyl purpurogallin

O-tetramethyl purpurogallin (0.1 g) was heated on a water bath with 2 *N* hydrochloric acid (10 ml). The substance melted and long yellow needles soon appeared. When all the oil had disappeared the crystals were recovered and recrystallised from alcohol, m. p. 174—176°, undepressed in admixture with an authentic sample of the trimethyl ether of purpurogallin obtained by methylation of purpurogallin with diazomethane.

SUMMARY

From the heart wood of western red cedar (*Thuja plicata* D. Don.) grown in Sweden, »dehydroperillic acid», $C_{10}H_{12}O_2$, together with two isomeric, crystalline compounds which have been termed α- and γ-thujaplicin have been isolated. In addition to »dehydroperillic acid» Anderson and Sherrard in 1932 working with American grown wood have isolated a compound which is identical with γ-thujaplicin. One of the liquid fractions obtained by them had crystallised in the meantime and from this material we isolated another isomeride $C_{10}H_{12}O_2$ which has been termed β-thujaplicin.

The structure of γ-thujaplicin, which is highly toxic to a great variety of fungi and to fish, has been elucidated. It contains a seven membered ring and is 1-*iso*propyl*cyclo*heptatrien-(1,3,6)-ol-(4)-one-(5), or on the basis of Dewar's suggestion to term this particular *cyclo*heptatrienolone system tropolone, γ-*iso*propyltropolone.

We wish to express our sincere thanks to Dr. A. B. Anderson, Portland, Oregon for providing us with products isolated by him from American *Thuja plicata*. Generous grants from *Statens Tekniska Forskningsråd* are also gratefully acknowledged.

The analyses were carried out by W. Kirsten, Upsala.

REFERENCES

1. Erdtman, H. *Svensk Papperstidn.* **46** (1943) 226, *Svensk Kem. Tid.* **56** (1944) 2, *Svensk Kem. Tid.* **56** (1944) 26, *Svensk Kem. Tid.* **56** (1944) 95.
2. Cahn, R. S., Penfold, A. R., and Simonsen, J. L. *J. Chem. Soc.* (1931) 3134.

3. Nozoe, T. *Bull. chem. Soc. Japan* **11** (1936) 295 *(Chamaecyparis)*, Kitajima, K. *Extr. from the Bull. Imp. Forestry Exp. Station* No. 2 (1933) 13 *(Thujopsis)*.
4. Trikojus, V. M., and White, D. E., *J. Proc. Roy. Soc. N. S. Wales* **66** (1933) 284.
5. Anderson, A. B., and Sherrard, E. C., *J. Am. Chem. Soc.* **55** (1933) 3813.
6. Ichikawa, N. *Bull. chem. Soc. Japan* **11** (1936) 759, **12** (1937) 267.
7. Grimal, E. *Compt. rend.* **139** (1904) 927.
8. Blasdale, W. C. *J. Am. Chem. Soc.* **29** (1907) 539.
9. Hägglund, E., Holmberg, J., and Johnson, T. *Svensk Papperstidn.* **39** (1936) Special-nummer 37, Erdtman, H. *Ann.* **539** (1939) 116, *Svensk Papperstidn.* **46** (1943) 226.
10. Dewar, M. J. S. *Nature* **155** (1945) 479.
11. Iinuma H. *J. Chem. Soc. Japan* **64** (1943) 742, *Chem. Abstr.* **41** 1947) 4731.
12. Birkinshaw, J. H., Chambers, A. R., and Raistrick, H. *Biochem. J.* **36** (1942) 242.
13. Dewar, M. J. S. *Nature* **155** (1945) 50.
14. Dewar, M. J. S. *Nature* **155** (1945) 141.
15. Arnstein, H. R. V., Tarbell, D. S., Huang, H. T., and Scott, G. P. *J. Am. Chem. Soc.* **70** (1948) 1669.
16. Barltrop, J. A., and Nicholson, J. S. *J. Chem. Soc.* (1948) 116.
17. Nierenstein, M. *J. Chem. Soc.* **115** (1919) 1328.
18. Nierenstein, M., and Swanton, A. *Biochem. J.* **38** (1944) 373.
19. Perkin, A. G., and Steven, A. B. *J. Chem. Soc.* **83** (1903) 192.
20. Southam, C. H. *Proc. Soc. Exptl. Biol. Med.* **61** (1946) 391, *Chem. Abstr.* **40** (1946) 4409.
21. Vavon, G., and Callier, A. *Bull. soc. chim. France* [4] **41** (1927) 677.
22. Cook, J. W., Philip, R., and Somerville, A. R. *J. Chem. Soc.* (1948) 164.
23. Paternò, E., and Mazzara, G. *Gazz. chim. ital.* **8** (1878) 389, Beilstein (4 Ed.) X. 271.

Received July 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 639—643

Antibiotic Substances from the Heart Wood of *Thuja plicata* D. Don

III.* The Constitution of α-Thujaplicin

JARL GRIPENBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

As briefly mentioned in the preceding paper[1] a compound, m. p. 34° has been isolated from the heart wood of Western red cedar *(Thuja plicata* D. Don) grown in Sweden (Böda, Öland) in addition to »dehydroperillic acid» and γ-thujaplicin. This substance is an isomeride of the latter compounds possessing the formula $C_{10}H_{12}O_2$. For reasons given below it has been termed α-thujaplicin.

The well crystalline substance is less acidic than γ-thujaplicin. It exhibits exactly the same green ferric colour reaction and similar green chloroform soluble copper complex as does γ-thujaplicin. Their ultra violet absorption curves are also very similar (Fig. 1 in the previous paper).

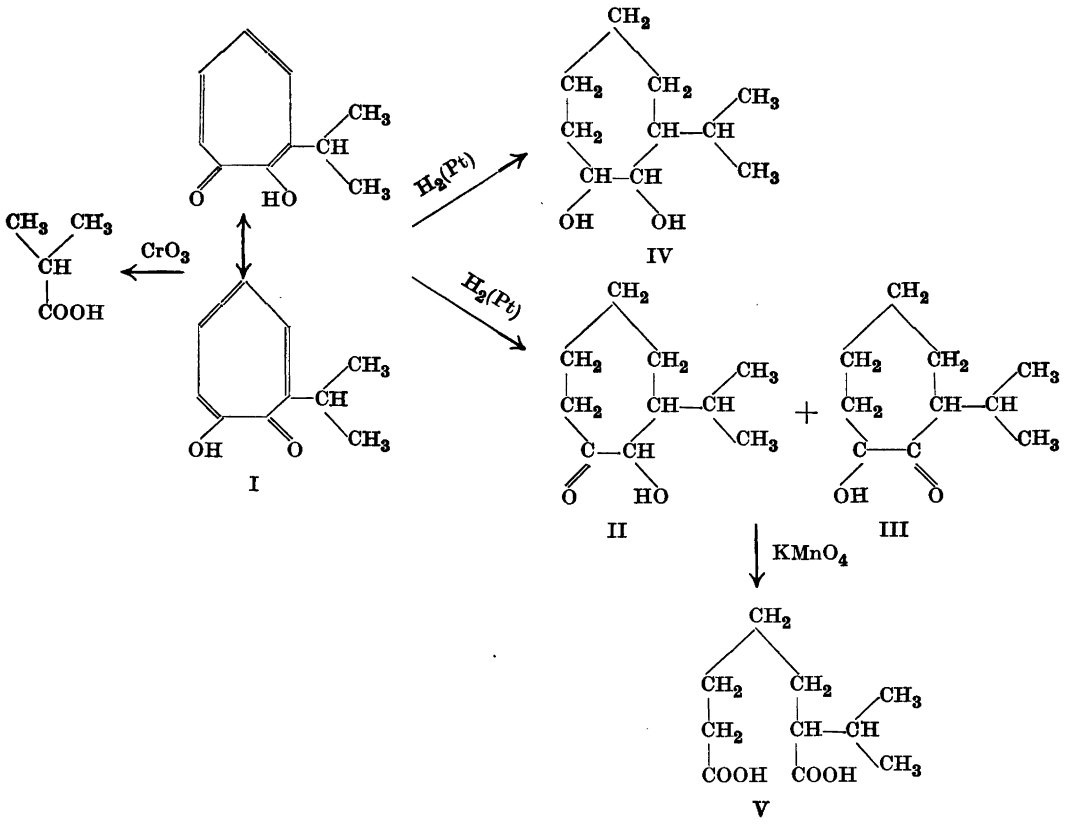
It seemed justifiable, therefore, to assume a closely analogous structure for α-thujaplicin and consequently the degradation experiments were carried out correspondingly.

Depending upon the quality of the platinum oxide catalyst α-thujaplicin on catalytic hydrogenation absorbs three or four moles of hydrogen in alcoholic solution. In the first case the resulting hydrogenation product is an oil which yields a yellow crystalline precipitate with 2,4-dinitrophenylhydrazine. It reacts with periodic acid and hence probably contains the grouping —CO—CHOH—. On oxidation with potassium permanganate an acid $C_{10}H_{18}O_4$ was obtained, which did not crystallise but was characterised as the well crystalline diamide. Attempts to characterise the acid as its benzylthiuronium salt failed since, probable due to hydrolysis, the salt could not be obtained with a constant melting point. When four moles of hydrogen are consumed

* Part II. *Acta Chem. Scand.* 2 (1948) 625.

during the hydrogenation of α-thujaplicin an oily diol is formed. Like γ-thujaplicin, α-thujaplicin on carefully conducted oxidation with chromic acid yields *iso*butyric acid. Hence, by analogy with the degradation of γ-thujaplicin to γ-*iso*propylpimelic acid one would expect the acid $C_{10}H_{18}O_4$ to be α- or β-*iso*propylpimelic acid. The former was synthesised and found to be identical with the acid obtained by degradation of α-thujaplicin.

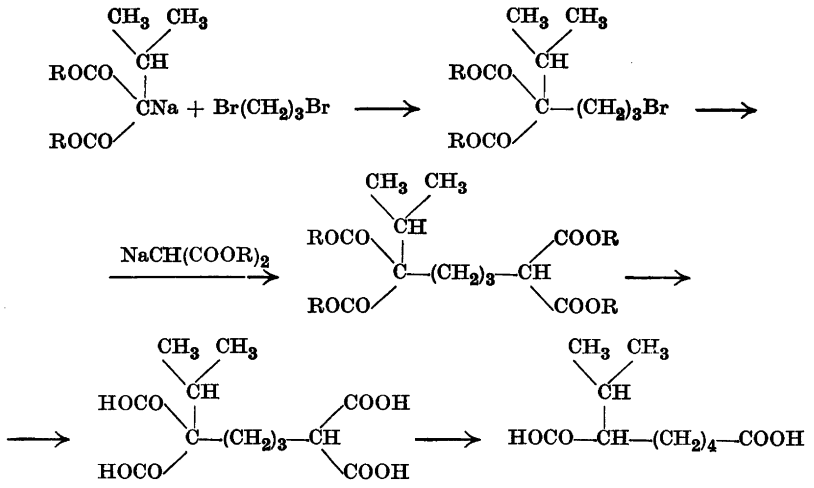
It follows that α-thujaplicin possesses structure I and the hydrogenation and oxidative fission of the hydrogenation product may be outlined in the scheme:



The name α-thujaplicin defines the position of the *iso*propyl group as being α relative to the oxygen atoms.

The oily hydrogenation product of α-thujaplicin most probably is a mixture of the keto alcohols II and III and the glycol IV. On oxidation they are oxidised to α-*iso*propylpimelic acid.

The synthesis of *α-iso*propylpimelic acid was accomplished by a method closely analogous to that of Francke and Kroupa [2] for the synthesis of *α-n*-propylpimelic acid.



V

The acid V was only obtained as an oil but furnished a diamide, the m. p. of which was undepressed when mixed with the diamide of the acid obtained by degradation.

Regarding the physico-chemical aspects of the α-thujaplicin molecule, so similar to those of γ-thujaplicin, it is sufficient to refer to the previous paper [1].

Like γ-thujaplicin, α-thujaplicin is a potent antibiotic. It appears, however, to be somewhat less active than γ- and β-thujaplicin. A full account of these properties will be published shortly by Docent E. Rennerfelt.

EXPERIMENTAL

The extraction of the heart wood and the separation of α-thujaplicin from the accompanying γ-thujaplicin has been described in the previous paper. The α-thujaplicin thus obtained was distilled in a vacuum and recrystallised from light petroleum, m. p. 34°.

$C_{10}H_{12}O_2$ (164.1)	Calc.	C 73.13	H 7.37
	Found.	» 73.27	» 7.41

Oxidation of α-thujaplicin

α-Thujaplicin was oxidised in the same manner as γ-thujaplicin, 0.2 g α-thujaplicin yielding 0.615 millimole of volatile acid (50 % of theoretical amount). The salt solution

was evaporated to dryness and the acid converted to its *p*-bromophenacyl ester. After recrystallisation from dilute alcohol this had m.p. 76—77° alone or admixed with an authentic sample of *iso*butyric acid *p*-bromophenacyl ester.

Hydrogenation of α-thujaplicin

a) α-Thujaplicin (1 g) was dissolved in alcohol and hydrogenated with Adams' PtO_2-catalyst. The hydrogen uptake amounted to 430 ml (the consumption of three moles of hydrogen requires 410 ml). The alcohol was removed in a vacuum and the product distilled. B.p. 100—110°/17 mm. With 2,4-dinitrophenylhydrazine a yellow precipitate appeared immediately.

$C_{10}H_{18}O_2$ (170.2)	Calc.	C 70.51	H 10.68
	Found.	» 69.67	» 10.92

b) α-Thujaplicin (0.2 g) was hydrogenated with Adams' PtO_2-ctalyst [of better quality than in a)] in alcohol. 110 ml of hydrogen were absorbed (calculated for 4 moles 110 ml). The alcohol was removed in a vacuum. The remaining very viscous oil was distilled in a high vacuum.

$C_{10}H_{20}O_2$ (172.2)	Calc.	C 69.69	H 11.73
	Found.	» 69.30	» 11.55

Oxidation of the hydrogenation product

The hydrogenation product from a) (0.35 g) was suspended in water, and a small amount of sodium carbonate added followed by potassium permanganate (0.4 g) in small portions. The reaction mixture was acidified and the manganese dioxide dissolved with sulphur dioxide. The acid was extracted with ether and the ether shaken with sodium hydrogen carbonate solution. The acid was regenerated with mineral acid and recovered by ether extraction. After evaporation of the ether 0.22 g of an oil was obtained, which did not crystallise.

The acid was converted into its diamide through the acid chloride in the usual manner. The diamide was recrystallised from ethyl acetate m.p. 167—168°.

$C_{10}H_{20}O_2N_2$ (200.2)	Calc.	N 13.99
	Found.	» 14.02

Synthesis of α-*iso*propylpimelic acid

α-*Iso*propyl-α,α'-dicarboxypimelic acid was prepared following the directions of Francke and Kroupa [2] for the preparation of α-*n*-propyl-α,α'-dicarboxypimelic acid, using *iso*propyl bromide instead of *n*-propyl bromide. The acid was recrystallised from light petroleum—ether, decomposition point 180—185°. It can also be recrystallised from conc. hydrochloric acid. Prolonged heating with the mineral acid, however, causes decarboxylation.

$C_{12}H_{18}O_8$ (290.2)	Calc.	C 49.62	H 6.27
	Found.	» 49.93	» 6.47

*α-Iso*propylpimelic acid was obtained by heating the tetracarboxy compound (0.5 g) to 190° in an oil bath until carbon dioxide evolution ceased. The product was distilled in a vacuum and obtained as an oil which could not be induced to crystallise. It was converted into its amide which on recrystallisation from ethyl acetate, had m. p. 166—167°, undepressed on admixture with the amide of the acid obtained by degradation of α-thujaplicin.

$C_{10}H_{20}O_2N_2$ (200.2)	Calc.	N 13.99
	Found.	» 13.97

SUMMARY

The structure of α-thujaplicin, one of the toxic principles of the heart wood of *Thuja plicata* D. Don, grown in Sweden, has been elucidated. It is 1-*iso*propyl*cyclo*heptatrien-(1,4,6)-ol-(2)-one-(3) *viz.* 1-*iso*propyl*cyclo*heptatrien-(3,5,7)-ol-(3)-one-(2), or *α-iso*propyltropolone.

Generous grants from *Statens Tekniska Forskningsråd* are gratefully acknowledged. The analyses were carried out by W. Kirsten, Upsala.

REFERENCES

1. Erdtman, H., and Gripenberg, J. *Acta Chem. Scand.* 2 (1948) 625.
2. Francke, A., and Kroupa, A. *Monatsh.* 69 (1936) 167.

Received July 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 644—650

Antibiotic Substances from the Heart Wood of *Thuja plicata* D. Don

IV.* The Constitution of β-Thujaplicin

ARTHUR B. ANDERSON** and JARL GRIPENBERG

*Forest Products Laboratory ***, Forest Service U. S. Department of Agriculture, Madison, Wisconsin, U. S. A.* and
Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In a joint communication with Sherrard [1] one of the present authors (A. B. A.) described the isolation of »dehydroperillic acid» and a »phenol», m. p. 82°, $C_{10}H_{12}O_2$, now termed γ-thujaplicin [2], from the heart wood of western red cedar (*Thuja plicata* D. Don). The crystalline »phenol», however, constituted only part of the »phenolic» fraction of the volatile oil obtained by steam distillation of the wood. The remainder was an oil possessing approximately the same composition as γ-thujaplicin. In the course of time (16 years have elapsed) the oil had partly crystallised. Purification of the crystals readily yielded a pure compound m. p. 52—52.5° having the composition $C_{10}H_{12}O_2$. Hence, it is an isomeride of »dehydroperillic acid», α-and γ-thujaplicin and has been termed β-thujaplicin for reasons given below. It exhibits the same green ferric colour reaction as α- and γ-thujaplicin and gives a chloroform soluble copper complex. Its U. V. absorption curve (Fig. 1, Part II) is also very similar to those of α- and γ-thujaplicin. Consequently a close structural relationship between β-thujaplicin and the two other thujaplicins was expected.

The degradation of β-thujaplicin, therefore, followed the route outlined in Part II. β-Thujaplicin, like γ-thujaplicin, on catalytic hydrogenation consumed 4 moles of hydrogen, yielding a thick oil, which could not be obtained

---

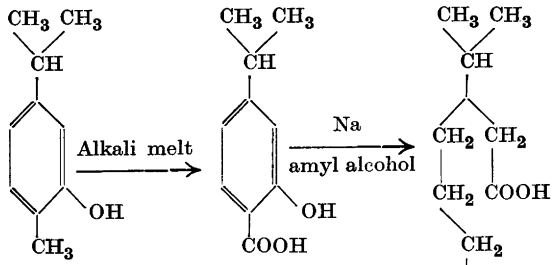
* Part III. *Acta Chem. Scand.* 2 (1948) 639.

** Present address: Western Pine Association, Research Laboratory, Portland, Oregon. U. S. A.

*** Maintained at Madison, Wisconsin, in cooperation with the University of Wisconsin.

crystalline. This oil may be a mixture of diastereoisomeric octahydro-β-thujaplicins. The composition of this product corresponded to $C_{10}H_{20}O_2$ (II). It reduced periodic acid and on oxidation with potassium permanganate it yielded a dicarboxylic acid $C_{10}H_{18}O_4$ (III). This also was obtained only as an oil, but was characterised as the bis-benzylthiuronium salt, m. p. 152.5—153°. Dry distillation of the barium salt of the acid yielded an oil showing ketonic properties and a smell resembling that of menthone. From this a 2,4-dinitrophenylhydrazone, m. p. 139—140° and a semicarbazone, m. p. 187—188° were obtained. These figures agree fairly well with those given by Whitmore and Pedlow [3] (139—140° and 189—190° resp.) for the corresponding derivatives of 3-*iso*propyl*cyclo*hexanone (IV).

β-Thujaplicin like α- and γ-thujaplicin gives *iso*butyric acid on careful oxidation with chromic acid. Therefore it could be anticipated that the acid $C_{10}H_{18}O_4$ should be the hitherto unknown β-*iso*propylpimelic acid. This was synthesised by the following route:



III

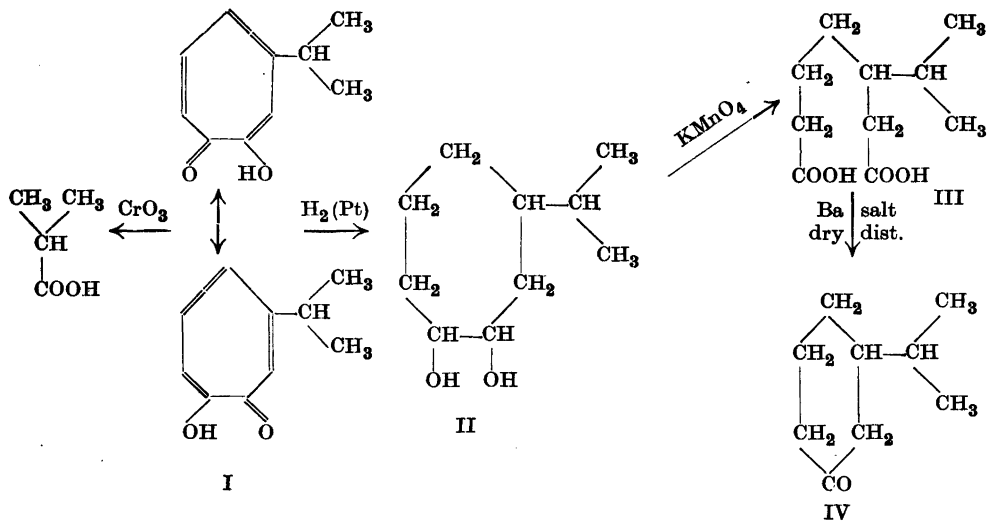
The oily dicarboxylic acid obtained, was characterised as its bis-benzylthiuronium salt m. p. 152.5—153°, undepressed when admixed with the corresponding salt of the acid obtained by degradation of β-thujaplicin.

It follows that β-thujaplicin possesses structure I and the degradation reactions are outlined in the scheme on page 566.

The name β-thujaplicin refers to the position of the *iso*propyl group relative to the oxygen atoms.

Regarding the physico-chemical aspects of the formulation of β-thujaplicin compare Part II [2].

Like α- and γ-thujaplicin, β-thujaplicin is highly toxic towards wood destroying fungi. A full account of the mycological aspect of these substances will be published elsewhere by E. Rennerfelt. As a matter of fact β-thujaplicin appears to be slightly more toxic than α- and γ-thujaplicin.



As already pointed out in Part II the yield of the thujaplicins varies considerably in different samples of heart wood. A study of the distribution of the thujaplicins (β- and γ-thujaplicin) in two different trees was carried out by one of us (A. B. A. 1932) in the Forest Products Laboratory, Madison, Wisconsin, in connexion with the early investigation by Anderson and Sherrard [1]. Sections from a tree were obtained from Portland, Oregon, which had been in a mill pond for some time and the other tree was obtained from Snoqualmie Falls, Washington. The latter tree was immediately sent to the laboratory after being felled.

The presence of the thujaplicins was determined by testing the toxicity to *Fomes annosus* of the steam distillate of the samples obtained as described in the experimental part.

The results are given in the following tables:

Table 1. Distribution of toxic principle in tree from Snoqualmie Falls, Washington.

Distance from center of butt of log	Colour with alcoholic $FeCl_3$	Killing or inhibition point (10 ml = 1 g wood)	Section 15 feet from butt of log (distance from center)	Colour with alcoholic $FeCl_3$	Killing or inhibition point (10 ml = 1 g wood)
Inches		ml	Inches		ml
0—4	Intense green . . .	10	0—4	Intense green . .	10
4—8	. . .do . . .	12	4—8	. . .do . .	10
8—12	. . .do . . .	12	8—12	. . .do . .	10
12—16	. . .do . . .	10	12—16	. . .do . .	10
16—20	. . .do . . .	10	16—20	. . .do . .	10

Table 2. Distribution of toxic principle in tree from Portland, Oregon.

Distance from center of butt of log	Colour with alcoholic $FeCl_3$	Killing or inhibition point (10 ml = 1 g wood)	Section 15 feet from butt of log (distance from center)	Colour with alcoholic $FeCl_3$	Killing or inhibition point (10 ml = 1 g wood
Inches		ml	Inches		ml
0—3	No colour . .	Nontoxic . .	0—3	No colour . .	Nontoxic
3—6	. . . do . . .	. . . do . . .	3—6	. . . do . . .	. . . do . . .
6—9	Slight yellow green . . .	50	6—9	Slight yellow green	50
9—12	Intense green	15	9—12	. . . do . . .	50
12—16	. . . do . . .	15	12—16	Intense green . . .	15

The results in Table 1 refer to the tree analysed immediately after felling: The amount of toxic principles in this tree does not vary appreciably, being very uniform throughout the cross section of the heart wood. The results in Table 2 refer to the tree which had been in the mill pond for some time. In this case the highest toxicity was found in the heart wood adjoining the sapwood, decreasing toward the center of the cross section. The center of the heart wood contained no volatile toxic substances. The amount of volatile material present in the heart wood adjoining the sapwood did not vary with the height of the tree.

The results show that the amount of toxic principles in these two trees varies greatly. It is, of course, uncertain whether these differences are due to the different treatment of the trees prior to laboratory investigation or to racial or other causes.

In 1941 Cartwright [4] found variability in resistance to decay of the heart wood of home grown (English) Western red cedar in different parts of the log. That extracts of Thuja plicata inhibits the growth of different fungi has been reported by Sowder [5] and Hubert [6].

EXPERIMENTAL

Isolation of β-thujaplicin (A. B. A.)

Sawdust of heart wood of American grown western red cedar was distilled with steam in a copper cylinder, capacity about 5 kg. The distillation was continued until the distillate no longer gave a green ferric coloration. The first 20 liters of the distillate

were quite cloudy, a very small amount of volatile oil finally settling at the bottom. After separating the volatile oil, the total distillate was made alkaline with a concentrated barium hydroxide solution. The yellow precipitate that formed was filtered off and the alkaline solution then reduced to a volume of about 300 ml in large evaporating dishes over a gas flame. After cooling the yellow precipitate was filtered. The yellow barium salts were combined, decomposed with dilute sulphuric acid and the resulting brown oil extracted with ether. The ether solution was then extracted three times with 5 per cent sodium hydroxide solution. The dark coloured alkaline solution was then saturated with carbon dioxide and the thujaplicins thus liberated extracted with ether and distilled b.p. 145—147°/12 mm. The oil partly crystallised, the crystals being γ-thujaplicin. The remaining liquid part showed a green ferric colour reaction and gave the following analytical figures:

$C_{10}H_{12}O_2$ (164.1)	Calc.	C 73.13		H 7.37	
	Found.	» 72.29,	72.47	» 7.35,	7.46

After several years this oil had partly crystallised. The crystals (β-thujaplicin) were removed by filtration and recrystallised from light petroleum, m. p. 52—52.5°.

$C_{10}H_{12}O_2$ (164.1)	Calc.	C 73.13		H 7.37	
	Found.	» 73.07,	73.27	» 7.42,	7.43

Oxidation of β-thujaplicin

The oxidation was carried out in the same manner as the oxidation of γ-thujaplicin[2], 0.2 g β-thujaplicin yielding 0.567 millimoles of volatile acid (46 % of the theoretical amount). The acid was converted into the *p*-bromophenacyl ester, m. p. 76—77°, undepressed in admixture with authentic *iso*butyric acid *p*-bromophenacyl ester.

Catalytic hydrogenation of β-thujaplicin

β-Thujaplicin (2 g) in alcohol was hydrogenated with Adams' platinum oxide catalyst. 1150 ml. hydrogen were absorbed (calculated for 4 moles 1100 ml). The catalyst was filtered off and the alcohol removed under reduced pressure. The residue was distilled, yielding a thick oil of octahydro-β-thujaplicin b.p. 152—153°/12 mm, which did not crystallise.

$C_{10}H_{20}O_2$ (172.2)	Calc.	C 69.69	H 11.73
	Found.	» 69.74	» 11.69

Oxidation of octahydro-β-thujaplicin

The hydrogenation product (0.87 g) was suspended in water (50 ml) and potassium permanganate (1.6 g) added in small portions. The solution was acidified and the manganese dioxide dissolved with sulphur dioxide. The acid was extracted with ether and the ether solution shaken with sodium hydrogen carbonate solution. The acid was again

liberated with dilute sulphuric acid and transferred into ether. On evaporation of the latter an oil remained. This was converted into its bis-benzylthiuronium salt which, after recrystallisation from alcohol—ethyl acetate, had m. p. 152—153°.

$C_{26}H_{38}O_4N_4S_2$ (534.5)	Calc.	C 58.38	H 7.19
	Found.	» 58.08	» 7.14

3-*Isopropylcyclohexanone*

The oily acid (0.2 g) was converted into its barium salt. On dry distillation this yielded a mobile oil with a smell resembling that of menthone. The oil was divided in two parts and converted into the 2,4-dinitrophenylhydrazone and the semicarbazone.

The 2,4-dinitrophenylhydrazone was recrystallised from alcohol and formed orange red leaflets, m. p. 139—140°.

$C_{15}H_{20}O_4N_4$ (320.2)	Calc.	N 17.50
	Found.	» 17.64

The semicarbazone was recrystallised from alcohol m. p. 187—188°.

$C_{10}H_{19}ON_3$ (197.2)	Calc.	N 21.30
	Found.	» 21.30

Synthesis of *β-iso*propylpimelic acid

2-Hydroxy-4-*iso*propylbenzoic acid was prepared by the method of Jacobsen [7]. This acid (0.6 g) was dissolved in 75 ml of amyl alcohol. To the boiling solution sodium (5 g) was added. When the sodium had dissolved the amyl alcohol was removed by distillation with steam. The remaining alkaline solution was acidified and again subjected to steam distillation in order to remove any unreacted 2-hydroxy-4-*iso*propylbenzoic acid. The nonvolatile acid was extracted with ether in a continuous extractor. The acid thus obtained, however, still contained appreciable amounts of the starting material. By digestion with small portions of water the *β-iso*propylpimelic acid could be obtained free from starting material which is much less readily soluble in water. The *β-iso*propylpimelic acid thus obtained was an oil and was directly converted into its bis-benzylthiuronium salt. This was recrystallised from alcohol—ethyl acetate, m. p. 152.5—153°. Mix. m.p. with the above salt 152.5—153°.

$C_{26}H_{38}O_4N_4S_2$ (534.5)	Calc.	C 58.38	H 7.19
	Found.	» 57.99	» 7.23

Distribution of the toxic principles in western red cedar (A. B. A.)

Sections of two virgin-growth trees were obtained for this investigation. One tree was obtained from Portland, Oregon. It had been in a mill pond for some time. The other tree was sent to the laboratory from Snoqualmie Falls, Washington, directly after felling and it had not been in a mill pond.

The cross section of the butt of each log about 8 inches thick and another cross section about 15 feet towards the top were used for sampling. A strip about 7 inches wide was cut through the center of each cross section. This piece of wood, from the center of the heart wood to the sapwood, was then cut into five sections of equal length. Each section in turn was cut up into sawdust, well mixed, and a weighed quantity of sawdust thus taken from each section to determine the toxicity of its steam volatile components.

A weighed amount (50 g) of sawdust was put into a 2-liter round bottom flask and steam distilled until 500 ml of distillate were collected. Thus 10 ml of steam distillate represented 1 g of wood. Portions of 100, 50, 25, 15, 10 and 5 ml of steam distillate were tested for their toxicity. When the distillate gave no colour with alcoholic ferric chloride the distillate was found to be nontoxic. The results are given in Tables 1 and 2 (pp. 646 and 647).

SUMMARY

The structure of β-thujaplicin, one of the toxic principles of the heart wood of *Thuja plicata* D. Don grown in U. S. A., has been elucidated. It is 1-*iso*propyl*cyclo*heptatrien-(2,5,7)-ol-(3)-one(4) *viz.* 1-*iso*propyl*cyclo*heptatrien--(1,4,6)-ol-(4)-one(3) or β-*iso*propyltropolone.

A short account of the distribution of the toxic principles in the heart wood of two samples of *Thuja plicata* is also given.

One of us (A. B. A.) wishes to acknowledge his indebtedness to R. H. Baechler and C. A. Richards of the *Forest Products Laboratory*, Madison, for their cooperation in carrying out the toxicity tests. The isolation of the toxic fraction was carried out in the same laboratory. The other author wishes to thank *Statens Tekniska Forskningsråd* for a grant which defrayed the costs of his part of this work.

The analyses were carried out by W. Kirsten, Upsala.

REFERENCES

1. Anderson, A. B., and Sherrard, E. C. *J. Am. Chem. Soc.* **55** (1933) 3813.
2. Erdtman, H., and Gripenberg, J. *Acta Chem. Scand.* **2** (1948) 625.
3. Whitmore, F. C., and Pedlow, G. R. *J. Am. Chem. Soc.* **63** (1941) 758.
4. Cartwright, K. St. G. *Forestry* **15** (1941) 65, *Chem. Abstr.* **36** (1942) 4857.
5. Sowder, A. M. *Ind. Eng. Chem.* **21** (1929) 981.
6. Hubert, E. E. *An outline of forest pathology* New York (1931) p. 467.
7. Jacobsen, O. *Ber.* **12** (1879) 429.

Received July 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 651—656

On the Preparation of Optically Active Phenyl-*p*-tolyl-methanesulphonic Acid and its Racemisation by Sodium Ethoxide

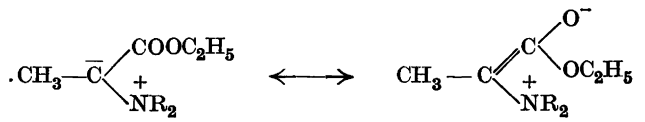
EVALD EGELUND PEDERSEN and KAI ARNE JENSEN

Chemical Laboratory of the University of Copenhagen, Denmark

In a series of papers Biilmann and coworkers [1] have investigated the racemisation of optically active propiobetaineesters of the formula:

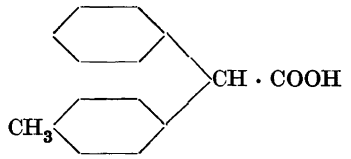
$$CH_3 \cdot \overset{+}{C}H(\overset{+}{N}R_3) \cdot COOC_2H_5$$

and related compounds containing sulphur, selenium, phosphorus and arsenic in stead of nitrogen. It was found, that these compounds were racemised immediately by sodium ethoxide and with measurable velocity by different tertiary amines. A plausible explanation of this observation is that a proton may be comparatively easily separated from the asymmetric carbon atom, because of the repelling (acidifying) effect of the positively charged group. The anion formed by this process will be mesomeric to an enolate ion, which is planar, due to the double bond, and accordingly the optical activity will be lost:



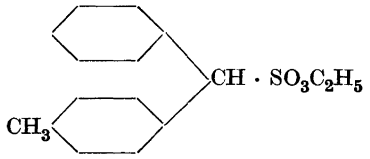
We were interested in investigating an analogous compound containing a sulphonic group in stead of the carboxyl group. In that case no enolization would be expected and accordingly the negative ion would possibly be optically stable.

However, due to difficulties in preparing the desired compounds, we have investigated a compound of a different type: Optically active phenyl-*p*-tolyl-acetic acid (and its esters):



are rapidly racemised too by alkalies, but not by tertiary amines. Ives [2] has shown by experiments with optically active phenyl-*p*-tolyl-deuteroacetic acid that this racemisation proceeds by an ionisation mechanism.

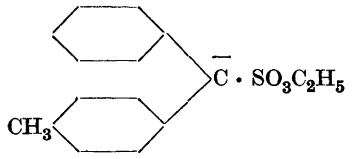
We have therefore studied the racemisation of an ester of the analogous sulphonic acid, phenyl-*p*-tolyl-methanesulphonicacid:



The acid was prepared by the reaction of *p*-methylbenzhydrylbromide with sodium sulphite and resolved through the strychnine salt. The ester was prepared without racemisation by reaction of the silver salt of the sulphonic acid with ethyl iodide.

This ester was found to racemise practically immediately by the action of sodium ethoxide, and accordingly it behaved in the same way as the carboxyl derivative.

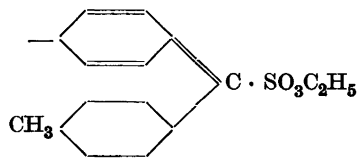
According to the experiments by Ives this racemisation would be expected to proceed through ionisation by which a negative ion is formed:



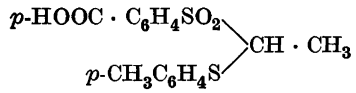
but while the racemisation of the carboxyl derivative may be easily explained by the formation of a planar enolate ion, an analogous explanation is in this case less likely. From the octet theory it was originally concluded that the sulphonyl group does not contain any double bonds and this conclusion was substantiated by several experimental data. However Pauling [3] and Sutton [4] have stated some reasons for accepting the classical formula, but according to Arndt and Eistert [5] their objections to the octet formulas cannot be considered as decisive. Although the validity of the octet theory for sulphur compounds

cannot be theoretically deduced, most of the experimental observations point to it as being practically valid in this case.

Other possibilities for explaining our experiments may, however, be considered too: the negative ion might be stabilized by resonance with quinoid forms, which would tend to flatten the ion:

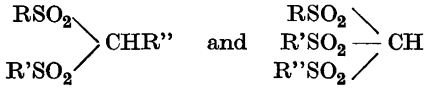


or the negative ion, that is isosteric with an amine, might be instable due to easy inversion, as in the case of amines of the type RR'R"N, which cannot be prepared in optically active forms. Kipping[6] has resolved the compound



and shown the active form to be easily racemised by alkali. In this case it is not possible to explain the racemisation by the formation of quinoid forms.

We are inclined, therefore, to explain our results as indicating that the negative ion, R_3C^-, is not optically stable in this case, but is easily inverted. The possibility that the sulphonyl group may be enolized cannot, however, be definitely rejected. In this connexion it may be mentioned, that all attempts to resolve compounds of the types:



have failed[7]. In the literature the only indication of the existence of an optically active $RR'R''C^-$ -ion is the result obtained by Wallis and Adams[8], which however we do not consider as conclusive.

EXPERIMENTAL

Sodium *p*-methylbenzhydrylsulphonate

To a solution of 260 g of $Na_2SO_3 \cdot 7H_2O$ in 260 ml of water was added 216 g of *p*-methylbenzhydrylbromide and the mixture was refluxed for two hours. On cooling the oily layer becomes very viscous and crystallisation of the sodiumsalt of the sulphonic

acid takes place. To avoid this the hot reaction mixture was poured into a preheated separatory funnel and separated rapidly. The water layer was cooled and the sodium salt filtered off, dried, and shaken twice with ether in order to remove the remaining traces of oil. Then the sodium salt was dissolved in 400 ml of absolute ethanol, and a small amount of sodium sulphite removed by filtration. The alcoholic solution was evaporated to dryness and the residue (68 g) recrystallised from water. Yield 59 g ~ 22 %.

$C_{14}H_{13}O_3SNa \cdot 2H_2O$ Calc. Na 7.18 Found Na 7.23

The oily byproduct (105 g) was found to be a mixture of *p*-methylbenzhydrol and di-*p*-methylbenzhydryl ether. By means of constant boiling hydrobromic acid it was easily converted to *p*-methylbenzhydrylbromide, which was used in the next preparation of the sodium salt.

Di-*p*-methylbenzhydryl ether

A sample of the oily byproduct was distilled under reduced pressure. During this treatment the alcohol was converted into the ether and all distilled at 9 mm and 275°. The ether solidified to a colourless, glassy mass that did not crystallise.

$C_{28}H_{26}O$	Calc.	C 88.85	H 6.92
	Found.	» 89.19	» 6.84

p-Methylbenzhydrylsulphonic acid

Twenty g of the sodium salt were dissolved in 40 ml of hot water, and the acid precipitated by means of 100 ml of conc. hydrochloric acid. The acid was filtered off and recrystallised twice from each time 20 ml of water. Yield 12 g ~ 64 %. The acid forms colourless leaves with m. p. 124—125° (dec.).

$C_{14}H_{14}O_3S \cdot 2H_2O$ Calc. Equiv. wt. 290.3 Found Equiv. wt. 299.3

Resolution of the sulphonic acid into optically active components

The cinchonine and brucine salts of the acid crystallised poorly. Recrystallisations of the *l*-menthylammonium salts from water and ethanol and of the *l*-α-phenethylammonium salts from water, ethanol, methanol, chloroform and dioxane did in no case lead to any resolution.

The resolution was effected, however, through the strychnine salts. Strychnine (40.9 g = 0.12 mole) was dissolved in 127 ml of 1 *N* sulfuric acid plus 1 liter of water. The solution was heated to 100° and, under stirring, dropped into a solution of 78 g of sodium *p*-methylbenzhydrylsulphonate (0.24 mole) in 1 liter of water, heated to 80°. The mixture was cooled, and the precipitate (75 g) was filtered off. The mother liquor was heated again to 80°, and 0.12 mole of strychnine sulphate was added dropwise. After cooling 54 g of strychnine salt was filtered off. The first fraction (75 g) was boiled for 1.5 hours with 1800 ml of ethanol, which dissolved only part of the salt. The mixture was cooled and filtered, and the precipitate was reboiled 8 times in the same way, namely with 1450, 1250, 1100 and 5 times 1000 ml of alcohol respectively. At this stage 49.6 g

of strychnine salt remained. The second fraction of strychnine salt (54 g) was boiled three times with 900 ml of alcohol each time; 40.6 g remained.

The sulphonic acid was liberated in the following way: 12 g of the strychnine salt from the first crop was shaken in a separatory funnel with 100 ml of 0.2160 *N* barium hydroxide, 600 ml of CO_2-free water and 150 ml of chloroform. By this treatment the chloroform dissolved the strychnine, while the barium salt of the sulphonic acid remained in the aqueous solution. The aqueous layer was extracted twice with 50 ml of chloroform. The chloroform remaining in the aqueous layer was removed by evaporation under reduced pressure. A little excess of 1 *N* sulphuric acid was added, and the mixture was left overnight. The barium sulphate was filtered off and the filtrate concentrated to 50 ml under reduced pressure at 40°. In the same way the acid was liberated from two other 12 g-portions of strychnine salt, and to the combined fractions of sulphonic acid 500 ml of conc. hydrochloric acid were added. The sulphonic acid was filtered off and dried in the air, then it was put in a desiccator and the last trace of hydrogen chloride removed by repeated evacuation; finally it was dried over potassium hydroxide. Yield 16.2 g or 90 % of the theoretical amount. M.p. 115.0°—119.5°. $[\alpha]_D = +7.7°$.

$C_{14}H_{14}O_3S \cdot 2H_2O$ Calc. Equiv. wt. 298.3 Found Equiv. wt. 297.1

The second fraction of strychnine salt yielded the levorotatory acid with $[\alpha]_D = -7.1°$.

Ethyl *p*-methylbenzhydrylsulphonate

Thirteen g of the sulphonic acid were dissolved in 32 5ml of water and 3 % more than the calculated amount of freshly precipitated silver oxide added during two hours under vigorous stirring. The stirring was continued for three hours, the reaction mixture being protected from light. Most of the silver salt was filtered off together with the excess of silver oxide, and the rest of silver salt was isolated from the mother liquor by evaporation to dryness under reduced pressure at a bath temperature of 35°. The silver salt (16 g) was added to a solution of 15 g of ethyl iodide in 110 ml of ether, and the mixture was shaken for 29 hours. Before filtering of the silver iodide 100 ml of benzene was added in order to increase the solubility of the ester. The filtrate was evaporated to dryness under reduced pressure at ordinary temperature. The ester was recrystallised three times from alcohol. Yield 7.8 g ~ 62 %.

$C_{16}H_{18}O_3S$ Calc. S 11.04 Found (Grote-Krekeler) S 10.96

The ester is slightly soluble in ether, cold ethanol and cold methanol, very soluble in benzene and toluene.

Optical rotatory power of the ester ($l = 2$ dm):

Solvent	Concentr. g/10 ml	$t°$	α_D	$[\alpha]_D$
toluene	1	12.50	+ 0.64	+ 3.2
toluene	1	24.98	+ 0.59	+ 2.95
toluene	0.5	24.98	+ 0.30	+ 3.0
benzene	0.5	24.95	+ 0.54	+ 5.4
ethanol	0.12	12.50	+ 0.06	+ 2.5
ethanol	0.12	25.20	+ 0.05	+ 2.1

The *l*-ester and the *d,l*-ester were prepared in the same way. For the *l*-ester in benzene solution was found $[\alpha]_D^{20} = -5.6°$. The melting points of the *l*-ester and the *d,l*-ester are 89.0—89.3° and 65.0—65.5° respectively.

Racemisation of the *d*-ester

Two grams of *d*-ester were dissolved in 166 ml of ethanol, freed from water and carbon dioxide by distillation over magnesium ethoxide and barium oxide successively. Alcoholic sodium ethoxide (the amount equivalent to the amount of ester) was added. The racemisation was very rapid, as stated below, *t* being the time in minutes calculated from the moment of adding the ethoxide:

t :	4	6	10
α :	+ 0.01	0.00	0.00
(l = 2 dm)			

The uncertainty was 0.01°.

The solution was acidified by means of 0.83 g of glacial acetic acid, concentrated to about 30 ml, diluted by 50 ml of water and extracted twice with 10 ml of benzene. The benzene solution was washed with 5 ml of water and dried over sodium sulfate. The solution showed no optical rotation. By evaporation it yielded 1.73 g of ester, giving a calculated $\alpha = +0.92°$, provided no racemisation had occurred. The regenerated ester had the m. p. 62.0—64.2°. As mentioned above the m. p. of the pure *d,l*-ester is 64.8—65.5°. Mixed m. p.: 62.5—64.9°.

SUMMARY

Phenyl-*p*-tolyl-methanesulfonic acid has been prepared and resolved in optically active components. The ethyl ester of this acid is racemised practically instantaneously by the action of sodium ethoxide. The possible explanations of this racemisation has been discussed.

REFERENCES

1. Biilmann, E., and Berg, N. *Bull. soc. chim. France* [5], **1** (1935) 1645, 1653. Biilmann, E., Jensen, K. A., and Jensen, H. B. *Ibid.* [5], **1** (1935) 1661; [5], **2** (1936) 2295.
2. Ives, D. J. G., and Wilks, C. G. *J. Chem. Soc.* (1938) 1455.
3. Pauling, L. *The nature of the chemical bond* New York (1939) p. 221.
4. Lister, M. W., and Sutton, L. E. *Trans. Faraday Soc.* **35** (1939) 495.
5. Arndt, F., and Eistert, B. *Ber.* **74** (1941) 451.
6. Kipping, F. B. *J. Chem. Soc.* (1933) 1506.
7. Gibson, D. T. *J. Chem. Soc.* (1931) 2637.
8. Wallis, E. S., and Adams, F. H. *J. Am. Chem. Soc.* **55** (1933) 3838.

Received July 9, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 657—668

Studies of the Hydrogen Bond

I. Influence of Double Bond on Associates between Ethers and Water

LARS EHRENBERG and INGA FISCHER

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

A comparison of the solubility in water of diethyl ether and the corresponding unsaturated compound, divinyl ether, exhibits a striking difference: at 37° C the solubilities are

diethyl ether [1]	0.632 mole l^{-1}
divinyl ether [2]	0.056 mole l^{-1}

The low solubility of the unsaturated compound is also found in other cases, *e. g.*

cyclopropyl ethyl ether [3]	(27° C) 0.25 mole l^{-1}
cyclopropyl vinyl ether [4]	(25° C) 0.08 mole l^{-1}

A more lipophilic character of divinyl ether is consistent with the fact that it has a higher oil/water distribution coefficient, and that it can anesthetize at a lower concentration than diethyl ether [2]. In this connection its ability to induce liver necrosis is of interest. This ability is not present in diethyl ether but is present in more lipophilic compounds, *e. g.* chloroform [5], benzene [6], and furan [7].

This study comprises an experimental orientation in the properties of the two-component systems diethyl ether — water and divinyl ether — water. From the data collected an attempt will be made to explain the effect of the double bond on the solubility and to elucidate the »hydrogen bond» concept *.

* For the discussion on this point see the following publication [8].

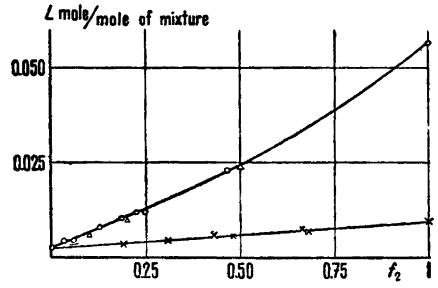


Fig. 1. Solubility of water, L, in benzene — ether mixtures as a function of the mole fraction of ether, f_2.
×——× *Benzene — divinyl ether as solvent.*
Benzene — diethyl ether as solvent:
○——○ *Measured values*
△ *Values calculated from equ. (3).*

SOLUBILITY DETERMINATIONS

The solubility of water in ether — benzene mixtures was determined. In Fig. 1 the solubility of water, L, expressed as moles per mole of mixture, is plotted against the mole fraction of ether, f_2. The greater solubility of water in the diethyl ether mixtures is noted. Further, it is interesting that the benzene — diethyl ether curve is convex, whereas the benzene — divinyl ether curve cannot be distinguished from a straight line. This fact makes it probable that in the latter case the solubility of water in the two solvent components might be chiefly due to the same causes. In the former case, however, a formation of molecular complexes between the ether and water might explain the curved line. We assume that water, W, and ether, E, form a binary complex, EW, the equilibrium constant of which is

$$K = \frac{a_{EW}}{a_E \cdot a_W} \tag{1}$$

The solubility of (free) water in the mixture, l_W, is assumed to be a linear function of f_2:

$$l_W - l_{Wb} = k_1 f_2 \tag{2}$$

where l_{Wb} is the solubility of water in pure benzene and k_1 is a constant [(2) holds in the case of divinyl ether]. If a_E is approximated to f_2 and a_W to l_W we obtain the total solubility:

$$L = a_{EW} + l_W = (K f_2 + 1)\,(k_1 f_2 + l_{Wb}) \tag{3}$$

i. e. an equation of the second degree. The constants in (3) cannot be evaluated with any accuracy without more careful measurements, but using a K-value estimated from the measurements of dipole moments (see below), (3) can be shown to be correct in principle.

The assumption of a binary complex is reasonable because in dilute solutions of HCl and of HBr in ether the phase diagrams show the existence of 1 : 1 complexes [9–11]. Further, dimethyl ether and HCl are known to form a rather stable complex 1 : 1 [12, 13, 11]. Complexes E_2W are of course possible but will not be taken into account at present.

DEGREE OF ASSOCIATION IN PURE ETHERS

In order to elucidate the varying behavior of the ethers towards water it is necessary to know the association in the pure liquids.

Several authors have claimed that ethyl ether is weakly associated. Summing up different data benzene — diethyl ether mixtures are to be regarded as almost ideal solutions:

1. The heat of solution is zero at all concentrations [14] (in decalin — ether solutions it is very small [15]).

2. The total vapor pressure is a linear function of the mole fraction (Schmidt [14]; Tahvonen [16] finds small positive deviations; *cf.* Schulze [16]).

3. The activities of the components, a, calculated from Pickering's [17] and Yamamura's [18] freezing point data and the equation:

$$\ln a_1 = (T - T_0)\, q/RTT_0$$

are identical with the mole fractions. This keeps within the limits of the uncertainty of $\frac{\partial q}{\partial T}$ (*cf.* Ferche [19], Bridgman [20]).

4. Löfgren's [21] surface tension function, $\gamma^{5/6}$, varies linearly with temperature.

5. If an associated liquid is mixed with benzene, it dissociates. As a rule, this leads to a volume expansion (*cf.* Hildebrand [22, pp. 59, 115]). The very small contraction found by the present authors (Table 1) and by others [14, 23] might be due to experimental errors through the difficulty of filling the pycnometers without evaporation of ether (*cf.* Niini [24]: from his measurements extrapolated value of the partial molar volume of ether 103.3; calc. 103.8). In fact, ether — heptane mixtures, where the components have about the same density, show no contraction [25].

6. The molar polarization of ethyl ether, P_2, determined in the present investigation is nearly constant (Table 1), which is also found by other authors [26–28] (*cf.* Rolinski [23], who calculates a small association from his P_2 data). In heptane solutions P_2 is independent of the concentration.

As to divinyl ether we found the same degree of constancy in P_2 (*cf.* Smyth

Table 1. Measurements on benzene solutions at 20° C.

f_2	ε_{12}	V_{12} cm_3	ΔV_{12} cm^3	P_{12} cm^3	P_2 cm^3
			Diethyl ether		
0.05596	2.3834	89.7232	+ 0.0259	28.317	51.71
0.10266	2.4751	90.3905	— 0.0107	29.794	54.82
0.25021	2.7568	92.6380	+ 0.0130	34.214	56.04
0.45907	3.1527	95.3844	— 0.3883	39.850	55.07
0.71658	3.6903	99.2712	— 0.3825	46.934	54.84
1.00000	4.3578	103.9253	—	54.887	54.89
$P_1 = 26.93 \pm 0.15$ cm³; $\frac{\partial P_{12}}{\partial f_2} = 28.06 \pm 0.37$ cm³; $P_2 = 54.99 \pm 0.52$ cm³;					
$MR_D = 22.41$ cm³;[46] $\mu = 1.23 \pm 0.01$ D					
			Divinyl ether		
0.10823	2.4337	89.0936	+ 0.0502	28.810	45.70
0.13875	2.4766	89.1784	+ 0.0815	29.415	45.96
0.32035	2.7698	89.4242	+ 0.0095	33.180	46.82
0.47979	3.0173	89.5774	— 0.1164	36.016	46.06
0.49863	3.0799	89.7198	— 0.0070	36.735	46.77
0.72918	3.4970	90.1851	+ 0.0548	40.966	46.24
1.00000	4.1269	90.6042	—	46.240	46.24
$P_1 = 26.75 \pm 0.08$ cm³; $\frac{\partial P_{12}}{\partial f_2} = 19.50 \pm 0.14$ cm³; $P_2 = 46.25 \pm 0.21$ cm³;					
$MR_D = 21.94$ cm³;[29] $\mu = 1.06 \pm 0.01$ D					

and Walls [29]) and a small deviation from the ideal molar volume (Table 1). As will be seen, these deviations are of the same order of magnitude as in the case of diethyl ether and are probably due to the same causes.

To sum up: both ethers are probably unassociated liquids which form ideal solutions with benzene. *If* there is any association it is of the same order of magnitude in the two compounds.

THE DIPOLE MOMENTS OF THE ETHERS

From the measurements of the dielectric constant, ε, and the specific volume, v, the molar polarization of the mixture, P_{12}, was calculated. P_{12}

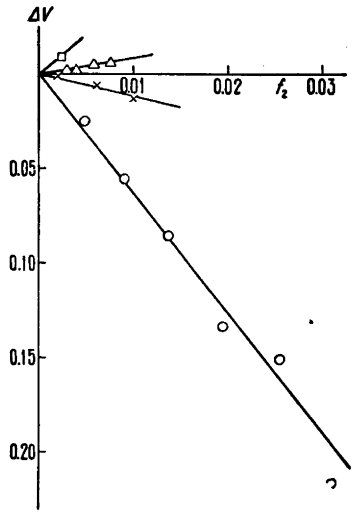


Fig. 2. Deviations from the ideal molar volumes of the solutions, ΔV, as a function of the mole fraction of water, f_2. ΔV in ml.
Solvent:
○——○ *Diethyl ether.*
×——× *Benzene — diethyl ether, 1 : 1.*
△——△ *Divinyl ether.*
□ *Benzene, value from Niini.*[32]

plotted against f_2 gave straight lines, from which P_1 and $\partial P_{12}/\partial f_2$ were obtained by the method of least squares. From

$$P_2 = P_1 + \frac{\partial P_{12}}{\partial f_2}$$

the dipole moments, μ, of the ethers were calculated assuming $P_E + P_A = 1.05\ MR_D$ [30]. The μ-values (see Table 1) for both ethers agree well with those of earlier measurements, *cf.* Fischer and Ehrenberg [8, Table 1].

ETHER-WATER SYSTEMS

Partial molar volume

The essential difference between the abilities of the two ethers to dissolve water (*cf.* above) is illustrated in an interesting manner by the partial molar volumes of the ether — water mixtures. If the deviations from the volumes calculated for one mole of ideal mixture, ΔV, are plotted against the mole fraction of water, f_2, water in diethyl ether exhibits a strong *contraction* (Fig. 2), the partial molar volume of water, V_2, being only 11.2 ml (the ideal value at 20° C is 18.0479 ml). A similar contraction is found in water — dioxane and water — tetrahydrofuran mixtures [31]. With benzene — ether mixtures, 1 : 1, as solvent the contraction is smaller, V_2 being about 16 ml; this value is, however, more uncertain. In divinyl ether, on the other hand, water exhibits a

Table 2. Measurements on ether solutions at 20° C.

f_2	ε_{12}	v_{12} cm^3 g^{-1}	P_{12} cm^3	P_2 cm^3
		Water in diethyl ether		
0.002565	4.3724	1.4065	55.065	
0.004729	4.3910	1.4062	55.103	63.68
0.009002	4.4420	1.4053	55.276	78.75
0.013886	4.4714	1.4044	55.253	66.96
0.019465	4.5527	1.4032	55.558	79.25
0.031093	4.6353	1.4008	55.547	69.85
$\varepsilon_1 = 4.3433 \pm 0.0127$; $\frac{\partial \varepsilon_{12}}{\partial f_2} = 10.3 \pm 1.1$; $v_1 = 1.4071 \pm 0.0001$; $\frac{\partial v_{12}}{\partial f_2} = -0.196 \pm 0.005$				
$P_{2_0} = 86 \pm 9$ cm^3; $MR_D = 3.75$ cm^3; $\mu = 1.99 \pm 0.11$ D				
		Water in divinyl ether		
0.002004	4.0132	1.29951	45.572	49.86
0.003165	4.0196	1.29942	45.578	49.98
0.004096	4.0247	1.29934	45.582	49.99
0.005886	4.0348	1.29926	45.594	50.68
0.007650	4.0449	1.29914	45.605	50.91
$\varepsilon_1 = 4.0020 \pm 0.0001$; $\frac{\partial \varepsilon_{12}}{\partial f_2} = 5.56 \pm 0.03$; $v_1 = 1.29963 \pm 0.00003$; $\frac{\partial v_{12}}{\partial f_2} = -0.0646 \pm 0.0002$				
$P_{2_0} = 51.62 \pm 0.24$ cm^3; $MR_D = 3.75$ cm^3; $\mu = 1.51 \pm 0.01$ D				

weak *expansion*, V_2 being 18.9 ml. This should be compared with the expansion exhibited by water in saturated benzene solution (Fig. 2, from Niini [32]). In agreement therewith (Hildebrand [22, p. 59]) the water vapor pressures over benzene solutions deviate positively from Raoult's law [33]. Though such data can only be interpreted with caution (*cf.* Hildebrand [22, Chap. VI.], Schmidt [14]), it is in accordance with the solubility curves above (Fig. 1) to suppose the main reaction of water in benzene or divinyl ether to be its entassociation, whereas in diethyl ether the solvation of the water molecules predominates.

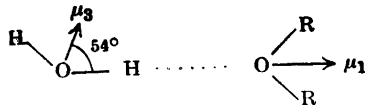
Table 3. Dipole moments measured in different solvents.

Solute	Solvent	t °C	μ recalcd. 10^{-18}e.s.u.	Given originally		Investigator
				P_2 cm^3	μ 10^{-18}e.s.u.	
Water	Gas	—	—	—	1.84	Sänger[44]
»	Diethyl ether	17	—	—	1.97	Hassel and Uhl [45]
»	» »	10	2.2	67.2	1.71	Linton and Maass [35]
»	» »	20	1.77	83	1.9	Higasi [26]
»	» »	20	1.99	86	—	Present invest.
»	Divinyl ether	20	1.51	51.6	—	Present invest.
»	Benzene	25	1.51	—	—	Williams [36]
»	Dioxane	25	—	79.1	1.90	Linton and Maass [35]
Ethyl alcohol	Diethyl ether	20	1.89	83	1.8	Higasi [26]
» »	Benzene	20	1.80	76	1.73	Stranathan [47]
Acetic acid	Diethyl ether	20	—	61.7	—	Smyth and Rogers[37]
» »	Benzene	20	—	22.8	—	Smyth and Rogers[37]

Dipole moment

The dielectric constants and the specific volumes of the ether — water mixtures were determined (Table 2). The dipole moment of water was calculated according to the method of Halverstadt and Kumler[34]: $\frac{\partial \varepsilon_{12}}{\partial f_2}$, $\frac{\partial}{\partial f_2}$ $_{12}$, ε_1 and v_1 are calculated by the method of least squares using the points for the solutions and omitting those for the pure solvent, whereby moisture and other impurities of the solvent are regarded. Earlier measurements of the dipole moment of water in diethyl ether solution [26, 35] were recalculated by the same method (Table 3). The values obtained in this manner agree rather well with ours. The difference between the P_2 values of water when dissolved in different solvents is striking. P_{2_0}[34] in diethyl ether is significantly higher than P_{2_0} in divinyl ether. The latter value agrees well with P_2 in benzene (Table 3; Williams'[36] μ value recalculated regarding the diminished density found by Niini[32]). The variation of P_2 with f_2 in divinyl ether is probably due chiefly to the formation of complexes: $n\ H_2O = (H_2O)_n$, and P_{2_0} is the polarization of free water molecules. The higher P_{2_0} value in diethyl ether is consistent with the existence of a certain proportion of ether-water complexes: W + E = EW, assumed above. In this case the equilibrium constant might be calculated from the dipole moments, if the dipole moment of the complex can be estimated. $\mu_W = \mu_3$ is supposed to be 1.51 D (the value in divinyl ether or ben-

zene), $\mu_E = \mu_1 = 1.23$ D, and a complex is supposed to be formed through a hydrogen bond:



for which $\mu_4 = 2.44$ D is calculated. If f_1 is the mole fraction of ether, f_3 that of water, and f_4 that of the complex, we get from equation (1), as

$$f_3 + f_4 = f_2 \text{ and } f_4 \ll f_1:$$

$$f_3 = \frac{f_2}{K+1}; \quad f_4 = \frac{f_2 K}{K+1}; \quad f_1 = 1 - f_2.$$

If these values are put into the expression for the total polarization

$$P_{134} = f_1 P_1 + f_3 P_3 + f_4 P_4$$

we obtain

$$P_{134} = P_1 + f_2 \left(-P_1 + \frac{1}{K+1} P_3 + \frac{K}{K+1} P_4\right),$$

and, because the molar refraction, R, is additive,

$$P_{134} = P_1 + f_2 \left[-P_1 + R_2 + A\left(\frac{1}{K+1}\mu_3^2 + \frac{K}{K+1}\mu_4^2\right)\right] \tag{4}$$

where A is a constant $= 4\ \pi N/9kT$. Now we have measured

$$P_{12} = P_1 + f_2 \left(-P_1 + R_2 + A\mu_{\text{eff}}^2\right) \tag{5}$$

where μ_{eff} is the dipole moment calculated for water in diethyl ether (1.99 D). As P_{134} is approximately equal to P_{12}, P_{12} being calculated from values extrapolated to infinite dilution, K can be estimated from

$$\mu_{\text{eff}}^2 = \mu_3^2 \frac{1}{K+1} + \mu_4^2 \frac{K}{K+1}$$

to 0.833. If this value is put into equation (3) above (k_1 being computed from the solubility in pure ether), the theoretical solubility curve can be constructed.

Table 4. Work of adhesion between some organic liquids and water at 20° C.

Organic liquid	γ_A dyn cm^{-1}	γ_{AB} dyn cm^{-1}	W_{AB} dyn cm^{-1}
Diethyl ether	16.99	10.44	79.30
Divinyl ether	17.94	24.15	66.54
Benzene [39]	28.88	35.00	66.63
n-Octyl alcohol [39]	27.5	8.5	91.8

The calculated points of Fig. 1 and the curvature of the line agree well with the experimental ones. Hence, the assumptions made and the order of magnitude of the constants seem to be reasonable.

It is interesting that water has a still higher μ-value when dissolved in dioxane, which is totally miscible with water [35] (Table 3).

In the case of alcohols there seems also to be a similar complex formation with diethyl ether (Table 3). Confer also the fact that acetic acid has a higher P_2-value in diethyl ether than in benzene [37].*

Surface tension

The surface tensions of the pure ethers, γ_A, and the ether—water interfacial tensions, γ_{AB}, were determined. These measurements we obtained by the courtesy of Mr. L. E. Tammelin, Assistant at Tekniska Högskolan,Stockholm. Methods of measuring and calculation were those used by Tammelin and Löfgren [38]. Using a tabulated value of the surface tension of water [39], $\gamma_B = 72.75$ dyn cm^{-1}, the work of adhesion [40], $W_{AB} = \gamma_A + \gamma_B - \gamma_{AB}$, was calculated (Table 4). For comparison tabulated values of benzene and *n*-octyl alcohol [39] are given. The values show clearly a similarity between divinyl ether and benzene, while the behavior of diethyl ether is intermediate between the hydrocarbon and the alcohol. These results are in accordance with the assumption made above that diethyl ether but not divinyl ether forms complexes with water to a measurable extent.

* We must also refer to the most interesting paper by Weith, Hobbs, and Gross[47], which did not become known to us until the present paper was in press. They found a large positive increment of the dipole moments of HF, HCl, and HBr when measured in dioxane solution compared to measurements in hydrocarbon solutions. They also found μ_{H_2O} in dioxane to be 1.89 D (*cf.* our Table 3).

EXPERIMENTAL

Diethyl ether (Abs. ether from AB Syntes, Nol, Sweden) and

Divinyl ether (»Vinethene» from Merck and Co. Inc. N. J., containing 3.5 % ethanol and 0.01 % phenyl-α-naphthylamine) were purified through treatment with and distillation over freshly cut sodium pieces in dry N_2 atmosphere.

Benzene (Baker's Analyzed, thiophene free) was dried through the same procedure.

The solubility determinations were performed principally as done by Marie and Lejeune [41]. Freshly redistilled water was titrated from a microburette (scale division 0.0002 ml) into stoppered flasks, containing weighed amounts of benzene and ether in different proportions, until the mixtures, after vigorous shaking, became opalescent in transmitted light. The temperature was kept at 20 ± 0.5 °C. The flasks were weighed after the titrations were completed. From the weight loss and the vapor pressures of the components the alteration of the mole fractions could be estimated. However, the errors due to evaporation were insignificant. — Because of the tendency of water to form larger drops in pure diethyl ether, the method failed to give a correct value for this case. Instead, Niini's refractometrically determined value [32] is used.

The dielectric constants were measured at a frequency of 2000 kilocycles with a crystal controlled heterodyne beat apparatus built in this laboratory according to a design published by Hudson and Hobbs [42]. The measuring condenser was a modification of one described by Arrhenius [43]. The difference between two settings of the condenser was determined with an accuracy of 0.05 %. The details of apparatus and calibration will appear in a later publication. The solutions were made up of freshly distilled liquids, care being taken to avoid moisture and evaporation. For the density measurements, performed on the same solutions as the dielectric constant measurements, an Ostwald-Sprengel pycnometer fitted with carefully ground caps was used. The temperature was kept constant at 20.00 ± 0.05° C.

CONCLUSIONS

Diethyl ether and divinyl ether are uni-molecular liquids. Data from measurements of solubility, partial molar volume, and dipole moment of water dissolved in the ethers and of surface and interfacial tensions are explained by the assumption that diethyl ether associates with water, about 50 % of the water molecules in diethyl ether solution being present as a (hypothetical) binary complex with $\mu = 2.44$ D, whereas divinyl ether in this respect is more like benzene, forming no obvious complex with water. Probably, this different behavior depends on different ability to form hydrogen bonds (see the following publication [8]).

The apparatus for the determination of dielectric constants was built with a grant from *Statens Tekniska Forskningsråd*. We are indebted to Dr. O. Friberg and to Leg. Apot. P. Thorsell who supplied us with vinethene.

LITERATURE

1. Klobbie, E. A. *Z. physik. Chem.* **24** (1897) 615 (see p. 620).
2. Ruigh, W. L., and Erickson, A. E. *Anesthesiology* **2** (1941) 546.
3. Krantz, J. C. Jr., Carr, C. J., Evans, W. E. Jr., Forman, S. E., and Wollenweber, H. L. *J. Pharmacol. Exptl. Therap.* **72** (1941) 233.
4. Krantz, J. C. Jr., Evans, W. E. Jr., Forman, S. E., and Wollenweber, H. L. *J. Pharmacol. Exptl. Therap.* **75** (1942) 30.
5. Beecher, H. K. *The physiology of anesthesia* New York (1938). *Cf.* pp. 250—253.
6. Everett, M. R. *Medical biochemistry* 2nd ed. New York and London (1946) p. 254.
7. Henderson, V. E., and Smith, A. H. R. *J. Pharmacol. Exptl. Therap.* **57** (1936) 394.
8. Fischer, I., and Ehrenberg, L. *Acta Chem. Scand.* **2** (1948) 669.
9. McIntosh, D. *Bull. Chem. Soc. Japan* **3** (1928) 82.
10. a: Maass, O., and McIntosh, D. *J. Am. Chem. Soc.* **33** (1911) 70; b: McIntosh, D. *Ibid.* **33** (1911) 71.
11. Maass, O., and McIntosh, D. *J. Am. Chem. Soc.* **35** (1913) 535.
12. Friedel, M. C. *Bull. soc. chim. France* (2) **24** (1875) 160.
13. Maass, O., and Morrison, D. M. *J. Am. Chem. Soc.* **45** (1923) 1675.
14. Schmidt, G. C. *Z. physik. Chem.* **A 121** (1926) 221.
15. Weissenberger, G., Henke, K., and Sperling, E. *Sitzber. Akad. Wiss. Wien, Math.-naturw. Klasse Abt. II b* **134** (1925) 483; also *Monatsh.* **46** (1926) 483.
16. Tahvonen, P. E. *Soc. Sci. Fennica Commentationes Phys.-Math.* **10** (1938) no. 2. The values of Schulze, A., are discussed.
17. Pickering, S. U. *J. Chem. Soc.* **63** (1893) 998 (see p. 1021).
18. Yamamura, S. *Bull. Chem. Soc. Japan* **1** (1926) 183.
19. Ferche, J. *Wied. Ann. Phys. Chem.* **44** (1891) 265.
20. Bridgman, P. W. *Phys. Rev.* **6** (1915) 94.
21. Löfgren, N. *Svensk Kem. Tid.* **53** (1941) 359.
22. Hildebrand, J. H. *Solubility of non-electrolytes* 2nd ed. New York (1936).
23. Rolinski, J. *Physik. Z.* **29** (1928) 658.
24. Niini, A. *Ann. Acad. Sci. Fennicae A* **46** (1936) no. 1.
25. Briegleb, G. *Z. physik. Chem. B* **14** (1931) 97.
26. Higasi, K. *Sci. Papers Inst. Phys. Chem. Research Tokyo* **24** (1934) 57.
27. Lange, L. *Z. Physik* **33** (1925) 169.
28. Williams, J. W., and Krchma, I. J. *J. Am. Chem. Soc.* **49** (1927) 1676.
29. Smyth, C. P., and Walls, W. S. *J. Am. Chem. Soc.* **54** (1932) 3230.
30. *Cf.* Smyth, C. P. in Weissberger, A. *Physical methods of organic chemistry* New York (1946) p. 993.
31. Wolf, K. L., and Klapproth, H. *Z. physik. Chem.* **B 46** (1940) 276.
32. Niini, A. *Suomen Kemistilehti* **A 11** (1938) 19.
33. Greer, E. J. *J. Am. Chem. Soc.* **52** (1930) 4191.
34. Halverstadt, I. F., and Kumler, W. D. *J. Am. Chem. Soc.* **64** (1942) 2988.
35. Linton, E. P., and Maass, O. *Canad. J. Research* **7** (1932) 81.
36. Williams, J. W. *Physik. Z.* **29** (1928) 204.
37. Smyth, C. P., and Rogers, H. E. *J. Am. Chem. Soc.* **52** (1930) 1824.
38. Tammelin, L. E., and Löfgren, N. *Acta Chem. Scand.* **1** (1947) 871.
39. *Handbook of chemistry and physics* 29th ed. Cleveland, Ohio (1945).
40. Adam, N. K. *The physics and chemistry of surfaces* 3rd ed. Oxford (1941) p. 8.

41. Marie, C., and Lejeune, G. *Monatsh.* **53** (1929) 69.
42. Hudson, B. E., and Hobbs, M. E. *Rev. Sci. Instruments* **13** (1942) 140.
43. Arrhenius, S. *Diss.* Uppsala (1940).
44. Sänger, R. *Physik. Z.* **31** (1930) 306.
45. Hassel, O., and Uhl, A. H. *Z. physik. Chem.* **B 8** (1930) 187.
46. Voellmy, H. *Z. physik. Chem.* **127** (1927) 305.
47. Stranathan, J. D. *Phys. Rev.* **31** (1928) 653.
48. Weith, A. J. Jr., Hobbs, M. E., and Gross, P. M. *J. Am. Chem. Soc.* **70** (1948) 805.

Received July 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 669—677

Studies of the Hydrogen Bond

II. Influence of the Polarizability of the Heteroatom

INGA FISCHER and LARS EHRENBERG

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

The association of liquids and several solubility phenomena have been explained by the assumption of a hypothetical hydrogen bond [1—4, 5a, 6a, 7]. Some authors have sought to explain these phenomena as an electrostatic attraction between more or less deformed dipoles [8—12]; regarding the smallness of the H atom the X—H partial moments (X being the negative heteroatom) are easily available for an arrangement from which a strong attraction follows [9a]. Hence, an interpretation of the association phenomena on the basis of electrostatic attraction is not excluded, as a rule. The schools maintaining this opinion have been supported by the fact that their calculations agree better with the experimental data than does the attempt to calculate the H bond energy on quantum mechanical basis as a resonance phenomenon [13]. However, Harms [10] and others [11] are forced to assume distances between the dipoles much shorter than those calculated from van der Waals' radii (Table 3) in order to get the right magnitude of the energy. Moelwyn-Hughes [8] bases his calculations on the interaction of dipoles instead of that of separated charges, which is hardly permissible (*cf.* Bauer and Magat [14]). Pauling [6a] interprets the hydrogen bond mainly as an electrostatic attraction of the proton to the two negative atoms linked through the bond. He states that this explanation overbridges the discrepancies between the different interpretations of association. It seems, however, to be rather difficult to draw any definite conclusions without detailed calculations.

The closest agreement with experimental values is obtained by Bauer and Magat [14], who calculate the energy of water association with due consideration to separate charges and polarizability effects. Their method is doubtless adequate so far as it concerns the association of water and other simple mole-

cules, as for instance ammonia (calculations made by van Velden and Ketelaar [16]) and hydrogen fluoride. This method is not opposed to the quantum mechanical one, but is a suitable approximation when the polarizabilities of the groups of atoms in question are known from experiments. Otherwise, the polarizabilities might be calculated, if possible, with the aid of quantum mechanics, or the whole problem might be treated through a direct study of the wave functions involved. However, such calculations have not been made as yet.

Summing up, it may be said that no satisfactory general treatment of the hydrogen bond has been made hitherto.

In the event of association between two molecules, similar or different, both carrying an active H atom the cause of association is difficult to interpret because one or the other or both H atoms might partake in the H bond formation. In the case of association between two molecules, only one of which contains an active H atom there is only one possibility of H bond formation. Gordy *et al.*[15] have studied such simple cases with infrared absorption measurements. Following a similar line we have, in a preceding paper [88], investigated solutions of water in diethyl ether and divinyl ether (solubility, surface tension, partial molar volume, and polarization measurements). The present paper will treat these and some other systems with uni-directional H bond formation, *viz.* the effect of double bond on the water solubilities of ethers, amines, and halides. From this study conclusions concerning the properties which encourage H bond formation can be drawn.

ETHERS

Our experimental data [88] could be explained by the assumption that diethyl ether associates with water, about 50 % of the water molecules in ether solution being present as a (hypothetical) binary complex with $\mu = 2.44$ D, whereas divinyl ether is more like benzene in its ability to dissolve water.

The size of the dipole moment, *cf.* Table 1, and the steric configuration at the oxygen atom of the two compounds being almost the same the different affinity for water cannot be due to a different ability to attract water electrostatically. Regarding physical data of the ethers and their mixtures with indifferent solvents we were able to conclude that the pure liquids are practically uni-molecular and that, *if* there is any association, it is of the same order of magnitude in the two compounds. Their uni-molecularity (*cf.* Harms [10]) is further illustrated by the similarity in boiling points and entropies of vaporization between the ethers and the hydrocarbons of corresponding molecular weight and degree of unsaturation (Table 1, *cf.* also Table 4). Both

Table 1. Physical data of ethers.

Substance	Boiling point °C	Entropy of vaporization cal · T^{-1} · mol^{-1}	Solubility in water mol · l^{-1}	Dipole moment 10^{18} e. s. u.	r_D cm^3	Exaltation of MR_D cm^3	Heat of hydrogenation cal · mol^{-1}
Diethyl ether	34.6 [33]	20.7 [37]	1.010 (20°) [41b] 0.632 (38°) [41a] 1.2 (25°) [35b]	gas: 1.14 [44—46] 1.10 [47] benzene soln: 1.22 [48—50] 1.15 [51] 1.27 [52] 1.23 [88]	0.3041 [53]	+ 0.22	—
Ethylvinyl ether	35.7 [34]		scarcely soluble [42]				26740 [34] *
Divinyl ether	28.3 [87]	20.8 [38]	0.11 (25°) [35b] 0.056 (37°) [43]	benzene soln: 1.06 [87, 88]	0.3130 [87]	+ 0.56	57236 [34] *
Cyclopropyl-ethyl ether	68 [35a]		0.25 (27°) [35a] 0.23 (25°) [35b]				
Cyclopropyl-vinyl ether	67 [36]		0.08 (25°) [36]				
Diethyl methane	36.2 [33]	20.0 [39]	0.005 (16°) [25]	0 [75, 82]	0.3504 [75]	0	
Divinyl methane	26.0 [33]	21.0 [40] (isoprene)					

the low heat of hydrogenation and the high specific refraction, r_D, of divinyl ether show that the electron configuration of this compound is similar to those of molecules with conjugated double bonds. Electrons from the oxygen atom must be involved in the conjugation. Hence, the electron cloud around the O atom becomes less polarizable than in diethyl ether which is the possible cause of the diminished miscibility with water.

Table 1 contains also data to show that the diminished attraction for water molecules seems to be a general property of ethers with a double bond in the vicinity of the oxygen atom. Gordy *et al.*[15] have found a smaller frequency shift of the O—D bond in the system CH_3OD-ether in the cases of phenetol and β, β-dichloroethyl ether compared to diethyl ether. They assume a diminished negativity of the oxygen to be the cause.

* *Cf.* allyl alcohol: 31458 cal . mol^{-1} [34].

Table 2. Physical data of heterocyclic compounds.

Substance	Boiling point °C	Entropy of vaporization cal. T^{-1} . mol^{-1}	Solubility in water at 20° C mol . l^{-1}	Dipole moment (benzene solution) 10^{18} e. s. u.
Furan	31.3 [87]	20.8 [55]	insoluble [58]	0.63 [68]; 0.67 [54]; 0.71 [87]
Tetrahydrofuran	64.1 [87]		∞ (22°) [59]; 4.2 (25°) [60]	1.68 [54]; 1.71 [87]
Thiophene	84 [54]		0.002—0.005 [61]	0.54 [54]; 0.63 [69]
Tetrahydrothiophene	120 [54]		insoluble [62]	1.87 [54]
Pyridine	115 [33]	22.0 [37]	∞ [63]	2.11 [49, 50]; 2.26 [70]; 2.21 [71]
Piperidine	106 [33]	19.9 [56]	∞ [64]	1.17 [70]
Pyrrole	131 [33]		insoluble [22]	1.80 [54]
Pyrroline	90 [33]		very soluble [65]	1.42 [54]
Pyrrolidine	88.5 [33]		∞ [66]	1.57 [54]
Trimethyl amine	2.9 [33]		∞ [63]	gas: 0.60 [72]; 0.82 [73]
Triethyl amine	89.4 [33]	22.5 [57]	1.41 [67]; ∞ (< 18.6°) [67]	gas: 0.76 [74]; 0.82 [73]

HETEROCYCLIC COMPOUNDS

A similar difference as to the ability to associate with water is found in a comparison between furan and tetrahydrofuran, Table 2. In this case the involvement of the oxygen electrons of furan is even more marked because of the aromatic structure. This will lead to an electron displacement towards the centre of the molecule, which is a probable cause of the diminished dipole moment. The small dipole moment of furan cannot account for the insolubility of the compound, because molecules (the tertiary amines of Table 2) with the same dipole moment, about the same molecular weight, about the same cohesion in the pure liquid (entropy of vaporization, boiling point), the same or greater steric hindrance, and a somewhat larger radius of the negative atom * (Table 3) are very soluble in water. A comparison between thiophene and tetrahydrothiophene shows the same peculiarities of dipole moment and electron displacement, but since the sulphur atom does not form H bonds to

Table 3. Van der Waals' radii of atoms [6b].

H	N	O	F	P	S	Cl
1.2 Å	1.5	1.40	1.35	1.9	1.85	1.80

* It might be supposed, *cf.* pyrrole [17], that the oxygen atom of furan were positive. From the dipole moment of furfural, 3.61 D [18], the moment of the aromatic aldehyde group, — 2.95 D [19], and the bond angles of furan from electron diffraction measurements [20] the oxygen atom of furan is shown to be negative.

Table 4. Physical data of halides.

Substance	Boiling point [33] °C	Entropy of vaporization cal · T^{-1} · mol^{-1}	Solubility in water at 20° C mol · l^{-1}	Dipole moment 10^{18} e. s. u.
Ethyl fluoride	— 37.7		0.09 (14°) [33]	1.92 [80]
Vinyl fluoride	— 51		insoluble [76]	
Ethyl chloride	12.2	20.9 [84]	0.089 [77, 25]	2.02 [81]
Vinyl chloride	— 13.9		0.014 [33a]	1.44 [27]
Ethyl bromide	38	21.1 [85]	0.083 [78]; 0.088 (17.5°) [25]	2.02 [80]
Vinyl bromide	15.8		insoluble [33]	1.48 [74]; 1.41 [27]
Ethyl iodide	72.2	20.6 [37]	0.026 [78]; 0.025 (22.5°) [25]	1.90 [80]
Vinyl iodide	56		insoluble [33]	1.26 [27]
Butane	— 0.6		0.007 (17°/772 mm) [33]	0
1,3-Butadiene	— 3	21.6 [86]	0.011 (25°/520 mm) [79]	0 [83]

any appreciable extent [5b] (larger atomic radius, Table 3) both compounds are clearly insoluble. In the case of pyridine and piperidine both are very soluble in water. The polarizability of the unshared electron pair of the nitrogen atom is not much changed in the aromatic state: like amines, pyridine can easily donate an electron pair in proton addition, forming the pyridonium ion. A similar proton addition to furan leads to a breakdown of the molecule. In his interesting work, Gordy [15] has found a relation between basicity constants of amines and frequency shifts of the O—D band (see above). The importance of this relation has also been regarded by Bernal [21].

In the pyrrole series the aromatic compound carries no unshared electron pair. Accordingly, pyrrole cannot act as electron donor in a supposed H bond and is, compared to its hydrogenated derivatives, very slightly soluble. Like furan it is incapable of forming a stable positive ion through proton addition. Naturally, pyrrole might be a proton donor in an H bond (*cf.* Harada *et al.* [22], Ingold *et al.* [23], and Gordy *et al.* [24]). The high solubilities in water of di-, tri-, and tetrazoles are understood on similar grounds [17].

ETHYL AND VINYL HALIDES

Unsubstituted alkanes are incapable of H bond formation and, consequently, are not very soluble in water [25], the solubility being chiefly due to dispersion forces (*cf.* London [26]). In this case the introduction of double bonds will increase the molecular polarizability and the mutual solubility (*cf.* butane-butadiene, Table 4; as to the solubility of water in hydrocarbons,

see the recent measurements by Black *et al.*[89]). This is a common effect, though immeasurably small when the solubility is dominated by the presence of a heteroatom capable of H bond formation. In the latter case the introduction of a double bond in the vicinity of the heteroatom will have the opposite effect on the water solubility. The unshared electrons of the heteroatom become partly involved in interaction with the π-electrons of the C = C bonds.

The halides show the same solubility peculiarities as the ethers. In this case, too, the decreased availability of the electrons of the heteroatom might be responsible for the decreased solubility of the unsaturated compounds (Table 4). In comparison to ethers, a more marked electron displacement occurs in the halides [27–30], *cf.* their dipole moments.

DISCUSSION

The examples mentioned above demonstrate that attraction between dipoles is not solely responsible for the water solubility of polar compounds (*cf.* Hildebrand [4]). This is shown most clearly in a study of the pair diethyl ether-divinyl ether. Hence, the interpretation of association phenomena on the basis of electrostatic attraction between dipoles is not sufficient.

The present examples show that the availability of unshared electrons at the heteroatom is greatly significant for the water solubility.

This fact has been mentioned casually by several authors [6c, 15] but it has not been regarded in the interpretation of the phenomena in question. It should be mentioned that this view as to »H acceptor atoms» is contrary to that of Huggins [31], who states that decreased electron availability means increased strength of the H bond, generally.

An involvement of the electrons of the heteroatom through conjugation or displacement, *i. e.* a decreased polarizability of the electron cloud around the heteroatom, will diminish the attraction of molecules with an active H atom. Consequently, data make it fitting to explain association phenomena with the concept of a hydrogen bond.

The energy of the hydrogen bond has been calculated by several authors [13, 14, 31, 32] using more or less modified Morse functions. This treatment seems, as a rule, to give the right order of magnitude of the energy, but it is to coarse for any estimations of such properties as those concerned in solubility and similar phenomena. Probably, experimental facts might be sufficiently accounted for only through more refined calculations of the energy of the system on the basis of quantum mechanics with due consideration to the change of the electronic orbitals effected partly by the rest of the molecule

and partly by the contiguity of the other molecule of the complex. Such calculations will be tried on the case of associates between water and different ethers.

SUMMARY

The water solubilities of ethers, tertiary amines, and halides are treated. Unsaturated or aromatic compounds, where the electron cloud of the heteroatom is less polarizable (through conjugation or displacement), are less soluble in water. It is fitting to explain the association phenomena studied with the concept of a hydrogen bond, for the formation of which the availability of unshared electrons at the heteroatom is important.

Acknowledgement: Thank you, Brother Arrhenius, for a cup of coffee and a good discussion!

LITERATURE

1. Latimer, W. M., and Rodebush, W. H. *J. Am. Chem. Soc.* **42** (1920) 1419.
2. Rodebush, W. H. *Chem. Revs.* **19** (1936) 55.
3. Huggins, M. L. *J. Org. Chem.* **1** (1936) 407.
4. Hildebrand, J. H. *Science* **83** (1936) 21.
5. Lassettre, E. N. *Chem. Revs.* **20** (1937) a: 259 ff., b: 267.
6. Pauling, L. *The nature of the chemical bond* 2nd ed. Ithaca (1940) a: 284 ff., b: 189, c: 289.
7. Hoyer, H. *Z. Elektrochem.* **49** (1943) 97.
8. Moelwyn-Hughes, E. A. *J. Chem. Soc.* (1938) 1243, (1940) 850.
9. Wolf, K. L. a: *Z. physik. Chem.* **B 43** (1939) 20, b: *Theoretische Chemie* Part 3. Leipzig (1943).
10. Harms, H. *Z. physik. Chem.* **B 43** (1939) 257.
11. Briegleb, G. *Z. physik. Chem.* **B 51** (1941) 9.
12. Prigogine, I. *Bull. soc. chim. Belg.* **52** (1943) 1.
13. Gilette, R. H., and Sherman, A. *J. Am. Chem. Soc.* **58** (1936) 1135.
14. Bauer, E., and Magat, M. *J. phys. radium* [7] **9** (1938) 319.
15. Gordy, W., and Stanford, S. C. *J. Chem. Phys.* **8** (1940) 170.
16. van Velden, P. F., and Ketelaar, J. A. A. *Chem. Weekblad* **43** (1947) 401.
17. Jensen, K. A., and Friediger, A. K. *Danske Videnskab. Selskab Math.-fys. Medd.* **20** (1943) no. 20.
18. Shott-L'vova, E. A., and Syrkin, Ya. K. *J. Phys. Chem. (USSR)* **12** (1938) 479 from *Chem. Abstr.* **33** (1939) 4839.
19. Coomber, D. I., and Partington, J. R. *J. Chem. Soc.* (1938) 1444.
20. Beach, J. Y. *J. Chem. Phys.* **9** (1941) 54.
21. Bernal, J. D. *Trans. Faraday Soc.* **36** (1940) 912.
22. Harada, M., and Titani, T. *Bull. Chem. Soc. Japan* **11** (1936) 465.
23. Ingold, C. K., and Wilson, C. L. *Z. Elektrochem.* **44** (1938) 62.
24. Gordy, W., and Stanford, S. C. *J. Am. Chem. Soc.* **62** (1940) 497.

25. Fühner, H. *Ber.* **57** (1924) 510.
26. London, F. *Z. Physik* **63** (1930) 245.
27. Hugill, J. A. C., Coop, I. E., and Sutton, L. E. *Trans. Faraday Soc.* **34** (1938) 1518.
28. Ketelaar, J. A. A. *Rec. trav. chim.* **58** (1939) 311.
29. Brockway, L. O., Beach, J. Y., and Pauling, L. *J. Am. Chem. Soc.* **57** (1935) 2693.
30. Walsh, A. D. *Trans. Faraday Soc.* **41** (1945) 35.
31. Huggins, M. L. *J. Phys. Chem.* **40** (1936) 723.
32. Cross, P. C., Burnham, J., and Leighton, P. A. *J. Am. Chem. Soc.* **59** (1937) 1134.
33. *Handbook of chemistry and physics.* 29th ed. Cleveland (1945), a: 1207.
34. Dolliver, M. A., Gresham, T. L., Kistiakowsky, G. B., Smith, E. A., and Vaughan, W. E. *J. Am. Chem. Soc.* **60** (1938) 440.
35. a: Krantz, J. C. Jr., Carr, C. J., Evans, W. E. Jr., Forman, S. E., and Wollenweber, H. L. *J. Pharmacol. Exptl. Therap.* **72** (1941) 233; b: Cone, N. M., Forman, S. E., and Krantz, J. C. Jr. *Proc. Soc. Exptl. Biol. Med.* **48** (1941) 461.
36. Krantz, J. C. Jr., Evans, W. E. Jr., Forman, S. E., and Wollenweber, H. L. *J. Pharmacol. Exptl. Therap.* **75** (1942) 30.
37. Mathews, J. H. *J. Am. Chem. Soc.* **48** (1926) 562.
38. Miles, F. T., and Menzies, A. W. C. *J. Phys. Chem.* **37** (1933) 425.
39. Griffiths, E., and Awbery, J. H. *Proc. Phys. Soc. (London)* **44** (1932) 121.
40. Landolt-Börnstein *Physikalisch-Chemische Tabellen* 5th ed. Erg. II : 2. Berlin (1931) p. 1489.
41. a: Klobbie, E. A. *Z. physik. Chem.* **24** (1897) 615, see p. 620, b: Schuncke, J. *Z. physik. Chem.* **14** (1894) 334.
42. *Beilstein's Handbuch d. org. Chemie* **I** (1918) 433.
43. Ruigh, W. L., and Erickson, A. E. *Anesthesiology* **2** (1941) 546.
44. Sänger, R. *Physik. Z.* **31** (1930) 306.
45. Stuart, H. A. *Z. Physik* **51** (1928) 490.
46. Fuchs, O. *Z. Physik* **63** (1930) 824.
47. Sänger, R., and Steiger, O. *Helv. Phys. Acta* **2** (1929) 136.
48. Williams, J. W., and Krchma, I. J. *J. Am. Chem. Soc.* **49** (1927) 1676.
49. Lange, L. *Z. Physik* **33** (1925) 169.
50. Rolinski, J. *Physik. Z.* **29** (1928) 658.
51. Meyer, L. *Z. physik. Chem.* **B 8** (1930) 27, see p. 45.
52. Hassel, O. *Z. Elektrochem.* **36** (1930) 735.
53. Voellmy, H. *Z. physik. Chem.* **127** (1927) 305.
54. de Vries Robles, H. *Rec. trav. chim.* **58** (1939) 111.
55. Mathews, J. H., and Fehlandt, P. R. *J. Am. Chem. Soc.* **53** (1931) 3212.
56. Werner, A. *Z. anorg. Chem.* **15** (1897) 1.
57. Timmermans, J., and Hennaut-Roland, Mme. *J. chim. phys.* **29** (1932) 529.
58. a: Koizumi, M., and Titani, T. *Bull. Chem. Soc. Japan* **13** (1938) 95; b: Limpricht, H. *Ann.* **165** (1873) 253, see p. 281; c: Koch, E. M., and Cahan, M. H. *J. Pharmacol. Exptl. Therap.* **26** (1925/26) 281.
59. Klapproth, H. *Nova Acta Leopoldina* **9** (1940) 305.
60. Stoughton, R. W., and Robbins, B. H. *J. Pharmacol. Exptl. Therap.* **58** (1936) 171.
61. Östergren, G., and Levan, A. *Hereditas* **29** (1943) 496.
62. Grischkewitsch-Trochimowski, E. *J. Russ. Phys. Chem. Soc.* **48** (1916) 901, from: *Chem. Zentr.* (1923) I, 1502.
63. Pickering, S. U. *J. Chem. Soc.* **63** (1893) 141.

64. Ewert, M. *Bull. soc. chim. Belg.* **46** (1937) 90.
65. Ciamician, G. L., and Dennstedt, M. *Ber.* **16** (1883) 1536.
66. Gabriel, S. *Ber.* **24** (1891) 3231.
67. Rothmund, V. *Z. physik. Chem.* **26** (1898) 433.
68. Hunter, E. C. E., and Partington, J. R. *J. Chem. Soc.* (1931) 2062.
69. Hassel, O., and Naeshagen, E. *Tids. Kjemi Bergvesen* **10** (1930) 81.
70. Rau, M. A. G., and Narayanaswamy, B. N. *Z. physik. Chem.* **B 26** (1934) 23.
71. Bergmann, E., Engel, L., and Meyer, H. *Ber.* **65** (1932) 446.
72. Steiger, O. *Physik. Z.* **32** (1931) 425.
73. Ghosh, P. N., and Chatterjee, T. P. *Phys. Rev.* **37** (1931) 427.
74. Höjendahl, K. *Physik. Z.* **30** (1929) 391.
75. Dornte, R. W., and Smyth, C. P. *J. Am. Chem. Soc.* **52** (1930) 3546.
76. Swarts, F. *Bull. acad. roy. Belg.* (1901) 383, from: *Chem. Zentr.* (1901) II, 804.
77. van Arkel, A. E., and Vles, S. E. *Rec. trav. chim.* **55** (1936) 407.
78. Rex, A. *Z. physik. Chem.* **55** (1906) 355.
79. McBain, J. W., and O'Connor, J. J. *J. Am. Chem. Soc.* **63** (1941) 875.
80. Smyth, C. P., and McAlpine, K. B. *J. Chem. Phys.* **2** (1934) 499.
81. Sänger, R. *Helv. Phys. Acta* **3** (1930) 162.
82. Fuchs, O., and Wolf, K. L. *Dielektrische Polarisation, Hand- u. Jahrb. d. chem. Physik* **6 I B** Leipzig (1935).
83. Hannay, N. B., and Smyth, C. P. *J. Am. Chem. Soc.* **65** (1943) 1931.
84. Yates, G. W. C. *Phil. Mag.* [7] **2** (1926) 817.
85. Wirtz, K. *Wied. Ann. Physik Chem.* **40** (1890) 438, see p. 446.
86. Vaughan, W. E. *J. Am. Chem. Soc.* **54** (1932) 3863, see p. 3874.
87. Smyth, C. P., and Walls, W. S. *J. Am. Chem. Soc.* **54** (1932) 3230.
88. Ehrenberg, L., and Fischer, I. *Acta Chem. Scand.* **2** (1948) 657.
89. Black, C., Joris, G. G., and Taylor, H. S. *J. Chem. Phys.* **16** (1948) 537.

Received July 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 678—682

Dipole Moments of α-, β-, γ-, and δ-Hexachlorocyclohexane and of Tetrachlorocyclohexane (m.p. 174° C)

ENOK HETLAND

Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

The following is a report of measurements of dipole moments of α-, β-, γ- and δ-hexachlorocyclohexane and of tetrachlorocyclohexane in benzene at 25°.

The measurements of the dielectric constant differences were carried out in an oil thermostat regulated to $25.00 \pm 0.02°$ using the heterodyne beat method. The solvent was purified by fractional distillation, the middle fraction only being used for the measurements.

EXPERIMENTAL

The oscillator. The oscillator consisted of two generators capable of producing electric vibrations of frequency of the order of 5 megacycles per second. The second harmonic was always used and the precision obtained in the reading was about one per cent.

The cell condenser. The test condenser contained in the glass vessel shown in Fig. 1A, in the following referred to as the cell condenser, consists of concentric gold-plated brass cylinders which were held in position by glass rods, indicated in Fig. 1B. Solute could be added to the cell through the opening H from a weighing tube Fig. 1C, the bent form of which made the transfer easy.

The level of the solution can, generally without difficulty, be adjusted to coincide with the marks MM owing to the fact that the mixing procedure intensely increases the evaporation which further causes a lowering of the miniscus. If a rising of the miniscus is desired pure benzene may simply be added.

Reference condenser. It was found very helpful to use a reference condenser with a capacity which could be set very close to that of the cell condenser when filled with pure benzene. It consisted of a precision air condenser in a glass vessel placed in the thermostat. The oscillator could be connected either to the cell condenser or to the reference condenser. Small changes in the stability of the oscillator could thus be detected and corrected for.

The measuring condenser. The measuring condenser consisted of a small variable precision air condenser which was wired parallel to the cell. A difference in the capacity

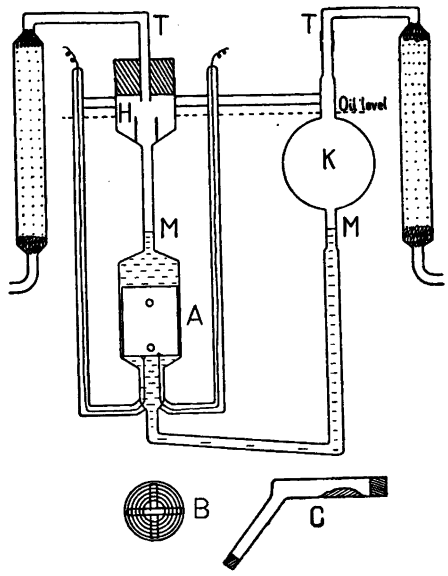


Fig. 1. Cell condenser (test condenser). The pressure inside the cell could be varied using the glass tubes filled with calcium chloride, and, by aid of the bulb K, mixing of the solution obtained.

of the cell condenser, due to an increment of the concentration, could thus be compensated and measured. The measuring condenser was adjusted by means of a variable high precision air condenser manufactured by H. W. Sullivan Ltd., London, two settings of which had been determined by comparison with a high precision condenser belonging to the Norwegian Broadcasting Station. The variation of the capacity of the measuring condenser could with sufficient accuracy be regarded as linear.

Procedure. Successively increasing concentrations were made up in the cell and the differences in the scale readings between that of pure benzene and that of the respective solutions in a series were found. The stability of the oscillator was always checked with the reference condenser.

Symbols and formulae [1] used in the calculations:

c = 99.57 picofarads, capacity of air-filled cell condenser.
k = 0.025813 sensitivity of measuring condenser (picofarads/scale divisions).
$\Delta c = k \cdot d$, increment of capacity due to dissolved solute.
d = reading of the measuring condenser due to dissolved solute.
ε = 2.2727 dielectric constant of benzene at 25° [2].

$$\Delta\varepsilon = \frac{\Delta c}{c}$$

$$\varepsilon_1 = \varepsilon + \Delta\varepsilon$$

$$p = \frac{\varepsilon - 1}{\varepsilon + 2} = 0.29786 \text{ for benzene}$$

$$p' = \frac{\varepsilon_1 - 1}{\varepsilon_1 + 2}$$

Q = weight of solvent in cell (33.110 g benzene sp. w. 0.8739).
q = weight of solute added, in grams.
M_1 = molecular weight of solvent (78.108 for benzene).
M_2 = molecular weight of solute.
f_2 = mole fraction of solute.
Δn_E = difference in refractive index, extrapolated. ($\Delta n_E = f_2 \cdot 0.1085$ used, for hexachlorocyclohexane; $\Delta n_E = f_2 \cdot 0.0404$, used for tetrachlorocyclohexane).

$$p_E = \frac{n^2 - 1}{n^2 + 2}$$

$$\Delta p_E = \frac{6n}{(n^2 + 2)^2} \Delta n_E.$$ Obtained by derivation of p_E.

$n = 1.5024$ refractive index used.
$V = 37.888$ cm^3, volume of the cell condenser at 25°.

$$C = \frac{1000 \Sigma q}{M_2 V}$$ concentration of solution in grammol/litre at 25°.

$$P_{A+O} = \frac{1000}{C} (p - p' - \Delta p_E)$$ sum of atomic and orientation polarization.

P^{∞}_{A+O} = sum of atomic and orientation polarization, extrapolated.

μ = dipole moment $= 1.273 \cdot 10^{-20} \sqrt{T \cdot P^{\infty}_{A+O}}$
$T = 298.2°$ absolute temperature.

RESULTS

Fig 2. gives the observed values d as a function of the weights q of the solute added to the cell.

Tables 1 and 2 is based on data taken from the graph Fig. 2. In the determination of P^{∞}_{A+O} a greater number of points were used than given in the tables.

Table 1.

Hexachlorocyclohexane, M = 290.8.

d	ε_1	p'	Δp_E	C	P_{A+O}
		α-isomere (m. p. 156.3°).			
50	2.2857	0.3000	0.00010	0.2015	100.7
100	2.2986	0.3021	0.00020	0.4021	100.7
		$P^{\infty}_{A+O} = 100.6$; $\mu = 2.20 \cdot 10^{-18}$ e. s. u.			
		β-isomere (m. p. 308°).			
		$P^{\infty}_{A+O} \sim 0$; $\mu = 0$.			
		γ-isomere (m. p. 113.3°).			
100	2.2986	0.30210	0.00012	0.02388	172.6
200	2.3246	0.30629	0.00023	0.04784	171.4
		$P^{\infty}_{A+O} = 173.3$; $\mu = 2.89 \cdot 10^{-18}$ e. s. u.			

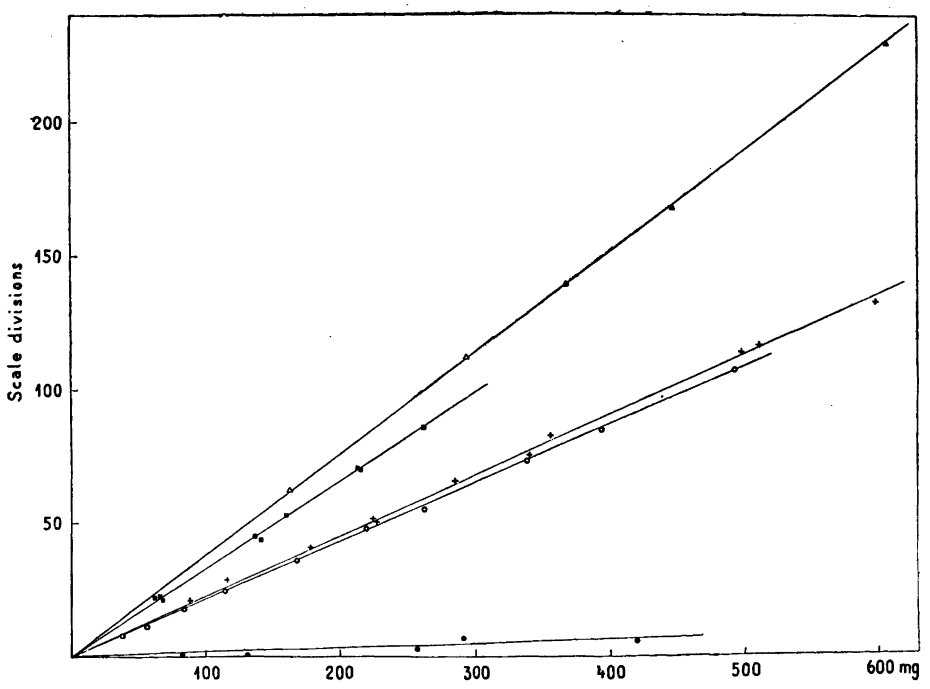


Fig. 2. Observed values (scale divisions) of the measuring condenser plotted agains the weights of solute added to the cell.

■ *Tetrachlorocyclohexane*
\+ *α-Hexachlorocyclohexane*
● *β-Hexachlorocyclohexane*
△ *γ-Hexachlorocyclohexane*
○ *δ-Hexachlorocyclohexane*

δ-isomere (m. p. 138.2°).

50	2.2857	0.3000	0.00010	0.02097	97.3
100	2.2986	0.3021	0.00020	0.04193	96.3

$P^{\infty}_{A+O} = 97.2$; $\mu = 2.17 \cdot 10^{-18}$ e. s. u.

Table 2.

Tetrachlorocyclohexane, M = 221.95 (m. p. 174°).

d	ε_1	p'	Δp_E	C	P_{A+O}
50	2.2857	0.3000	0.00003	0.01813	116.3
100	2.2986	0.3021	0.00006	0.03621	115.4

$P^{\infty}_{A+O} = 115.6$; $\mu = 2.36 \cdot 10^{-18}$ e. s. u.

Δp_E is found from the experimental values of Δn (difference between the refractive index of solution and solvent) for different wave lenghts. By plotting the experimental Δn values against the corresponding frequencies the value Δn_E at zero frequency may be obtained by graphical extrapolation. A Pulfrich refractometer, fitted with a divided cell was used for the determination of Δn [3].

The γ- and δ-compounds were plased at our disposal by Imperial Chemical Industries Ltd.

The obtained values for α- and β-hexachlorocyclohexane show good agreement with those of Hassel and Næshagen [4] and also with those of Williams [5] for the α-isomere. B. Melander [6] reports $\mu = 3.6 \cdot 10^{-18}$ for the γ-isomere and $\mu \sim 0$ for the α-isomere at 17°.

I wish to express my gratitude to *Nansenfondet*, to *Norsk Varekrigsfond* and to *Houens Legat* for grants which made this research possible. I am indebted to Professor Dr. O. Hassel for placing room and apparatus at my disposal and for many helpful suggestions.

SUMMARY

A cell, convenient for dielectric constant measurements of solutions, has been described.

Measurements of the sum P^{∞}_{A+O} of the atomic and orientation polarization at infinite dilution of α-, β-, γ- and δ-hexachlorocyclohexane and of tetrachlorocyclohexane (m. p. 174° C) have been carried out on solutions in benzene at 25°, and the corresponding dipole moments have been computed.

REFERENCES

1. Hassel, O., und Næshagen, E. *Z. Phys. Chem.* **B 4** (1929) 217.
2. Phillips, G. M., Hunter, J. S., and Sutton, L. E. *J. Chem. Soc.* (1945) 151.
3. Højendahl, K. Diss., Copenhagen (1928).
4. Hassel, O., und Næshagen, E. *Tids. Kjemi Bergvesen* **10** (1930) 126.
5. Williams, J. W., and Fogelberg, J. M. *J. Am. Chem. Soc.* **53** (1931) 2096.
6. Melander, B. *Svensk Kem. Tid.* **58** (1946) 231.

Received June 17, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 683—692

On the Reaction between Carbon Monoxide and Alcohol Catalyzed by Alcoholate

II. The Propyl- and Butyl Alcohols

Appendix: The Solubility of Carbon Monoxide in Secondary and Tertiary Butyl Alcohol

J. CHR. GJALDBÆK

Department of Inorganic Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark

In an earlier paper [1] the kinetics of the reactions of carbon monoxide with methanol and ethanol solutions of the corresponding alcoholates, giving alkyl formates, were investigated. The present paper deals with the kinetics of the analogous reactions in the two propyl alcohols and the four butyl alcohols.

MATERIALS

Carbon monoxide was prepared by adding anhydrous formic acid to concentrated sulphuric acid. It was passed through soda lime and collected in a glass bulb gasometer over concentrated sulphuric acid. By analysis in a micro-gas-burette [2] the gas was found to contain more than 99 % carbon monoxide, the rest being air.

The alcohols were dried by slow distillation in a wire-gauze column [3]. The boiling points (corrected to 760 mm) were: normal propyl alcohol 97.4°, isopropyl alcohol 82.4°, normal butyl alcohol 117.7°, isobutyl alcohol 107.9°, secondary butyl alcohol 99.5° and tertiary butyl alcohol 82.4° (m. p. 25.6°).

The alcoholates were prepared by dissolving sodium in the alcohols in question except in the case of tertiary butyl alcohol where potassium was used on account of the slight solubility of sodium butylate in tertiary butyl alcohol.

PROCEDURE

The velocities of the reactions were determined in a Brønsted shaking apparatus. For descriptions of the apparatus, the procedure, and the method of treatment of the data see the paper cited [1]. The volume of the reaction flask was 108.4 ml and the dead space from the neck of the flask to the surface of the mercury in the manometer was 17.5 ml; in each experiment 25.00 ml of catalyst solution was introduced.

In the earlier paper all pressure readings were corrected, since it was observed in blank experiments with pure alcohol in both flasks that on addition of carbon monoxide to the reaction flask the pressure did not immediately rise to its final value. Blank experiments were also carried out with the propyl and butyl alcohols, but since the increase of pressure in all cases was less than 0.2 cm (no doubt because of the smaller vapour pressure of these alcohols) corrections were not necessary.

EXPERIMENTAL RESULTS AND DISCUSSION

A survey of the experimental results is given in Tables 1 and 2 where the velocity constants are calculated on the basis of natural logarithms and seconds.

All the reactions were found to be first order with respect to carbon monoxide. At every temperature the experiments were carried out at several con-

Table 1. $CO + C_3H_7OH$; catalyst C_3H_7ONa.

	Temp. °C	$c_{cat.}$	Mol ester / Mol $CO_{abs.}$	$k \cdot 10^5$ s^{-1}	$\frac{k \cdot 10^5}{c_{cat.}}$	$k^\circ \cdot 10^5$ s^{-1}	$k' \cdot 10^5$ s^{-1}
Normal	20.0	0.9846	—	3.089	3.137	2.262	53.89
		0.4672	1.00	1.233	2.639		
	30.0	1.013	0.95	9.650	9.526	6.780	157.2
		0.5044	0.98	3.990	7.911		
		0.2632	0.98	1.965	7.466		
	40.0	0.9870	1.01	28.97	29.35	19.77	443.0
		0.4766	1.01	11.20	23.51		
		0.2903	1.06	6.520	22.45		
Iso	20.0	0.2038	1.05	1.589	7.798	7.577	168.7
		0.0989	0.99	0.7597	7.681		
	40.0	0.2410	1.02	11.63	48.24	40.47	836.3
		0.1492	1.06	6.730	45.11		

Table 2. $CO + C_4H_9OH$; catalyst C_4H_9ONa, except in the experiments with tertiary C_4H_9OH where tertiary C_4H_9OK was used as catalyst.

	Temp. °C	$c_{cat.}$	Mol ester / Mol $CO_{abs.}$	$k \cdot 10^5$ s^{-1}	$k \cdot 10^5 / c_{cat.}$	$k^\circ \cdot 10^5$ s^{-1}	$k' \cdot 10^5$ s^{-1}
Normal	30.0	0.8477	1.02	7.409	8.740	5.991	149.4
		0.4847	1.05	3.684	7.601		
		0.3347	1.03	2.302	6.878		
	40.0	0.4907	—	10.71	21.82	16.98	411.5
		0.4579	0.96	9.899	21.62		
		0.2911	1.07	5.756	19.77		
	50.0	0.4725	1.02	25.98	54.98	40.38	951.9
		0.2418	—	11.97	49.51		
Iso	20.0	0.3180	0.96	1.919	6.033	4.604	111.5
		0.2080	—	1.143	5.495		
	40.0	0.2722	—	12.52	46.00	39.04	884.8
		0.1914	0.95	8.394	43.86		
Secondary	20.0	0.3922	1.02	5.284	13.47	9.375	237.6
		0.2934	1.01	3.534	12.04		
		0.1740	1.05	1.911	10.98		
	40.0	0.5813	—	63.12	108.6	62.23	1473
		0.3485	0.90	30.43	87.31		
		0.2893	—	23.48	81.17		
		0.1539	0.97	11.13	72.30		
Tertiary	26.1	0.7382	1.02	25.94	35.14	17.78	391.9
		0.4964	1.05	13.85	27.90		
		0.2327	1.00	5.142	22.10		
	30.0	1.025	—	64.46	62.89	26.82	568.6
		0.5552	—	25.04	45.09		
		0.2501	—	8.058	32.22		
	40.0	0.5534	1.01	47.20	85.29	56.38	1098
		0.2835	1.09	19.65	69.30		
		0.2344	1.12(?)	15.81	67.44		

centrations of the catalyst and as in the earlier paper the velocity constants were extrapolated to $c_{cat.} = 0$ according to the equation

$$\log \frac{k}{c_{cat.}} = \log k^{\circ} + \beta \cdot c_{cat.}$$

In quantity k' is given by the equation

$$k' = k^{\circ} \cdot \left(\frac{V_g}{V_l} + \alpha \right) \frac{1}{\alpha} * \qquad (1)$$

where V_g and V_l are the volume of the gas phase and liquid phase, respectively, and α is the distribution coefficient of carbon monoxide between the liquid and the gas phase (*i. e.* the Ostwald absorption coefficient). The values of the absorption coefficient for carbon monoxide in the propyl alcohols and in normal and isobutyl alcohol have been determined by the author [4]; for secondary and tertiary butyl alcohol see the appendix to this paper.

The values of $k' \cdot 10^5$ for methanol and ethanol are calculated to be

	35.05°	45.05°	54.16°
Methanol	53.28	136.0	358.3
Ethanol	180.9	475.6	1052

In an experiment with 0.1682 molar sodium butylate in tertiary butyl alcohol at 30° the value $k \cdot 10^5/c_{cat.} = 44.5$ was found; thus with sodium alcoholate the reaction is a little faster than with potassium alcoholate. The same result was obtained in an experiment with 0.6208 molar potassium butylate in secondary butyl alcohol at 40° where $k \cdot 10^5/c_{cat.}$ was found to be 96.7 (*cf.* Table 2).

After each experiment 5.00 ml of the cooled reaction mixture was diluted with 50 ml of carbon dioxide-free water, allowed to stand long enough for complete hydrolysis of the ester, and titrated with 0.1 *N* hydrochloric acid. As in the earlier paper the ester concentrations so found are approximately equivalent to the amount of carbon monoxide absorbed; the results are given in Tables 1 and 2.

In nearly all the experiments small amounts of a sodium formate sediment appeared. When dissolved in water and neutralized the sediment reduced mercuric chloride to mercurous chloride and on distillation with sulphuric

* The corresponding equation though without the α in the parenthesis was used in the earlier paper. Professor Verner Schomaker of the California Institute of Technology, Pasadena, who has been kind enough to correct the translation into English, called attention to this mistake.

acid it evolved formic acid. A 0.672 g sample of the washed and dried sediment after heating neutralized 10.00 ml of 0.1 *N* hydrochloric acid (theoretical 10.20 ml). Since it was difficult to determine the small amounts of sediment quantitative analysis was carried out in only a few cases. It can be estimated that the amount of sediment in all experiments was less than about 5 % of the amount of sodium alcoholate. The formation of sodium formate can be explained according to the equation

$$HCOOR + RONa = R_2O + HCOONa$$

where R denotes the alcohol radical. This equation implies that the concentration of the catalyst decreases as the reaction proceeds. In most of the experiments the time of earliest appearance of sediment was noted; however, in no case did the formation of the sodium formate appear to influence the velocity constant, undoubtedly because the alteration in concentration of the catalyst was only slight.

In many of the experiments the reaction proceeded to an equilibrium considerably short of complete absorption of the carbon monoxide. Since the concentration of the ester was determined and the concentration of carbon monoxide is known from the final pressure the equilibrium constant of the gross reaction CO + ROH = HCOOR can be calculated; however, in many of the experiments the final pressure of carbon monoxide was too small to give more than a crude estimate. At 40° the equilibrium constant for ester-formation in tertiary butyl alcohol may be estimated to be about 4 l/M, in normal and secondary butyl alcohol about 6, in isobutyl alcohol about 8 and in propyl alcohol about 11.

Table 3.

Alcohol	Methyl	Ethyl	Normal propyl	Iso-propyl	Normal butyl	Iso-butyl	Sec. butyl	Tert. butyl
A	4357	4037	4198	3191	3941	4129	3637	2960
H	10.85	10.36	11.05	8.12	10.19	11.14	9.78	7.50
Solubility * 20°	> 1	> 1	> 1	0.22	> 1	0.63	0.66	0.34 $^{27°}$

* Approximate molarity of saturated solution of sodium alcoholate in the corresponding alcohol.

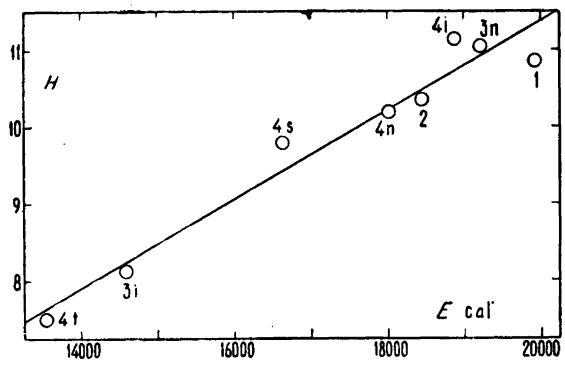


Fig. 1. 1: CH_3OH; 2: C_2H_5OH; 3n: n-C_3H_7OH; 3i: iso-C_3H_7OH; 4n: n-C_4H_9OH; 4i: iso-C_4H_9OH; 4s: sec.-C_4H_9OH; 4t: tert.-C_4H_9OH.

The quantities A and H of the equation

$$\log k' = -\frac{A}{T} + H$$

were calculated by the method of least squares. The results are recorded in Table 3 together with values for the earlier methyl and ethyl alcohol data [1] recalculated according to the new solubility measurements [4] and to the corrected equation (1). The values of A and H for isopropyl and isobutyl alcohol are the least exact since these values are based on only four experiments in each alcohol. In Fig. 1 the frequency exponent H is plotted as ordinate and the energy of activation E ($E = 4.571\ A$) as abscissa, the straight line representing the equation

$$H = \frac{E}{1720} - 0.24$$

obtained by least squares on the assumption of linear dependence.

Calculation of the number of collisions S from the formula

$$S = \sigma_{1,2}^2\, n_1 n_2 \sqrt{8\pi RT \frac{M_1 + M_2}{M_1 M_2}}$$

where $\sigma_{1,2}$ denotes the sum of the collision radii of the molecules, n the number of molecules per ml, and the M's the molecular weights, leads to values of log S at unit concentrations (1 M/l) between 11.1 and 11.4 for all the alcohols,

Table 4. Values of densities and vapour pressures of the alcohols used in the calculations.

Temp.	Secondary butyl alcohol		Tertiary butyl alcohol	
	$d\frac{t^5}{4}$	Vapour pressure mm Hg [6]	$d\frac{t}{4}$	Vapour pressure mm Hg [9]
20.0	0.8073	16.2		
26.0			0.7804 [7]	44.3
30.0	0.8000	27.7	0.7762 [7]	56.9
40.0	0.7922	45.9	0.7630 [8]	102.3
50.0	0.7834	74.0	0.7522 [8]	174.3

S being expressed in M/l per s. Since the literature, as far as the author is aware, does not contain data for the radii of the propyl and butyl alcohols, the radii were estimated with the help of Fisher-Hirschfelder-Taylor atom models of the alcohols on the basis of the value 2 Å known for methanol from its viscosity; the estimates all lie between 2 and 3.5 Å. The further values $\sigma_{CO} = 1.6$ Å and $T = 318$ were used. Only for isopropyl alcohol, secondary butyl alcohol and tertiary butyl alcohol are the values of H markedly different from the calculated log S.

Saturated solutions of the alcoholates were titrated with 0.1 N hydrochloric acid; the approximate molarities are recorded in Table 3.

APPENDIX: THE SOLUBILITY OF CARBON MONOXIDE IN SECONDARY AND TERTIARY BUTYL ALCOHOL

By a procedure and in an apparatus earlier described [4] the solubilities of carbon monoxide in secondary and tertiary butyl alcohol were determined at different temperatures. The boiling points of the alcohols used are recorded on p. 683 of this paper. For values of densities and vapour pressures used in the calculations see Table 4. The solubilities expressed by means of Ostwald's absorption coefficient are recorded in Table 5. Smoothed values at different temperatures are recorded in Table 6, where also u and log a according to the equation

$$\log l = \frac{u}{RT} + \log a$$

are given. The extraordinary high temperature coefficient for the solubility in tertiary butyl alcohol should be noticed.

Table 5. Solubility of carbon monoxide in the butyl alcohols.

Secondary			Tertiary		
t	*l*	*l*	*t*	*l*	*l*
20.05	0.168		27.56	0.194	
20.10		0.164	27.75		0.195
35.40	0.173		35.57	0.206	
35.50		0.170	35.60		0.208
49.98	0.180		49.98	0.226	
50.25		0.176	50.28		0.227

Table 6. Smoothed values of the solubility of carbon monoxide in the butyl alcohols.

Temp.	20°	26°	30°	40°	50°	*dl/dt*	*u*	log *a*
Secondary	0.166		0.170	0.174	0.178	0.00040	— 438	— 0.45
Tertiary		0.192	0.198	0.213	0.226	0.00142	— 1320	+ 0.24

SUMMARY

1. The reactions between carbon monoxide and six alcohols (the two propyl and the four butyl alcohols), catalyzed by the corresponding alcoholates, are investigated manometrically. Esters of formic acid and the alcohol in question are formed. The rate is found to be of the first order with respect to carbon monoxide. The rate is shown to be determined by the reaction

$$CO + RO^- = (COOR)^-$$

the complex formed reacting to give HCOOR and RO^- which thus acts as a catalyst, R denoting the alcohol radical.

2. The velocity constant increases somewhat faster than proportional to the concentration of the catalyst and is approximately described by the equation

$$\log \frac{k}{c_{cat.}} = k^\circ + \beta \cdot c_{cat.}$$

3. All the experiments are carried out at two or three temperatures and a survey of the results is given in Tables 1 and 2. Using the method of least squares A and H are calculated according to the equation

$$\log k' = -\frac{A}{T} + H$$

where $4.571 \cdot A$ is the energy of activation (E) and H the frequency exponent. The results are together with recalculated values for the reaction in methanol and ethanol recorded in Table 3. Linear dependence between E and H is shown in Fig. 1.

4. In most of the experiments small amounts of sodium formate sediment appeared (less than 5 % of the amount of sodium alcoholate). The formation can be explained according to the equation

$$\mathrm{HCOOR} + \mathrm{RONa} = \mathrm{R_2O} + \mathrm{HCOONa}$$

involving a decrease of the concentration of the catalyst which, however, did not alter the velocity constant notably.

5. In many of the experiments the reaction proceeded to an equilibrium. The equilibrium constant

$$K = \frac{c_{\mathrm{ester}}}{c_{\mathrm{CO}} \cdot c_{\mathrm{alcohol}}}$$

was in tertiary butyl alcohol roughly estimated to be about 4, in normal and secondary butyl alcohol about 6, in isobutyl alcohol about 8 and in normal propyl alcohol about 11 at 40°.

6. Approximate solubilities of the sodium alcoholates, determined at 20°, are recorded in Table 3.

7. Appendix. The solubility of carbon monoxide in secondary and tertiary butyl alcohol is determined at different temperatures ranging from 20° to 50°. Smoothed values of the solubilities are recorded in Table 6.

My thanks are due to *Professor Emil Koefoeds Mindelegat, Etatsraad, cand.pharm. C. A. Olesens Studielegat* and *Danmarks farmaceutiske Højskoles Jubilæumsfond* for scholarships which made it possible to carry out this work. I also wish to thank Professor J. A. Christiansen for helpful discussions of the subject.

REFERENCES

1. Christiansen, J. A., and Gjaldbæk, J. Chr. *Kgl. Danske Videnskab. Selskab Mat.fys. Medd.* **XX** (1942) no. 3.
2. Christiansen, J. A., and Wulff, I. *Kgl. Danske Videnskab. Selskab Mat.fys. Medd.* **XXII** (1945) no. 4.
3. Klit, A. *Fraktioneret Destillation* København (1943).
4. Gjaldbæk, J. Chr. *Kgl. Danske Videnskab. Selskab Mat. fys. Medd.* **XXIV** (1948) no. 13.
5. Pickard, R. H., and Kenyon, J. *J. Chem. Soc.* **99** (1911) 45.
6. Gilliland, E. R., and Sherwood, T. K. *Ind. Eng. Chem.* **26** (1934) 519.
7. Timmermans, J., et Delcourt, Y. *J. chim. phys.* **31** (1934) 85.
8. Paterno, E., e Mieli, A. *Atti reale accad. Lincei* (5) **16** (1907) 153.
9. Parks, G. S., and Barton, B. *J. Am. Chem. Soc.* **50** (1928) 25.

Received July 15, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 693—701

A Spectrophotometric Study on the Orcinol Reaction with Carbohydrates

ERIK VASSEUR

Wenner-Grens Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden

The colour reaction of carbohydrates with orcinol and sulphuric acid was studied in detail by Sørensen and Haugaard [1]. They heated the samples for 5 to 30 minutes at 80° in a water bath and the developed colours were read in a Pulfrich step photometer, usually with filters S 43 and S 53 (maximum transmission at 430 and 530 mμ resp.). The ratios of the densities with these filters were plotted against the time of heating. The curves obtained were considered characteristic for the different carbohydrates.

The present study was carried out with the Warburg and Negelein objective spectrophotometer [2] and with the Beckman spectrophotometer model DU. The absorption spectra thus obtained allowed both a qualitative and a quantitative characterisation of many different carbohydrates, and proved to be useful for the study of the chemistry of the egg coat in sea-urchins.

METHODS

In a detailed investigation of the orcinol reaction, Sørensen and Haugaard [1] studied the effect of variations in the orcinol and sulphuric acid concentrations, the temperature, and the time of heating. It was accordingly not judged necessary to deviate from the conditions which they found to be optimum. However, since it is difficult to prevent a 2 per cent solution of orcinol in 20 per cent sulphuric acid (by volume) from crystallising, a 1 per cent orcinol solution in the same strength of acid was used (Rimington [3] employed a 1.6 per cent orcinol solution in 30 per cent sulphuric acid). The concentration of the stock sulphuric acid was changed from 6 : 4 to 7 : 3, *i. e.*, 7 volumes of concentrated sulphuric acid to 3 volumes of distilled water, in order to maintain as far as possible the same final concentrations of orcinol and sulphuric acid

as used by Sørensen and Haugaard and to employ simple volume quantities of the components. Furthermore, 2 ml instead of 1 ml of the carbohydrate solutions were used, as a convenience in testing mixtures of different sugars. The approximate final orcinol and sulphuric acid concentrations according to the modifications of Sørensen and Haugaard (S-H), Rimington (R), and the author (V) are as follows:

	S-H	R	V
H_2SO_4 concentration in milliequiv. per ml sample	20.0	19.6	19.4
Orcinol concentration in mg per ml sample	2.22	2.16	2.00

Reagents

Carbohydrate solutions. Solutions of the various sugars were made up to contain 10 micromoles of monosaccharide per 2 ml, *e. g.*, for glucose, 90.0 mg per 100 ml; for dextran (a polysaccharide consisting solely of glucose units, $(C_6H_{10}O_5)_n$, $M = n \cdot 162$), 81.0 mg per 100 ml. These were the stock solutions from which other concentrations were prepared.

Orcinol solution. 1.000 g orcinol was dissolved in about 50 ml distilled water, a cooled mixture of 20 ml of concentrated sulphuric acid (sp. gr. 1.84) and 30 ml of distilled water was added, and the solution adjusted to 100 ml with distilled water. Titration gave approximately 7.4 equivalents of H_2SO_4 per liter.

Sulphuric acid solution. 700 ml of concentrated sulphuric acid was mixed with 300 ml of distilled water under careful chilling. Sp. gr. 1.699 at 20°; titration gave approximately 26.8 equivalents per liter.

Procedure

Into an ice-chilled test tube* provided with a stirrer** was pipetted 2 ml of the carbohydrate solution, followed by 3 ml of the orcinol solution. When the mixture had cooled, 10 ml of the sulphuric acid solution was added, and the sample was cautiously stirred to keep the temperature as low as possible***. The sample, still provided with the stirrer, was heated in a water bath at $80 \pm 0.5°$ for exactly 2, 5, or 20 minutes. While heating, the samples may not be stirred. In order to quickly and thoroughly cool the sample, the tube was circulated about in ice water while occasionally stirring the contents. Photo-

* Jena Geräte glass; 180 × 18 mm.

** 5 mm glass tubing with a bulb of about 12 mm blown at one end.

*** After thorough cooling, the sample may be kept in ice water, preferably in the dark, for as long as 12 hours without deterioration. After heating, the density is the same as for freshly prepared samples.

meter measurements of the 2 and 5 minute samples were taken as soon as possible after heating, whereas the 20 minute samples could be kept in ice water for some time without any appreciable change of the density.

The colour displayed by fructose is especially labile and gradually fades. By cooling the 2 minute samples for 5 minutes after heating, and measuring every two minutes at 485 mμ for some time, the density values obtained by extrapolation to zero time were directly proportional to the concentration of fructose.

For each carbohydrate solution investigated, a blank without orcinol was also measured. The orcinol solution was replaced by 20 per cent by volume sulphuric acid. Similarly, when new orcinol or sulphuric acid solutions were employed, a blank without carbohydrate was also determined. It was found advisable to use orcinol and sulphuric acid of as pure a grade as possible in order to minimise the values of samples without carbohydrate. It was also expedient to prepare large volumes of sulphuric acid solution at a time and to store it in vessels free of dust. The orcinol solution could be used for at least six months if stored in a brown bottle kept in the dark except when adding to a sample. In this manner the blank values could be kept constant.

Since the colour in the blank without carbohydrate, if the orcinol is pure, is probably due to traces of pentosans in the sulphuric acid, the analyses must be corrected accordingly. On the other hand, it is not quite certain that the whole density for the blank without orcinol should be subtracted from the density of the sample. Upon heating fructose and rhamnose, and to a lesser extent sorbose and mannose, without orcinol (2 ml of carbohydrate solution, 3 ml of 20 per cent sulphuric acid, and 10 ml of sulphuric acid solution), a colour was formed with maximum absorption at 460 mμ. This colour appears to be suppressed in the presence of orcinol (*cf.* also Sørensen [4]). Very probably these carbohydrates contain the same kind of impurity, probably of aromatic character, which produces a colour with the furfural derivative formed, but more slowly than does orcinol. The correct procedure in this case would be to subtract only a fraction of the density of the blank without orcinol. Since it is hardly possible at present to calculate this fraction, it is advisable to omit this correction for the blank in most cases.

EXPERIMENTAL

From the theory of the absorption of light in a coloured solution [5], $\log \frac{I_0}{I} = D = k \cdot c \cdot d$, and $\log D = \log k + \log c + \log d$ (where I_0 = incident intensity, I = transmitted intensity, D = density, k = specific density = $\frac{D}{c \cdot d}$, c = concentration of the coloured substance, and d = thickness of the liquid layer) it becomes evident that the curve sof $\frac{I}{I_0}$ or $\log \frac{I_0}{I}$ plotted against wavelength, λ, or frequency, ν, change their shape depending on the concentration, c, and thickness, d. If however, $\log D$ or $\log k$ is plotted as ordinate, curves of the same shape are obtained, regardless of the values of c or d.

The colour curves for 20 minutes heating are based on experiments using 1 micromole of monosaccharide as measured in the Warburg and Negelein apparatus. The measurements of the densities were generally performed with a cell thickness sufficient to produce density values ranging from about 0.160 to 0.600, with $d = 0.1$ cm for the strongest densities, and up to 3 cm for the weakest. Concentrations of 0.5 micromoles and, in general, 5 micromoles of carbohydrate as well gave curves in accordance with these standard curves, at least between 470 and 530 mμ; 10 micromoles gave a more or less pronounced turbidity which elevated the density at 560 mμ but depressed it at 425 mμ. It was therefore advantageous to employ a concentration of carbohydrate in the neighbourhood of 1 micromole per sample for the 20 minute curves. On the other hand, 5 micromoles was convenient for the 5 minute curves of the aldohexoses, while 10 micromoles was most useful for their 2 minute curves; under these conditions cells of about 1 cm thickness could be used. For the curves of the ketohexoses and pentoses and for the 5 minute and 20 minute curves of the methylpentoses, 1 micromole was satisfactory and 5 micromoles for the 2 minute curves of the methylpentoses. The curves obtained with the Beckman apparatus during the later part of the study were in good agreement with those obtained with the Warburg and Negelein apparatus.

The maximum at 425 mμ of the 20 minute curves is too sharp to be used for quantitative measurements when photometers with filters are employed (*e. g.*, Pulfrich, Klett, Spekker) since the density values vary too much with variations in concentration and cell thickness. On the other hand, portions of the curves between 480 and 520 mμ, especially of the aldohexoses, slope more gradually and may be used for the estimation of carbohydrate concentration. The Pulfrich step photometer, for example, gives good results with filter S 50 (maximum transmission at 500 mμ) in the determination of blood glucose.

Table 1. Density values with one micromole glucose per sample; 20 minutes heating; measurements with the Beckman apparatus.

λ mμ	Date							Mean
	21/1	22/1	23/1	24/1				
490	0.236	0.230	0.225	0.241	0.246	0.222	0.241	0.2344 ± 0.0034
510	0.225	0.215	0.217	0.230	0.237	0.215	0.232	0.2244 ± 0.0034
520	0.214	0.205	0.208	0.218	0.220	0.208	0.218	0.2130 ± 0.0023

Table 2. Density values with one micromole rhamnose per sample; 5 minutes heating; measurements with the Beckman apparatus.

λ mμ	Sample		Deviation of sample 1 from sample 2 %
	1	2	
370	1.057	1.080	2.1
375	.958	.990	3.2
380	.998	1.025	2.6
385	1.060	1.100	3.6
390	1.140	1.170	2.6
400	1.260	1.300	3.1
410	1.380	1.400	1.4
420	1.350	1.370	1.5
430	1.170	1.200	2.5
440	.880	.919	4.2
450	.598	.621	3.7
460	.378	.393	3.8
470	.271	.280	3.1
480	.233	.238	2.1
490	.230	.235	2.1
500	.239	.241	0.8
510	.229	.234	2.1
520	.195	.200	2.5
530	.154	.160	3.7
540	.115	.122	5.7
550	.084	.090	6.7
560	.063	.069	8.7
580	.035	.039	10.2
Mean of deviation (per cent)			2.8

The accuracy and reproducibility of the method is fairly good as seen from Tables 1 and 2. Table 1 represents some values on glucose obtained with the Beckman spectrophotometer by a probationer.

The carbohydrates investigated in this work were glucose, mannose, galactose, fructose, sorbose, xylose, arabinose, rhamnose, potassium gluconate, sodium glucuronate, mannitol, glucosamine, dextran *, mannan from yeast **, and nucleic acid from yeast. The results obtained with glucose, mannose, galactose, rhamnose, xylose, and fructose are summarised in Fig. 1.

* Generously supplied by Dr. Björn Ingelman.

** Generously supplied by Dr. Gösta Lindstedt.

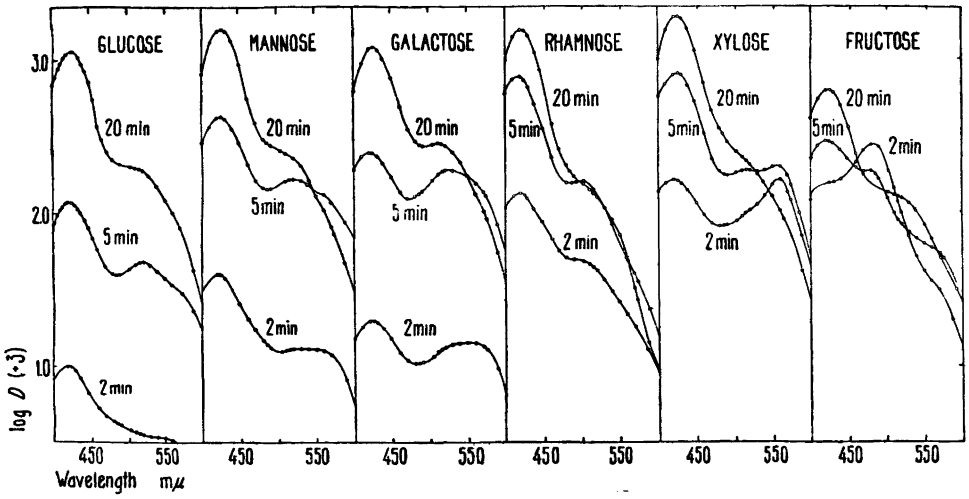


Fig. 1. Colour curves between 400 and 600 mμ for 1 micromole of glucose, mannose, galactose, rhamnose, xylose, and fructose in 15 ml orcinol and sulphuric acid sample after 2, 5, and 20 minutes heating.

Naturally, potassium gluconate and mannitol, like glucosamine, failed to give any colour because the colour development depends on the transformation to a furfural derivative which reacts with orcinol. The preparation of sodium glucuronate tested was a brown mass exhibiting a strong brownish colour when dissolved in water. In spite of this, the blank without orcinol was almost colourless. The colour curves obtained were somewhat similar to both the pentose and the galactose curves. For more reliable values, purer samples of glucuronic acid are necessary; the values obtained in the present investigation are therefore not shown. The ketohexose, sorbose, behaved similar to fructose, and the pentose, arabinose, similar to xylose. The results with dextran, mannan, and yeast nucleic acid are discussed below.

DISCUSSION

The colour curves of the aldohexoses, ketohexoses, pentoses, and methylpentoses differ distinctly from each other. Within each group of carbohydrates, however, the form of the curves is very similar. The aldohexoses have maxima at about 425, 515, and 560 mμ, and a minimum at about 485 mμ (at 470 mμ for galactose after 5 minutes heating). The ketohexoses exhibit a pronounced maximum at about 485 mμ after 2 minutes heating; after 20 minutes heating, their curves are more similar to those of the aldohexoses but

with lower intensities. The pentoses are characterised by well-defined maxima at about 560 mμ for heating times of 2 and 5 minutes, whereas the 20 minute curves are still steeper between 470 and 530 mμ than those of the aldohexoses. The methylpentose, rhamnose, is almost devoid of the maximum at 560 mμ, even for the shorter heating times. The other maxima are displaced toward the short wave region of the spectrum. They are situated at 418 instead of 425 mμ, and at 505 instead of 515 mμ.

The 20 minute curves of the three aldohexoses differ from each other in the following respects. The mannose curve has the steepest slope between 470 and 530 mμ, the glucose curve slopes less steeply in this region, whereas the galactose curve actually rises between 490 and 510 mμ. With the aid of these dissimilarities it is easy to distinguish between them and also between their derivatives. Mannan, for example, gives a 20 minute curve which almost completely corresponds with that of mannose, and the dextran curve nearly parallels that of the glucose analogue. It is remarkable, however, that the densities of these polysaccharides only amount to about 90 per cent of those of the monosaccharides. This may partially depend upon the time required for hydrolysis of the polysaccharides. Furthermore, their 5 minute curves are too low and closely resemble the 2 minute curves of the monosaccharides. If however, the samples are heated for 6 or 7 minutes, the curves nearly attain the shape of the 5 minute curves of mannose and glucose, respectively. The 21 and 22 minute curves rather than the 20 minute curves of these polysaccharides are in good agreement with the 20 minute curves of the monosaccharides. Yeast nucleic acid shows curves similar to those of the pentoses, but the colour development is even more delayed than in the case of mannan and dextran. For the quantitative determination of carbohydrates in macromolecular compounds, this retardation must be kept in mind.

Another fact worth pointing out is that aldohexoses produce no significant colour after 2 minutes heating, even in concentrated solutions, whereas fructose even after 1 minute exhibits a yellow colour of measureable density. After 2 minutes heating, samples containing methylpentoses also show a yellow colour which however is paler than that given by the same amount of ketohexoses. Finally, pentoses give a purple colour after 2 minutes heating. As compared with the density values at 485 mμ for fructose after 2 minutes heating, the values, under the same conditions, for xylose are only 1/3, for rhamnose 1/5, for galactose 1/25, for mannose 1/18, and for glucose 1/60 as intense. On the other hand, xylose has a strong absorption at about 560 mμ after 2 minutes heating, compared to which fructose is 1/5, rhamnose 1/7, galactose 1/10, mannose 1/13, and glucose 1/50 as great. Unfortunately, the colours developed under these conditions are not very stable and their intensities vary

with small differences in the dimensions of the test tubes. The 2 minute extinctions are therefore difficult to use for quantitative determinations. If sufficient care is taken to avoid variation in size of tubes, etc., the use of the 2 minute or even the 1 minute extinctions may be very useful. Thus, by using this method with only 1 minute heating and reading the densities with different filters of the Pulfrich step photometer, Dr. Anders Westgren demonstrated that the reducing sugar in human semen consists chiefly of fructose *.

With this method, it is possible to characterise small amounts of carbohydrates such as are present in limited amounts of biological material. With only a few milligrams of pure carbohydrate, a complete set of colour curves for 2, 5, and 20 minutes heating can be obtained. In more complicated cases, it is advantageous to employ the principle of Dische [6] using different colorimetric reactions. In this way, various sugars exhibit greater dissimilarities than is possible to obtain with only one method. A detailed study of other colorimetric reactions would well compliment the usefulness of the method of Sørensen and Haugaard [1] and the modifications outlined in this paper.

SUMMARY

The colour reaction of carbohydrates with orcinol and sulphuric acid has been studied with the Warburg and Negelein and the Beckman spectrophotometers. The colour curves obtained after 2, 5, and 20 minutes heating are characteristic for aldohexoses, ketohexoses, pentoses, and methylpentoses. These differences permit a distinction of the kind of sugar present even in small samples of biological material. In most cases, a quantitative estimation of the carbohydrate is also possible with this method. The carbohydrates investigated were glucose, mannose, galactose, fructose, sorbose, xylose, arabinose, rhamnose, potassium gluconate, sodium glucuronate, mannitol, glucosamine, dextran, mannan, and yeast nucleic acid.

During the investigation I have received financial aid from the *Rockefeller Foundation* and from the *Hierta-Retzius stipendiefond.*

REFERENCES

1. Sørensen, M., and Haugaard, G. *Biochem. Z.* **260** (1933) 247; *Compt. rend. trav. lab. Carlsberg* **19** (1933) no. 12; *cf.* also Haugaard, G. *Kolorimetrische Zuckerbestimmungen* in Bamann, E., and Myrbäck, K. *Die Methoden der Fermentforschung* Leipzig (1941) p. 1080.

* This was demonstrated independently of the work by T. Mann, *Nature* **157** (1946) 79.

2. Warburg, O., and Negelein, E. *Biochem. Z.* **214** (1929) 64.
3. Rimington, C. *Biochem. J.* **34** (1940) 931.
4. Sørensen, M. *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **21** (1936) 123.
5. Heilmeyer, L. *Medizinische Spektrophotometrie* Jena (1933); Brode, W. R. *Chemical spectroscopy* New York (1939).
6. Dische, Z. *Mikrochemie* **7** (N. F. 1) (1929) 33; *cf.* also Ozaki, G. *J. Biochem. (Japan)* **24** (1936) 73.

Received September 18, 1948.

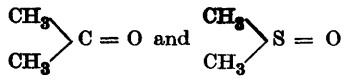
ACTA CHEMICA SCANDINAVICA 2 (1948) 702—706

A Comparison between the Structures of Acetone and Methyl Sulphoxide

O. BASTIANSEN and H. VIERVOLL

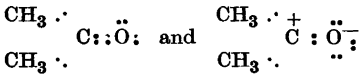
Universitetets Kjemiske Institutt, Blindern — Oslo, Norway

According to the classical theory of valency the two compounds acetone and methyl sulphoxide should have similar formulae



and a similar atomic arrangement might be expected in the two cases. The modern theory of valency, however, leads to a decisive difference between the two structures.

In order to explain the great dipole moment of the acetone molecule, Pauling has introduced the assumption of resonance between the two structures [1],



the contribution of each of them to the normal state of the molecule being about 50 %. Considering the general rules for estimating the relative stabilities of different structures which occur in a resonating molecule *, the 50 % contribution seems rather surprising. The first structure for acetone has the adventage of 1) having the octet arrangement for the central carbon atom maintained, 2) having the largest number of bonds, and 3) having no formal charges. For both these structures, however, a plane arrangement of the bonds originating from the central carbon atom must be expected. In both cases the electron configuration of the central carbon atom is assumed to contain one »promoted» electron, leading to an sp^2-configuration. In the first structure

---

See for instance: Wheland, G. W., *The theory of resonance* (1944) pp. 15—17.

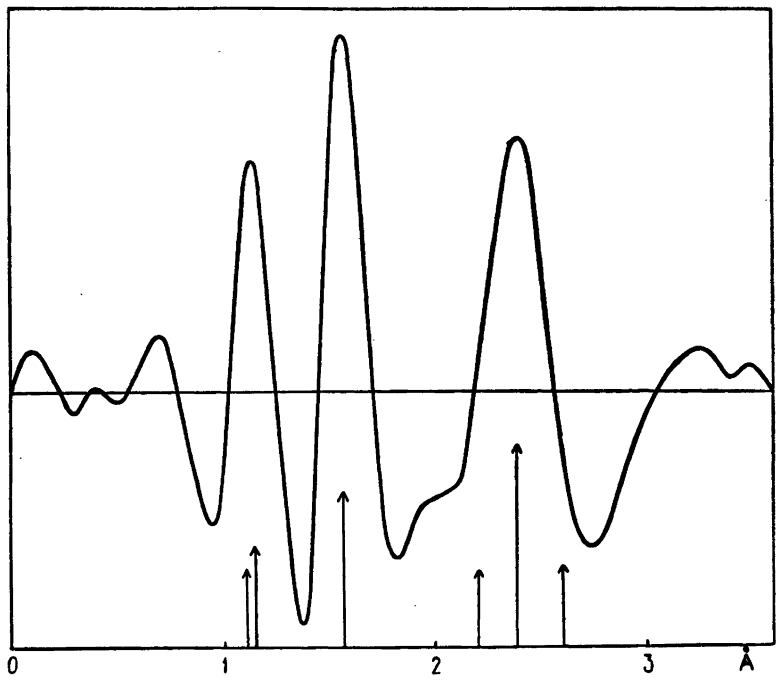
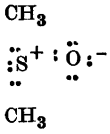


Fig. 1. Experimental $\frac{\sigma(r)}{r}$*-curve for acetone.*

the fourth electron of the central carbon atom is responsible for the additional π-bond, and in the second structure the fourth electron is given to the oxygen atom to complete its shell.

The most probable configuration for methyl sulphoxide by the light of modern theories is indicated by the formula:



The bond between the sulphur atom and the oxygen atom is a dative covalent bond, *i. e.* both the electrons forming the bond originate from one atom, here the sulphur atom. The sulphur atom has a lone pair, *i. e.* it has in its valency shell an electron pair which does not take part in the formation of any bond.

The electron configuration of the sulphur atom in its ground state, omitting the closed shells, is s^2p^4. Here no promotion of an s-electron to a p-orbital is

to be expected. The two s-electrons can therefore not take part in the formation of bonds, but will form the lone pair. — We can now imagine that the dative covalent sulphur-oxygen bond is formed in the following two steps: 1) The sulphur atom gives one of its electrons to the oxygen atom, resulting in a positive charge on the sulphur atom and a negative charge on the oxygen atom. 2) The formation of a usual two-electron bond takes place. From that point of view the electron configuration of the sulphur atom responsible for the bonds is p^3, *i. e.* the symmetry of the bonds should be trigonal pyramidal. Such a pyramidal structure has been observed in the thionyl halides [2].

To demonstrate the difference between the structures of acetone and methyl sulphoxide, these compounds have been studied by means of the electron diffraction sector method [3]. Fig. 1 gives the experimental $\frac{\sigma(r)}{r}$-curve for acetone. The line diagram indicates the position of the distances occuring in the acetone molecule. Because of the uncertainty in the position of the hydrogen atoms the O—H and the greatest C—H-distances are omitted. The contribution of these distances to the $\frac{\sigma(r)}{r}$-curve is, however, small compared with the contribution of the C—C and C—O distances. The experimental $\frac{\sigma(r)}{r}$-curve leads to a plane structure. The atomic distances and the CCO angle are given in Table 1.

Table 1. Distances and angle occuring in the acetone molecule.

C — H	1.09 Å	CCO	123° ± 3°
C — C	1.56 »		
C — O	1.14 »		

The value for the C—C distance is in good agreement with that calculated by the visual method [4].

In Fig. 2 the upper curve, Exp., — is the experimental curve for methyl sulphoxide. The curves A—F are theoretical curves for various structures of the molecule, the S—H-distance and the greatest C—H-distances having been omitted. The observed distances are listed in Table 2.

Table 2. Distances occuring in the methyl sulphoxide molecule.

C — H	1.08 Å
C — S	1.82 »
S — O	1.47 »

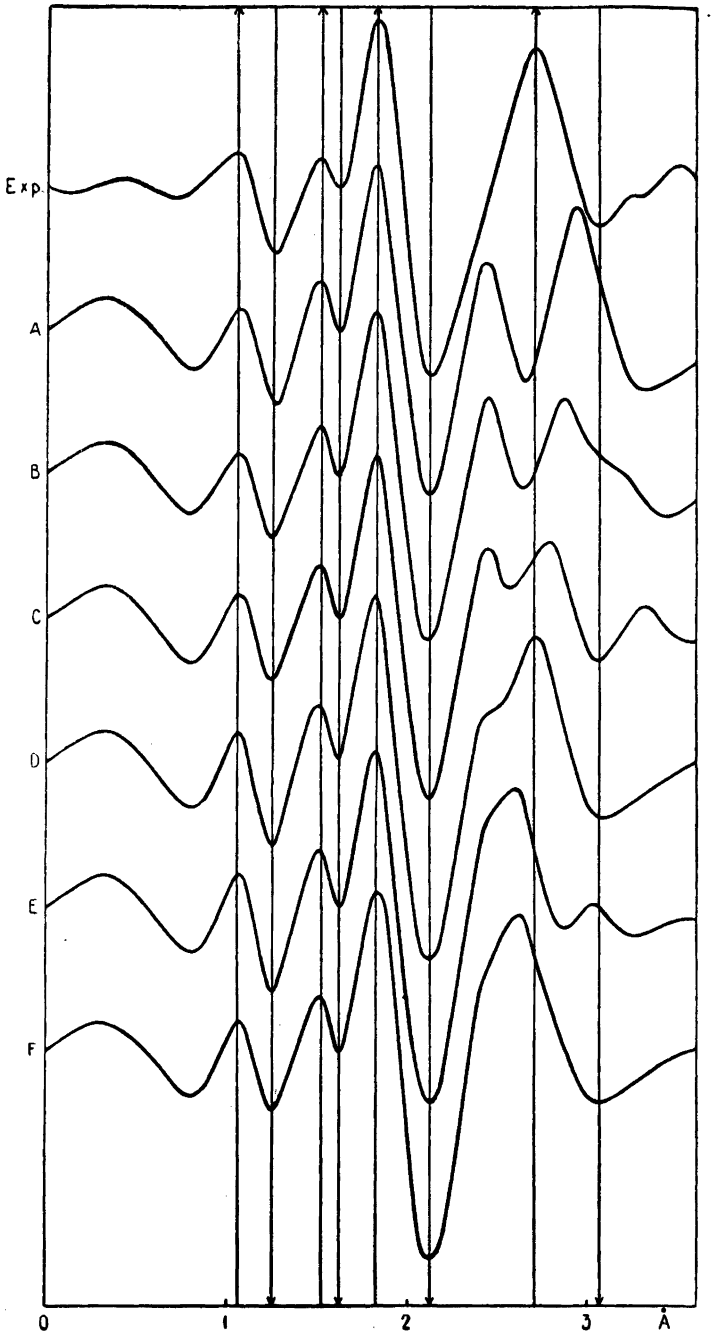


Fig. 2. The upper curve is the experimental $\frac{\sigma(r)}{r}$*-curve for methyl sulphoxide. The curves A—F are theoretical* $\frac{\sigma(r)}{r}$*-curves for methyl sulphoxide based on various values for the CSC- and CSO-angles.*

These distances are used in all the theoretical curves. The angles for the different models are listed in Table 3.

Table 3. CSC- and CSO-angles corresponding to the curves A—F in Fig. 2. The values in the last column correspond to the model of methyl sulphoxide which gives the best agreement with the experiments.

	A	B	C	D	E	F	
CSC	110°	120°	130°	100°	110°	100°	100° ± 5°
CSO	125°	120°	115°	110°	105°	105°	107° ± 5°

A comparison of the theoretical $\frac{\sigma(r)}{r}$-curves and the experimental one leads to exclusion of the plane structures A, B and C. The structures D and F seem to give the best agreement with the experimental $\frac{\sigma(r)}{r}$-curve. A more accurate determination of the angles is not possible in this case; the values tabulated in the last column of Table 3 will, however, within the limits indicated give the correct magnitude.

SUMMARY

A comparison of the structures of acetone and methyl sulphoxide has been carried out by the aid of the electron diffraction sector method. The investigation leads to a plane structure for the acetone molecule and a pyramidal structure for the methyl sulphoxide molecule.

We wish to express our gratitude to Prof. O. Hassel of the University of Oslo for his helpful advice and for having read the manuscript. We also wish to thank Prof. Bror Holmberg who has placed a sample of methyl sulphoxide at our disposal.

LITERATURE

1. Pauling, L. *The nature of the chemical bond* (1939) p. 75.
2. Palmer, K. J. *J. Am. Chem. Soc.* **60** (1938) 2360.
3. Viervoll, H. *Acta Chem. Scand.* **1** (1947) 120; Hassel, O., and Viervoll, H. *Acta Chem. Scand.* **1** (1947) 149.
4. Hengstenberg, J., and Bru, L. *Anales soc. espan. quim.* **30** (1932) 341.

Received June 25, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 707—712

A Method for Preparing Boron of High Purity

ROLAND KIESSLING

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

Commercial boron has a low degree of purity, varying between 65 and 80 % of boron [1]. It contains impurities of different kinds, for instance oxides of boron, aluminium, magnesium and iron, borides of aluminium and magnesium, and silicon compounds. Some of the impurities may be removed by treating the boron with acids and potassium hydroxide followed by heat treatment in vacuum, but the product still contains impurities amounting to 5—15 %.

Many methods have been suggested and used to obtain a pure product. These have been reviewed by Laubengayr and his associates [2]. Later Laubengayr *et al.* [3] prepared pure crystalline boron by reducing boron tribromide with hydrogen on a hot tungsten or tantalum filament. All the methods described, however, only give very small amounts of boron and in most instances a rather complicated apparatus is necessary. In connection with the studies on metal borides, carried out at this institute, a method for preparing boron of high purity has been worked out. It consists essentially of the vapor phase reduction of boron tribromide with hydrogen in a quartz tube at 750—800° C. The use of a quartz tube has the advantage of giving a much greater hot surface than a filament, and at the temperatures used quartz doesn't seem to be attacked at the reaction. The yield is 1—2 g of boron for each run, but the apparatus can be constructed for greater capacity.

APPARATUS

The apparatus is shown in Fig. 1. It consists of two reaction tubes (1 and 3) with condensation vessels (2 and 4). In the first tube boron tribromide is prepared by the action of bromine on commercial boron at 700° C. The reaction tube is composed of pure fused silica (translucent vitreosil) having a length of 700 mm and a diameter of 10 mm. The glass details are made of Pyrex, sealed to the tube by picein. All of these materials are resistant to the

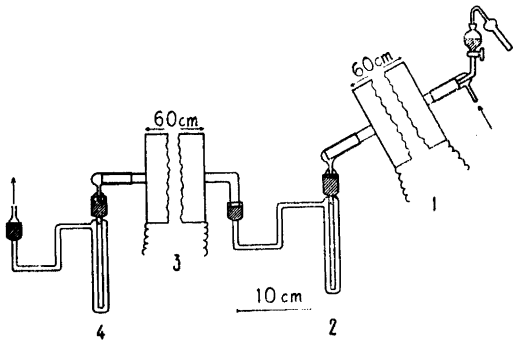


Fig. 1. Apparatus.

1. *Furnace with quartz tube for preparation of boron tribromide.*
2. *Condensation vessel.*
3. *Furnace with quartz tube for reduction of boron tribromide with hydrogen.*
4. *Condensation vessel.*

vapors of boron tribromide. Ground joints are avoided on the boron bromide side of the apparatus as most stopcock greases are attacked by the vapors, forming a mass which causes the joints to stick together. The connections between different parts of the apparatus are tightened by mercury in a manner, shown in Fig. 2. (Mercury is not attacked by boron tribromide, even at elevated temperatures [4].) The use of mercury tightenings has the further advantage over ground joints in that they give a less rigid apparatus.

In the second tube (3) (Fig. 1) boron tribromide vapors are reduced by hydrogen to give pure boron. The reduction is carried out in a quartz tube (translucent vitreosil) having a length of 600 mm and a diameter of 10 mm. One end is bent and connected with the condensation vessel (2). The other end of the tube is provided with a Pyrex socket, sealed to the tube by picein and connected to the condensation vessel (4) as shown in Fig. 2. The condensation vessels are made of Pyrex, each having a volume of about 25 ml.

PROCEDURE

As boron tribromide is decomposed even by slight traces of humidity, the apparatus must be carefully dried by a stream of hydrogen, purified by passage over copper at 500° C and dried over cooled silica gel and phosphorous pentoxide. Boron tribromide is prepared by the action of dry bromine on commercial boron * (containing about 75 % of boron) at 700° C, principally according to the method given by Meyer and Zappner [5]. Instead of filling the tube with powdered boron, however, the boron is first pressed to pastilles to avoid subsequent stoppage of the tube. The bromide, having a boiling point of about 90° C, is condensed in vessel (2), cooled by a salt-ice mixture.

* To remove some impurities (iron oxide, part of the boron nitride and so on) the commercial boron is previously treated with hydrochloric acid.

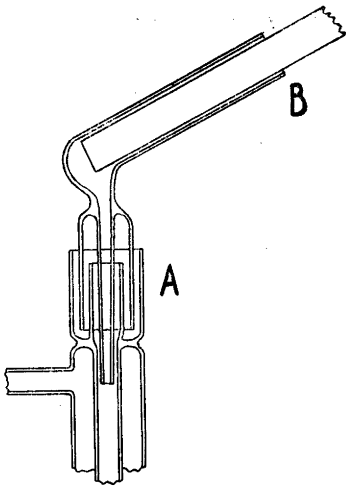


Fig. 2. Connections between different parts of apparatus.

The connections are tightened by means of mercury at A and by means of picein at B.

When 5—10 ml have been obtained, the bromine flow is interrupted and all bromine, remaining in the tube, is carefully driven out by hydrogen. With careful work, the preparation of the bromide takes about half an hour, giving a colourless liquid without any trace of bromine. Then the temperature of vessel (2) is raised to room temperature and the stream of hydrogen regulated to 2—4 bubbles per second. The hydrogen picks up the vapors of the bromide and the mixture is allowed to pass through the second quartz tube (3), where the reduction is carried out at 800° C. The elementary boron is deposited on the wall. At 800° C about half the initial amount of boron bromide has not taken part in the reaction and is condensed in vessel (4). The vessels are then changed and the procedure continued. When the boron bromide has been consumed, a new amount of bromide is prepared and the procedure repeated as above. The amount of commercial boron in tube (1), about 15 g, yields about 75 ml of the bromide. It is more convenient to work with 5—10 ml portions as described rather than preparing the whole amount of bromide at once because of the difficulty in storing the bromide for any longer time. If so stored, part of it often solidifies to a glassy colourless solid. This is probably caused by traces of humidity.

The deposited boron seems to catalyze the reduction. This is shown by Table 1. It thus is advantageous not to remove the boron from quartz tube (3) too early.

Reaction temperatures between 600° and 800° C have been tried, the upper limit depending on the permeability of the quartz tube for hydrogen at higher temperatures. The yield at different temperatures is given in Table 2. It thus seems to be of advantage to work at as high a temperature as possible.

Table 1. Yield of boron, using different amounts of boron tribromide each time starting with a clean reaction tube.

Temperature 800° C, reaction time about 1 hour/ml bromide.

Amount of BBr_3 ml	Yield g	Efficiency %
5	0.220	39
10	0.550	48
14	1.250	78

For best performance of an apparatus, working continuously and with as small losses of bromide as possible, it would no doubt be necessary to have the hydrogen input in vessel (2). The mixture then should pass a quartz tube, the dimensions of the tube and the composition of the mixture having previously been chosen to give as complete a reaction as possible. The construction of such an apparatus, however, would necessitate a further study of the reaction constants, an investigation which lies beyond the purpose of this work.

PURITY AND PROPERTIES OF BORON

The product obtained is washed with hot water to remove traces of boron tribromide and dried. A yellish-brown powder is obtained, which reacts vigorously and in an inflammatory way with concentrated nitric acid. To determine the boron content, samples of 50—100 mg were completely oxidized by nitric acid 1 : 1, and boric acid titrated in the precence of mannitol in the usual way. Values between 98.0 and 100.0 % of boron have been obtained with a mean value of 98.9 %. The possible impurities are silicon, oxygen and hydrogen and those metals, present in commercial boron which form easily vaporized bromides, especially aluminium. To determine the content of silicon, aluminium and magnesium, about 400 mg were brought into solution and evaporated in the precence of sulfuric acid. Boric acid was evaporated with methyl alcohol and the silicon content of the insoluble residue determined by the usual method. The value was compared with results, obtained from colorimetric determinations of soluble silicon.[6] In the filtrate aluminium was precipitated as 8-hydroxyquinolate in slightly acid solution and determined by titration with bromate. Magnesium, if present, should form a precipitate with the same reagent in the filtrate from the aluminium determination if the solution was made slightly alkaline. No precipitate, however, was formed.

Table 2. Yield of boron at different temperatures.

Amount of bromide, used at each preparation 5 ml, reaction time about 1 hour/ml bromide.

Temperature ° C	Yield g	Efficiency %
600	0.003	0.5
650	0.079	14
700	0.110	19
750	0.165	29
800	0.220	39

The results showed, that most of the impurities were concentrated in the first 5 ml of boron tribromide formed by each successive filling of commercial boron in tube (1). If this portion was neglected, a mean value of 0.4 % aluminium and 0.1 % silicon was obtained. The silicon, present in the boron, does not seem to originate from the quartz tube, which after a run of about 150 hours was apparently unattacked. It seems to be generated by the reduction of silicontetrabromide present as an impurity of boron tribromide. A further purification was obtained by distilling the tribromide once in slightly modified vessels. For the purpose of this investigation, however, the purity of the boron was considered to be high enough and for this reason the tribromide was used undistilled.

Oxygen, if present, must form oxides of boron, silicon or aluminium. To determine the content of these oxides, samples of about 100 mg were heated in a stream of dry chlorine [1]. In no case was any weighable residue left. To determine the content of hydrogen, samples of 25—50 mg were burnt in oxygen and the water thus formed absorbed by magnesiumperchlorate (anhydron) and weighed. No weighable amounts were obtained.

The mean values of the analyses of the final product are

Boron	98.9 weight %
Aluminium	0.4 » »
Silicon	0.1 » »
Magnesium	less than 0.1 weight %
Oxygen	» » 0.1 » »
Hydrogen	» » 0.1 » »

SUMMARY

A method for preparing boron of high purity by reduction of boron tribromide vapor with hydrogen in a quartz tube is described.

A description of the apparatus is given as well as the yield obtained at different temperatures and for different reaction times.

At 800° C an amount of 1—2 g of boron may be obtained after about 14 hours reaction time and with an efficiency of about 80 % according to the amount of boron tribromide used.

The product has a mean purity of 98.9 %, containing 0.4 % aluminium and 0.1 % silicon as impurities.

Grateful acknowledgement is made for a grant from *Statens Tekniska Forskningsråd,* which has supported this investigation. Also, I wish to thank Professor G. Hägg and Dr. F. Nydahl for valuable advice and continued interest throughout the course of the work.

REFERENCES

1. Winslow, E. H., and Liebhafsky, H. A. *J. Am. Chem. Soc.* **64** (1942) 2725.
2. Laubengayer, A. W., Newkirk, A. E., and Brandaur, R. L. *J. Chem. Education* **19** (1942) 382.
3. Laubengayer, A. W., Hurd, D. T., Newkirk, A. E., and Hoard, J. L. *J. Am. Chem. Soc.* **65** (1943) 1924.
4. Stock, A., and Kuss, E. *Ber.* **47** (1914) 3113.
5. Meyer, F., and Zappner, R. *Ber.* **54** (1921) 550.
6. *Standard methods of water analysis* 9th ed. (1946) p. 44.

Received October 26, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 713—726

The Potentiometric Titration of Fatty Acids with Silver Nitrate

OLAVI HARVA and PER EKWALL

Institute of Physical Chemistry, Åbo Akademi, Åbo, Finland

Ekwall and Juup[1] found that the potentiometric determination of the higher fatty acids can be achieved by precipitation of their sodium salts with silver nitrate in aqueous solution. In this manner the individual acids could be estimated quantitatively, the titration error not exceeding — 5 %. These authors calculated the solubility products of the silver salts of different acids from the inflexion points of the titration curves and obtained the following values, which refer to 70° C.

Silver laurate	L Ag L	$= 2.7 \times 10^{-6}$
Silver myristate	L Ag My	$= 4.0 \times 10^{-9}$
Silver palmitate	L Ag Pl	$= 5.3 \times 10^{-10}$
Silver stearate	L Ag St	$= 6.9 \times 10^{-11}$
Silver oleate	L Ag Ol	$= 6.0 \times 10^{-10}$

They also succeeded in the quantitative estimation of fatty acids in a number of binary mixtures. The titration was more difficult when the chains of the saturated fatty acids differed in length by only two CH_2-groups.

The method has been subjected to further study and investigations have been continued in various directions. For example, P. Ekwall, Kjell Andersson-Groth and Margaretha Witting (at the Institute of Physical Chemistry of the University of Uppsala) investigated the method in the case of certain saturated fatty acids and paid particular attention to the titration of binary mixtures. Harva and Ekwall reinvestigated the titration of single acids, extended the study to include capric and lower fatty acids and investigated the possibility of estimating quantitatively the acids in different types of mixtures. The titration of salts and mixtures of salts of the rosin acids has also been studied by Ekwall and Ralph Andresen.

The following is a summary of the results obtained.

METHODS

The fatty acids were determined as their sodium and potassium salts in aqueous solution. In most experiments a known weight, between 50—120 mg of acid or acid mixture, was dissolved in absolute alcohol and neutralized by an equivalent amount or a slight excess of sodium or potassium hydroxide. After the neutralization, the alcohol was expelled and the salt dissolved in water.

In some experiments the fatty acids were neutralized in aqueous solution with potas sium hydroxide, and in others a known amount of the sodium salt of the fatty acid was dissolved in water. All solutions contained a small excess of alkali to prevent hydrolysis.

The electrode used was a silver wire which had been silvered according to the method of Brown [2]. As reference electrode either a saturated or a 1 *M* calomel electrode was used; this was connected to the titration solution through an agar-agar bridge saturated with potassium nitrate. Titrations were performed with 0.05 *N* or 0.1 *N* silver nitrate. The electromotive force (E. M. F.) of the cell was determined with a valve potentiometer. All the E. M. F. values given below refer to a 1 *M* calomel electrode ($E_{cal} = 0.283$ V). The adherence of the silver soap to the electrode, which resulted in erratic E. M. F. values, was avoided by efficient stirring and by allowing the mixer blade to brush the electrode.

THE TITRATION OF THE INDIVIDUAL FATTY ACIDS

When the sodium salts of the fatty acids were titrated individually at 70° C, the solubility products of silver stearate, palmitate, myristate and laurate calculated from the inflexion points were found to conform with the values given by Ekwall and Juup [1]. The results obtained with sodium stearate, palmitate and laurate were found to be too small by 3—4 %, 2—3 %, and, 0.5—1.5 %, respectively. At 20° C the titration curve of sodium laurate was steeper than at 70° C and hence the accuracy was higher. The calculated solubility product of silver laurate at 20° C was found to be

$$\text{L Ag L} = 5 \times 10^{-10} \text{ (E. M. F. = 0.257 V)}$$

This value is slightly greater than the value, 3×10^{-10} reported by Ekwall and Juup [1] but lies in the interval $3—7 \times 10^{-10}$ obtained by Ekwall and Harva [3] with the silver/silver laurate electrode.

The titration curves of sodium caprate (NaCi), sodium caprylate (NaCy) and sodium caproate (NaCo) obtained at 20° C are shown in Fig. 1. In the titration of the caprate a definite jump was observed in the titration curve but the solubility of silver caprylate and silver caproate was so high that a precipitation titration of these acids was not possible. The calculated solubility product of silver caprate at 20° C was found to be

$$\text{L Ag Ci} = 1.9 \times 10^{-8} \text{ (E. M. F. = 0.303 V)}$$

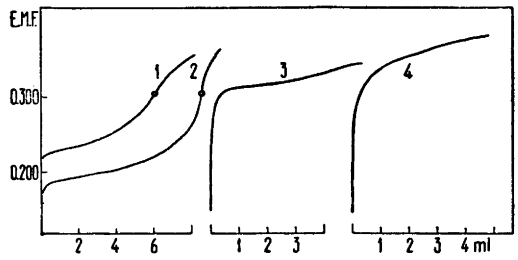


Fig. 1. 1. *Titration of 0.0118 N NaCi with 0.1 N $AgNO_3$ at 70° C. Error: + 1.4 %.*
2. *Titration of 0.01702 N NaCi with 0.1 N $AgNO_3$ at 20° C. Error: — 0.4 %.*
3. *Titration of 0.00419 N NaCy with 0.1 N $AgNO_3$ at 20° C.*
4. *Titration of 0.00405 N NaCo with 0.1 N $AgNO_3$ at 20° C.*

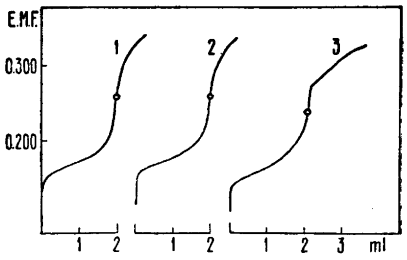


Fig. 2. 1. *Titration of 0.00204 N NaL with 0.1 N $AgNO_3$ at 20° C. Error: — 0.5 %.*
2. *Titration of 0.00205 N NaL with 0.1 N $AgNO_3$ at 20° C; the solution contained 23.3 mol-% NaOH. Error: — 0.9 %.*
3. *Titration of 0.00207 N NaL with 0.1 N $AgNO_3$ at 20° C; the solution contained 72 mol-% NaOH. Error: — 0.8 %.*

The titration error with sodium caprate varied in different experiments between — 1.5 % and + 2.5 % at 20° C.

At 70° C the solubility of silver caprate was so high and the variation of the curve was so slight that the point of inflexion was difficult to determine. The calculated value of the solubility product of silver caprate at 70° C was

$$\text{L Ag Ci} = 2.7 \times 10^{-7} \text{ (E. M. F. = 0.304 V)}$$

The effect of excess of alkali on the titration curves may be seen in the titration of mixtures of sodium laurate and sodium hydroxide (Fig. 2). When the solution contains 20 mol-% sodium hydroxide the titration curve is still quite regular, but when the alkali concentration exceeds 40 mol-% precipitation of silver hydroxide occurs with an accompanying change in the colour of silver laurate from white to grayish brown; at the same time the titration curve is deflected at or above the inflexion point of sodium laurate. Hence an excess of alkali from 10—20 mol-% (pH $<$ 10.8) may be used in the titration without any alteration in the form of the titration curve. Even the theoretical error in the titration of sodium laurate does not exceed — 0.8 % in this case.

If the solution contains sodium chloride, silver chloride precipitates with the silver salts of palmitic, myristic and lauric acids and the amounts of the component acids in the mixture cannot be estimated from the titration curve (Fig. 3, curve 1). In titrating a mixture of sodium chloride and sodium stearate at 70° C a jump corresponding to silver stearate is observed but the titration error is large (Fig. 3, curve 2) owing to the simultaneous precipitation of silver chloride and silver stearate; theoretically the coprecipitation should not occur. Both components of a mixture containing sodium caprate and sodium chloride can, however, be estimated at 20° C (Fig. 3, curve 3).

An attempt was made to reduce the titration error which possibly arises from the coprecipitation of the soap with the silver salt by using various emulsifiers to effect the suspension of the silver soap. Tylose, mersolate and starch were found to be of no use in this respect.

The effect of added electrolytes in the titration was also studied. Theoretically the titration curve should run higher in the solution containing added electrolytes than in a solution containing no added electrolytes. The addition of electrolyte effects in part an increase in the solubility of the silver soap and in part an increase in the degree of association of the soap; the latter causes a decrease in the fatty acid anion concentration. Both of these effects increase the activity of the silver ion. When the soap has been precipitated the electrolyte effects a decrease in the activity coefficient of the silver ions in excess, and, hence, the titration curve of the solution containing added electrolyte is lower than that of the solution containing no additional electrolyte. In Fig. 4 the extent of the effect of added electrolyte in the titration of potassium stearate is shown. As the concentration of the electrolyte is increased the jump in the titration curve becomes smaller and the curve is flatter. The titration result is not affected by the presence of added electrolyte, although the determination of the end point of the titration becomes less accurate.

THE TITRATION OF BINARY FATTY ACID MIXTURES

As Ekwall and Juup [1] have shown the different values of the solubility products of the silver soaps make it possible to separate quantitatively fatty acids in binary mixtures when these acids differ in structure by more than two CH_2-groups but difficulties are encountered when two successive fatty acids having even numbers of carbon atoms are present.

Theoretically the titration result varies with the relative solubility products of the components, their relative proportions in the mixture, and the total concentration of the solution. When the concentration c_2 of the soap of the lower fatty acid is approximately

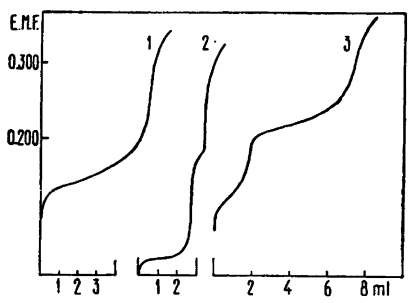


Fig. 3. 1. *Titration of 0.0066 N NaL + 0.002766 N NaCl with 0.1 N Ag NO_3 at 43° C. Total titration error: — 0.6 %.*
2. *Titration of 0.00415 N NaSt + 0.002766 N NaCl with 0.1 N $AgNO_3$ at 70° C. Total titration error: + 3.9 %.*
3. *Titration of 0.01081 N NaCi + 0.003897 N NaCl with 0.1 N $AgNO_3$ at 21° C. Total titration error: — 0.65 %.*

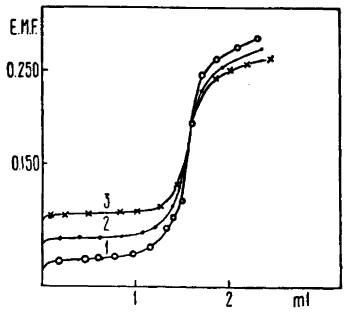


Fig. 4. Titration of 0.00159 N KSt with 0.05 N $AgNO_3$ at 65° C:
1. *Without KNO_3;*
2. *Solution 0.02 N in KNO_3;*
3. *Solution 0.2 N in KNO_3. Error: —0.8 %.*

P. Ekwall, K. Andersson-Groth, and Margaretha Witting.

$$c_0 = \frac{P_2}{\sqrt{P_1}} \tag{1}$$

where P_1 and P_2 are the activity products of the silver soaps of higher and lower molecular weight, respectively, the silver salt of the lower acid begins to precipitate at the equivalence point of the higher acid. When the concentration c_2 is less than c_0, its silver salt begins to precipitate only after the equivalence point of the higher acid has been passed and when $c_2 > c_0$ this precipitation occurs before the equivalence point of the higher salt is reached. In these cases the magnitude of the error which arises in the titration of the higher salt also depends on the proportion of the components in the mixture. The magnitude of the error in mol-% ($\triangle$) can be calculated theoretically by using, for example, the equation:

$$\triangle = \frac{P_1 c_2^2 f_2^2 - P_2^2}{P_2 f_2 c_2 c_1} \cdot 100 \tag{2}$$

where

$\triangle$, the mol-% of the original amount of the higher soap which is still in solution when the silver salt of the lower soap begins to precipitate;

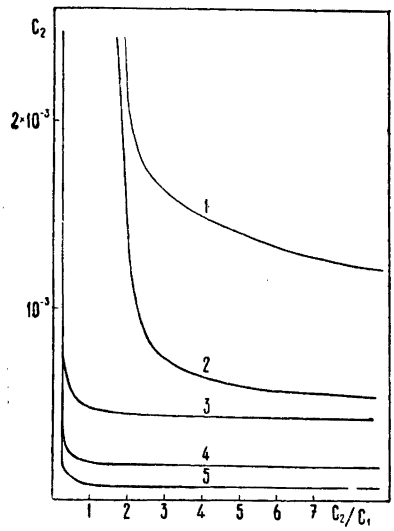


Fig. 5. Allowed concentration ranges at 70° C in the titration of:
1. NaPl + NaL;
2. NaSt + NaMy;
3. NaMy + NaL;
4. NaPl + NaMy;
5. NaSt + NaPl.

P_1 and P_2 are the activity products of the higher and lower soap, respectively; c_1 and c_2 their respective concentrations;
f_2, the activity coefficient referring to the concentration c_2 of the lower soap; the activity coefficient of the higher salt is assumed to be approximately unity.

When the value of $\triangle$ calculated from equation (2) is negative, this means that the silver salt of the lower fatty acid begins to precipitate when the equivalence point of the higher acid has been passed.

From the equation given above it may be seen that only when the concentration of the lower soap is greater than the c_0-value given in Table 1 an error is to be expected in the estimation of the component of higher molecular weight. When the molar ratio of the components is unity the maximum value which this error may attain is that given by the expression P_1/P_2. This error arises when $c_2 \geqq 10 \cdot c_0$. It may be seen from Table 1 that the greatest error occurs when a mixture of sodium palmitate and laurate is titrated, in which case the maximum error is — 1.96 mol-%. When the concentration of the lower component increases relative to that of the higher component, and its value is at least ten times c_0, the theoretical titration error also increases in the same proportion.

Table 1. Maximum titration errors under certain conditions.

Mixture	c_0	P_1/P_2
NaSt — NaMy	4.8×10^{-4}	1.72×10^{-2}
NaSt — NaL	3.25×10^{-3}	2.55×10^{-3}
NaPl — NaL	1.2×10^{-3}	1.96×10^{-2}

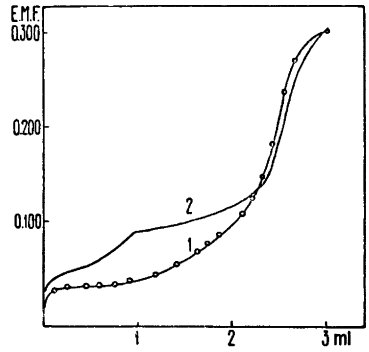


Fig. 6. 1. Titration of 0.0011 N NaSt + 0.00145 N NaPl with 0.1 N $AgNO_3$ at 70° C. Total titration error — 2.4 %.
2. Theoretical curve for the same mixture.

Fig. 5 indicates in what ranges of the concentration of the lower fatty acid (c_2) and of the concentration ratio (c_2/c_1) of the two components the titration can be conducted at 70° C without incurring a theoretical error exceeding — 3 % in the value of the higher fatty acid. The allowed concentration ranges lie to the left of and below the curve referring to the acid pair in question. The conditions in our experiments have been such that the concentrations and the concentration ratios were in these areas and hence it was found possible to estimate both components in the binary mixtures stearate-myristate, stearate-laurate, and palmitate-myristate, the titration error being between the limits — 3 % and + 1 %. For example, this was the case in the series of titrations of palmitate-laurate mixtures in which the concentration ratio, c_2/c_1, varied from 0.4 to 8, and in a series of titrations of stearate-myristate mixtures in which the ratio varied from 0.14 to 7.

If the titration is continued at a lower temperature after the higher soap has been precipitated at a higher temperature the equivalence point of the lower soap can be determined more accurately since the slope of the curve becomes steeper when the solubility of the silver salt of the lower acid decreases (Fig. 7, curve 1).

The theoretical possibility of titrating mixtures containing successive fatty acid salts having even numbers of carbon atoms in the chain is shown by the data given in Table 2.

Table 2. Maximum titration errors under certain conditions.

Mixture	c_0	P_1/P_2	*Temp.*
NaSt — NaPl	6.4×10^{-5}	1.3×10^{-1}	70° C
NaPL — NaMy	1.7×10^{-4}	1.4×10^{-1}	70° C
NaMy — NaL	4.3×10^{-4}	1.5×10^{-1}	70° C
NaL — NaCi	8.5×10^{-4}	2.6×10^{-2}	20° C

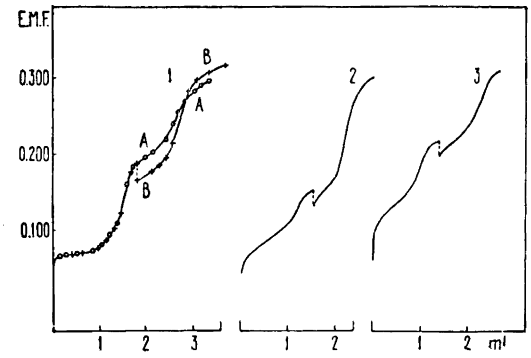


Fig. 7. *1. Titration of 0.000795 N KSt + 0.000555 N KMy with 0.05 N $AgNO_3$:*
A) at 65° C. Error in KSt — 2.5 % (theoretical — 0.28 %) and in KMy — 3.6 %.
B) KSt at 65° C and KMy at 45° C. Error in KSt — 2.5 % and in KMy — 0.3 %.
2. Titration of 0.00064 N KPl + 0.000555 N KMy with 0.05 N $AgNO_3$ at 65 and 45° C. Error in KPl — 4 % (theoretical — 10 %) and KMy — 6 %.
3. Titration of 0.000555 N KMy + 0.00062 N KL with 0.05 N $AgNO_3$ at 65° C. Error in KMy — 10 % (theoretical — 8.6 %) and in KL + 2.5 %.
P. Ekwall, K. Andersson-Groth and Margaretha Witting.

A titration error occurs even when the concentration of the lower soap is as small as 6.0×10^{-5}—10^{-4} *M*. When the molar ratio of the components is unity, a minimum error ranging from — 10 to — 15 mol-% may be expected in the titration of the higher fatty acid salt. If the theoretical titration error is not to exceed — 3 %, it is necessary, as shown by Figure 5 (curves 3, 4, 5), to conduct the titration in very dilute solution. It may be expected that the estimation of both components in these mixtures is then very difficult and conducted experiments confirm this. When a mixture of sodium stearate and sodium palmitate was titrated no inflexion point corresponding to the stearate was noted, and, hence, the stearate could not be estimated (Fig. 6, curve 1). The calculated titration curve (Fig. 6, curve 2) indicates, however, that it should be possible to separate the stearate and the palmitate. If activities were employed in the calculations instead of concentrations (as we have done above) the jump due to the precipitation of the stearate should be more distinct, as Ekwall and Juup [1] have been able to show. The reason for the discrepancy between theory and practice has not been found. It may be that the concentration of the solution under titration exceeded the critical concentration of the soap mixture for micelle formation and hence the formation

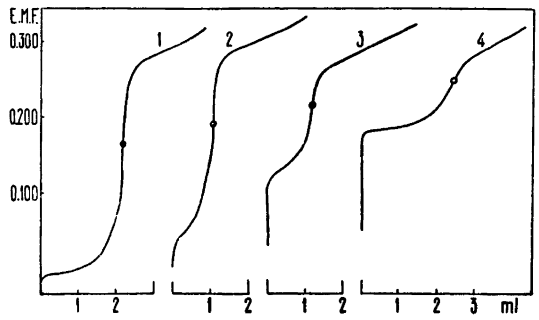


Fig. 8. 1. *Titration of 0.002288 N NaSt + 0.001488 N NaCi with 0.1 N $AgNO_3$ at 60° C. Error in NaSt — 2.6 %.*
2. *Titration of 0.001093 N NaPl + 0.001204 N NaCi with 0.1 N $AgNO_3$ at 60° C. Error in NaPl + 0.36 %.*
3. *Titration of 0.001153 N NaMy + 0:002105 N NaCi with 0.1 N $AgNO_3$ at 60° C. Error in NaMy + 0.8 %.*
4. *Titration of 0.002395 N NaL + 0.001157 N NaCi with 0.1 N $AgNO_3$ at 60° C. Error in NaL + 1.5 %.*

of mixed stearate-palmitate micelles led to a coprecipitation of both the component fatty acids.

On the other hand, the separation of the components of mixtures of other successive even saturated fatty acids by titration at 70° C was feasible. The precipitation of the silver salt of the lower soap before the equivalence point of the higher soap was reached had in this case a considerable effect on the titration. The titration error is thus great.

In such cases the concentration of the titration at a lower temperature after the higher component has been titrated offers considerable advantages; this procedure was therefore followed in most of the titrations. Using this procedure it was found possible to estimate both components in palmitate-myristate and myristate-laurate mixtures. The concentrations were as a rule so high that the theoretical titration error considerably exceeded — 3 %; the experimentally found titration error was of the same magnitude (Fig. 7, curves 2, 3). It should be noted that in these cases the total concentration of the solution under titration was so low that it did not exceed the critical concentration of the mixture for micelle formation, and, hence, mixed micelles were not formed.

In the case of equimolar binary mixtures of laurate and caprate at 20° C, $c_0 = 8.5 \times 10^{-4}$ and $P_1/P_2 = 2.63 \times 10^{-2}$. It should thus be possible to estimate both acids separately; this was confirmed by experiments.

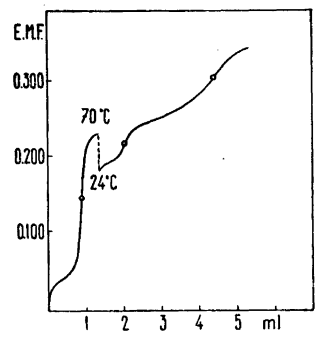


Fig. 9. Titration of mixture containing 0.000975 N NaSt + 0.00105 N NaL + 0.00240 N NaCi with 0.1 $AgNO_3$ at 70° C and after precipitation of NaSt at 24° C. Error in NaSt — 5 %, in NaL + 2.4 % and in NaCi + 2.2 %.

When the mixture 0.00128 *N* in NaL and 0.00138 *N* in NaCi was titrated the error in NaL was + 0.8 % and in NaCi + 0.2 %. According to theory the precipitation of silver caprate should occur 0.5 mol-% before the equivalence point of sodium laurate.

When titrating binary mixtures containing sodium caprate as one component and sodium stearate, palmitate, myristate and laurate as the other component at 60° C the solubility of silver caprate was so great that the titration curve continued almost linearly when the other component had been precipitated, and hence caprate could not be estimated (Fig. 8). However, the other component could be determined quantitatively, the titration error varying from — 2 % to — 3 % with sodium stearate and amounting to — 1 % with the other salts.

The same behaviour was noted with binary mixtures containing sodium caprylate and their titration could be conducted at 20° C without any inflexion point due to the precipitation of silver caprylate being observed.

THE TITRATION OF TERNARY AND POLYCOMPONENT MIXTURES

All fatty acids in ternary mixtures could be estimated separately when two successive fatty acids of the even series C_{14}—C_{18} were not present (Fig. 9). The titration error of each acid was actually larger than in the case of binary mixtures, but in no case greater than 5 %. In order to increase the accuracy of the determination of the inflexion point with the lower fatty acid salts the latter part of the titration was conducted between 20—25° C, whereas the higher salts were titrated at 60—70° C.

When the acid mixture contained several acids varying in structure by less than two CH_2-groups the individual fatty acids could not be separated as may be seen from Fig. 10. The end point of the titration was difficult to determine

since no distinct inflexion point was evident in the titration curve. If the end point of the titration was taken to be the volume in ml of silver nitrate corresponding to the E. M. F. value of silver laurate at 60° C, the error in the total acid was less than — 2 %.

The fatty acid mixture for which the titration curve is curve 1 of Fig. 10 had an acid value (AV) of 220.2 mg KOH/1 g. 69.92 mg of the mixture consumed 2.75 ml of 0.1 N silver nitrate (to the E. M. F. value 0.268 V), which corresponds to a calculated acid value (AV_p) of 218.8 mg KOH/1 g, the titration error being — 0.2 % when the results are expressed in these units.

The precipitation of the silver salts of capric and other acids of lower molecular weight was not evident in the titration curves obtained at 60° C due to the high solubility of the silver salts of these acids. This made it possible to estimate the total amount of the higher acids in mixtures containing capric and lower acids. (Fig. 10, curves 2 and 3).

Table 3. Titration of some soap mixtures.

	Solution titrated (normality)	Total acid value (AV) of fatty acid mixtures	Calculated acid value (AV_c) of acids higher than C_{10} in the mixture	AV_p obtained potentiometrically	Error
No. 1.	NaPl : 0.00104 NaMy : 0.000816 NaL : 0.000925 NaCi : 0.000669	256.8	207.0	204.5	— 1.2 %
No. 2.	NaSt : 0.0003231 NaPl : 0.0003435 NaMy : 0.0003945 NaL : 0.0007864 NaCi : 0.0005094 NaCy : 0.000765	279.9	165.6	158.7	— 4.2 %
No. 3.	NaSt : 0.000726 NaPl : 0.000592 NaOl : 0.0004448 NaMy : 0.0003596 NaL : 0.000324 NaCi : 0.0007328 NaCy : 0.0004832	247.1	165.0	165.3	+ 0.18 %

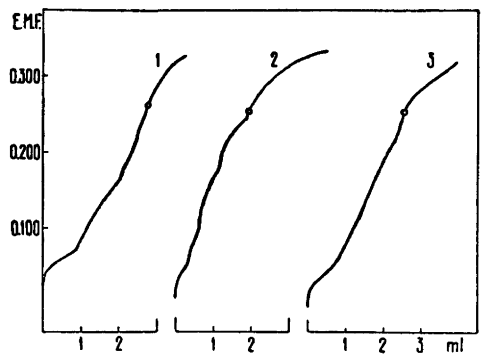


Fig. 10. 1. Titration of mixture containing 0.0003912 N NaSt + 0.000777 N NaPL + 0.0007332 N NaOl + 0.0004418 N NaMy + 0.000404 N NaL with 0.1 N $AgNO_3$ at 60° C.
2. Titration of mixture No. 2 of Table 3 with 0.1 N $AgNO_3$ at 60° C.
3. Titration of mixture No. 3 of Table 3 with 0.1 N $AgNO_3$ at 60° C.

If we take for the end point of the titration at 60°C the volume of silver nitrate corresponding to the E.M.F. 0.253 V (the E.M.F. corresponding to the inflexion point of the titration curve of NaL when capric and lower acids were present) the acid value AV_p calculated from this silver nitrate consumption corresponds to the acid value of the higher acids beginning from C_{12}, as may be seen from Table 3. The difference between the total acid value and AV_p is a measure of the fatty acid C_{10} and lower and can be rapidly determined by this procedure. For example, the fatty acids in cocoanut oil, the total acid value AV of which was 274 mg KOH/1 g, gave on potentiometric titration an acid value AV_p 217.6 mg KOH/1 g. The literature [4] gives for the corresponding average values of cocoanut oil AV 272 mg KOH/1 g and AV_p 218.5 mg KOH/1 g.

THE TITRATION OF ROSIN ACID AND ITS MIXTURES WITH FATTY ACIDS

Preliminary titration of rosin soaps with silver nitrate was also conducted at temperatures from 20 to 70° C. The titration curves of sodium abietate and talloil rosin soap were similar to those obtained with sodium laurate. The solubility product of the silver salt of rosin acid at 70° C calculated from the inflexion point of the titration curve was

$$\text{L Ag Ro} = 5\text{—}9 \times 10^{-8}$$

The titration result is, however, unsatisfactory, since the error is — 30 to — 36 mol-%. A part of the rosin acids evidently are not precipitated, possibly the oxidized rosin acids present in the rosin soap. Although talloil rosin is a mixture of rosin acids, its titration curve resembles that of sodium abietate. Evidently the solubilities of the silver salts of the various rosin acids are of the same magnitude.

When sodium abietate is titrated in binary mixtures containing sodium palmitate, myristate, laurate or oleate, each component causes a jump in the titration curve, but the titration error is greater. A similar behaviour was observed in titrating mixtures of sodium palmitate, oleate and abietate and of sodium myristate, oleate and abietate.

The curves obtained in the titration of the sodium salt of talloil containing 10 % rosin acids revealed jumps due to rosin acid and the various fatty acids and it seems possible that the method might be improved to allow an approximate determination of the rosin acids in talloil.

SUMMARY

1. The solubility products of the silver salts of the higher fatty acids (C_{18}—C_{12}) in aqueous solution were redetermined from the inflexion points of the titration curves of the acids obtained by titration with silver nitrate, and the values of the solubility product of silver caprate at 20° C and 70° C were established. The solubility of silver caprylate and silver caproate was so high between 20° and 60° C that the precipitation titration could not be used to evaluate the solubility of these silver salts. A preliminary value of the solubility product of the silver salt of rosin acids (abietic acid and talloil rosin acid) was obtained.

2. The saturated fatty acids (C_{18}—C_{10}) and oleic acid can be individually estimated quantitatively by potentiometric precipitation titration with silver nitrate, the titration error not exceeding — 4 %.

3. Both components in binary mixtures of saturated fatty acids containing an even number of carbon atoms can be quantitatively estimated by potentiometric precipitation titration with silver nitrate with an error not exceeding — 4 % if the chains of the two fatty acids differ by more than two CH_2-groups. Both laurate and caprate could also be estimated quantitatively together in their binary mixtures (error ± 1 %). The estimation of both components in binary mixtures of myristate-laurate and palmitate-myristate mixtures is also possible, but in these cases the titration error is rather great (— 10 %). Stearate and palmitate could not be estimated separately in the same solution.

4. The fatty acids in a ternary mixture can be determined separately if the mixture does not contain successive even fatty acids between C_{14} and C_{18}. When the number of components in the mixture is increased it is no longer possible to obtain a separation of the acids but their total amount can be evaluated with an accuracy of 2 % when the E. M. F. of the inflexion point of (sodium) laurate at 60° C is taken for the end point of the titration. (E. M. F. = 0.268 V with reference to the calomel electrode + 0.283 V.)

5. Owing to the relatively high solubility of the silver salts of capric and lower acids at higher temperatures the presence of these acids in the fatty acid mixtures has very little effect on the titration curve obtained in the titration with silver nitrate at 60° C. The total amount of C_{12} and higher acids can in this case be determined by titration to the E. M. F. value of the inflexion point of laurate (E. M. F. = 0.253 V). When the results are expressed in terms of the acid value (AV) the maximum error found was — 5 %. The difference between the total acid value of the acid mixture and the potentiometrically determined acid value gives a measure of the fatty acids C_{10} and lower contained in the mixture and their amount can thus be rapidly evaluated by this method.

REFERENCES

1. Ekwall, P., and Juup, G. *The Svedberg 1884—1944* (1944) p. 104.
2. Brown, A. *J. Am. Chem. Soc.* **56** (1934) 646.
3. Ekwall, P., and Harva, O. *Finska Kemistsamfundets Medd.* **3—4** (1943) 257.
4. Hefter, G., and Schönfeld, H. *Chemie und Technologie der Fette und Fettprodukte* Bd *I.* Vienna (1939) p. 85.

Received September 10, 1948.

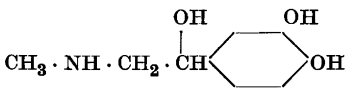
ACTA CHEMICA SCANDINAVICA 2 (1948) 727—732

Thiazole Derivatives of Aminopropanes

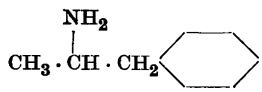
TORSTEN EKSTRAND

The Central Laboratories, Astra, Södertälje, Sweden

Several compounds related to adrenaline (I) possess a similar pharmacodynamic effect and some of these have been employed clinically, *e. g.* 1-phenyl-2-amino-propane (Amphetamine, II).



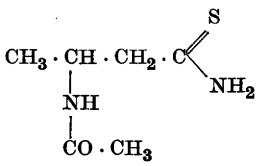
I



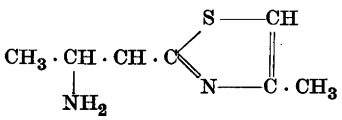
II

A large number of amphetamine derivatives have been tried and clinically used (for a review see Hartung [1]). Both the side chain and the benzene nucleus have been modified. However, no simple analogues appear to have been reported, containing a thiazole nucleus instead of a benzene nucleus. Therefore it seemed to be worth while preparing some thiazole substituted aminopropanes in order to study their potential sympathomimetic effect.

By addition of ammonia to allyl cyanide according to the method of Bruylants [2] 1-cyano-2-amino-propane was obtained. This compound was acetylated with acetic anhydride and hydrogen sulphide added to the resulting cyano compound. The 2-acetylamino-thiobutyramid (III) obtained was convertep into the thiazole derivative in the usual way by means of chloroacetone. After deacetylation 1-(4′-methyl-thiazolyl-2′)-2-amino-propane, IV, was obtained.



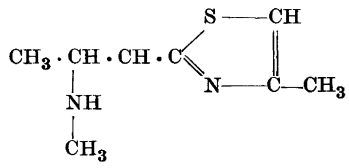
III



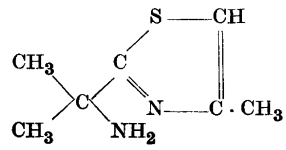
IV

The corresponding methylamino derivative V was prepared in a similar way by addition of methylamine to allyl cyanide. In this case, however, the methylamino group was benzoylated.

An isomeride of IV, 2-amino-2-(4'-methyl-thiazolyl-2')-propane, VI, was also prepared. Acetone and ammonium cyanide yielded α-amino-isobutyronitrile which after acetylation and addition of hydrogen sulphide gave α-acetylamino-thioisobutyramide [3], which with chloroacetone and subsequent deacetylation furnished VI.



V



VI

The compounds IV, V and VI have been tested according to the method of Clymer and Seifter [4]. IV and V had a certain stimulating effect on the cerebrum. However, this effect was weaker than that of II, which was used as a reference substance. IV and V however were less toxic than II. The compound VI had no stimulating effect. On the contrary it possessed a sedative effect.

EXPERIMENTAL PART

1-Cyano-2-acetylaminopropane

To 22.3 g of 1-cyano-2-amino-propane, prepared according to the method of Bruylants [2], 27.2 g of acetic anhydride was cautiously added. The mixture was cooled at intervals in ice-water. The following day it was distilled in high vacuum. The main fraction was obtained as an oil, which soon solidified. M. p. 40—45°, b. p. 190° 0.06 mm*. Yield 29.6 g.

$CH_3 \cdot CH(NH \cdot CO \cdot CH_3) \cdot CH_2 \cdot CH$	Calc.	C 57.2	H 7.93	N 22.2
	Found.	» 57.3	» 8.02	» 21.8

2-Acetylamino-thiobutyramide (III)

0.5 g of sodium was dissolved in 155 ml of ethanol. The ice-cooled solution was saturated with hydrogen sulphide to which was added 20 g of 1-cyano-2-acetylaminopropane and the mixture kept in a sealed bottle at 70° over night. The ethanol was

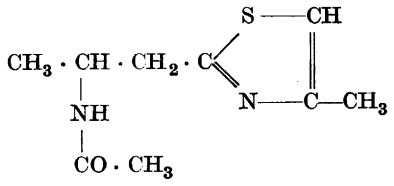
* The temperature given is the bath temperature.

distilled off at reduced pressure and the solid residue washed with petrol ether. Yield 27 g. After repeated recrystallisations from ethanol m. p. 138—140°.

$CH_3 \cdot CH(NH \cdot CO \cdot CH_3) \cdot CH_2 \cdot CSNH_2$ Calc. C 45.0 H 7.56 N 17.5 S 20.0
Found. » 45.0 » 7.58 » 17.3 » 19.3

1-(4'-Methyl-thiazolyl-2')-2-acetylamino-propane (VII)

10 g of 2-acetamido-thiopropionamide was suspended in a mixture of 6.5 g of chloroacetone and 50 ml of ethanol and the suspension shaken for 24 hours at room temperature. The clear solution was evaporated under reduced pressure. The resulting oil was distilled at 0.05 mm. After three distillations at a bath temperature of 190° 7.1 g of an almost colourless oil was obtained.



VII

Calc. N 14.0
Found. » 13.9

1-(4'-Methyl-thiazolyl-2')-2-amino-propane (IV)

11 g of the crude acetylated compound was boiled with 35 ml of hydrochloric acid for one hour. The solution was cooled and neutralized with 60 ml of 5 *N* NaOH and then extracted with chloroform. After drying the extract was evaporated and the resulting oil distilled at 130° 0.08 mm. 1.5 g of an almost colourless oil was obtained.

$C_7H_{12}N_2S$ Calc. N 17.9 S 20.5
Found » 17.7 » 20.0

Dihydrochloride. The oil was dissolved in abs. ether and the solution saturated with dry hydrogen chloride. After recrystallisation from abs. ethanol very hygroscopic crystals m. p. 178—180° were obtained.

$C_7H_{12}N_2S \cdot 2HCl$ Calc. Cl 30.9 Found Cl 30.7

1-Cyano-2-(N-benzoyl)-methylamino-propane

55 g of sodium hydroxide was dissolved in 110 ml of water and 60 g of 1-cyano-2-methylamino-propane [2] was added to the solution. The mixture was kept below 15° and 96 g of benzoylchloride added through a dropping funnel with stirring. The resulting precipitate was collected, washed with water and recrystallised from 500 ml of hot water, the yield was 95 g. M. p. 69—71°.

$C_6H_5 \cdot CO \cdot N(CH_3) \cdot CH(CH_3) \cdot CH_2 \cdot CN$ Calc. N 13.9 Found N 13.8

2-(N-Benzoyl)-methylamido-thiobutyramide

This compound was prepared in the same manner as 2-acetamido-thiobutyramide above. From 30 g of 1-cyano-2-benzoyl-methylamino-propane 28 g of the thioamide was obtained. It was dissolved in 80 ml of boiling ethanol and to this solution 200 ml of light petroleum was added. 25 g of a crystalline material was obtained. M. p. 144—145°.

$C_{12}H_{16}ON_2S$	Calc.	N 11.8	S 13.5
	Found	» 11.6	» 13.1

1-(4'-Methyl-thiazolyl-2')-2-(N-benzoyl)-methylaminopropane

This compound was prepared in the same manner as VII. From 11.8 g of the thioamide and 8.0 g of chloroacetone in 60 ml of ethanol 13.7 g of the crude thiazolecompound was obtained. This was distilled twice at 100° 0.003 mm and 7.8 g of a light yellow oil was obtained.

$C_{15}H_{18}ON_2S$	Calc.	C 65.7	H 6.61	N 10.2	S 11.7
	Found	» 64.8	» 6.63	» 10.1	» 11.3

1-(4'-Methyl-thiazolyl-2')-2-methylamino-propane (V)

The crude benzoyl compound was boiled for six hours with a mixture of 75 ml of conc. hydrochloric acid and 50 ml of water. After cooling the benzoic acid formed (5.5 g, m. p. 121°: calc. 6.1 g) was removed by filtration. The mother liquor was made alkaline with 5 *N* NaOH and the oil which separated extracted with chloroform. After drying and evaporation of the solvent the rest was distilled. At 50° 0.05 mm 3.8 g of a light yellow oil was obtained.

$C_8H_{14}N_2S$	Calc.	N 16.5	S 18.6
	Found	» 16.5	» 18.5

Dipicrate. From ethanolic solutions of the base and picric acid. Recrystallised from ethanol. M. p. 135—136°.

$C_{20}H_{20}N_8SO_{14}$	Calc.	C 38.3	H 3.18	N 17.8	S 5.10
	Found	» 38.3	» 3.08	» 17.8	» 4.92

Dihydrochloride. By saturating an ethereal solution of the base with dry hydrogen chloride. Recrystallised from ethanol. M. p. 161—162°. The substance was very hygroscopic.

$C_8H_{16}ON_2Cl_2S$	Calc.	N 11.5	Cl 29.2
	Found	» 11.5	» 28.4

As the hydrolytic removal of the benzoyl group gave rather poor yields, the acetylated cyanocompound and thioamide were also prepared. Unfortunately the deacetylation experiments resulted in no better yields.

1-Cyano-2-(N-acetyl)-methylaminopropane

20 g of 1-cyano-2-methylamino-propane was acetylated with 20.8 g of acetic anhydride. After standing over night the mixture was distilled. 27.8 g of the acetyl compound distilled at 190° 0.1 mm. It was redistilled twice at the same temperature and pressure.

$C_7H_{12}ON_2$ Calc. N 20.0 Found N 19.3

2-(N-Acetyl)-methylamino-thiobutyramide

This was prepared in the same way as the thioamides mentioned above. From 20 g of the cyano compound 13 g thioamide was obtained. After precipitation of an ethanolic solution with light petroleum the substance had m.p. 125—126°.

$C_7H_{14}ON_2S$	Calc.	C 48.3	H 8.10	N 16.1
	Found	» 48.3	» 8.28	» 16.0

2-Acetamino-2-(4′-methyl-thiazolyl-2′)-propane

This was prepared in the same manner as the thiazole compounds mentioned above. From 3.4 g α-acetamino-thioisobutyramid [3] and 2.2 g chloroacetone 4.0 g of the thiazole was obtained. After repeated recrystallisations from acetone it had m.p. 192—195°.

$C_9H_{14}ON_2S$	Calc.	N 14.1	S 16.2
	Found	» 13.9	» 16.0

2-Amino-2-(4′-methyl-thiazolyl-2′)-propane (VI)

10 g of the acetylcompound above was boiled for one hour with 50 ml conc. hydrochloric acid. The reaction mixture was made alkaline with 5 *N* NaOH and extracted with chloroform. The residue was distilled at 130° 0.03 mm. Yield 2 g. After one redistillation the resulting oil was colourless.

$C_7H_{12}N_2S$	Calc.	C 53.8	H 7.74	N 18.9
	Found	» 53.7	» 7.63	» 17.9

Dihydrochloride. Prepared as above. M.p. 177—179°.

$C_7H_{14}N_2Cl_2S$ Calc. Cl 30.9 Found Cl 30.5

SUMMARY

Three thiazole derivatives of 2-aminopropane have been prepared and tested for sympathomimetic activity.

REFERENCES

1. Hartung, W. H. *Ind. Eng. Chem.* **37** (1945) 126.
2. Bruylants, P. *Bull. Soc. Belg.* **32** (1923) 256.
3. Johnson, T. B., and Gatewood, E. *J. Am. Chem. Soc.* **51** (1929) 1815.
4. Clymer, N. V., and Seifter, J. *J. Pharm. Exp. Ther.* **89** (1947) 149.

Received August 24, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 733—739

On the Solubilization of Carcinogenic Hydrocarbons by Association Colloids

PER EKWALL and KAI SETÄLÄ

Institute of Physical Chemistry, Åbo Akademi, Åbo, and Department of Anatomy, University of Helsinki, Helsinki, Finland

The polycyclic aromatic hydrocarbons that possess carcinogenic properties are fat-soluble, but are practically insoluble in water. When homogeneous solutions of these substances have been needed in experimental cancer research it has been found necessary to dissolve them in liquids such as benzene, acetone, sesame oil, tricaprylene etc.[1]. In some cases suspensions of the carcinogenic hydrocarbons in water or in glycerol have been employed. Attempts have been made to disperse these substances in as fine a state and as stably as possible; for instance by letting an acetone solution of the hydrocarbon to drip into distilled water and then removing the acetone by dialysis into pure water [2] (Feigenbaum), or by rapid evaporation at low pressures [3] (Weigert and Mottram). Several investigators have stabilized the suspension with the aid of a colloid. Berenblum [4] and Boyland [5] started with a pyridine solution of the hydrocarbon and diluted it carefully with an aqueous solution of acacia gum, after which they removed the pyridine by dialysis, whereas O'Hara and Pollia [6] dialyzed the pyridine solution directly into the aqueous acacia gum solution. Lorentz and Andervont [7], Shimkin [7] and others used horse serum, Lasnitzki and Woodhouse [8] gelatin as the stabilizing colloid. Stamer [9] produced suspensions by means of dissolving the carcinogenic hydrocarbon in melted »Postonal», a derivative of poly-ethylene oxide and then stirring the mixture in hot distilled water. Thus suspensions were obtained containing up to 2.5 % 1,2,5,6-dibenzanthracene. The suspensions were, however, unstable and had to be used within one hour. — Winterstein and Vetter [10] treated 1,2-benzpyrene in acetone with a 20 % aqueous solution of sodium desoxycholate and found that after removal of the acetone, the hydrocarbon

had dissolved to the extent of 2 mg per ml. Fieser and Newman [11] investigated the formation of molecular compounds of desoxycholic acid with some polynuclear aromatic hydrocarbons and found that in absolute alcohol solutions of the components crystals of »choleic acids» containing four molecules of desoxycholic acid and one molecule of methylcholantrene or 1,2,5,6-dibenzanthracene were formed. The sodium salts of these choleic acids are soluble in water *. — Beck [12] added an ether solution of a carcinogenic hydrocarbon to a highly concentrated solution (1.8 g/ml) of an ether soap, the identity of which was not disclosed. This comprised the storage solution, and solutions of desired concentrations were prepared by diluting with water and vaporizing the ether. *E. g.* solutions containing 7.5 % by weight of soap and 0.1 mg per ml of hydrocarbon were prepared in this manner. The fluorescence spectra of the hydrocarbon in these aqueous solutions and in acetone solution were identical. However, these solutions were unstable, for even within a few hours the limpid solutions were dimmed by opalescence. But Beck found that even in this state the hydrocarbon particles were much smaller than those present in the colloid solutions previously used. Graffi [13] mentions incidentally that the sodium salts of the higher fatty acids are able to dissolve benzpyrene to a certain extent. — Recently Weil-Malherbe [14] found that the solubility of carcinogenic hydrocarbons is considerably increased in the presence of purines, and that the solubilizing effect of the purines varies with their molecular structure. Apparently the solutions are homogeneous, but the solubilization process seems to result in a quenching of the fluorescence characteristic of the carcinogenic hydrocarbon.

As it would be advantageous in certain cases if carcinogenic hydrocarbons could be handled in the form of their stable and absolutely homogeneous aqueous solutions we have investigated the possibility of solubilizing these hydrocarbons with the aid of association colloids. A characteristic of these colloids, among which common soap is the best known type substance, is that when they are dissolved in water their molecules and ions aggregate when a certain concentration is exceeded to form micelles that are able to solubilize considerable amounts of fat-soluble substances such as hexane, cyclohexane, benzene, toluene *etc.* Also naphthalene, anthracene and phenanthrene are solubilized in slight amounts by these colloids [15]. We have now found that carcinogenic hydrocarbons, in spite of the rather large size of their molecules, dissolve in detectible amounts in these colloids yielding clear, absolutely homogeneous and stable, more or less fluorescent, aqueous solutions [16].

---

* Lately Weil-Malherbe reported experiments conducted with sodium deoxycholate-water solutions of benzpyrene (*Brit. Journ. of Cancer* **1** (1947) 423).

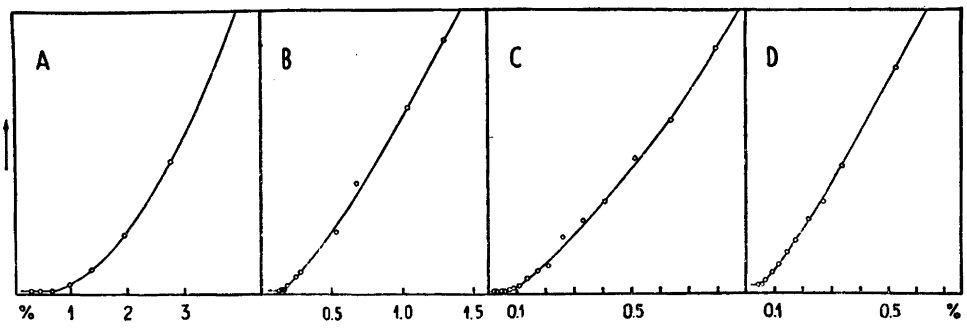


Fig. 1. The fluorescence of colloid solutions saturated with 20-methylcholanthrene. The relative values of the fluorescence intensity as measured with a Pulfrich Step-photometer employing one of the solutions as reference solution has been plotted against the concentration of the colloid in the solution in weight per cent. A. Sodium cholate; B. Sodium myristate; C. Sodium myristyl sulphate; D. Triton NE.

Several colloid-forming substances have been investigated respecting their ability to solubilize carcinogenic hydrocarbons: *e. g.* sodium oleate and myristate, sodium myristyl sulphate, sodium cholate and the commercial compounds Mersolate, Gardinol WA and Triton NE. Mersolate is an alkyl sulphonate, Gardinol is probably an aliphatic alcohol sulphonate and Triton NE is an alkyl polyether alcohol, the last-mentioned differing from the others in being a non-ionogenic association colloid. The colloid solutions were shaken with the finely powdered hydrocarbons in a thermostat at 40° C until the solutions were saturated with the hydrocarbons. The solubilities of the following three hydrocarbons have been investigated: 20-methylcholanthrene, 9,10-dimethyl-1,2-benzanthracene and 1,2,5,6-dibenzanthracene.

The solubilization ability of the association colloid becomes evident only when its concentration exceeds a definite limit, see Fig. 1. The figure illustrates the fluorescence of solutions of sodium cholate, myristate, myristyl sulphate and Triton which have been saturated with methylchloranthrene. In dilute solution the fluorescence is nil, as in the pure colloid, but at a definite concentration fluorescence appears and the intensity of the fluorescence increases rapidly, indicating a corresponding increase in the solubility of the hydrocarbon. The inception of the fluorescence coincides with the attainment of the critical concentration for micelle formation of the association colloid; at this point the colloidal substance begins to develop. This implies that the solubilization of the polycyclic hydrocarbon is coupled with the formation of micelles, and leads one to suppose that the rather large hydrocarbon molecules are enclosed in the hydrocarbon part of the micelles as are benzene *etc.*

Table 1. Approximate solubility values of the carcinogenic hydrocarbons in mg per 1000 ml solution. Colloid concentration in weight per cent.

	2 %	4 %	8 %	20 %
	Sodium cholate solutions			
1,2,5,6-Dibenzanthracene	0.84	3.9	14	
20-Methylcholanthrene	4.3	17	65	390
9,10-Dimethyl-1,2-benzanthracene	27	145	450	
	Sodium oleate solutions			
1,2,5,6-Dibenzanthracene	2.9	5.9	11	
20-Methylcholanthrene	21	45	83	
9,10-Dimethyl-1,2-benzanthracene	81	165	308	
	Triton NE solutions			
1,2,5,6-Dibenzanthracene	5.5	11	22	
20-Methylcholanthrene	15	33	59	157
9,10-Dimethyl-1,2-benzanthracene	89	180	360	700—1100

The method nearest at hand for the determination of the solubilities of the hydrocarbons is the measurement of their fluorescence intensity. A linear relation between the intensity of the fluorescence and the hydrocarbon concentration is however to be found only at comparatively low concentrations (*e. g.* up to ca. 40 mg methylchloranthrene per liter in 8 % sodium cholate solutions). Also the intensity is affected by many factors such as the solvent's own absorption, the quenching effect of the fluorescent substance, of the colloid and of other foreign substances (*e. g.* oxygen). Consequently identical hydrocarbon concentrations may give rise to fluorescence of varying intensity in different solvents, *i. e.* in solvents containing various concentrations of one colloid or in solvents containing different colloids. It seems that in some cases the hydrocarbon itself is changed in some way during the solubilization or during the storage of the solutions. We have not as yet been able to overcome these difficulties, which affect the determination of the concentration. Furthermore, the instrument at our disposal (Pulfrich Step-photometer) has not enabled us to attain the desired accuracy in the fluorescence measurements. Hence the solubility values given above are to be regarded only as approximate.

The values given in Table 1 confirm that the solubilities of the hydrocarbons increase with the concentration of the colloid. There is, however, a marked difference between the increase in the solubility in the sodium oleate and Triton solutions on one hand, and in the sodium cholate solutions on the other. In the former group the solubility increases in the same proportion as

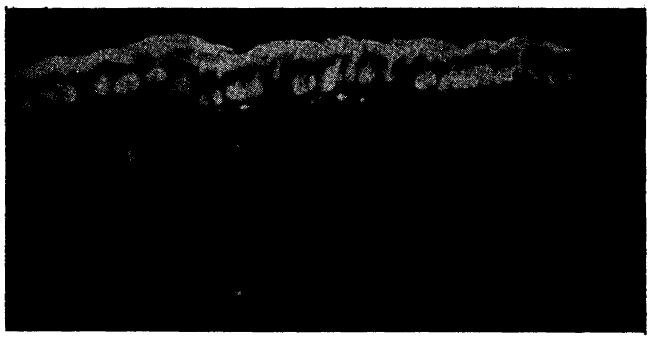


Fig. 2. A microphotograph taken in ultraviolet light of a frozen section from the skin of a mouse painted with a 20 % Triton NE solution saturated with 20-methylchloranthrene, unstained section. The specimen was taken 1 hour and 45 min after the painting of the skin. The fluorescent substance is clearly visible.

the concentration of the colloid, a behaviour that is also found in the case of the solubility of lowmolecular hydrocarbons in these and other association colloids over a wide concentration interval (in the range of »small micelles», »Kleinmicellen»). In the cholate solutions the increase in solubility is more rapid; there is a practically linear relation between the solubility and the quadrate of the cholate concentration. A consequence of this is that the solubility of the hydrocarbon in the more concentrated cholate solutions is higher than in the corresponding oleate and Triton solutions, whereas the reverse is true in the more dilute solutions. Whether this behaviour is due to the different manner in which the colloid is formed from the substances with straight chains as compared with its formation from the cholate with its four-ringed molecule and three hydroxyl groups, or to differences in the solubilization mechanism, is a question that must be left open.

The solubilities of the three hydrocarbons in the same colloid differs widely. In all cases 1,2,5,6-dibenzanthracene shows the slightest and 9,10-dimethyl-1,2-benzanthracene the highest solubility. The greatest variations are found in the cholate solutions. Also in benzene the first-mentioned hydrocarbon is the least and the last-mentioned the most soluble. It is interesting to note that 1,2,5,6-dibenzanthracene has the least carcinogenic effect, while 9,10-dimethyl-1,2-benzanthracene has proved to be in many cases the most active in this respect. In the fifteen-step scale introduced by Berenblum [17] 9,10-dimethyl-1,2-benzanthracene is characterized by the cipher X, 20-methylcholanthrene by VII and 1,2,5,6-dibenzanthracene by VI when skin tumours are concerned. It remains, however, to be found whether a relation exists

between the different solubilities of the hydrocarbons in the micelles and their carcinogenic action.

Experiments with animals have been undertaken to study the effect of the association colloid solutions of the carcinogenic hydrocarbons. When a small amount of such a solution is painted on the skin of a mouse the carcinogene is rapidly absorbed and with the aid of a fluorescence microscope the hydrocarbon has been found distributed in about the same places as when applied by painting with an acetone solution of the carcinogene. Fig. 2 is a microphotograph of a cut from the skin after painting with methylchloranthrene in Triton solution. The fluorescent carcinogene is found in the epidermis, and in the sebaceous glands with their contents. We have also been able to produce skin tumours by application of these association colloid solutions of the hydrocarbons (unpublished data).

The experiments are being continued in several directions. Animal experiments in which association colloid solutions of the carcinogenes are being introduced by subcutaneous and intravenous injection and with food are in progress.

SUMMARY

When the concentrations of association colloids of a number of types exceed their respective so-called critical concentrations for micelle formation they are able to bring carcinogenic hydrocarbons into solution. The solutions obtained are clear, absolutely homogeneous and stable. In some cases the solubilities of the hydrocarbons increase in the same proportion as the concentration of the colloid (oleate, Triton NE) and in other cases as the quadrate of the colloid concentration (cholate). There is some parallelism between the solubilities of the three hydrocarbons investigated in a given colloid and their carcinogenic action.

We wish to express our indebtedness to Messrs. Lars Sjöblom, Ph. M., and Leon Tigerstedt for valuable aid with the solubility measurements.

REFERENCES

1. Greenstein, J. R. *Biochemistry of cancer* New York (1947) p. 40.
2. Feigenbaum, J. *Nature* **155** (1945) 207.
3. Weigert, F., and Mottram, J. C. *Canc. Res.* **6** (1946) 97.
4. Berenblum, I. *Lancet* **2** (1932) 1107.
5. Boyland, E. *Lancet* **2** (1932) 1108.
6. O'Hara, P. H., and Pollia, J. A. *Am. J. Cancer* **37** (1937) 493.
7. Lorenz, E., and Andervont, H. B. *Am. J. Cancer* **26** (1936) 783; Shimkin, M. B. *Arch. Path.* **29** (1940) 229, 239.

8. Lasnitzki, A., and Woodhouse, D. L. *Nature* **150** (1942) 660.
9. Stamer, S. *Acta Path. Microbiol. Scand.* **22** (1945) 65.
10. Winterstein, A., and Vetter, H. *Hoppe-Seyler's Z. physiol. Chemie* **230** (1934) 169.
11. Fieser, L. F., and Newman, M. S. *J. Am. Chem. Soc.* **57** (1935) 1602.
12. Beck, S. *Nature* **152** (1943) 537.
13. Graffi, A. *Z. Krebsforsch.* **49** (1939) 477.
14. Weil-Malherbe, H. *Biochem. J.* **39** (1945) xxxvi; **40** (1945) 351, 361.
15. Ekwall, P. Unpublished experiments.
16. Ekwall, P. *Sjätte nordiska kemistmötet* Lund (1947) p. 179.
17. Berenblum, I. *Canc. Res.* **5** (1945) 561.

Received October 13, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 740—758

A Thermodynamic Investigation on the Racemization of Tartaric Acid

THOMAS ROSENBERG

Physico-Chemical Institute, University of Copenhagen, Denmark

The basis of the thermodynamic treatment of the racemization process is the energetic identity of the optical antipodes. On this basis, Brønsted[1] in a paper on the racemization of potassium tartrate has shown and realized various possibilities for the determination of the affinity of the process, *inter alia* by means of solubility determinations of the optically active and the racemic forms. This method is well suited for such determinations, provided that the gas laws hold for the saturated solutions in question. In the mentioned investigations, this condition has been fulfilled, a strong salt solution being used as a solvent in which the solubility of potassium tartrate and racemate was very low.

In the present investigation the racemization of crystalline tartaric acid into racemic acid (*dl*-tartaric acid) is investigated; the process is represented by the scheme

$$d \longrightarrow r \qquad (1)$$

One mole of *d*-tartaric acid, *l*-tartaric acid, and racemic acid are denoted by *d*, *l*, and *r*, respectively, and identical values are used for the molecular weights of racemic acid and tartaric acid. A hydrate of racemic acid, which is obtained by crystallization in aqueous medium and which contains 1 mole of H_2O per 150 g acid, is denoted by *rh*.

While in the case of potassium tartrate the deviations from the ideal conditions almost exclusively are caused by the fact that the ions, in view of their electric charge, affect each other, the conditions in the system tartaric acid — racemic acid are different. The electrolytic dissociation of tartaric acid is relatively low ($K_1 \sim 10^{-3}$), and its solubility in water is rather high, much higher than the solubility of racemic acid. The deviations from the ideal conditions are, therefore, preponderantly due to a solvent effect in addition to

the mutual influence of the molecules, their association, etc., factors which cannot be eliminated in the aqueous medium. Consequently, determinations according to the mentioned principle necessitate the choice of another well suited solvent. A determination of the racemization affinity on the basis of the knowledge of the saturation conditions in aqueous medium can, however, be performed if the degree of deviation from the ideal conditions can be determined quantitatively, for example, by determining the activity coefficients.

THEORETICAL BASIS

Racemization of an optically active crystalline compound generally does not occur spontaneously at ordinary temperature. Without changing the energetic conditions we can, however, assume that half of the observed quantity of the *d*-compound, for example ½ mole, is converted into the *l*-compound and, instead of (1), we may write

$$\tfrac{1}{2}\, d + \tfrac{1}{2}\, l \longrightarrow r \qquad (2)$$

This process, which is thermodynamically equivalent to (1), will occur spontaneously if a small amount of a solvent is present, the effect of which may be considered as catalytic.

Different possibilities exist for the determination of the affinity of process (2), *viz.*

1. It can be treated analogously to the double salt formation whose formal similarity to (2) is immediately obvious.

2. It can be understood by analogy to the allotropic transformation, the mixture of equal parts of the *d*- and the *l*-form being the one modification which is converted into the stable racemic form.

Brønsted [2] has described a method for the affinity determination in double salt formation which, in the present work, is used for the determination of the racemization affinity. The method is based upon the following principle;

Let us assume that process (2) is performed by transferring ½ mole *d* from a solution saturated with respect to *d* (s—*d*) and ½ mole *l* from a solution saturated with respect to *l* (s—*l*) into a solution saturated with respect to *r* (s—*r*). The affinity for this process is given by

$$A^* = \tfrac{1}{2}\,(\mu_{d\,(s-d)} - \mu_{d\,(s-r)}) + \tfrac{1}{2}\,(\mu_{l\,(s-l)} - \mu_{l\,(s-r)}) \qquad (3)$$

where the chemical potentials are denoted by μ. Thus, $\mu_{d(s-d)}$ is the chemical potential of *d*-tartaric acid in a solution saturated with *d*-tartaric acid. The

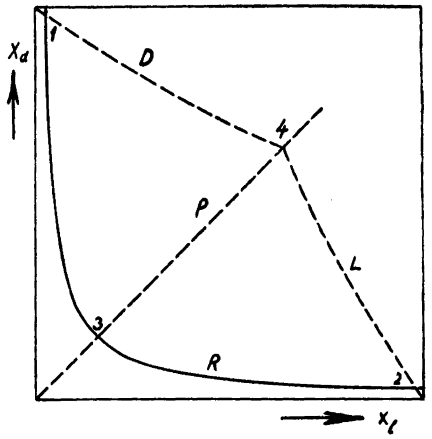


Fig. 1. Schematic solubility diagram in a system with optically active components.

conditions are most clearly illustrated by means of a solubility diagram (Fig. 1).

In Fig. 1, the concentrations of d and l as mole fractions are are the coordinates. The three solubility curves D, L, and R give the composition of the solutions in equilibrium with solid d, l, and r, respectively. The points of intersection 1, 2, and 4 denote the composition of solutions in equilibrium with two crystalline compounds. If the racemic compound is dissolved in the pure solvent until saturation is reached, a solution of composition 3 is obtained. The line P divides the diagram into two symmetrical parts in view of the thermodynamic identity of the optically active forms. The transferring process mentioned above can obviously be performed from arbitrary points on the curves D and L to an arbitrary point on the curve R. In the present case, it is most advantageous to transfer d and l from 1 and 2, respectively, to 3. Hence, we obtain for the affinity, according to (3),

$$A^* = \tfrac{1}{2}\,(\mu_{d(1)} - \mu_{d(3)}) + \tfrac{1}{2}\,(\mu_{l(2)} - \mu_{l(3)}) \tag{4}$$

For symmetry reasons, however, the two potential differences in the equation (4) are equal and (4) can be simplified to

$$A^* = \mu_{d(1)} - \mu_{d(3)} \tag{5}$$

From (5) it results that the affinity determination presupposes the knowledge of the change in potential of the d-form along curve R between 1 and 3.

The present case involves the complication that racemic acid is present as a hydrate in equilibrium with the solutions, so that R may represent the

saturation curve of *rh*. If $^1/_2$ mole *d* and $^1/_2$ mole *l* are transferred to 3, apart from one mole *r*, 1 mole H_2O will disappear from the solution, which must be replaced by addition of 1 mole H_2O from outside to 3. The affinity of this process which obviously has to be included in the affinity of the total process depends on from where this water is taken. If it is taken from a mixture of *r* and *rh* (*r*—*rh*), the total process will consist in the formation of 1 mole *r*, in agreement with (2).

In view of this addition of H_2O the expression (5) for the affinity must be extended to

$$A^* = \mu_{d(1)} - \mu_{d(3)} + (\mu_{H_2O(r-rh)} - \mu_{H_2O(3)}) \tag{6}$$

Along the saturation curve of *rh* we have

$$d\mu_{rh} = \tfrac{1}{2} d\,\mu_d + \tfrac{1}{2} d\,\mu_l + d\,\mu_{H_2O} = 0 \tag{7}$$

and, furthermore, Gibbs-Duhem's equation

$$n_d\, d\mu_d + n_l\, d\mu_l + n_{H_2O}\, d\mu_{H_2O} = 0 \tag{8}$$

where *n* is the number of moles of the respective component in the solution.

Consequently, we obtain

$$\mu_{d(1)} - \mu_{d(3)} = \int_3^1 d\mu_d = \int_1^3 \frac{n_{H_2O} - 2n_l}{n_d - n}\, d\mu_{H_2O}$$

and by means of (6):

$$A^* = \int_1^3 \frac{n_{H_2O} - 2n_l}{n_d - n_l}\, d\mu_{H_2O} + (\mu_{H_2O(r-rh)} - \mu_{H_2O(3)}) \tag{9}$$

A rough calculation by means of van der Waals' equation for water vapour shows that the gas laws can here be applied. (The constants *a* and *b* are taken from Landolt-Börnstein: $a = 5.47$ and $b = 0.0305$, *p* being given in atmospheres and *v* in litres.) For the vapour, we have

$$\left(\frac{d\mu}{dp}\right)_T = v = RT\left(\frac{1}{p} + \frac{d\ln f}{dp}\right)$$

where *f* is the activity coefficient of the water vapour, and $\frac{d\ln f}{dp}$ is calculated by means of a and b to -0.00815 atm.$^{-1}$. In the pressure range observed, this correction term is of the order of magnitude of $0.2\,^0/_{00}$ and, thus, without significance.

Introducing the relation between the chemical potential and the vapour pressure in (9), we obtain

$$A^* = RT \left[\int_1^3 \frac{1 - x_A - 2x_l}{x_A - 2x_l} \, d\ln p + \ln \frac{p_{r-rh}}{p_3} \right] \tag{10}$$

where

$$x_A = \frac{n_d + n_l}{n_{H_2O} + n_d + n_l} \text{ and } x_l = \frac{n_l}{n_{H_2O} + n_d + n_l}$$

SOLUBILITY DETERMINATIONS

Equation (10) shows that for the calculation of the affinity we must know the interdependence of vapour pressure and composition for solutions which are saturated with respect to *rh* and in which $n_d \geqq n_l$. The method applied in the present work leads only to corresponding values of the vapour pressure and the total quantities of *d*, *r*, and H_2O in the system containing a certain amount of *rh* in solid form. In order to arrive at the composition of the solution from these total quantities, solubility determinations of *rh* had to be performed in solutions with varying x_d. Two methods have been used, *viz.*

1. At relatively low *d*-concentrations ($x_d < 0.05$), weighed quantities of *d*, *r*, and H_2O are shaken for 1—2 days in a thermostat at 20° C. Subsequently, a weighed quantity of the saturated solutions is titrated with 0.1 *N* NaOH and phenolphtalein as an indicator. The distribution of the weighed substances between solution and solid phase is seen from the following table, where the quantities of the substances are given in g, and *M* denotes the molecular weight.

	Tartaric Acid (*t*)	Racemic Acid	H_2O
Total amounts taken	*a*	*b*	*e*
Amounts in the solid phase	—	$y \cdot M_r/M_{H_2O}$	*y*
Amounts in the solution	*a*	$b - y \cdot M_r/M_{H_2O}$	$e-y$

By titration we find the sum of the tartaric acid concentration and the racemic acid concentration, *viz.*

$$c_A = \frac{g \cdot \text{total acid}}{g \cdot H_2O} = \frac{a + b - y \dfrac{M_r}{M_{H_2O}}}{e - y}$$

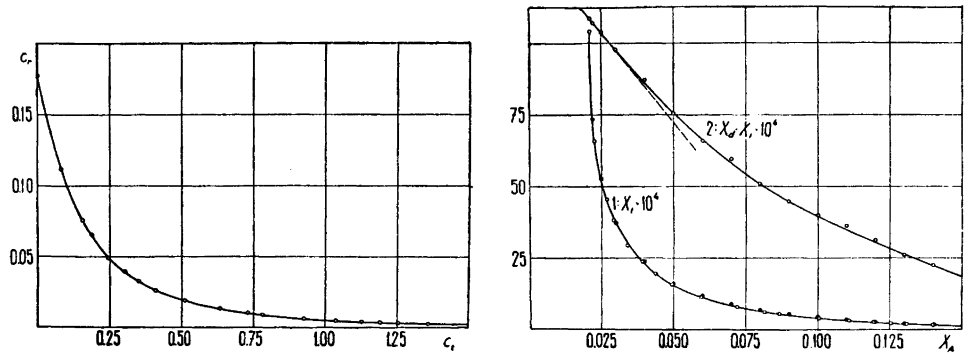


Fig. 2a and 2b. Solubility of racemic acid in solutions of tartaric acid.
○ : experimental values
● : calculated from (15)

From this, we derive

$$y = \frac{a + b - e \cdot c_A}{\frac{M_r}{M_{H_2O}} - c_A}$$

and, for the solubility of the racemic acid in the same concentration units, we obtain

$$c_r = \frac{b - y\frac{M_r}{M_{H_2O}}}{e - y} = \frac{c_A\left(\frac{M_r}{M_{H_2O}} \cdot e - b\right) - a\frac{M_r}{M_{H_2O}}}{\frac{M_r}{M_{H_2O}} \cdot e - b - a}$$

With increasing x_d the solubility of the racemic acid decreases and, consequently, the inaccuracy of the titration method increases. For $x_d > 0.05$, the following procedure was therefore used.

2. Known amounts of *d* and *r* are placed in a weighed flask. Somewhat less H_2O than necessary to dissolve all racemic acid is added. The flask is rotated in the thermostat at 20° C and water is added by the drop until all racemic acid is dissolved. By renewed weighing of the flask the added amount of water is found and, thereby, the composition of the solution.

The results of the two experimental series are given in Figs. 2a and 2b. The preparations used were Kahlbaum's *p. a.* The racemic acid was repeatedly recrystallized. Titration proved that the preparations were pure (100 %).

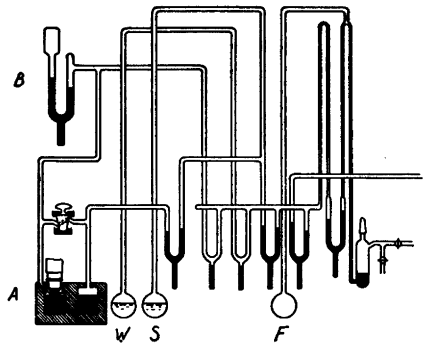


Fig. 3. Apparatus for the measurement of vapour pressures.

A Mercury manometer
B Mercury manometer
F Recipient
S Flask containing the system under investigation
W Flask containing water

The solubility determinations of racemic acid were continued in the range supersaturated with respect to tartaric acid.

Furthermore, the solubility of tartaric acid in H_2O was determined by titration to be $c_d = 1.36$.

MEASUREMENTS OF VAPOUR PRESSURES

Measuring principle

A flask containing weighed quantities of tartaric acid and racemic acid and an unknown quantity of H_2O is evacuated and, then, connected with the manometer by means of which the vapour pressure is measured. Subsequently, part of the water is distilled off and weighed. The vapour pressure at the new concentration is measured and the procedure is continued until the solution is saturated with both acids. The apparatus is shown in Fig. 3 which gives the details.

The connections in the system are made with mercury valves which are operated by means of a pressure-vacuum-system (not shown in the figure). *A* is a mercury manometer drilled out of an iron block and kept at 25° C by means of an interior thermostat arrangement, in order to prevent condensation of water. Readings are made by focussing a microscope upon the picture of a radiating slit, which it is itself projecting upon the mercury surface (Prytz's optical contact principle [3]). The accuracy is 0.002 mm Hg.

The 2 flasks *W* and *S* contain water and the system under investigation, respectively. They are placed in a thermostat at 20° C and connected with a shaking mechanism. To measure the vapour pressure, the 2 flasks are connected to each side of *A*. *B* is a mercury manometer with microscope reading and nonius graduation. It is connected to *W* and serves as a control. Water

is distilled from S to the recipient F, which is kept cold in ice. After the last reading, S is weighed in order to determine the amount of remaining water. Using this value, we can calculate the composition for all previous measurements in the same series.

Measurements

First, three series were made with solutions of *d*-tartaric acid without addition of racemic acid. The measurements extend to the range of supersaturation. The results, given in Fig. 4, are later applied to a calculation of the solubility of racemic acid in solutions of tartaric acid.

The pressure is always given in mm Hg. The vapour pressure of the solution is calculated on the basis of $p_0 = 17.535$ mm for pure H_2O. The activity coefficient of water is obtained from the relation

$$f_{H_2O} = \frac{p}{(1-x)\,p_0}$$

In the homogeneous system *d*-tartaric acid — water, the calculation of the composition of the solutions is performed directly from the total quantities. In systems which besides tartaric acid contain racemic acid in solid and in dissolved form, the determination of the composition of the liquid, on the basis of the total amounts, is made graphically by means of the solubility curve shown in Fig. 2a as follows, the amounts of the respective substances being given in g and the concentrations (c) in g dissolved substance per g H_2O in solution:

	Tartaric acid (t)	Racemic acid	H_2O
Total amounts in the system	a	b	e
Amounts in the solid phase	—	$y\frac{M_r}{M_{H_2O}}$	y
Amounts in the solution	a	$b-y\frac{M_r}{M_{H_2O}}$	$e-y$

$$c_r = f\,(y) = \frac{b - \frac{M_r}{M_{H_2O}} \cdot y}{e-y}$$

$$c_t = F\,(y) = \frac{a}{e-y}$$

$$c_r = \psi\,(c_i) = c_i \cdot \left(\frac{b}{a} - \frac{M_r}{M_{H_2O}} \cdot \frac{e}{a}\right) + \frac{M_r}{M_{H_2O}}$$

The relation $c_r = \psi\,(c_i)$ represents a straight line containing the point which gives the composition. The dependence between c_i and c_r on the basis of the solubility determination, however, is represented by the curve Fig. 2a. Consequently, the composition of a given solution is obtained from the point of intersection of this curve and the straight line $c_r = \psi\,(c_i)$.

Two series of measurements were performed, the results of which are shown in Fig. 4, where they are plotted together with the results from the tartaric acid series.

The results indicate that the vapour pressure within the limits of the experimental accuracy depends only on the total acid concentration, irrespective of the quantities of acid present in the *d*-form and in the *l*-form, respectively. This result suggests that the specific forces between the *d*- and the *l*-form, which are preponderant in the racemic crystals, are of no significance in the solutions investigated, or — less plainly spoken — that the racemic acid is not present in demonstrable amounts as a racemic compound in solution. Raoult [4], when examining this question by means of the more sensitive freezing point method, has found that a solution of 14.23 g anhydrous racemic acid in 100 g H_2O has a freezing point of — 1.807° C, while a tartaric acid solution of the same concentration has a freezing point of — 1.862° C. From this result he calculated that 6 per cent of the racemic acid at this concentration is present as a racemic compound. Apart from the fact that Raoult in his calculations erroneously uses $M_r = 376$ instead of 300, the solubility of racemic acid in water at 0° C., according to the available solubility determinations [5, 6] amounts to 8.25 g anhydrous racemic acid in 100 g H_2O; thus, Raoult's racemic acid solution has been considerably supersaturated. His result may probably be due to beginning crystallization and, therefore, it cannot be taken as a proof of the existence of a racemic compound in solution.

Furthermore, the vapour pressure of the saturated *rh*-solution without addition of *d* (p_3) and of a mixture of crystalline *r* and *rh* (p_{r-rh}) was measured. The first was determined in the same apparatus as described above, the second one by means of an ordinary manometer filled with liquid paraffin, the specific gravity of which was determined to be 0.888 g/cm³. The results were as follows

$$p_3 = 17.149 \text{ mm Hg}$$
$$p_{r-rh} = 19.88 \text{ cm paraffin} = 13.03 \text{ mm Hg}$$

The vapour pressure measurement of *r*—*rh* incipiently gave low and slowly increasing values and it took several weeks until equilibrium was reached.

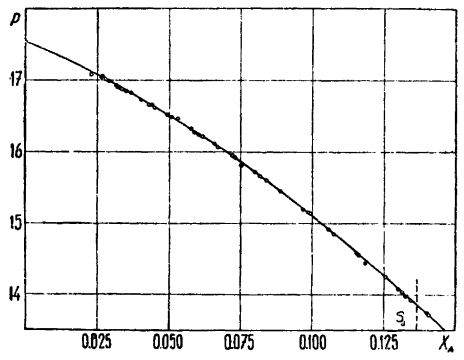


Fig. 4. Vapour pressure of solutions of d-tartaric acid in water (○) and in saturated racemic acid solutions (●).

This can be explained by assuming that the transformation of the crystal lattice of the hydrate to that of the anhydrous substance is a rather slow process. The equilibrium value was checked by repeatedly increasing and decreasing the temperature, by which procedure the above value was corroborated. Partington[7] has determined p_{r-rh} by means of a dynamic method at various temperatures. From his results we find by interpolation to 20° C, the value of 5.42 mm Hg, which is less than half of the value obtained in the present work. Already when taking into consideration that the racemic acid hydrate effloresces under normal conditions, Partington's result appears to be very dubious. If we, moreover, keep in mind the conditions observed at the adjustment of equilibrium, it becomes obvious that a dynamic method is inapplicable to the measurement of the vapour pressure of this system.

CALCULATION OF THE RACEMIZATION AFFINITY

For the calculation of the integral in equation (10), the interdependence of $\log p$ and $y = \frac{1 - x_A - 2x_l}{x_A - 2x_l}$ must be known. This is represented in the curve of Fig. 5.

For $p = p_3$, $y = \infty$, so that for pressure values near p_3, the integral cannot be calculated without further assumptions. Therefore, the integration was performed by calculating two partial integrals I_1 and I_2. For the range $0.03 \leqq x_A \leqq 0.1367$, the following empirical formula was used:

$$\log p = 1.2391 - \frac{2.14}{6}\frac{1}{y} - \frac{122}{72}\frac{1}{y^2} \qquad (11)$$

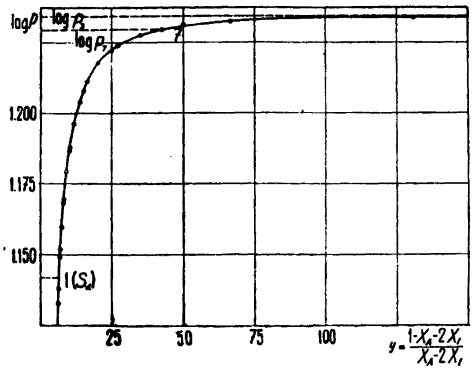


Fig. 5. Vapour pressure of solutions of d-tartaric acid in saturated racemic acid.
○ : experimental values
● : calculates from (11)

The values calculated according to (11) are plotted in Fig. 5. For the integral in equation (10) we obtain, for the part of the curve considered, by insertion of (11)

$$I_1 = \int_1^7 \frac{1 - x_A - 2x_l}{x_A - 2x_l} d\ln p = \int_1^7 2.303 y \frac{d \log p}{\mathrm{d}\ y} dy$$

$$= \int_1^7 2.303 \left(\frac{2.14}{6} \frac{1}{y} + \frac{244}{72} \frac{1}{y^2} \right) dy$$

Insertion of $y_1 = 6.370$ and $y_7 = 42.63$ gives

$$I_1 = 2.604$$

From Fig. 2b, curve 2, it appears that the product $x_d \cdot x_l$ to a good approximation varies linearly with x_A between $x_A = 0.02$ and $x_A = 0.03$. This relation was used in the calculation of the other partial integral, I_2. From

$$(x_A - x_l)\ x_l = -\frac{b}{4} x_A - \frac{a}{4} \tag{12}$$

we obtain

$$x_l = \frac{1}{2} \left(x_A - \sqrt{a + b x_A + x_A^2} \right) \text{ and}$$

$$x_A - 2x_l = \sqrt{a + b x_A + x_A^2} = \sqrt{z}$$

Insertion of this expression gives, for I_2,

$$I_2 = \int_7^3 \frac{1 - x_A - 2x_l}{x_A - 2x_l} \frac{d\ln p}{dx_A} dx_A = \int_7^3 \frac{1 - 2x_A + \sqrt{Z}}{\sqrt{Z}} \frac{d\ln p}{dx_A} dx_A$$

For $$\frac{d\ln p}{dx_A} = -\frac{1}{1 - x_A} + \frac{d\ln f_{H_2O}}{dx_A}$$ we insert

$$\frac{d\ln p}{dx_A} = k - 1 - x_A - x_A^2$$

using

$$\frac{1}{1 - x_A} = 1 + x_A + x_A^2 \text{ and}$$

$$\frac{d\ln f_{H_2O}}{dx_A} = k \text{ (cf. Fig. 6, curve 1).}$$

Consequently, we obtain for the whole expression

$$I_2 = \int_7^3 \frac{(1 - 2x_A + \sqrt{Z})\ (k - 1 - x_A - x_A^2)}{\sqrt{Z}} dx_A$$

where $Z = a + bx_A + x_A^2$.

In the integration we take no account of a cubic term:

$$I_2 = \left(k - 1 - \frac{b}{2} + bk + \frac{3}{8}b^2 - \frac{a}{2}\right) \ln\left(\sqrt{Z} + x_A + \frac{b}{2}\right)$$

$$+ \left(1 - 2k + \frac{x_A}{2} - \frac{3b}{4}\right)\sqrt{Z} + kx_A - x_A - \frac{x_A^2}{2} - \frac{x_A^3}{3}\Bigg|_{x_7}^{x_3}$$

If we introduce the limiting values $x_7 = 0.03$ and $x_3 = 0.02087$ and also

$$a = -5.403 \cdot 10^{-4}$$
$$b = 5.04 \cdot 10^{-3}$$
$$k = 1.61 \cdot 10^{-1}$$

which three values we obtain from Fig. 2b, curve 2, and Fig. 6, we get

$$I_2 = 0.969$$

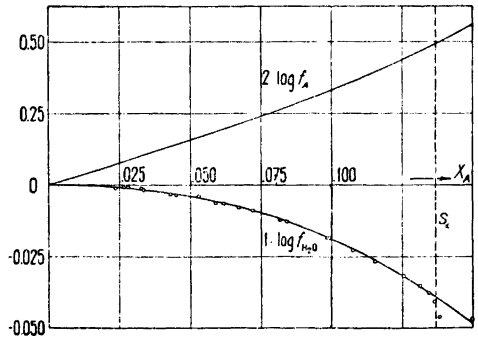


Fig. 6. Activity coefficients of tartaric acid and of water. Curves drawn according to (17) and (18).
O : experimental values

Hence, for the first term on the right side of equation (10) we get:

$$RT\ (2.604 + 0.969) = A_2^* = 2080 \text{ cal}$$

A_2^* is the affinity for the formation of 168 g *rh* from 75 g *d*, 75 g *l*, and 18 g H_2O, which are taken from solution 3 saturated with *rh*.

Consequently we obtain for the total affinity,

$$A^* = 2080 + RT \ln \frac{13.03}{17.15} = \mathit{1920} \text{ cal}$$

CALCULATION OF THE SOLUBILITY OF RACEMIC ACID IN TARTARIC ACID SOLUTIONS

The vapour pressure of mixtures of *d*- and *l*-solutions depends — as shown — upon the total acid concentration only and not on the relative quantities of the components. Consequently, the activity coefficients for both forms in a given mixture must be equal. Therefore, the solubility of racemic acid in tartaric acid solutions of varying concentration can be calculated on the basis of the vapour pressure curve of tartaric acid solutions and of the solubility of racemic acid in water. According to the law of mass action, we have for a solution saturated with respect to *rh*:

$$x_d \cdot f_d \cdot x_l \cdot f_l\ (1 - x_A)^2\ f_{H_2O}^2 = K'_a \tag{13}$$

an equation which also may be written as

$$(x_A - x_l)\ x_l \cdot f_A^2 \cdot p^2 = K_a \tag{14}$$

where K'_a and K_a are constants.

From (14) we derive, for the dependence between x_l and x_A,

$$x_l = \frac{x_A}{2} - \frac{1}{2}\sqrt{x_A^2 - \frac{4Ka}{f_A^2 \cdot p^2}} \tag{15}$$

f_A is found from $f_{H_2O} = \frac{p}{(1-x_A)\, p_o}$ by means of Gibbs-Duhem's equation

$$x_A \frac{d \log f_A}{dx_A} + (1-x_A) \frac{d \log f_{H_2O}}{dx_A} = 0$$

$$\log f_{A(i)} = \int_0^{x_{A(i)}} - \frac{1-x_A}{x_A} \frac{d \log f_{H_2O}}{dx_A} dx_A \tag{16}$$

The values of $\log f_{H_2O}$ are plottet against x_A, giving a curve (Fig. 6), which may be represented by the empirical equation

$$\log f_{H_2O} = -1.60\ x_A^2 - 164\ x_A^{4.85} \tag{17}$$

According to (16) and (17) we obtain

$$\log f_A = \log f_{H_2O} + 3.20\ x_A + 207\ x_A^{3.85} \tag{18}$$

From the solubility of *rh* in pure H_2O we find $K_a = 0.04347$, the pressure being expressed in mm Hg. By means of this value, x_l was calculated for a series of values of x_A. The results of these calculations together with the results found in the solubility determinations are given in Fig. 2b, curve 1. The excellent agreement between these values confirms the absence of a specific association of the *d*- and *l*-compound.

Racemic acid is seen to be very slightly soluble in saturated tartaric acid, its solubility being about 100 times lower in tartaric acid than in water. This fact can be utilized in the preparation of racemic acid from tartaric acid. After racemization of tartaric acid with NaOH the separation of racemic acid from simultaneously formed meso-tartaric acid and non-converted tartaric acid usually occurs via the Ca-salts and the acid K-salts [8]. This rather troublesome and not very efficient procedure can be replaced by the following. After racemization, HCl is added to the mixture until the color of added methyl orange has changed. *d*-tartaric acid is added until saturation, whereby racemic acid crystallizes almost quantitatively, while meso-tartaric acid remains in the

solution. The precipitate is filtered off and washed with a solution of 100 g of tartaric acid in 100 g water, and is then recrystallized from alcohol in which — as will be shown later — the solubility of tartaric acid can be assumed to be about 13 times higher than that of racemic acid.

OTHER METHODS OF AFFINITY CALCULATION

A calculation of the affinity, which in formal respect is somewhat simpler than that previously applied, can be performed on the basis of the principle of allotropic transformations. According to (2), a mixture of equal parts of *d*- and *l*-tartaric acid can be regarded as an instable modification of racemic acid, and the transformation affinity

$$\begin{aligned} A^* &= \tfrac{1}{2}\mu_{d(d)} + \tfrac{1}{2}\mu_{l(l)} - \mu_{r(r)} \\ &= \tfrac{1}{2}\mu_{d(d)} + \tfrac{1}{2}\mu_{l(l)} - (\tfrac{1}{2}\mu_{d(r)} + \tfrac{1}{2}\mu_{l(r)}) \\ &= \mu_{d(s-d)} - \mu_{d(s-r)} \end{aligned} \tag{19}$$

is identical with the racemization affinity.

According to (6), we have

$$A^* = \mu_{d(s-d)} - \mu_{d(s-rh)} + \mu_{H_2O(r-rh)} - \mu_{H_2O(s-rh)} \tag{20}$$

Hereby, $\mu_{d\,(s-r)}$, the chemical potential of *d* in the instable solution saturated with anhydrous racemic acid in water, becomes accessible to calculation. From (19) and (20) we obtain

$$\mu_{d(s-r)} - \mu_{d(s-rh)} = \mu_{H_2O(s-rh)} - \mu_{H_2O(r-rh)} \tag{21}$$

which can be transformed to

$$RT\ln\frac{x_{d(s-r)}\,f_{d(s-r)}}{x_{d(s-rh)}\,f_{d(s-rh)}} = RT\ln\frac{p_3}{p_{(r-rh)}} \tag{22}$$

By insertion of the values $x_{d\,(s-rh)} = 0.01044$, $f_{d\,(s-rh)} = 1.166$, $p_3 = 17.15$, and $p_{(r-rh)} = 13.03$ we obtain

$$x_{d\,(s-r)}\,f_{d\,(s-r)} = 0.01602 \tag{23}$$

If we insert this value and $x_{d\,(s-d)} = 0.137$ and $f_{d\,(s-d)} = 3.13$ (according to equation (18)) into equation (19) transformed to

$$A^* = RT\ln\frac{x_{d\,(s-d)}\ f_{d\,(s-d)}}{x_{d\,(s-r)}\ f_{d\,(s-r)}} \tag{24}$$

we obtain $A^* = 1910\ cal$ in agreement with the previously calculated value. The solubility of r in H_2O is calculated from (23) to

$$x_{r\,(s-r)} = 2x_{d\,(s-r)} = 0.0264$$

After transformation of the precipitate in the instable solution into the hydrate, the concentration decreases to $x_{r\,(s-rh)} = 0.0209$.

SOLUBILITY IN NON-AQUEOUS SOLVENTS

If the ideal laws can be applied to the saturated solutions, the equation (24) can be simplified to

$$A^* = RT\ln\frac{x_{d\,(s-d)}}{x_{d\,(s-r)}} = RT\ln\frac{2\ x_{d\,(s-d)}}{x_{r\,(s-r)}} \tag{25}$$

It is a supposition for the applicability of (25) that the solubilities in the solvent concerned are low and that no mutual association of the tartaric acids occurs in the solutions. On the other hand, an association of the tartaric acids with the molecules of the solvent can be advantageous to the applicability of (25) by counteracting the mutual association. It is, therefore, natural to use a solvent of polar character, since the association tendency of tartaric acid must be assumed to originate from the presence of carboxyl groups and hydroxyl groups. The question whether the conditions for the validity of (25) are fulfilled is best decided by performing solubility experiments in different solvents, as the affinity and consequently the ratio $\frac{x_{d\,(s-d)}}{x_{d\,(s-r)}}$ must be independent of the solvent.

Solubility determinations were performed in glacial acetic acid and in isobutyl alcohol. *C.* 15 g of the solvent were shaken in a thermostat with an excess of the respective acid. *C.* 10 g of the saturated solution were weighed accurately and evaporated in vacuo. The solubility was calculated from the evaporation residue. The results are given in Table 1.

From the values at 20° C the solubility quotient was calculated:

$$\underset{\text{glacial acetic acid}}{\frac{x_{d\,(s-d}}{x_{r\,(s-r)}}} = 12.80 \text{ and } \underset{\text{isobutyl alcohol}}{\frac{x_{d\,(s-d)}}{x_{r\,(s-r)}}} = 12.82$$

Table 1. Solubilities of tartaric acid and racemic acid in non-aqueous solvents.

Solute	Solvent	Temp °C	g acid / g sol.	x_A
tartaric acid	glac.acetic acid	20	0.01395	.005633
racemic acid	» » »	20	0.00110	.000440
tartaric acid	» » »	25	0.01662	.006716
racemic acid	» » »	25	0.00130	.000523
tartaric acid	iso-butylalcohol	20	0.04644	.02349
racemic acid	» »	20	0.00372	.00183

The agreement between these values is in favour of the applicability of equation (25). For the affinity we obtain, consequently,

$$A^* = RT\ln\ 25.62 = 1890\ cal$$

The agreement with previously found values must be considered satisfactory.

HEAT OF RACEMIZATION

The relation between racemization affinity and heat of racemization ΔH is given by the equation

$$\Delta H = T^2 \frac{d\left(\frac{A^*}{T}\right)}{dT} \tag{26}$$

or, according to (25), by

$$\Delta H = RT^2 \frac{d\ln \frac{x_{d\,(s-d)}}{x_{r\,(s-r)}}}{dT} = q_d - q_r \tag{27}$$

where q_d and q_r according to the method of derivation are the differential heats of solution per mole acid in the saturated solutions in question. The heat of racemization can, therefore, be determined on the basis of the temperature

coefficients of the solubilities. From the solubilities in glacial acetic acid at 20° C and 25° C given in Table 1 we calculate

$$q_d = 6150 \text{ cal} \qquad \text{and} \qquad q_r = 6000 \text{ cal}$$

Hence,

$$\Delta H = 150 \text{ cal}$$

This value is, however, rather uncertain, as ΔH is obtained as a difference between two much higher values. From these values, therefore, we can only conclude that the heat of racemization is low, much lower than the affinity. A transition temperature, if any, between $d + l$ and r, is therefore very far from the temperatures used here.

SUMMARY

1. The theoretical basis for the determination of the racemization affinity is discussed.

2. The solubility of racemic acid in tartaric acid solutions of varying concentrations is determined.

3. The vapour pressure of tartaric acid solutions and of saturated racemic acid in tartaric acid solutions is measured. The results show that no detectable specific association of the *d*- and *l*-form is found in solution.

4. The affinity of the process $d \longrightarrow r$ is calculated on the basis of the experimental results according to the principle of double salt formation. The value found is 1920 cal/150 g.

5. The solubility of racemic acid in tartaric acid solutions of different concentrations is calculated from the vapour pressure curve of tartaric acid and from the solubility of racemic acid in water. A method is outlined for the preparation of racemic acid, which is based upon the low solubility of racemic acid in strong tartaric acid solutions.

6. The racemization affinity is calculated according to the principle of allotropic transformation on the basis of the solubility of tartaric acid and of anhydrous racemic acid in water, glacial acetic acid, and isobutyl alcohol, respectively. The values found are 1910 cal/150 g and 1890 cal/150 g, respectively.

7. The heat of racemization is calculated on the basis of solubility determinations in glacial acetic acid to be 150 cal/150 g.

The author is indebted to the late Professor Y. N. Brønsted for his interest in this work.

REFERENCES

1. Brønsted, J. N., *Kgl. Danske Videnskab. Selskabs., Naturvidenskab. math. Comm.* Series 7 **XII**, No. 6 (1915) 243.
2. Brønsted, J. N. *Kgl. Danske Videnskab. Selskab., Mat.-fys. Medd.* I, **5** (1918) 3.
3. Prytz, K. *Ann. Physik* **4** (1905) 16, 735.
4. Raoult, F. M. *Z. physik. Chem.* **1** (1887) 186.
5. Leidie, E. *Z. anal. Chem.* **22** (1883) 269.
6. Kuhn, R., and Wagner-Jauregg, T. *Ber.* **61** (1928) 487.
7. Partington, J. R. *J. Chem. Soc.* **99** (1911) 466.
8. *Cf. e. g. Org. Syntheses* **6** (1926) 82.

Received November 14, 1947.

ACTA CHEMICA SCANDINAVICA 2 (1948) 759—769

Hydrolysis of Glycogen with Hydrochloric Acid

BIRGER CARLQVIST

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

Both amylopectin and glycogen are now regarded as polysaccharides built up of ramified glucose chains with α-glucosidic linkages. In most cases, methylated glycogen from different animals yields about 9 % of tetramethylglucose[1-4]. Bell[5] and Haworth[6a] found, however, certain samples containing only 6 % of end-groups. Among the hydrolysis products of methylated glycogen the only tri-derivative formed is 2,3,6-trimethylglucose. The yields of di- and tetramethylglucose should be equal, but the values for the former substance are unreliable since it cannot be obtained in a pure state. Haworth *et al.*[6a] found that nitric acid oxidized the dimethylglucose fraction mainly into *d*-dimethoxy succinic acid, which must have come from 2,3-dimethylglucose. In a recent paper, Bell[6b] states that trimethylglucose can also produce this compound, and since it is very difficult to separate the various methylglucoses the result obtained by Haworth is inconclusive as to the position of the interchain linkage (the point of ramification). Most of the linkages in glycogen are thus of the 1,4-type as in amylopectin, but there is a marked difference between these polysaccharides in the end-group content and the chain-length. Glycogen does not contain any »amylose fraction» with unbranched chains as in the case of starch[7].

In these experiments, a preparation of Glycogen Hoffmann-La Roche was used. A 5 % solution was prepared and precipitated by alcohol. The precipitate was centrifuged off and dried *in vacuo*. The ash content was 0.27 %. $[\alpha]_D = 196.5° - 197.5°$ ($c = 1$—2). The consumption of iodine, measured by Willstätter and Schudel's method, corresponded to a reducing power of about 1 %, calculated as glucose, and was the same as that of the unprecipitated preparation. The reducing power determined by means of a copper method was less than 0.1 %. (Different samples of starch also showed a small consumption of iodine corresponding to nearly 0.5 % glucose.)

ACID HYDROLYSIS

The hydrolysis of chain molecules has been studied by many investigators (summary by Sillén [17]). Freudenberg *et al.* [8, 9] found that maltose was more rapidly broken down with 50 % sulfuric acid than was starch. They showed also that the velocity of hydrolysis increased in the order cellulose < cellotetraose < cellotriose < cellobiose. In 1930 Kuhn [10] deduced some formulae for hydrolysis of chain molecules using two velocity constants. In an investigation of starch at this institute [11], we found that the best agreement was obtained if we assumed one of the two terminal linkages to be ruptured at the rate of 1.7 k and the other linkages at the rate of k. Myrbäck and co-workers [12] found that starch dextrins containing 1,6-linkages were hydrolyzed more slowly than starch itself.

Experiments

Glycogen was hydrolyzed with hydrochloric acid, and the progress of the reaction was measured by means of iodometric titration.

The reducing power of a dextrin or a sugar solution was determined by Willstätter and Schudel's method, modified by Myrbäck and Örtenblad [13]. The solution, which must be neutral, was mixed with about twice the necessary amount of 0.15 N iodine solution. A slight excess of 0.2 N NaOH was added drop by drop for five minutes. The solution was shaken continuously and, after 15 minutes in all, acidified with 4 N sulfuric acid and titrated with 0.1 N thiosulfate solution. A blank containing no sugar was treated in the same way.

41 g of glycogen, precipitated by alcohol, was dissolved in 1991 ml of water and boiled for one hour over a sand bath in a flask fitted with a reflux condenser. 8.7 ml of conc. HCl was added. At various intervals of time, samples of about 200 ml were withdrawn and neutralized with solid sodium carbonate, cooled and kept under toluene.

A sediment appearing after a few days was filtered off. (The amount was about 0.3 % of the total substance.)

The total amount of carbohydrate in each of these solutions was measured in three ways.

1. The optical rotation was measured. Before the addition of acid the rotation was 3.58° (1 dm tube) and at the end of the reaction it was 1.09°. These values correspond to concentrations of 2.02 % and 2.08 % resp. as the specific rotation of glycogen is 177° (calculated for the hydrate) and that of glucose 52.5°. In this paper, the concentration of a solution is calculated for the hydrate (maltose as $C_{12}H_{22}O_{11} + H_2O$, glycogen as $C_6H_{10}O_5 + H_2O$ etc.).

2. 5 or 10 ml of a solution was evaporated to dryness, and after one day at 105°, the residue was weighed. As the substance is obtained in anhydrous form the measured values must be multiplied by a factor, which is 1 for glucose, 1.052 for maltose, 1.11 for glycogen etc. In this case, the values must also be corrected for the amount of salt. This method gave values between 2.00 % and 2.12 %.

3. The solution was hydrolyzed completely with N HCl for 3 hours at 100°, and the amount of glucose thus formed was titrated iodometrically. In this case, values between 2.03 % and 2.07 % were obtained. It can thus be considered the most accurate method.

The reducing power of the various solutions was determined. Finally, the degree of hydrolysis, α, was calculated (*i. e.* the ratio between the number of free, reducing groups and the total amount of glucose units).

At the beginning of the hydrolysis, the concentration of acid was 0.049 N, but this value had increased to 0.052 N after 23 hours. This may be due to formation of laevulinic acid or other acids. However, evaporation of water would have the same effect.

The unimolecular constant, k, was calculated from the formula

$$k = \frac{1}{t_2 - t_1} 2.303 \log_{10} \frac{1 - \alpha_1}{1 - \alpha_2}$$

The values of α and of k are given in Table 1.

Table 1. Hydrolysis of 2.05 % glycogen solution with 0.049 N HCl at 100°.

Fraction no.	t hours	Degree of hydrolysis %	k hours $^{-1}$
1	0.33	4.0	0.122
2	0.67	8.0	0.128
3	1.00	11.9	0.133
4	1.77	22.0	0.157
5	2.50	30.1	0.150
6	4.65	57.0	0.226
7	8.67	83.8	0.241
8	17.3	96.5	0.202
9	22.9	98.5	0.151
10	41.0	(101)	
		Extrapolated value $k_0 = 0.120$	

As can be seen from the table, the values of k increase at first, and at the end of the reaction they tend to decrease. During the course of hydrolysis, there are formed low-molecular dextrins which are broken down more rapidly, thus causing the increase in k. Branched dextrins containing more stable linkages than the 1,4-type must amount to a relatively larger portion at the end of the reaction, thus accounting for the decrease in the k values.

Freudenberg *et al.* [8] and Myrbäck *et al.* [11, 12] have obtained similar results in the case of starch. On the other hand, Swanson and Cori [14] found that k was nearly constant all the time on hydrolysis of amylose, amylopectin and glycogen. In those experiments, however, the temperature was lower and the concentration of the acid was much higher than in this work.

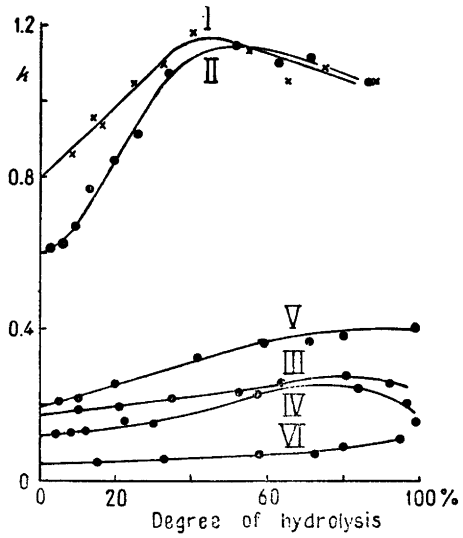


Fig. 1. The variation of the unimolecular constant, k, with the degree of hydrolysis.

I: Starch, 0.215 N HCl.
II: Glycogen, 0.215 » »
III: Starch, 0.049 » »
IV: Glycogen, 0.050 » »
V: Dextran, 0.21 » »
VI: » 0.05 » »

A hydrolysis experiment similar to the first one mentioned was carried out with slightly stronger acid (Table 2).

Table 2. Hydrolysis of 2.2 % glycogen solution with 0.063 N HCl at 100°.

No.	t hours	Degree of hydrolysis %	k hours $^{-1}$
I	0.38	5.6	0.151
II	1.00	14.8	0.173
III	2.20	31.8	0.185
IV	4.29	61.8	0.295
V	7.24	83.0	0.275
		Extrapolated value k_0	= 0.145

HYDROLYSIS OF MALTOSE, STARCH AND DEXTRAN

In order to study the relation between the velocity constant and different types of bonds, hydrolysis experiments on maltose, starch and dextran were also carried out under the same conditions. If the values of k are plotted against the degree of hydrolysis, curves of characteristic form are obtained (see Fig. 1). There is a marked difference between starch and glycogen (curves I—II and III—IV). In the beginning, the k values of glycogen increase more rapidly than those of starch. In the middle of the hydrolysis, there is only a small difference, thus indicating that the low-molecular dextrins from both

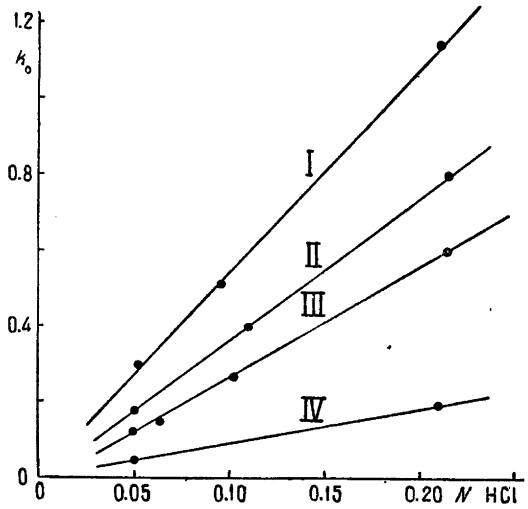


Fig. 2. The relation between the initial rate constant, k_0, and the acid strength.

I: Maltose.
II: Starch.
III: Glycogen.
IV: Dextran.

glycogen and starch are hydrolyzed with nearly the same velocity. The k values of dextran increase steadily during the course of the reaction (curves V and VI). In the case of maltose, k is nearly constant all the time.

The extrapolated value, k_0, from the curves in Fig. 1 is a measure of the initial velocity. From Fig. 2, it appears that k_0 is proportional to the concentration of acid. When the values of α in Table 1 or 2 are plotted against the time, a practically straight line is obtained up to about 50 % of breakdown. The slope of this line has a value that is only slightly higher than that of k_0. Similar results were obtained on hydrolysis of starch and dextran. The first phase of the hydrolysis seems thus to follow a zero-order reaction, but it is impossible to explain such a mechanism. Successive, unimolecular reactions with increasing velocity constants can also show the same effect.

The ratio between the k_0 of maltose and that of starch, glycogen and dextran is calculated for 0.050 N and 0.200 N acid (Table 3).

Table 3. The ratio between various k_0 values.

Concentration of the acid	$\frac{k_0 \text{ maltose}}{k_0 \text{ starch}}$	$\frac{k_0 \text{ maltose}}{k_0 \text{ glycogen}}$	$\frac{k_0 \text{ maltose}}{k_0 \text{ dextran}}$
0.050 N	1.54	2.24	6.00
0.200 N	1.48	1.93	6.00

Dextran formed by certain species of *Leuconostoc* contains mainly α-glucosidic 1,6-linkages. According to Table 3, dextran is hydrolyzed six times

more slowly than maltose. Under quite different conditions, Swanson and Cori [14] found the rate to be 11—15 % of that of maltose. It may be assumed that the 1,4-linkages in the interior parts of a long chain are split with the initial rate of hydrolysis of starch, and the same relation between the 1,6-linkages and dextran is also assumed. On these assumptions, the rate constant of a 1,4-linkage is about $6/1.5 = 4$ times greater than that of a 1,6-linkage. In the present investigation, I have reckoned with interchain linkages of the 1,6-type, since the rate constants have not been determined for any other type. The possibility of the presence of 1,3 and 1,2-linkages is not out of the question; in any case, however, the interchain linkages of glycogen are probably more stable than the 1,4-linkage. The number of interchain linkages must be almost the same as of the end-groups, *viz.* 9 % for glycogen and 4.5 % for starch. Hence one would expect the difference in the k_0 values to be $(9—4.5) \cdot 0.75$ or about 3 %, as the relative difference in the rate of hydrolysis between the two types of bonds is 1—0.25. The effect observed is, however, much greater, about 30 %.

CALCULATIONS BY MEANS OF TWO VELOCITY CONSTANTS

At first, it is convenient to consider only long, straight chains, assuming the one terminal linkage (as in maltose) to be hydrolyzed at the rate of k_0 $(1 + p)$ and the other linkages at the rate of k_0. The values 1.93 and 2.24 in Table 3 would then mean that p is about 1 in the case of glycogen. According to Sillén [17], there is the following relation between the degree of hydrolysis, α, and $k_0 t$

$$1 - \alpha = e^{p(1-x)}\, x^{1+p} \quad \text{where } x = e^{-k_0 t}$$

The values of $1 - \alpha$ obtained by means of this equation are compared with the experimental ones in Table 1. The agreement is good only when $p = 2$ (see Table 4), and from this fact one can conclude that the velocity »constant», k, increases more rapidly than the rate of formation of new terminal linkages. If we now take into consideration the lower rate of splitting the 1,6-linkages, this correction becomes $9 \cdot 0.75$ or about 7 %. Instead of k_0 the value 1.07 k_0 ought to be used, but even after this correction, the agreement is still not good when $p = 1$, which should be expected from the ratio of the k_0 values in Table 3.

Table 4. Comparison between the observed and the calculated values of 1—a.

No.	k_0t	1—a			
		Obs.	Calc. for		
			$p = 2$	$p = 1$	$p = 0.5$
2	0.081	0.920	0.914	0.915	0.920
3	0.120	0.881	0.880	0.881	0.885
4	0.212	0.780	0.780	0.789	0.798
5	0.300	0.699	0.685	0.715	0.721
6	0.558	0.430	0.431	0.508	0.542
7	1.04	0.162	0.161	0.238	0.290
8	2.08	0.035	0.018	0.042	0.085

FERMENTATION EXPERIMENTS

Glucose and maltose can be determined separately by fermentation with Swedish baker's yeast. At a pH of about 5, glucose is fermented much more rapidly than maltose. Myrbäck and Leissner [15] have shown that also maltotriose, containing two 1,4-linkages, is fermented with about the same velocity as maltose.

Method

The sugar solution was evaporated on a water bath to remove all traces of toluene. Small tubes were used, each containing the solution thus obtained (about 2 ml) and 1 ml of a yeast suspension. This was prepared by suspending 40 g of baker's yeast in 100 ml of a phosphate buffer (1/15 *M*, pH = 5.3). The tubes were connected with a gas buret, immersed into a thermostat at 30° and shaken mechanically. Readings were made after 5 minute intervals, and the amount of CO_2 was plotted against the time (Fig. 3). Parallel experiments with known amounts of glucose and maltose must be carried out, because the formation of carbon dioxide is not stoichiometric. (Many examples of differential fermentation in [11, 15, 16]).

Sillén [17] has derived the following equations relating to the change in the amount of fermentable sugar with the degree of hydrolysis:

$$\text{Glucose} = 1-(1-\alpha)\ (2-x) \text{ where } 1-\alpha = e^{p(1-x)}x^{1+p}$$
$$\text{Maltose} = 2(1-x)^2\ (1-\alpha)$$
$$\text{Maltotriose} = 3(1-x)^2\ (1-\alpha)x$$

The experimental values of fermentable sugar (Table 5) were plotted against the degree of hydrolysis, and the corresponding curves for the values calculated

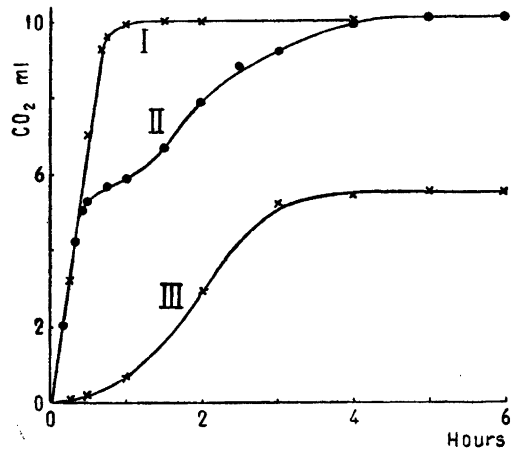


Fig. 3. Fermentation diagram. Fermentation of

I: 50.5 mg of glucose,
II: 10 ml of solution no. 4,
III: 29.4 mg of maltose hydrate.

by means of Sillén's formulae were drawn in the same figure. A few values from this graph are listed in Table 6.

Table 5. The amount of fermentable sugar in the solutions no. 1—8 and no. I—V. (Average of three similar experiments.)

No.	Glucose %	Maltose fraction %	No.	Glucose %	Maltose fraction %
1	1.0	—	8	93.1	—
2	(2.0)	3.1	I	1.2	2.0
3	1.8	6.8	II	6.3	7.4
4	11.1	12.0	III	18.0	15.6
5	19.0	16.9	IV	47.0	15.0
6	43.1	16.1	V	(78.1)	6.1
7	72.9	5.0			

Table 6. Comparison between the observed and the calculated amounts of fermentable sugar.

Degree of hydrolysis %	Glucose %			Maltose fraction %		
	Obs.	Calc. for		Obs.	Calc. for	
		$p = 2$	$p = 1$		$p = 2$	$p = 1$
20	9.0	6.3	5.0	10.5	10.9	11.6
30	16.1	13.0	11.0	16.4	18.1	20.6
40	24.8	21.0	19.0	19.9	24.5	28.9
50	34.5	30.4	28.5	19.6	28.5	34.2
60	44.9	42.0	39.6	16.0	30.0	36.0
70	57.0	54.0	52.5	11.4	28.5	34.0
80	69.4	67.6	66.8	7.2	23.6	27.2
90	83.9	83.1	82.9	3.1	15.0	15.9

Considering first the values of glucose, the agreement is best when $p = 2$. The experimental values of maltose + maltotriose are, however, much smaller than the calculated ones. This must be due to the branched, unfermentable dextrins.

As an example, the calculation of the chain-length for these branched dextrins is made in the case of solution no. 7. The unfermentable sugar amounts to 100—72.9—5.0 = 22.1 %, and the degree of hydrolysis is 83.8 % (Tables 1 and 5). An estimate of the ratio maltose/maltotriose by means of the above-mentioned formulae gives a value of about 2. The reducing power of the maltose fraction is then $2/3 \cdot 1/2 + 1/3 \cdot 1/3 = 4/9$ of the same amount of glucose. The reducing power of the unfermentable part is thus 83.8—72.9—$4/9 \cdot 5.0$ = 8.7 %, and the average degree of polymerization is $\bar{n} = 22.1/8.7 = 2.54$. There must therefore be a large amount of di and trisaccharides with linkages other than the 1,4-type at high degrees of hydrolysis.

CALCULATIONS BY MEANS OF THREE VELOCITY CONSTANTS

Sillén has also deduced more complicated formulae for branched molecules (not published), assuming the interchain linkages to be broken with a lower velocity constant, $q \cdot k_0$. As mentioned before, the 1,4-linkages are hydrolyzed about 4 times more rapidly than the 1,6-bonds, which means that the value of q is about 0.25. Numerical calculations of $1-a$ and of the fermentable sugar have been made for different values of p and q (Tables 7 and 8).

On comparison between Tables 6 and 8, it is clear that the effect of introducing the constant q is negligible in the case of glucose, but the calculated values of the maltose fraction are lowered considerably. By these calculations the amounts of all types of saccharides can be computed so that only unbranched di- and trisaccharides are considered. The calculated amounts of the

Table 7. Comparison between the observed and the calculated values of 1—a. Formulae with two or three velocity constants are used.

No.	$k_0 t$	$1-a$				
		Obs.	Calc. for			
			$p = 2,\ q = 0.2$	$p = 2$	$p = 1,\ q = 0.2$	$p = 1$
III	0.319	0.682	0.670	0.672	0.708	0.695
IV	0.622	0.382	0.400	0.382	0.505	0.458
V	1.05	0.170	0.224	0.165	0.302	0.234

Table 8. Comparison between the observed and the calculated amounts of fermentable sugar. Formulae with three constants are used.

Degree of hydrolysis %	Glucose %			Maltose fraction %			
	Obs.	Calc. for		Obs.	Calc. for		
		$p = 2$ $q = 0.2$	$p = 1$ $q = 0.2$		$p = 2$ $q = 0.2$	$p = 1$ $q = 0.2$	$p = 1$ $q = 0.5$
30	16.1	13.9	13.0	16.4	17.1	17.4	17.7
50	34.5	31.5	29.5	19.6	21.6	25.2	27.0
70	57.0	55.0	53.0	11.4	16.1	22.0	24.4
90	83.9	83.1	83.0	3.1	4.0	5.1	9.0

maltose fraction in Table 8 are only slightly larger than the observed ones when $p = 2$ and $q = 0.2$. Concerning the values of $1-a$, the agreement is, however, not so good for $p = 2$ and $q = 0.2$ as for $p = 2$ alone (Table 7).

These experiments show that the simple formulae that seem to be valid for starch do not hold good in the case of glycogen. The velocity of hydrolysis increases more rapidly than the rate of formation of the new, terminal linkages, and this shows that the other bonds are not split at the same rate as could be assumed in the case of starch. It is possible that, for instance, the linkages in the short interior chains, *i. e.* the chains between two ramifications, are more protected. (The average length of these chains is, according to Meyer and Fuld [7], only about 3 units.) These effects must be more obvious in the case of glycogen than of starch. It is of little value to introduce more complex formulae since there is so little known of the hydrolysis of different types of linkages.

On hydrolysis of glycogen, starch and dextran, the optical rotation was measured, and the specific rotation was plotted graphically against the degree of hydrolysis. Practically straight and coincident lines were then obtained so that, in this way, no difference between the linkages could be proved.

In a subsequent paper, the enzymatic experiments with glycogen will be described.

SUMMARY

The hydrolysis of glycogen, starch, dextran and maltose with hydrochloric acid has been studied. Maltose is hydrolyzed about twice as rapid as glycogen under the same conditions. Different formulae for hydrolysis of straight and branched chain molecules deduced by Sillén have been discussed.

REFERENCES

1. Haworth, W. N., and Percival, E. *J. Chem. Soc.* (1932) 2277.
2. Bell, D. J. *Biochem. J.* **31** (1937) 1683.
3. Meyer, K. H. *Naturwissenschaften* **29** (1941) 287.
4. Hassid, W. Z., and Chaikoff, J. L. *J. Biol. Chem.* **123** (1938) 755.
5. Bell, D. J. *Biochem. J.* **30** (1936) 1612, 2144.

6a. Haworth, W. N., Hirst, E. L., and Isherwood, F. A. *J. Chem. Soc.* (1937) 577.

6b. Bell, D. J. *Biol. Rev.* **23** (1948) 256.

7. Meyer, K. H., and Fuld, M. *Helv. Chim. Acta* **24** (1941) 375.
8. Freudenberg, K., Kuhn, W., Dürr, W., Bolz, F., and Steinbrunn, G. *Ber.* **63** (1930) 1510.
9. Freudenberg, K., and Blomqvist, G. *Ber.* **68** (1935) 2070.
10. Kuhn, W. *Ber.* **63** (1930) 1503.
11. Myrbäck, K., and Magnusson, B. *Arkiv Kemi, Mineral. Geol.* **A 20** (1945) no. 14.
12. Myrbäck, K., Örtenblad, B., and Ahlborg, K. *Biochem. Z.* **307** (1940) 53.
13. Myrbäck, K., and Örtenblad, B. *Svensk Kem. Tid.* **50** (1938) 72.
14. Swanson, M. A., and Cori, C. F. *J. Biol. Chem.* **172** (1948) 797.
15. Myrbäck, K., and Leissner, E. *Arkiv Kemi, Mineral. Geol.* **A 17** (1943) no. 18.
16. Myrbäck, K. *Arch. Biochem.* **14** (1947) 53.
17. Sillén, L. G. *Svensk Kem. Tid.* **55** (1943) 221.

Received September 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 770—782

Enzymatic Breakdown of Glycogen

BIRGER CARLQVIST

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

The acid hydrolysis of glycogen was described in a previous paper [1]. In this paper we shall deal with the enzymatic breakdown of glycogen and of partly acid hydrolyzed glycogen. Experiments with periodate oxidation have also been carried out and the »formic-acid method» for determining the end-group content of glycogen applied.

ENZYMATIC INVESTIGATIONS

Myrbäck and Sillén [2,3] have attempted to calculate the constitution of amylopectin and glycogen from simple assumptions on the mechanism of the enzymatic synthesis. Lengthening of the chains occurs at carbon atom 4 at a certain rate and ramification at carbon atom 6 at another (lower) rate. Assuming that the ratio between these rates is constant, they have shown that even at moderately low degrees of polymerization the degree of branching assumes a constant value. It is also possible to calculate the distribution of the glucose units in chains of different lengths, as well as the average chain-length. The following two extreme cases are considered:

A. The ramification may occur at every glucose unit having a free hydroxyl group at carbon atom 6.

The problem of the actual position of the branching is, however, of no importance for these calculations.

B. The ramification may occur at the terminal group only.

The following calculations refer to glycogen assuming the end-group content to be 9 %. Provided that at least 1 or 2 units of the end-chain must remain at the branching point, β-amylase will yield 53 % or 45 % of maltose (case A) and 39 % or 33 % of maltose (case B). Meyer and Jeanloz [4] found the yield to be 43—47 %, while the experiments in this paper give slightly lower values: 38—46 % of maltose. The theoretical values for the average chain-length of

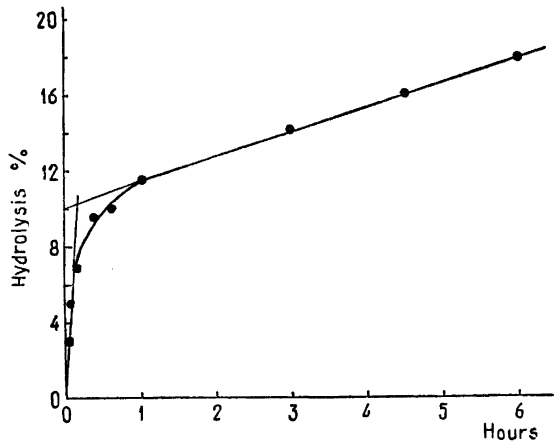


Fig. 1. The time course of the hydrolysis of glycogen with α-amylase.

the interior and the end-chains are 2.8 and 6.4 respectively (case A) and 4.3 and 4.8 respectively (case B). Meyer and Fuld [5] found that the β-dextrin contained 18 % of end-groups. Hence they concluded that the interior chains, *i. e.* the chains between two ramifications, have an average length of about 3 units and the end-chains of about 6 units.

From these results it is evident that the structure of a glycogen molecule corresponds to an intermediate state between the extreme states, but more close to A than to B. Similar results have been obtained in the case of starch (amylopectin) [3].

a) α-Amylase

In germinating barley there is formed or activated an enzyme, called dextrinogenic or α-amylase. According to Ohlsson [6], it is rather stable at increased temperatures and can easily be separated from β-amylase which is rapidly destroyed above 70°.

Experimental part

Preparation of α-amylase. 200 g of ground malt was extracted for 45 minutes with 1 l of cold water, and the suspension thus obtained was kept at 70° for 12 minutes. After filtration, alcohol was added so that the concentration became 70 % by volume. The mixture was kept at 0° for one hour. The precipitate was then centrifuged off, washed with 95 % alcohol and dried *in vacuo*. Finally, a water solution was prepared.

Performance of the experiments. 25 ml of 2—3 % substrate solutions, 5 ml of a phosphate buffer (1/15 *M*, pH = 5.2) and 1 ml of the enzyme solution were made up with water to 50 ml in a flask fitted with a glass stopper. A few drops of toluene were added, and the flask was kept at 30° in a thermostat. Just after adding the enzyme and at suitable intervals, 5 ml of the solution was pipetted into an iodine solution, and the reducing power was determined. A blank containing no substrate was treated in the same manner.

The result from an experiment with glycogen is shown in Fig. 1. The hydrolysis curve consists of two separate branches. This fact has been stated many times by Myrbäck and co-workers [7–10] in the case of starch and also of glycogen [3]. After about 10 % of loosened linkages, the reducing power increases very slowly. The practically straight lines are extended until they meet, and the point of intersection corresponds to 10.3 %. The mean value of four experiments was 10.1 ± 0.3 %.

The various dextrin preparations described in a previous paper [1] were also treated with α-amylase. The number of rapidly broken linkages was calculated from the curves of hydrolysis as the difference between the reduction value at the point of intersection and the original degree of hydrolysis (Table 1).

Table 1. Treatment of various dextrin solutions (hydrolyzates) with α-amylase.

No.	Original degree of hydrolysis %	Number of rapidly broken linkages %	No.	Original degree of hydrolysis %	Number of rapidly broken linkages %
1	4.0	8.0	6	57.0	—
2	8.0	5.9	I	5.6	7.4
3	11.9	3.5	II	14.8	4.0
4	22.0	1.9	III	31.8	1.5
5	30.1	1.0	IV	61.8	(0.5)

At degrees of hydrolysis > 30 %, the action of this amylase is insignificant. As described in a previous paper [1], the average chain-length of the unfermentable part of the dextrins can be estimated. Thus the value of $\bar{n} = 6.1$ is obtained for solution no. III, and according to these experiments, shorter chains contain no rapidly broken linkages. (Shorter chains can, of course, be ruptured during the slow, second phase, the saccharification). Myrbäck and Thorsell [10] found that the dextrins formed during the dextrination phase of *amylose* chiefly consisted of 4—7 units.

b) Fractionation of α-dextrins

In order to investigate the α-dextrins more closely, the reaction is interrupted when 10 % of the linkages are broken, and the dextrin mixture is fractionated with alcohol.

Method

2.81 g of glycogen was dissolved in 96 ml of water. 2 ml of the phosphate buffer (pH = 5.3) and 2 ml of the α-amylase solution were added. When the degree of hydrolysis

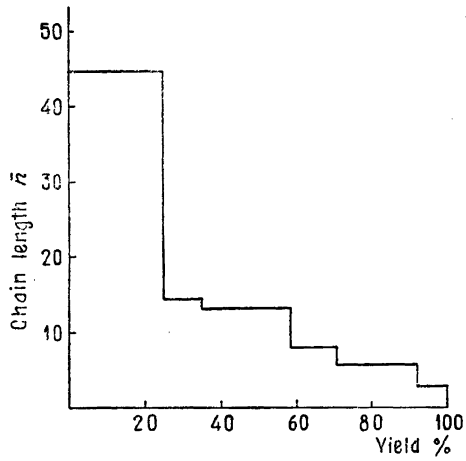


Fig. 2. Fractionation of α-dextrins. The corrected yield and the average chain-length of the various fractions.

was 10.6 % the solution was boiled for a few seconds. The dextrins were fractionated with alcohol of concentrations between 40 and 90 % by volume. The precipitates were centrifuged off, dried *in vacuo* and then dissolved in water. The concentrations of these solutions were determined in three ways: by means of total hydrolysis, by weighing of the dried substance and by the optical rotation. The reducing power was also determined by means of an iodometric method (see the previous paper[1]), and the average degree of polymerization, $\bar{n}$, could then be calculated. The loss of substance was 7 %, and the yields of the fractions were corrected for this.

Most of the dextrins have chain-lengths between 3 and 15 units, but there is also a fraction consisting of rather high-molecular dextrins, about $\bar{n} = 45$ (see Fig. 2). In the corresponding experiments with starch [9], no higher dextrins than $\bar{n} = 14$—19 occur. From this fact, it must be concluded that many interior chains in glycogen are so short as to remain intact; this is also proved later on in this paper. (On the other hand, Myrbäck and Sillén [3] have shown that the interior chains in starch [amylopectin] are ruptured about once.)

Part of the fraction $\bar{n} = 2.7$ was fermented, and the amount of glucose and of maltose + maltotriose was 1 % and 4 % respectively of the total amount of glycogen used. The formation of fermentable sugar during the course of dextrination is thus little or none as these saccharides can also be formed from the primary dextrins.

Part of the fraction $\bar{n} = 13.3$ was treated with β-amylase for 5 days. The reducing power remained constant after a breakdown of 39.4 %, calculated as maltose. The corresponding experiment on the fraction $\bar{n} = 5.8$ gave the value 89 % (as maltose). Obviously the lower dextrins consist to a large extent of unbranched chains, whereas the higher dextrins are more ramified since these are saccharified only partly. Myrbäck [11] has shown that most of the

α-dextrins from starch with chain-lengths of 6—7 units are normally built, while the larger dextrin molecules (about $\bar{n} = 12$—18) probably contain one branching point. The normal α-dextrins from both glycogen and starch are evidently very similar.

c) β-Amylase

Experiments with β-amylase from barley show that the enzyme splits off maltose from the non-reducing end of a chain with 1,4 - α-glucosidic bonds (summary by Myrbäck [8]). Thus only the end-chains of ramified molecules can be attacked, leaving behind a high-molecular residue, the β-dextrin, which contains the interior and fractions of the end-chains and the branching units.

Preparation of β-amylase from ungerminated barley

200 g of ground barley was extracted for 45 minutes with 1 l of cold water. After filtration, alcohol was added until the concentration became 75 % by volume, and the mixture was kept cold for one hour. The precipitate was then centrifuged off, washed with 95 % alcohol, dried *in vacuo* and finally dissolved in water. According to Blom [13], the traces of α-amylase which are always present were inactivated by adding acetic acid until pH = 3.6. The solution was kept at this pH and at 0° for two weeks. It was then decanted and sodium hydroxide was added to pH = 5.3.

These experiments were carried out as described above for α-amylase. The course of hydrolysis of glycogen and starch is shown in Table 2.

Table 2. Enzymatic breakdown of glycogen and potato starch with β-amylase. (Calculated as percentage of loosened linkages.)

Time (hours)	0.25	0.5	2	5	24	72	96	192
Glycogen	10.2	11.5	14.4	15.9	17.5	19.1	20.2	21.2
Starch	10.3	18.5	24.0	28.5	30.2	30.9	30.6	31.4

A number of glycogen experiments proceeding for 8—15 days gave values between 19 and 23 %. At first the rate of hydrolysis of glycogen is as high as that of starch, but when about 10 % of the linkages are broken it is much lower. As there are about 9 end-chains per 100 units, this fact means that one maltose group is rapidly split off from each branch, while the second or third one is loosened rather slowly.

The chains must be packed more closely in glycogen than in amylopectin (starch), and Meyer and Jeanloz [4] assume that the voluminous enzyme molecule cannot easily reach the interior parts of the glycogen molecule so

that certain end-chains remain unaffected. Measurements of osmotic pressure and of viscosity show that the glycogen molecule has a more or less spherical form, while the amylopectin molecule is more elongated. It is possible that this difference may also contribute to the low final velocity of hydrolysis in the case of glycogen.

The various hydrolyzates were treated with β-amylase for 5 days. The reducing power and the amounts of fermentable sugar were determined as previously described [1], and the values before treatment with the enzyme were obtained from that paper. In Table 3 it is shown that the glucose values

Table 3. Action of β-amylase on the hydrolyzates. Comparison between the increase in the reducing power and that of the maltose fraction.

No.	Before treatment with the enzyme	After treatment with the enzyme		
	Glucose %	Glucose %	Increase in the degree of hydrolysis, %	Increase in the maltose fraction, %
1	1.0	1.1	19.8	39.2
2	2.0	1.6	15.5	32.9
3	1.9	2.3	13.9	30.1
4	11.1	11.4	7.5	16.0
5	19.0	20.5	5.9	13.1
6	43.1	45.7	2.8	6.3

remain constant, while the increase in the maltose fraction is about double the increase in the reducing power. Thus only maltose can have been formed on action of β-amylase. This result is in conformity with what is known from starch experiments [7].

d) β-Dextrins

These dextrins were prepared by treatment with β-amylase for 5 days, after which the enzyme was inactivated by boiling for a few seconds. The dextrins were precipitated from a 90 % alcoholic solution, centrifuged off, dried *in vacuo* and dissolved in water. The reducing power was determined by iodometric titration and the concentrations of the solutions were measured in three ways as described in a previous paper [1], where the relation between the specific rotation, $[\alpha]_D$, and the degree of hydrolysis was also studied. The average degree of polymerization, $\bar{n}$, could then be calculated (Table 4).

Table 4. Comparison of the results from different methods for determining the concentration of β-dextrin solutions. The calculated values of $[\alpha]_D$ *and of* $\bar{n}$.

Method of determination	β-Dextrin from				
	glycogen	I	II	III	IV
	Concentrations, %				
Titration after total hydrolysis	1.60	0.71	0.98	1.25	0.73
Calculation from the dry weight	1.58	0.71	1.01	1.24	0.83
Calculation from the optical rotation	1.61	0.71	0.97	1.21	—
$[\alpha]_D$ (hydrates, x · $C_6H_{12}O_6$)	177°	175°	172°	158°	
$\bar{n}$	77	17.5	11.6	5.0	3.0

The results from the various methods do not differ more than ± 2 % (apart from no. IV). The values of $\bar{n}$ are surely too low, since all the traces of alcohol and other reducing substances cannot be removed, but the errors are considerable only at fairly low degrees of hydrolysis.

These β dextrins were then treated with α-amylase, and the percentage of easily hydrolyzable linkages was determined in the usual way (Table 5). On comparison with the values in Table 1, it is evident that α amylase has a lower affinity to the β-dextrins, which is quite natural as all the end-chains are lacking. As about 42 % of maltose is formed from glycogen, the yield of β-dextrin is 58 %, and the corresponding values for the dextrins from the hydrolyzates I, II and III are 55, 52 and 35 % respectively. By means of these figures the values in the last line of Table 5 are calculated. Assuming that

Table 5. Calculation of the number of rapidly broken linkages in the β-dextrins when treated with α-amylase.

Dextrin	Glycogen		I	II	III
Original degree of hydrolysis	1.3	1.0	5.7	8.6	19.8
The number of rapidly broken linkages in the β-dextrins, %	3.1	3.7	2.7	1.9	0.7
The number of rapidly broken linkages, calculated for the amount of glycogen started with, %	2.0		1.5	1.0	0.2

there are 9 interior chains per 100 units in glycogen, only about one fifth of these chains is thus rapidly broken with α-amylase. The β-dextrin III, the average chain-length of which is 5 (according to Table 4) contains only few rapidly broken linkages.

e) Calculations of the mechanism of α-amylase degradation

As mentioned before, Sillén and Myrbäck [2] have attempted to calculate the statistical distribution of different chain-lengths, and the following figures are extracted from that paper. Only case A is considered, *i. e.* the ramification can occur at every glucose unit.

Table 6. The number of interior and end-chains of a certain, minimum length, n, calculated for 100 units in glycogen. (According to Sillén and Myrbäck [2].)

$n \geqq$	2	3	4	5	6
Interior chains	4.71	3.57	2.72	2.08	1.57
End-chains	7.31	6.47	5.68	4.94	4.33
The sum of all chains	12.02	10.04	8.40	7.01	5.90

At first we can assume that every chain is ruptured once by α-amylase. The β-dextrin from glycogen contains 2 % of rapidly broken linkages (Table 5), and thus the best agreement is obtained if interior chains shorter than 5 units are assumed to be intact. When the sum of all chains is considered the best value of n is 3, as 10.1 % of the glycogen linkages can be split off rapidly.

Surely longer chains can be ruptured many times. As the normal, unbranched α-dextrins consist of 5—7 units, it may be assumed that chains containing 13—18 units are split twice and the longer ones three times. The values calculated in this way are given in Table 7.

Table 7. Calculation of the number of linkages which can be rapidly broken by α-amylase, assuming the longer chains to be broken many times.

Minimum chain-length, $n \geqq$	3	4	5	6
The number of broken linkages in the interior chains, %	3.81	2.96	2.32	1.83
The sum of all broken linkages, %	11.76	10.12	8.74	7.57

According to these calculations, α-amylase cannot rapidly split interior chains shorter than 5—6 units and end-chains shorter than 3—4 units.

The results mentioned above show that in dextrins with 5 or 6 units there are only a few rapidly broken linkages. Although *a*-amylase cannot rapidly split tri and tetrasaccharides, it could be assumed that the end-chains with 3 or 4 units in large molecules are broken by the enzyme. Myrbäck and Sillén's theories of branched molecules seem therefore to be consistent with the known facts concerning the enzymatic degradation of glycogen.

PERIODATE OXIDATIONS

Periodate or periodic acid is able to rupture a carbon chains between two adjecent hydroxyl groups, and two aldehyde groups are formed. If three adjecent cabon atoms have unblocked hydroxyl groups, the middle one is oxidized to formic acid. Accordingly, all the C—C linkages of glucose are broken, and 1 mole of formaldehyde and 5 moles of formic acid are formed, while 5 atoms of oxygen are consumed (*cf.* Fig. 3a). The oxidation of maltose proceeds as in the case of glucose. Ahlborg [13] found, however, that 6-*a*-glucosido-glucose only consumes 6 atoms of oxygen per mole (*cf.* Fig. 3b). According to Jackson and Hudson [14], starch and cellulose consumed slightly more than one atom of oxygen per glucose unit (abbreviated to O/glucose). Hence the pyranose ring is split between the carbon atoms 2 and 3. The non-reducing end-group of a glucose chain contains free hydroxyl groups in the positions 2, 3 and 4, and it gives thus rise to one mole of formic acid. A source of error in all periodate experiments is due to the reducing chain. Little is known of the properties of this chain.

a) Determination of the consumption of oxygen

The oxidations are carried out in more or less acid solution. Periodate is reduced to iodate, and the most simple method for determining the excess of periodate has been devised by Rappaport *et al.* [15], consisting of iodometric titration at pH = 7 in a phosphate buffer. In acid solution iodate also reacts, which makes the volume of thiosulfate unnecessarily large.

Experimental

The experiments were carried out at 30°, for the reaction did not come to a standstill at higher temperatures. 0.1 *M* periodate solution was prepared from 21.4 g of $NaIO_4$, 25 ml of 4 *N* H_2SO_4 and water made up to 1 l. A certain volume of 2 % carbohydrate solutions and 10 ml of the periodate solution were mixed with water in a glass-stoppered flask so that the volume became 50 ml. The excess of periodate must be at least 25 %. pH ranged from 1.8 to 2.4. At suitable intervals, 5 ml was transferred to a flask contain-

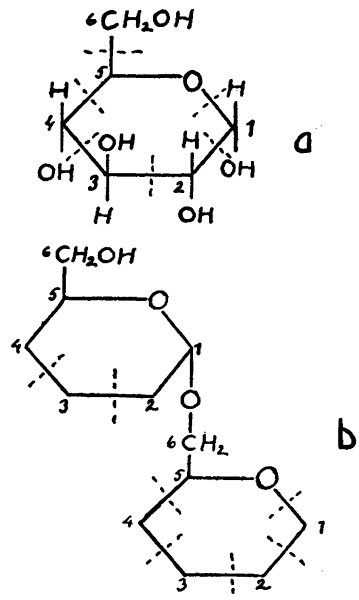


Fig. 3. The action of periodate on glucose (a) and on 6-glucosido-glucose (b).
(a) All C—C linkages in glucose are split by oxydation and the oxygen bridge is hydrolyzed.
(b) 6 C—C linkages in 6-glucosidoglucose are split. The oxygen bridge of the reducing glucose unit is hydrolyzed, whereas the two remaining ones are intact.

ing 20 ml of 1 % phosphate buffer (pH = 7.0). About 0.1 g of KI was added, and the iodine liberated was titrated with thiosulfate. A blank without carbohydrate was treated in the same way.

The results from oxidation of glycogen, starch and dextran are shown in Table 8. Further experiments on glycogen gave final values between 1.10 and 1.15 O/glucose. Apart from the reducing chain, the value would be 1.09

Table 8. Periodate oxidation of polysaccharides at pH about 2 and at 30°. The consumption of oxygen is indicated as O/glucose.

Time (hours)	0.5	1.0	3.0	5.0	10.0	24.0	48	96
Glycogen	0.53	0.66	0.82	0.87	0.92	1.05	1.09	1.12
Starch	0.60	0.75	0.89	0.94	0.96	1.00	1.08	1.07
Dextran	0.83	1.15	1.56	1.68	1.78	1.85	1.91	1.90

O/glucose for glycogen and 1.045 for starch if the interchain linkages are of the 1,6-type. Otherwise the calculated value would be 1.00 for both polysaccharides. The observed values seem therefore to indicate the presence of 1,6-linkages.

On complete oxidation of the amylose (about 20 % of starch) the calculated value is about $0.8 + 0.2 \cdot 5 = 1.8$ for starch.

It is evident that amylose is oxidized in almost the same way as amylopectin under these conditions. One would expect a 1,6-polysaccharide to consume about 2 O/glucose, and the observed value of 1.9 for dextran, which contains mainly such linkages, is therefore quite satisfactory.

b) Periodate oxidation of β-dextrins

Some β-dextrins were treated with periodate in the same way for 3 days. The end-gruop content of the dextrin from glycogen is about 15 %. The greater

Table 9. Periodate oxidation of β-dextrins for 3 days at 30°.

Dextrin from	Glycogen		I	II	III	3	4	7
O/glucose	1.25	1.17	1.72	1.86	3.08	1.60	1.80	3.60
Average chain-length			17.5	11.6	5.1			2.7

consumption of oxygen of this dextrin (Table 9) as compared with that of glycogen may be due to the higher end-group content in the case of branching at position 6. Otherwise the calculated value for the β-dextrins would also be near 1. The dextrins from the solutions no. 3, 4 and 7 have been prepared by removal of the fermentable sugar, and, hence, they can contain low-molecular substances as *iso*maltose and branched trisaccharides. The theoretical consumption of oxygen has been calculated for these compounds (Table 10).

Table 10. Theoretical consumption of oxygen of different di- and trisaccharides.

Type of linkage *Iso*maltoses	O/glucose	Types of linkage Trisaccharides	O/glucose
1,6	3.0	1,4 + 1,6	3.7 2.3 2.0
1,3	5.0	1,4 + 1,3	5.0 5.0 2.0
1,2	1.5	1,4 + 1,2	2.7 1.3 1.3

The β-dextrin preparation no. 7 is evidently a mixture of unfermentable di- and trisaccharides (and probably small amounts of higher dextrins). The observed value of 3.6 O/glucose seems to exclude the presence of 1,2-linkages, but is consistent with both the 1,3 and 1,6-structures.

c) Determination of the end-group content

Hirst and co-workers [16, 17] have developed a method based upon the fact that each end-group gives rise to one mole of formic acid. They showed that

the reducing chain was normally oxidized at low temperatures (15°) and when the periodate concentration was decreased by precipitating the bulk of $NaIO_4$ with potassium chloride. A number of glycogen samples from different animals were examined, and, in most cases, they found, the end-group content to be about 9 %, but in certain samples from rabbit liver the value was only 6 %.

Experimental

The experiments were carried out at 15° or at room temperature (20—22°). In this case periodate solution alone was used so that the pH was higher (about 4) than in the previous experiments. 10 ml of 0.1 *M* $NaIO_4$ was mixed with 10 ml of 1 % solutions of glycogen and starch in a stoppered flask, and in one series potassium chloride wes added. When the reaction was to be broken, 1 ml of ethylene glycol was added to destroy the excess of periodate. The precipitate (potassium periodate) was dissolved after a few minutes and then destroyed. The formic acid was titrated with 0.01 *N* NaOH and phenolphthalein as indicator. Blanks, containing the same amounts of the solutions, were also treated, but in this case the periodate was first detroyed by glycol.

As may be seen from Table 11, the formation of formic acid is fairly slow at 15°, and the velocity is further decreased when KCl is added. Experiments on starch gave values between 5.1 and 5.5 (3 days, room temperature). The

Table 11. Determination of formic acid formed by oxidation of glycogen. Calculated as moles of formic acid per 100 glucose units.

Time (days)	1	2	4	6	8
Without KCl (room temp.)	9.8	10.9	11.9		
» » (15°)	7.5	8.2	9.2	9.6	
KCl added (room temp.)	7.8	8.0	8.8	9.5	
» » (15°)	4.0	5.2	6.7	8.4	9.2

preparation of glycogen used in these investigations seems to have an end-group content which corresponds to the higher value of 9 %.

SUMMARY

Enzymatic breakdown of glycogen and of partly acid hydrolyzed glycogen with α- and β-amylase has been carried out. Various dextrin fractions have been prepared and the average chain-length calculated. When treating glycogen with β-amylase, the terminal maltose group of every branch is split off more rapidly than the second or third one. As in the case of starch, lower dextrins consisting of less than 5—6 units cannot be rapidly split by α-amylase. Only about one fifth of the interior chains in glycogen is rapidly broken, thus

indicating that such chains must be rather short as compared with amylopectin. The results have been compared with Myrbäck and Sillén's theories of enzymatic synthesis of ramified molecules, and according to these calculations, α-amylase cannot rapidly attack end-chains shorter than 3—4 units and interior chains less than 5—6 units.

Periodate oxidation of glycogen and of its dextrins has been carried out. The results seem to exclude the presence of 1,2-linkages, but it is scarcely possible to decide whether the linkages are of the 1,6 or 1,3-type or a mixture of both. The formic acid liberated corresponds to about 9 % of end-groups.

The author wishes to thank Prof. K. Myrbäck for the privilege of working at his institute and Prof. L. G. Sillén for his interest in this work. These investigations were aided by a grant from *Statens Naturvetenskapliga Forskningsråd.*

REFERENCES

1. Carlqvist, B. *Acta Chem. Scand.* **2** (1948) 759.
2. Sillén, L. G., and Myrbäck, K. *Svensk Kem. Tid.* **55** (1943) 294, 354.
3. Myrbäck, K., and Sillén, L. G. *Svensk Kem. Tid.* **55** (1943) 311; **56** (1944) 60.
4. Meyer, K. H., and Jeanloz, R. *Helv. Chim. Acta* **26** (1943) 1784.
5. Meyer, K. H., and Fuld, M. *Helv. Chim. Acta* **24** (1941) 375.
6. Ohlsson, E. *Z. physiol. Chem.* **189** (1930) 17.
7. Myrbäck, K., and Magnusson, B. *Arkiv Kemi, Mineral. Geol.* **A 20** (1945) no. 14.
8. Myrbäck, K. *J. prakt. Chem.* **162** (1943) 29.
9. Myrbäck, K., and Stenlid, G. *Svensk Kem. Tid.* **54** (1942) 103.
10. Myrbäck, K., and Thorsell, W. *Svensk Kem. Tid.* **54** (1942) 50.
11. Myrbäck, K. *Biochem. Z.* **311** (1942) 242.
12. Blom, J., Bak, A., and Braae, B. *Z. physiol. Chem.* **241** (1936) 273.
13. Ahlborg, K. *Svensk Kem. Tid.* **54** (1942) 205.
14. Jackson, E. L., and Hudson, C. S. *J. Am. Chem. Soc.* **59** (1937) 2049.
15. Rappaport, F., Reifer, J., and Weinmann, H. *Mikrochim. Acta* **1** (1937) 290.
16. Brown, F., Dunstan, S., Halshall, T. G., Hirst, E. L., and Jones, J. K. N. *Nature* **156** (1945) 785.
17. Halshall, T. G., Hirst, E. L., and Jones, J. K. N. *J. Chem. Soc.* (1947) 1399.

Received September 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 783—796

On the Paperchromatographic Analysis of Amino Acids and Peptides in Tissue Extracts and Enzyme Hydrolyzed Proteins

CARL-HENRIC de VERDIER and GUNNAR ÅGREN

Department of Medical Chemistry, University of Uppsala, Sweden

During the last year we have been working with the paper chromatographic method of Consden, Gordon and Martin [1] with the aim to apply it to analysis of amino acids and peptide mixtures in blood filtrate, tissue extracts and enzyme digested proteins. Some of our results are given in this communication.

EXPERIMENTAL AND RESULTS

When not otherwise stated the quick-filtering paper no. 0B supplied by Munktells Pappersfabriksaktiebolag, Grycksbo, was used. Dimensions 48 × 48 cm. This number was the best of the ten Munktell papers available in filter sheets, possibly with the exception of no. 00 in which, however, the fronts of the organic solvents moved very slowly. Two-dimensional parallel runs were also carried out with the Munktell paper no. 0B and the Whatsman paper no. 4 using phenol-cupron in one direction and a mixture of pyridine and amyl alcohol in the other. The resolving capacity of the two papers was about the same. The pair of solvents mostly used was phenol containing 0.1 % of cupron and a mixture of pyridine and amyl alcohol consisting of 35 % by volume of pyridine, 35 % by volume of technical amyl alcohol and 30 % by volume of water, (Edman [2]).

At the time when the experiments were started collidine, isovaleric acid or isobutyric acid were not available. In some of the runs with phenol a 0.15 % solution of ammonia was present in the tray at the bottom of the chamber. A typical result obtained with a two-dimensional run of a casein hydrolysate is given in Fig. 1. Phenol was used in the first direction and pyridine-amyl alcohol in the second. The R_F values are not given since they have not been found to be very consistent when calculated from different chromatograms

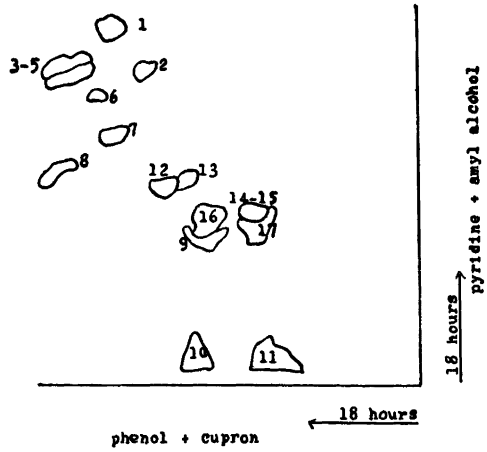


Figure 1. Phenol-cupron (0.1 %)/pyridine-amyl alcohol diagram, showing the positions of the amino acids from 1.75 mg of a casein hydrolysate fortified with 100 of tryptophane on a two-dimensional chromatogram.

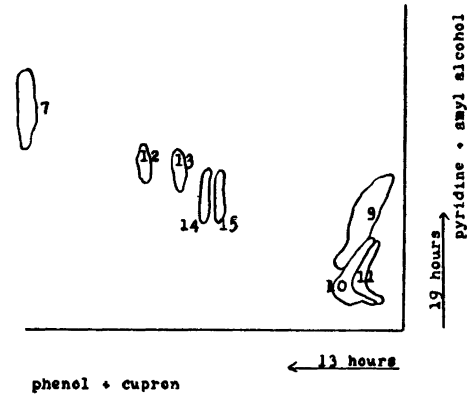


Figure 2. Pyridine-amyl alcohol/phenol-cupron (0.1 %) diagram showing the positions of different amino acids in amounts from 50—100 γ on a two-dimensional chromatogram.

1 = Try 5 = Leu 9 = His 13 = Thr 17 = Asp
2 = Tyr 6 = Met 10 = Arg 14 = Gly 18 = (CyS-)$_2$
3 = Phe 7 = Val 11 = Lys 15 = Ser 19 = Glu-NH$_2$
4 = Ileu 8 = Pro 12 = Ala 16 = Glu

Abbreviations according to Brand and Edsall[25]

(Pratt and Auclair [3]). The resolving capacity of this pair of solvents was comparatively good with some exceptions. The leucines and phenylalanine usually appeared as a single irregular spot. Further on histidine, serine and glycine were sometimes difficult to distinguish from glutamic and aspartic acid. When pyridine-amyl alcohol was run first and phenol afterwards a good separation of all these amino acids was obtained. A typical chromatogram is given in Fig. 2. In this way a better separation of alanine from threonine was also accomplished.

However, a good resolution of the other amino acids was not obtained in this way, possibly with the exception of the three basic amino acids. If, on the other side, ammonia was present in the cabinet when phenol was run in the first direction the R_F values of aspartic and glutamic acid decreased (Consden, Gordon and Martin [1]). This is easily seen by a comparison of Fig. 1 with Fig. 3 which presents such a chromatogram. The R_F values of arginine and lysine were both increased and the spots corresponding to the amino acids usually touched each other when this procedure was used. Later on when

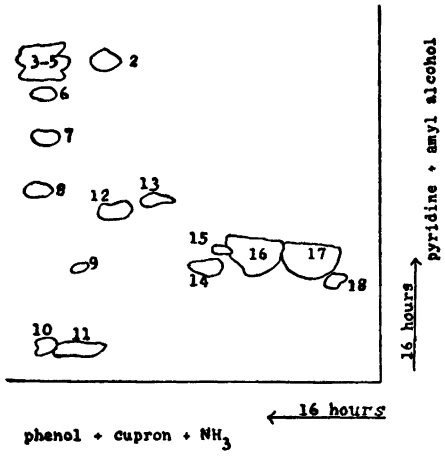


Figure 3. Phenol-cupron (0.1 %) — ammonia (0.3 %)/pyridine-amyl alcohol diagram showing the positions of amino acids from 0.56 mg of a casein hydrolysate (HCl + HCOOH) on a two-dimensional chromatogram.

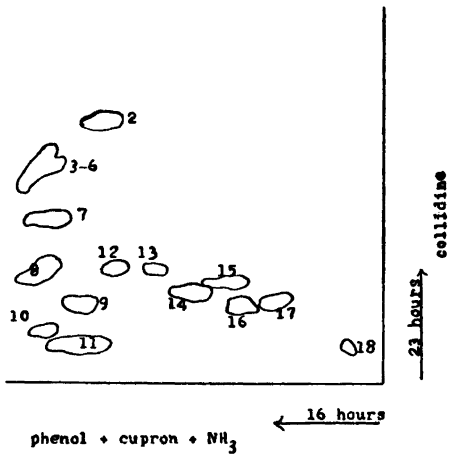


Figure 4. Phenol-cupron (0.1 %) — ammonia (0.3 %)/collidine diagram showing the positions of the amino acids from 0.56 mg of a casein hydrolysate (HCl + HCOOH) on a two-dimensional chromatogram.

collidine was available some experiments were carried out in which pyridine-amyl alcohol was exchanged for this solvent in two-dimensional runs with the Munktell paper no. 0B. A typical result is given in Fig. 4.

A comparison with Fig. 3 shows that collidine had some advantages over the pyridine-amyl alcohol mixture. More distinct spots were obtained for the dicarboxylic acids but the spot corresponding to methionine was usually covered by the leucine spot. Several attempts were made to resolve the leucine-isoleucine-phenylalanine group by one or two dimensional chromatograms using butanol with 3 % ammonia and benzylalcohol with 0.1 % cupron (Consden, Gordon and Martin [1]). The best results were obtained in two dimensional runs but clearly separated spots were never seen.

The organic solvents were removed from the papers by drying at 80°. After drying, the papers were sprayed with a solution of 0.1 % ninhydrin in butanol to which in some cases acetic acid was added to a final concentration of 1 % (Consden and Gordon [4]). The papers were then heated at 50° in a constant temperature chamber for 1 to 2 hours. It was observed that the spots usually appeared at quite different times and it occured to us that if these different times for development were characteristic constants for the various amino acids it would be able to use this circumstance in the identifi-

cation of amino acids with similar R_F values. To test this possibility 5 μl of amino acid solutions were placed on filter papers previously treated with phenol containing 0.1 % cupron and a pyridine-amyl alcohol mixture. The papers were coloured with ninhydrin and placed in the constant temperature chambers. For each of 19 amino acids the time was measured when the colour first appeared. Several series were carried out. However a comparison between the results of the different series showed a considerable spreading of the time values for each amino acid. Thus it seemed impossible to use this method to identify leucine in the presence of isoleucine and phenylalanine, nor would the method increase the possibilities to locate serine in the presence of glycine or alanine in the presence of threonine.

Similar experiments were also carried out with the following series of peptides which were synthezised in this laboratory (*cf.* Ågren [5]):

Glycyl-DL-leucine
Glycyl-DL-alanine
Glycyl-L-tyrosine
Glycyl-glycine
Alanyl-glycine
Glycyl-DL-alanyl-glycine
Glycyl-glycyl-glycine

In accordance with the results recently published by Consden, Gordon and Martin [6] we found that peptides in which glycine carried the free amino group on heating with ninhydrin gave first a yellow colour, then, grey and finally purple. The colour came slower for the two tripeptides than for the dipeptides.

Several attempts have been made to apply quantitative methods in the paper chromatographic procedure (*cf.* Consden [7]). In this laboratory it has been found advantageously to proceed in the following manner. One-dimensional runs were extracted in the apparatus described by Consden, Gordon and Martin [6] and on the small volume thus obtained, about 0.25 ml, the ninhydrin reaction was carried out as described by Moore and Stein [8]. The molar extinction coefficients for the different amino acids were determined and used for this purpose.

The preparation of blood filtrates

A paper chromatographic investigation of the free amino acids in blood plasma has so far only been carried out by Dent [9]. He prepared the blood filtrates by treating plasma or serum with ten times its volume of 95 % ethanol. Values for the amino nitrogen in the filtrates were not given, and the figures of the chromatograms only displayed 4—5 amino acids. Accordingly, determinations of the amino nitrogen in filtrates prepared by this method were carried out and compared with those obtained on filtrates prepared by

other methods. Cattle serum was used in these experiments. Zinc hydroxide precipitation according to Somogyi [10], tungstic acid precipitation according to Hier and Bergeim [11], ferric hydroxide precipitation according to Somogyi [12], dialysis and trichloracetic acid precipitation were carried out. The clear filtrates both from zinc hydroxide, ferric hydroxide and trichloracetic acid precipitations contained small amounts of protein which coagulated upon concentration. The largest amounts were found in the filtrates from zinc hydroxide precipitation. Since inorganic salts are known to be a disturbing factor in paper chromatographic analysis (Consden and Gordon [4]) the large amounts of sodium sulphate present in the filtrates after zinc hydroxide and ferric hydroxide precipitation must be removed if such an analysis should be carried out. The solubility of sodium sulphate in higher concentrations of ethanol is very low, the following figures being obtained at 2° when calculated per 100 ml of solvent: 27.5 mg in 70 %, 5.2 mg in 80 % and 3.6 mg in 90 % ethanol. The filtrates from zinc hydroxide and ferric hydroxide precipitation were accordingly partially desalted by addition of 95 % ethanol to a final concentration of 80 %.

In the trichloracetic acid precipitation 10—50 % solutions of the reagent were added to a final concentration of 5—10 % followed by rapid heating to boiling and subsequent cooling to room temperature. The surplus of trichloracetic acid was first removed by boiling under reflux (Eichloff and Lossen [13]) for 3 hours when all of the reagent had formed chloroform. During the boiling a brown colour appeared when the final concentration of reagent was 10 % mainly depending on destruction of tryptophane. When precipitation was carried out by addition of a 10 % solution of the reagent to a final concentration of 5 % the filtrate remained colourless during the reflux. Subsequent amino nitrogen determination showed, however, that the peptides present in these filtrates must have been hydrolyzed during the reflux (*cf.* Table 1). Accordingly the reagent was removed by ether extractions. Ninhydrin controls showed that amino acids were not removed in this procedure.

Dialysis was carried out in cellophane tubes, Ø = 5 mm, at 2° for 8 hours during constant stirring against distilled water which was exchanged three times. The different filtrates were analyzed by Van Slyke determinations. The values obtained on three different samples of serum are given in Table 1. Tungstic acid filtrates and ferric hydroxide filtrates contained about the same amounts of α-amino nitrogen, while the zinc filtrates gave lower values. Somogyi [14] investigating non-protein nitrogen in the same kind of filtrates also found the lowest values in the zinc filtrates while the nitrogen values of iron filtrates lay between those of zinc and tungstic acid filtrates. Agreeing α-amino nitrogen values were obtained in the filtrate series from 10 and 5 %

Table 1. Comparison of deproteinizing agents. α-amino nitrogen of various filtrates of cattle serum. Values are in mg α-amino nitrogen per 100 ml of serum and each value is an average of two or more analysis.

Sample	Serum no.		
	1	2	3
$Zn(OH)_2$-filtrate	3.95	4.10	4.0
$Fe(OH)_3$-filtrate	4.75	4.80	4.75
Tungstic acid filtrate	4.75	4.85	4.75
Filtrate from trichloracetic acid precipitation. Final reagent concentration 10 %. Reagent removed by boiling	9.50	9.80	9.60
Filtrate from trichloracetic acid precipitation. Final reagent concentration 10 %. Reagent removed by 4 ether extractions	5.25	5.40	5.30
Same as preceding but with final reagent concentration 5 %. Reagent removed by ether extraction	5.30	5.45	5.30
Dialyzable α-amino nitrogen	4.95	5.05	5.0
Filtrate from 80 % ethanol precipitation	3.80	3.90	3.80

trichloracetic acid precipitations where the surplus of reagent was removed by ether extractions. A comparison with the results of the filtrate series from which the reagent had been removed by boiling showes that these filtrates contained bound α-amino nitrogen. This explanation may also be valid for the dialysates which also contained more α-amino nitrogen than the ferric hydroxide and tungstic acid filtrates (*cf.* Christensen and Lynch[15]). Finally, the α-amino nitrogen in the ethanol filtrates lay mainly on the same level as in the zinc hydroxide filtrates. One possible explanation to the few amino acids found by Dent[9] in his chromatograms of the free amino acids in blood plasma could thus be the precipitation method and another an incomplete resolution in the runs. In this investigation the interest was mainly focused on the latter possibility. Precipitation with 80 % ethanol was used.

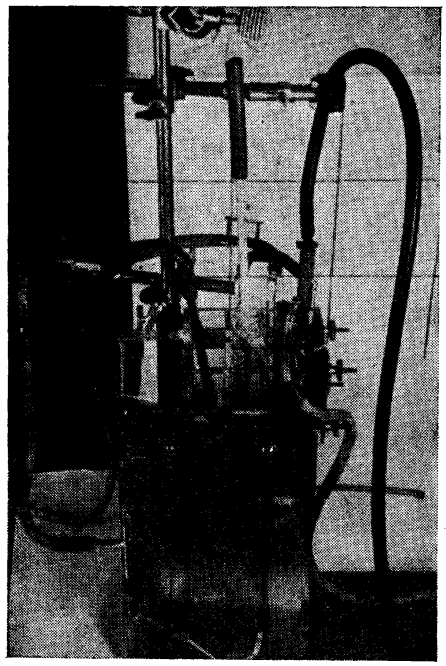


Figure 5. The desalting apparatus.

Results obtained on blood filtrates

When the first two-dimensional paper chromatograms were run with ethanol filtrates very unclear pictures were obtained. Extraction of the dried alcohol filtrates with dry ethyl ether removed some interfering substances but the results still were far from satisfactory. However, when the ether treated extracts were desalted more appropriate pictures were obtained. The apparatus used by us was a modification of the desalter described by Consden, Gordon and Martin [6] (*cf.* Fig. 5). Platina was prefered as anode and it was found favourable to applicate a cellophane bag around the anode in addition to the membrane which separated the middle compartment from the anode compartment *. A comparison was made between descending and ascending paper chromatography (Williams and Kirby [16]). As far as our experience goes we prefere to use the ascending procedure in this case. A typical chromatogram is shown in Fig. 6. This was carried out on a 48 × 48 cm paper with an ethanol filtrate from normal human plasma. Similar runs performed on 24 × 24 cm papers did not give the same good resolution of the amino acids when ascending chromatography was used. In some cases filtrates from 70 %

* A similar apparatus was used by H. T. Macpherson in Chibnall's laboratory.

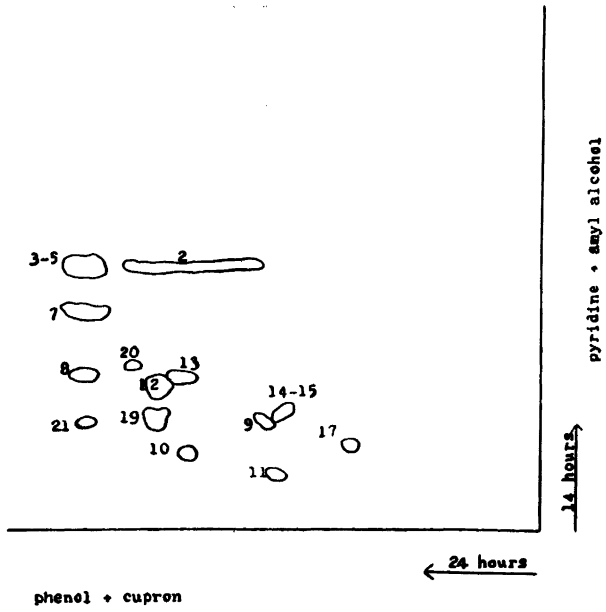


Figure 6. Phenol-cupron (0.1 %)/pyridine-amyl alcohol diagram, showing the positions of the amino acids from 1 ml of human plasma filtrate (80 % ethanol precipitation) on a two dimensional ascending chromatogram.
20 = α-amino-butyric acid? 21 = unidentified spot.

ethanol precipitation were also investigated. The same number of ninhydrin spots were obtained as with the filtrates from 80 % ethanol precipitation. However, with filtrates from the same amount of plasma, 1 ml, the ninhydrin colour of the spots were stronger when the lower ethanol concentration was used. In conclusion one reason to the discrepancy between our results and those of Dent's is that we removed some interfering lipids and inorganic salts.

Results with liver extracts

In connection with investigations of the growth effects of certain liver extracts and enzyme hydrolyzed proteins on lactic acid bacteria (Ågren [17]) paper chromatographic analysis were also performed. As described in that paper a highly purified liver extract (Heptomin) containing the antipernicious anemia liver factor could be divided in three microbiologically active fractions by electrodialysis. The anode fraction was subjected to the one-dimensional technic using a butanol acetic acid mixture (Partridge [18]). Four ninhydrin positive spots were observed. Using the method described by Dent [9] for

isolation of similar substances three of them seemed to increase the growth of *Lactobacillus casei* cultivated on the medium of Henderson and Snell [19]. The microbiological assay technic previously described (Ågren [17]) was used. Data from the assays will be published elsewhere.

The middle fraction run in a mixture of pyridine-amyl alcohol was resolved in at least three ninhydrin positive and four ninhydrin negative but fluorescing spots (Phillips [20]). The material corresponding to the positions of the spots was again isolated and tested for growth effect on *Lactobacillus lactis*. Two of the ninhydrin positive and one of the ninhydrin negativ but fluorescing spots stimulated the growth of this microorganism. The result is of interest since this microorganism was used in the standardization of vitamin B_{12} (Shorb [21]) and also depending on the presence in the middle fraction of the antipernicious anemia liver factor (Ågren [17]). The three substances giving the ninhydrin reaction after hydrolysis were again subjected to one-dimensional runs in the mixture of pyridine-amyl alcohol. All of them seemed to contain valine, leucines or phenylalanine, glycine or serine and perhaps aspartic acid.

A two-dimensional run with the cathode fraction of electrodialyzed Heptomin showed the presence of five ninhydrin positive spots. After hydrolysis of the cathode fraction the following amino acids were found in a two dimensional run with phenol and the pyridine-amyl alcohol mixture: leucine, isoleucine, phenylalanine, methionine, valine, threonine, alanine, histidine, arginine and lysine. The cathode fraction also contained a complex mixture of basic peptides. This fraction was the only one of the three fractions separated by electrodialysis which gave a biuret positive spot on paper (Killander [22]). Microbiological assays were not carried out on this fraction.

The Wilson liver fraction L was also electrodialyzed in three fractions which all stimulated the growth of *Lactobacillus casei* (Table 2). This is of interest with regard to the present discussion of the constitution of strepogenin (Wolley [23]). So far only the cathode fraction has been analyzed by paper chromatography. In two-dimensional runs with phenol and the pyridine-amyl alcohol mixture 5 ninhydrin spots were obtained. The substances corresponding to the position of the spots on the paper were isolated and each of them hydrolyzed and run again in one-dimensional chromatography with pyridine-amyl alcohol. The results indicated that the cathode fraction contained five polypeptides of different amino acid composition. Leucine or isoleucine was found in all of them. The two slowest running peptides contained comparatively large amounts of lysine and the peptides with the highest R_F values showed the largest content of leucines. In this preliminary survey microbiological assays of the different substances were not carried out.

Table 2. Growth of Lactobacillus casei after supplementing the basal medium of Henderson and Snell with electrodialyzed fractions from Wilson liver fraction L. Photoelectric readings and titrations after 24 hours of incubation.

	Anode fraction				Middle fraction				Cathode fraction			
pH at the end of electrodialysis	2.0				5.0				10.			
Total nitrogen added per tube in mg	0	1.0	0.1	0.01	0	0.5	0.05	0.005	0	0.8	0.08	0.008
Photoelectric readings *	4	69	25	20	4	160	105	20	3	105	65	25
Titration values **	0	3.4	0.6	0.1	0	4.9	2.0	0.2	0	3.5	1.4	0.4

Result with enzyme hydrolyzed casein

In the recent paper of Ågren [17] a commercial tryptic digest of casein (Aminosol) was used as a source of strepogenin for the microbiological work. This preparation was now resolved in three fractions by electrodialysis and all fractions stimulated the growth of *Lactobacillus casei*. The assay technic was the same as described in that paper. The results are given in Table 3. The anode fraction was run with one-dimensional chromatography using butanol, pyridine-amyl alcohol, collidine, phenol with addition of ammonia, hydrochloric acid or acetic acid, butanol with addition of acetic acid and finally a mixture of *iso*butyric acid and *iso*valeric acid. Only the three last mentioned organic solvents gave a resolution of the anode fraction in two spots, also when two-dimensional runs were performed. A comparison with simultaneously run amino acids showed that one of the spots coincided with aspartic acid, the other with glutamic acid.

Microbiological analysis with *Lactobacillus casei* showed that growth stimulating effect was present only in the spot laying on the same level as aspartic acid. The material of this »aspartic acid spot» was hydrolyzed before and after deamination according to Consden *et al.*[6] and analyzed with one- and two-dimensional technic using phenol in the first case and phenol and a pyridine-

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. Mean values of three tubes corrected for the blank titrations.

** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. Mean values from three tubes corrected for the blank titrations.

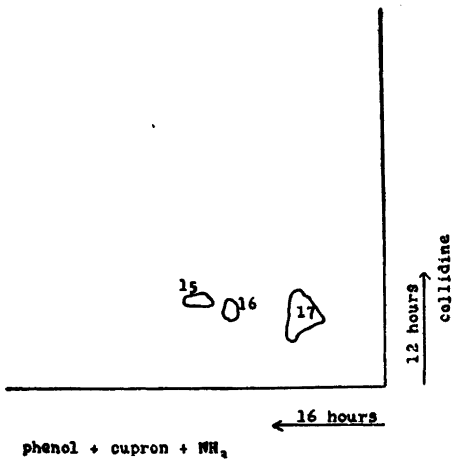


Figure 7. Phenol-cupron (0.1 %)-ammonia (0.3 %)/collidine diagram, showing the positions of the amino acids from hydrolyzed »aspartic acid spot» on a two-dimensional chromatogram.

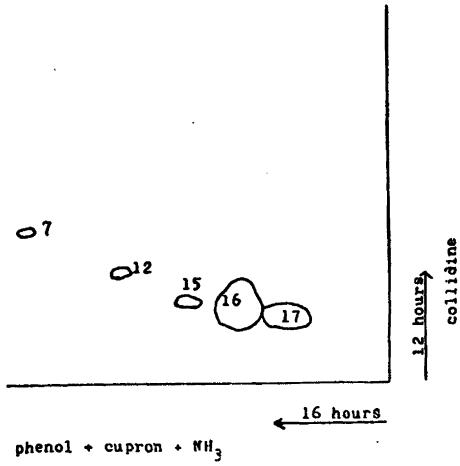


Figure 8. Phenol-cupron (0.1 %)-ammonia (0.3 %)/collidine diagram, showing the position of the amino acids from hydrolyzed »glutamic acid spot» on a two-dimensional chromatogram.

Table 3. Growth of Lactobacillus casei after addition of fractions from electrodialyzed Aminosol to the basal medium of Henderson and Snell. The amounts added are expressed as mg of total nitrogen. Photoelectric readings and titrations after 24 hours of incubation.

	Anode fraction				Middle fraction				Cathode fraction			
pH at the end of electrodialysis	3.0				4.8				10.0			
Total nitrogen added per tube	0	0.35	0.035	0.0035	0	0.52	0.052	0.0052	0	0.20	0.020	0.0020
Photoelectric readings *	2	99	22	10	3	73	15	10	2	62	20	9
Titration values **	0	1.84	0.72	0.20	0	1.30	0.44	0.20	0	1.30	0.58	0.10

amyl alcohol mixture in the second. The results of several of these analysis (*cf.* Fig. 7) indicated that the eluted material consisted of free aspartic acid

* Given as scale readings on the Klett Summerson photoelectric colorimeter. Mean values of three tubes corrected for the blank titrations.

** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. Mean values from three tubes corrected for the blank titrations.

and a seryl-glutamic acid peptide, probably containing several glutamic acid molecules per serine molecule (*cf.* Wolley [23]). The material from the »glutamic acid spot» was analyzed in the same way and seemed to be a mixture of glutamic acid and an asparagyl-peptide (Fig. 8) probably containing serine, alanine and valine. The results of the microbiological assays are given in Table 4. The technic is the same as previously described (Ågren [17]). The amino nitrogen in the anode fraction amounted to 57 % of the total nitrogen. 5 mg of substance was used in the chromatographic resolution and it was calculated that the amount of growth stimulating seryl-glutamic acid peptide added in the assays amounted to about 0.1 mg per tube, which gave a maximal growth effect.

Table 4. Growth of Lactobacillus casei on the medium of Henderson and Snell supplemented with substances isolated from the anode fraction of electrodialyzed Aminosol. Incubation time 24 hours.

	»Glutamic acid spot»		»Aspartic acid spot»	
Amount of substance added per tube	0 mg	4 mg	0 mg	1 mg
Ml of acid produced *	1.3	1.5	1.4	8.6
Photoelectric readings **	84	94	83	300

The cathode fraction of Aminosol was investigated with one-dimensional technic using the pyridine-amyl alcohol mixture. So far only peptides containing histidine and valine have been identified and may be responsible for the growth effect of this fraction. This result is of interest with regard to the present discussion of the chemical constitution of strepogenin (*cf.* Wolley [24]).

SUMMARY

1. Several details in the paper chromatographic procedure have been investigated. In two dimensional runs with phenol in the first direction we have compared the resolving capacity of collidine with that of a pyridine amyl

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are the mean values from three tubes and have been corrected for the blank titrations.

** Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading 0 corresponds to 100 per cent transmission. The values are the mean values from three tubes.

alcohol mixture in the second direction without finding any special advantage when using collidine.

2. Different methods for the preparation of blood serum and plasma filtrates containing the free amino acids have been compared with regard to the yield of α-amino nitrogen and fitness for use in paper chromatography. It was found necessary to remove lipids and inorganic salts which interfered with analysis. Ascending chromatography was prefered when working with desalted blood plasma filtrates.

3. A highly purified preparation containing the antipernicious anemia liver factor was investigated by means of electrodialysis followed by a combined paper chromatographic and microbiological analysis. In this way it could be demonstrated that the preparation was very far from pure. Each of the three fractions from electrodialysis was found to contain a mixture of peptides which in some cases showed a growth stimulating effect on *Lactobacillus casei* and *Lactobacillus lactis*.

4. A similar analysis of an enzyme hydrolyzed casein preparation was also carried out. The anode fraction from the electrodialysis was resolved in at least one biologically inactive peptide and one growth stimulating peptide which probably was a seryl-glutamic acid peptide. The cathode fraction, which also stimulated the growth of *Lactobacillus casei*, contained a mixture of peptides.

The investigation was supported by a grant for medical research from the University of Uppsala and from the Ferrosan Corporation. The technical assistance of Dr. G. Ekström and Ing. T. Eklund are gratefully acknowledged.

REFERENCES

1. Consden, R., Gordon, A. H., and Martin, J. P. *Biochem. J.* **38** (1944) 224.
2. Edman, P. *Arkiv Kemi, Mineral. Geol.* **A 22** (1945) no 3.
3. Pratt, J., and Auclair, J. *Science* **108** (1948) 213.
4. Consden, R., and Gordon, A. H. *Nature* **162** (1948) 180.
5. Ågren, G. *Acta Physiol. Scand.* **13** (1947) 347.
6. Consden, R., Gordon, A. H., and Martin, J. P. *Biochem. J.* **41** (1947) 590.
7. Consden, R. *Nature* **162** (1948) 359.
8. Moore, S., and Stein, W. *Fed. Proc.* **7** (1948) 174.
9. Dent, C. E. *Biochem. J.* **41** (1947) 240.
10. Somogyi, M. *J. Biol. Chem.* **86** (1930) 655.
11. Hier, S. W., and Bergeim, O. *J. Biol. Chem.* **161** (1945) 717.
12. Somogyi, M. *J. Biol. Chem.* **90** (1931) 725.
13. Eichloff, R., and Lossen, W. *Ann.* **342** (1905) 112.
14. Somogyi, M. *J. Biol. Chem.* **87** (1930) XXXII.
15. Christensen, H. N., and Lynch, E. L. *J. Biol. Chem.* **166** (1946) 87.

16. Williams, R. J., and Kirby, Helen *Science* **107** (1948) 481.
17. Ågren, G. *Acta Physiol. Scand.* (1948) In Press.
18. Partridge, S. M. *Biochem. J.* **42** (1948) 238.
19. Henderson, L. M., and Snell, Esmond E. *J. Biol. Chem.* **172** (1948) 15.
20. Phillips, D. M. B. *Nature* **161** (1948) 53.
21. Shorb, M. *Science* **107** (1948) 396.
22. Killander, A. *Uppsala Läkareförens. Förhandl.* (1948) In Press.
23. Woolley, D. W. *J. Biol. Chem.* **172** (1948) 71.
24. Woolley, D. W. *Fed. Proc.* **7** (1948) 200.
25. Brand, E., and Edsall, J. T. *Ann. Rev. Biochem.* **16** (1947) 223.

Received October 5, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 797—802

Microbiological Determinations of Amino Acids on the Medium of Henderson and Snell

GUNNAR ÅGREN

Department of Medical Chemistry, University of Uppsala, Sweden

Henderson and Snell [1] recently described a uniform medium for the determinations of amino acids with various microorganisms *. In that paper an account of the different lactobacilli selected for the determination of fourteen amino acids was also given. In this laboratory a similar investigation has been carried out where each of eighteen amino acids was assayed with several lactic acid bacteria for which the amino acid was indispensable. Use of more than one test organism for the determination of different amino acids seems advisable depending upon the well known interrelationships between certain vitamins and amino acids and the lack of an absolute requirement.

EXPERIMENTAL AND RESULTS

Cultures and procedure. The organisms used were *Lactobacillus arabinosus* 17—5 (8014), *Lactobacillus delbrückii* LD5 (9595), *Lactobacillus casei* (7469), *Streptococcus faecalis* R (8043), *Leuconostoc mesenteroides* P-60 (8042), *Lactobacillus fermenti* 36 (9338) and *Lactobacillus citrovorum* (8081). The assay technique was mainly that described by Henderson and Snell [1]. The total volume was 6 ml. and the lactic acid was titrated with 0.05 *N* NaOH using thymol blue as indicator in the citrate containing medium and bromthymol blue in the acetate containing medium. Other small variations for carrying stab cultures and inocula are described in a recent paper (Ågren [2] 1948). In that paper it was also pointed out that according to the experience in this laboratory higher lactic acid production was obtained with *Lactobacillus citrovorum* (8081) and *Lactobacillus delbrückii* LD5 when they were grown in a medium containing 0.5 times the concentration of all ingredients of the Henderson and Snell medium with the exception of Salt C which was kept at the original concentration. Accordingly, these two microorganisms were cultivated on this weaker medium. *Lactobacillus casei* was usely grown in the original

* I wish to express my appreciation to these authors for obtaining the composition of their basal medium prior to its publication.

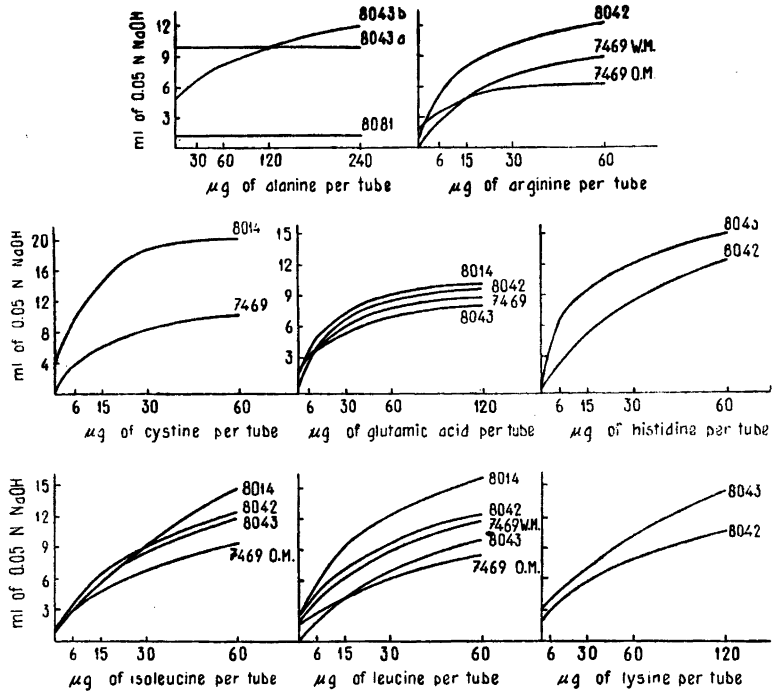


Figure 1. Standard curves on the medium of Henderson and Snell. The American Type Culture Collection number is used for each microorganism.

medium (O. M.) but for some amino acids more steep standard curves were obtained when the weaker medium was used (W. M.). The amino acids used were from Merck. DL-forms of alanine, aspartic acid, isoleucine, methionine, phenylalanine, serine, threonine and valine were employed. The natural isomers of the others were used. For some amino acids (alanine and glycine) the medium of Dunn *et al.*[3] was prefered.

Standard curves. Results obtained with the different amino acids tested on the medium of Henderson and Snell are given in Fig. 1 and 2. When *Streptococcus faecalis* R was grown on the original medium of Henderson and Snell, curve 8043 a, alanine was a dispensable amino acid for the microorganism. When the citrate buffer was exchanged for acetate, curve 8043 b, better growth curves were obtained. The high level of the curve may depend on the presence of the pyridoxine derivate in the medium (Lyman *et al.*[4] 1947). Dunn *et al.*[3] also reported that alanine was an essential amino acid for *Streptococcus faecalis* R but they also obtained a comparatively high blank value on the medium which contained acetate but no citrate. For corroboration of this result see Fig. 3. It appears that *Streptococcus faecalis* R has the enzymatic

mechanism for synthesezing alanine provided that the proper substrates are present. The poor growth curve with *Lactobacillus citrovorum* in the medium of Henderson and Snell was previously reported (*cf.* Ågren [2] 1948).

Comparing the standard curves obtained with different lactobacilli when arginine was obmitted from the medium *Leuconostoc mesenteroides* gave the steepest curves. *Lactobacillus casei* seemed to give a better curve when the weaker medium of Henderson and Snell was used. Of the seven microorganisms tested in the present investigation only *Leuconostoc mesenteroides* could be used for aspartic acid determinations (*cf.* Dunn *et al.*[3], Henderson and Snell [1]). The cystine standard curves obtained with *Lactobacillus arabinosus* were always much steeper than those obtained with *Lactobacillus casei.* In some experiments when *Lactobacillus arabinosus* was used for cystine determinations it was observed that more consistent results were obtained after 48 hours of incubation than after 72 hours.

The glutamic acid curves in Fig. 1 were all obtained in experiments were the microorganisms were cultivated on the weaker medium of Henderson and Snell adjusted to pH 7.0. In this case no lag period was encountered. Using the medium in the same concentration as given by Henderson and Snell the induction period observed by this authors was also noticed in this laboratory. The medium seemed not to be suitable for assays of glycine with *Leuconostoc mesenteroides* or *Lactobacillus citrovorum.* The same lactic acid production was obtained whether glycine was present in the medium or not. Exchanging citrate for acetate made no difference. Better assay curves were obtained on the medium of Dunn *et al.*[3] (Fig. 3). Subsequently acceptable curves were also obtained on the medium of Henderson and Snell when recrystallized alanine was used (*cf.* Henderson and Snell [1], Shankman [5]). With regard to histidine it may be mentioned that *Streptococcus faecalis* R was used by Henderson and Snell in their analysis of this amino acid. The same authors also reported severs drifts in assay values when *Lactobacillus arabinosus* was employed for isoleucine determinations. The levels of the leucine curves obtained with *Lactobacillus casei* were always higher when the diluted medium of Henderson and Snell was used (Fig. 1).

For several reasons (*cf.* Lyman *et al.*[4], Dunn *et al.*[3], Stokes *et al.*[6]) only *Streptococcus faecalis* R and *Leuconostoc mesenteroides* could be used for lysine determinations. Methionine assays carried out with several microorganisms on the diluted medium of Henderson and Snell always gave much lower standard curves than assays performed with the original medium. Only the latter curves are shown in Fig. 2. *Lactobacillus arabinosus* has been used by several investigators for determination of phenylalanine but this microorganism and *Streptococcus faecalis* (9790) may be able to synthesize phenylalanine (cf.

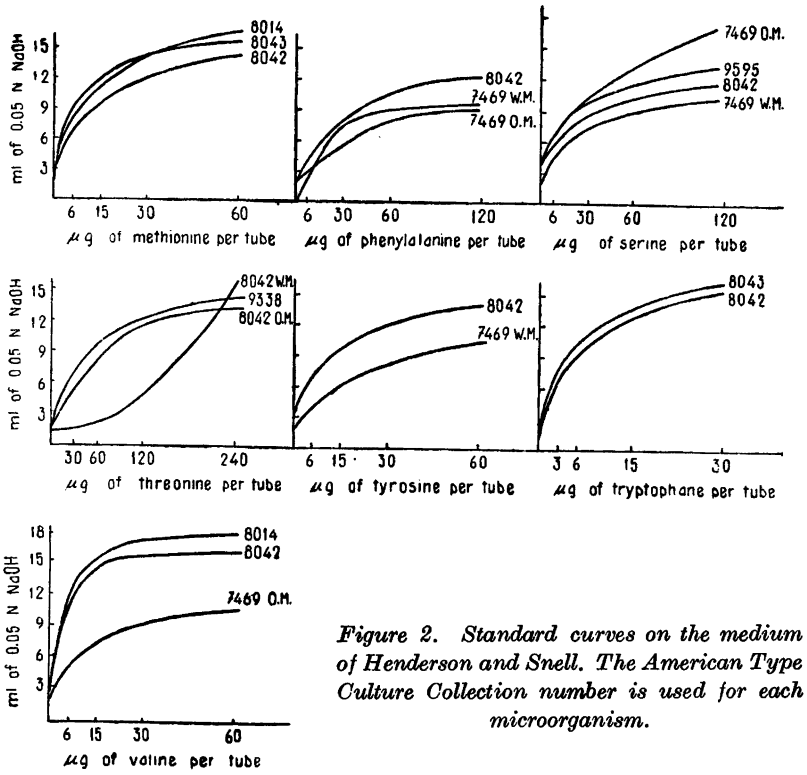


Figure 2. Standard curves on the medium of Henderson and Snell. The American Type Culture Collection number is used for each microorganism.

Stokes *et al.*[6], Lyman *et al.*[4]). A steeper phenylalanine curve was also obtained with *Lactobacillus casei* when the microorganism was cultivated on the diluted medium than on the original (*cf.* Fig. 2). Of the seven microorganisms employed in the present investigation only *Leuconostoc mesenteroides* seemed to be suitable for proline determinations (Snell [7]) and this lactobacillus was also used by Henderson and Snell. Dunn *et al.*[3] showed that on their medium this microorganism could not use hydroxyproline instead of proline.

Serine standard curves were carried out with *Lactobacillus casei* and *Leuconostoc mesenteroides*. The composition of the medium influences the types of curves obtained. Contrary to the previous experience with *Lactobacillus casei* a steeper curve was obtained when the microorganism was cultivated on the original medium of Henderson and Snell. Meinke and Holland [8] recently demonstrated that 2 mg of DL-threonine per 10 ml of the basal medium had an antagonistic effect on the utilization of serine by several lactic acid bacteria. The inhibitory effect was stronger when *Lactobacillus casei* or *Lactobacillus delbrückii* were used as test organisms while the effect

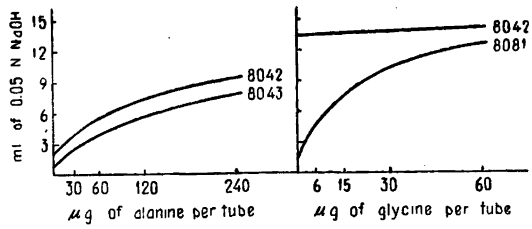


Figure 3. Standard curves on the medium of Dunn, Shankman, Camien and Block. The American Type Culture Collection number is used to denote each microorganism.

was less disturbing in assays with *Leuconostoc mesenteroides* or *Streptococcus faecalis*. In the medium of Henderson and Snell the inhibitory effect was also observed. When threonine was excluded from the medium much steeper standard curves were obtained with *Lactobacillus casei* and *Lactobacillus delbrückii*. Only the latter types of curves are shown Fig. 2.

The two different types of threonine curves obtained with *Leuconostoc mesenteroides* when it was grown in the two concentrations of the Henderson and Snell medium is of interest. Meinke and Holland [8] found that serine had an antagonistic effect on the utilization of threonine and that this amino acid inhibited the availability of serine for growth. Now, the threonine standard curves obtained by these authors with *Leuconostoc mesenteroides* shows the same type of induction period as the threonine curves obtained by us in the diluted medium of Henderson and Snell while this lag disappears when the original medium is used. Thus it would seem possible to eliminate this lag by using a suitable medium. The experience obtained in this laboratory is also that the serine-threonine antagonism is more pronounced on the medium of Meinke and Holland than on the medium of Henderson and Snell.

As mentioned before the exchange of citrate for acetate in the medium of Henderson and Snell made alanine an essential amino acid for *Streptococcus faecalis*. As the medium of Dunn *et al.*[3] only contained acetate it was considered of interest to carry out some alanine assays on that medium. Typical results are given in Fig. 3. The curves were of the same flat type as those obtained on the medium of Henderson and Snell but the blank values were lower. Attempts were also made to obtain suitable glycine standard curves on the medium of Dunn *et al.*[3]. *Leuconostoc mesenteroides* and *Lactobacillus citrovorum* were used as test organisms. The Merck DL-alanine preparation used in the medium was not recrystallized and the two glycine curves shown in Fig. 3 were carried out simultaneously on the same batch of medium. The curve obtained with *Leuconostoc mesenteroides* was of the same horizontal type as that obtained on the medium of Henderson and Snell. *Lactobacillus*

citrovorum on the latter medium also gave a simular curve. The reason for the different result obtained with *Lactobacillus citrovorum* on the medium of Dunn *et al.*[3] must depend on differences in the composition of the two media and not on a possible glycine contamination in the alanine preparate. Our results with the two microorganisms on the medium of Dunn *et al.*[3] are in agreement with those reported by these authors. One consequense is obviously that commercial alanine preparations may be used without crystallization for glycine assays if these are carried out on the medium of Dunn *et al.*[3] with *Lactobacillus citrovorum.*

SUMMARY

Using seven lactic acid bacteria and the uniform medium of Henderson and Snell several standard curves have been carried out for each of the eighteen amino acids which can be determined by microbiological methods. This was made to complete the investigation of Henderson and Snell which comprised fourteen amino acids and to obtain a conception of alternatively usable microorganisms. With one exception the reported results of Henderson and Snell included the use of one microorganism for each amino acid. Employment of more than one test organism seems advisable depending on the well known interrelationship between certain vitamins and amino acids and the lack of an absolute requirement. The implication of some results are discussed.

The investigation was aided by a grant from the *Medicinska Forskningsrådet* (Swedish Medical Science Research Council).

REFERENCES

1. Henderson, L. M., and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
2. Ågren, G. *Acta Physiol. Scand.* (1948) In press.
3. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H. *J. Biol. Chem.* **168** (1947) 1.
4. Lyman, C. M., Mosely, O., Wood, S., Butler, B., and Hale, F. *J. Biol. Chem.* **167** (1947) 177.
5. Shankman, S. *J. Biol. Chem.* **173** (1948) 809.
6. Stokes, J. L., and Gunnes, M. *Science* **101** (1945) 43.
7. Snell, E. E. *Ann. New York Acad. Sc.* **47** (1946) 161.
8. Meinke, W. W., and Holland, B. R. *J. Biol. Chem.* **173** (1948) 535.

Received October 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 803—812

Enzymatic Breakdown of Dextran

BJÖRN INGELMAN

Institutes of Biochemistry and Physical Chemistry, University of Uppsala, Uppsala, Sweden

In order to find enzymes which are able of breaking down the polysaccharide dextran, attempts have been made to prepare appropriate extracts of enzymes originating from various biological materials. During this work it was found that such extracts could be obtained from the bacterium *Cellvibrio fulva*.

Dextran is built up from glucose units and is formed if the bacterium *Leuconostoc mesenteroides* or closely related organisms grow on appropriate solutions of saccharose. (This polysaccharide is now produced in this way on an industrial scale. After partial hydrolysis it has found extensive medical use as a blood plasma substitute). Dextran is also formed by bacteria free extracts from *Leuconostoc mesenteroides*. Hehre has shown that such enzyme solutions can change saccharose into dextran and fructose [1].

A complete review of the literature on the structure of dextran will not be given here as Evans and Hibbert [2] have recently made a review of the field. It is sufficient to mention that Levi, Hawkins and Hibbert [3] have suggested a structure according to which dextran is built up of a main chain of considerable length, to which shorter side chains are attached. Evidence for this structure has been obtained by hydrolysis of methylated dextran and identification of the degradation products. Most of the glucopyranose units in the dextran molecule are joined by 1,6-D-glucosidic bonds, but 1,4-D-glucosidic bonds can also occur. It is assumed that these bonds have an α-configuration. By means of partial acid hydrolysis and acetylation of dextran from *Leuconostoc dextranicum* Georges, Miller and Wolfrom [4] were able to isolate a crystalline octaacetate of 6-α-D-glucopyranosido-β-D-glucose.

Dextran is a polymolecular substance. Its molecular weight is generally very high, the order of magnitude being several million [5,6]. Ultracentrifugation, viscosity measurements, etc. have shown that the dextran molecule has a threadlike shape. The largest of the molecules can be seen in the electron microscope [7–9]. The pictures obtained either in the usual way or after gold

shadowing show long, branched chains. A more homogeneous dextran can be prepared by fractionation of raw dextran or partially hydrolyzed dextran [6]. Molecular constants have been determined on these fractions. These measurements show that the fractions also consist of molecules which are more or less threadlike.

Dextran is not, or only very slightly, broken down by the common amylases, for instance salivary amylase [10] or malt amylase. Studies on this subject have been made not only in this country but also in many laboratories in other countries. Attempts to break down dextran by phosphorylases in the presence of phosphate have so far not given any results [10]. The stability of the dextran molecule against such enzymes is also confirmed by similar experiments in this laboratory (unpublished) which have been made in connection with investigations on the breakdown of dextran in the animal tissues. However, the very interesting observation has been made that dextran is able to activate phosphorylase for polysaccharide synthesis [11, 12]. There are indirect evidences that the partially hydrolyzed dextran, used as a plasma substitute, is broken down within the animal organism. The elimination of infused dextran from the organism, however, is a rather slow process and this is one of the reasons why dextran can be used as a plasma substitute [13, 14]. The mechanism of this elimination is as yet unknown; possibly it must be searched for in systems other than the now known amylase and phosphorylase systems.

As already mentioned, dextran is formed from saccharose by enzyme extracts from *Leuconostoc mesenteroides*. The equilibrium of this reaction is such that the formation of dextran and fructose is strongly favoured. As far as we know no reverse reaction, that is the breakdown of dextran in the presence of fructose and extracts of enzymes [1], has yet been observed.

It would be of interest to find new carbohydrases not only from an enzymatic point of view. In carbohydrate chemistry this is also of importance, as enzymatic inversions of carbohydrates among other things can supply information about the chemical structure of these compounds. This is well exemplified within the field of the chemistry of polysaccharides by all the investigations on the enzymatic breakdown of starch. In combination with other methods these experiments have made important contributions to the discussions concerning the structure of this substance (*cf.* a review given by Myrbäck [15]). In the elucidation of the structure of dextran and of certain other polysaccharides, the availability of such enzymes is also of importance. Furthermore it may be possible to use enzymes of this type to obtain the partially hydrolyzed dextran used for infusion purposes.

In a search for a suitable micro-organism capable of breaking down dextran it was found that the bacterium *Cellvibrio fulva* could grow on dextran solu-

tions, to which suitable inorganic salts had been added. The growth of the bacteria is evident from the facts that the culture liquid becomes yellow due to a dyestuff produced by the bacteria, and that the viscosity of the solution decreases as a consequence of the breakdown of dextran. It will be shown here that an enzyme extract can be obtained from cultures of these bacteria which also has a breakdown effect on dextran.

Cellvibrio fulva is known as a cellulose degradating bacterium. It grows on cellulose if suitable inorganic salts are added. This microorganism can also grow on starch.

It must be emphasized that not all cellulose degrading microorganisms are able to break down dextran. Thus enzyme extracts from the mould *Aspergillus niger* etc., tried on dextran solutions, did not give any decrease in viscosity. Investigating the breakdown of cellulose by *Cytophaga*, Fåhraeus [16] was able to show that *Cytophaga globulosa* did not have any effect on dextran.

In this investigation the action of *Cellvibrio fulva* on dextran has been studied by means of viscosity measurements and determinations of reducing sugar. The viscosity measurements were made in ordinary capillary viscosimeters according to Ostwald.

As Hultin [17] has shown the activity of α-amylases in the breakdown of starch can be calculated from viscosity data. He has deduced the following expression:

$$A = c^2 \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{1}$$

A is the enzyme activity, c is the substrate concentration, η_{sp} is the specific viscosity and t the time. As an abbreviation the following notation is introduced:

$$\frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} = p \tag{2}$$

Eq. (1) is derived on the assumption that Staudinger's law is valid (direct proportionality between intrinsic viscosity and molecular weight) for the substrate, and that the enzyme breaks all linkages between the basic molecules in the substrate at the same rate. Hultin [18, 19] has also used his formula to study the breakdown of other high-molecular substances. The formula has even been applied to the enzymatic breakdown of very high-molecular polymetaphosphates [20]. In this case, however, corrections must be made, because the viscosity is not only dependent upon the concentration of the substrate

but also to a high degree upon the salt content in the solution. Hultin's formula has also been applied to this investigation of the breakdown of dextran by enzymes from *Cellvibrio fulva*. As will be shown later the formula has been found valuable. Plotting $1/\eta_{sp}$ against time a linear relationship is obtained for the first stage of the breakdown. From the declination of these straight lines the p-values defined by equation (2) can be calculated.

PREPARATION OF ENZYME EXTRACT FROM CELLVIBRIO FULVA

For these experiments a culture of *Cellvibrio fulva* was used. This was kindly supplied by Dr. G. Fåhraeus, Lantbrukshögskolan, Ultuna, Uppsala. The bacteria were grown at about 26° C on solutions of the following composition: 0.5 g $NaNO_3$, 0.25 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 20 g partially hydrolyzed dextran (dextran plasma substitute) and 1 litre of tap water. After some months the culture liquid with the bacteria was mixed in a »Waring blendor». The solution was dialyzed against water for 2 days at + 5° C in a bag of cellulose nitrate. As the enzymes degradate cellulose, cellophane bags could not be used. After the dialysis the solution was cleared by centrifugation. Then it was filtered through a glass filter (Jena G 4). Filtration through filter paper was avoided during the preparation of the extract.

PREPARATION OF DEXTRAN

In these experiments the very viscous raw dextran was not use as a substrate as it has been found too difficult to handle. Partially hydrolyzed and fractionated dextran is a much better and more suitable substrate. It was prepared in the following way. Very high molecular raw dextran, formed from saccharose in the ordinary way by *Leuconostoc mesenteroides*, was dissolved in hot water. HCl was added and the hot solution was stirred until the viscosity had reached a suitable value. Then it was neutralized with NaOH and filtered. In order to get more homogeneous substances the dextran obtained was fractionated. This procedure is described elsewhere [6]. The partially hydrolyzed dextran was divided up into seven fractions. The first two (most high-molecular fractions) corresponded to about 35 per cent of the original amount of substance. The third fraction, F III, III, (13 per cent) was used for the enzyme experiments. The intrinsic viscosity of this fraction was $[\eta] = 0.80$ (measured at 25° C in a phosphate buffer of the following composition: 0.025 M Na_2HPO_4 and 0.025 M NaH_2PO_4; $[\eta] = \lim_{c=0} \eta_{sp}/c$, c in per cent). This fraction has a rather high molecular weight, about 2,000,000, which can be estimated from the $[\eta]$-value (*cf.* Ingelman *et al.*[6]).

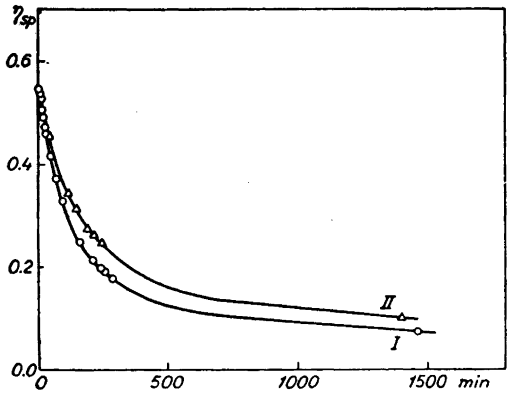


Fig. 1. Variation of η_{sp} with time during the enzymatic breakdown.

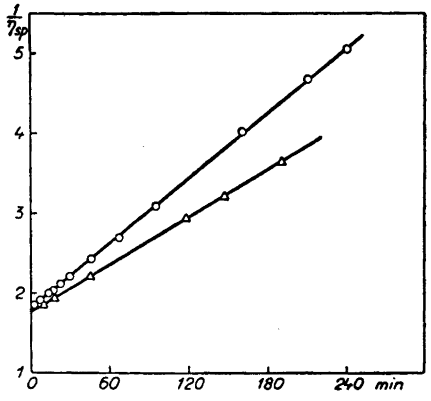


Fig. 2. Variation of $1/\eta_{sp}$ with time.

PRELIMINARY BREAKDOWN EXPERIMENTS

150 mg of dextran (F III, III) was dissolved in 21 ml of a phosphate buffer with an ionic strength of 0.1. NaCl was added to a total ionic strength of 0.3. The pH-value of the buffer was 5.22. An extract prepared from *Cellvibrio fulva* in the manner just described was used as an enzyme (*E* 1). Furthermore two heat treated solutions of this enzyme were used. One of these was heated for 5 minutes at 55° C (*E* 2) and the other for 5 minutes at 75° C (*E* 3). The experiments were performed directly in the viscosimeters and at a temperature of 25.0° C. The following mixtures of enzyme and substrate were used:

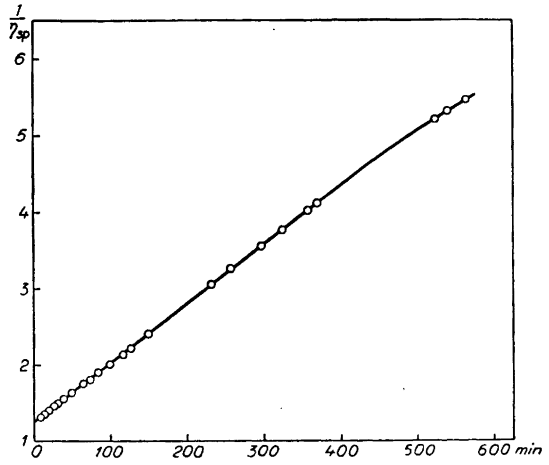


Fig. 3. Variation of $1/\eta_{sp}$ with time.

I. 5 ml dextran solution + 2 ml *E* 1.
II. 5 ml dextran solution + 2 ml *E* 2.
III. 5 ml dextran solution + 2 ml *E* 3.

The decrease in viscosity was most rapid for solution I. For solution II it was more moderate. The viscosity of solution III did not change at all. Evidently the heat treatment used for *E* 3 is sufficient to destroy the activity of the enzyme. In Fig. 1 the specific viscosity, η_{sp}, has been plotted against time, and in Fig. 2 the quantity $1/\eta_{sp}$ is shown as a function of time. (Time = time elapsed after mixing + half the time of outflow). As is seen from Fig. 2 a linear relationship exists between $1/\eta_{sp}$ and time during the first stage of the breakdown. This is in agreement with Eq. (1). The declination of the straight lines gives the p-values 138.10^{-4} min^{-1} and 98.10^{-4} min^{-1} for solutions I and II respectively.

BREAKDOWN STUDIED BY MEANS OF VISCOSITY MEASUREMENTS AND DETERMINATIONS OF REDUCING SUGAR

200 mg of dextran was dissolved in 20 ml buffer (the same buffer used in previous experiments, pH 5.22). To 15 ml of this dextran solution 6 ml of *Cellvibrio fulva* enzyme was added. The breakdown was studied by means of viscosity measurements and by determinations of reducing sugar. As a control the same measurements were made on two blank solutions consisting of dextran solution + water, and buffer + enzyme respectively. All experimenst were performed at 25.0° C. In some cases ultracentrifugal measurements were made in order to study the change in sedimentation constant with time. The determinations of reducing sugar were performed according to the well known method of Hagedorn and Jensen. The purpose of this was merely to show that the reducing power of the solutions was increased only very slightly during the first stage of the breakdown. In Fig. 3 $1/\eta_{sp}$ has been plotted against time. Even in this experiment a linear relationship is obtained. After some time, however, the curve becomes less steep. From the linear part of the curve the p-value 77.10^{-4} min^{-1} is calculated. Ultracentrifugal measurements were made on samples from the principal experiment, taken after certain time intervals. Determinations of reducing sugar were made on samples both from the principal experiment and from the blank series. All samples were heated for 15 minutes at 80° C in order to destroy enzymatic activity. The results are given in Table 1, which contains the time, the specific viscosity, the amount of reducing sugar set free and the sedimentation constant of dextran at the concentration existing in the mixture (0.7 per cent).

Table 1. Enzymatic breakdown of dextran studied by means of viscosity measurements, ultracentrifugation and determinations of reducing sugar.

Time in min	Specific viscosity	Reducing sugar in per cent of amount of dextran	Sedimentation constant in S-units
15	0.74	0.0	9.6
60	0.59	0.0	
120	0.46	0.4	6.3
240	0.32	0.5	
367	0.24	0.9	1.8
536	0.19	2.0	
1410	0.09	3.0	

The reducing sugar has been calculated as glucose even if the reduction is caused by larger split products than glucose. It has been expressed in per cent of the original amount of dextran.

The sedimentation constants are not very reliable. The measurements are rather inaccurate as the broken down dextran has a high polymolecularity. However, it is evident that the molecular weight decreases rapidly during the breakdown. It follows from the table that only very small amounts of reducing sugar are set free in spite of the pronounced decrease in viscosity. The experiments indicate that the breakdown gives comparatively large split products at the beginning. Thus the enzyme does not split off glucose or disaccharides from the ends of the dextran molecules. (Compare in this respect the breakdown of starch by α- and β-amylases.) It is, however, too early to say anything definite about the mechanism of the breakdown.

EXPERIMENTS AT DIFFERENT ENZYME CONCENTRATIONS

In order to investigate the influence of the enzyme concentration on the rate of degradation, experiments were made with more or less diluted enzyme solutions at a constant dextran concentration. The original concentration of the *Cellvibrio fulva* enzyme was arbitrarily called 8 *e*. The diluted enzyme solutions had then the concentrations 4 *e*, 2 *e* and *e* respectively. The dextran solution was made from 200 mg dextran and 28 ml buffer (the same phosphate buffer as was used above). The temperature was 25.0° C as before. 5 ml of the dextran solution was mixed with 2 ml of the different enzyme solutions. The breakdown was followed viscosimetrically. In the same way as before the quantity $1/\eta_{sp}$ was plotted against time, and from the linear part of the curves

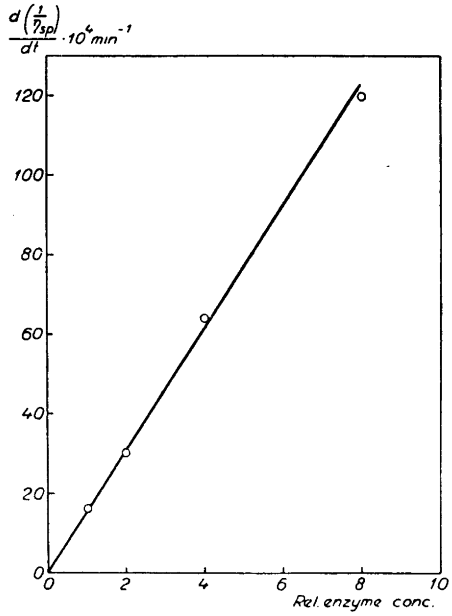


Fig. 4. Experiments at different enzyme concentrations.

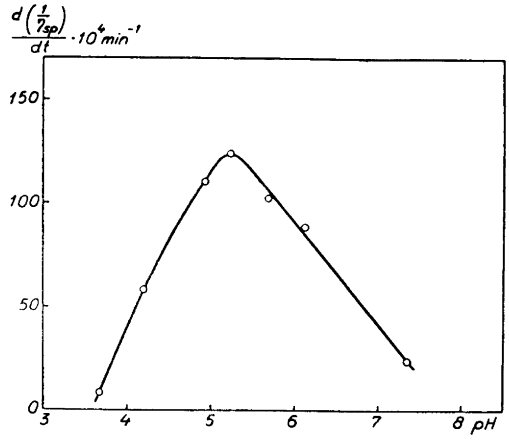


Fig. 5. The influence of pH upon the activity.

p-values for the four enzyme concentrations were calculated. In Fig. 4 the obtained p-values are plotted against the relative amount of enzyme. It is evident from the figure that during the present experimental conditions the p-values are directly proportional to the enzyme concentrations.

EXPERIMENTS AT DIFFERENT DEXTRAN CONCENTRATIONS

Hultin's formula (Eq. (1)) contains the substrate concentration squared. In order to prove whether or not this is valid even for the enzymatic breakdown of dextran Eq. (1) is written in the more general form represented by Eq. (3). Thus:

$$A = c^n \cdot p \tag{3}$$

Writing this equation in a logarithmic form:

$$\log A = n \log c + \log p \tag{4}$$

In order to determine the value of n, experiments have been made at a constant enzyme concentration but at varying dextran concentrations. The

Table 2. Experiments at different dextran concentrations.

Dextran concentration in per cent	$p \cdot 10^4$ min^{-1}	n
1.429	17.8	
0.879	43.6	
0.616	75.5	1.7
0.385	158.0	

measurements were made at 25.0° C in the same buffer (pH 5.22) as before. From the straight lines obtained from the relationship between $1/\eta_{sp}$ and time, the p-values have been calculated. The experimental c- and p-values have then been put into Eq. (4) and n has been calculated. The result follows from Table 2. The n-value is 1.7 and thus less than 2. Also in the enzymatic breakdown of polymetaphosphate [20] n-values less then 2 have been obtained.

DETERMINATION OF THE OPTIMUM pH OF THE ENZYME

In order to find out the optimum pH of the enzyme some experiments were made at different pH-values, but at constant dextran and enzyme concentrations. 50 mg dextran was dissolved in 7 ml each of different buffers. Sodium phosphate buffers of ionic strength 0.3 (ionic strength 0.1 coming from the buffer substances and 0.2 for NaCl) were used with different pH-values and also two sodium acetate/acetic acid buffers, one of ionic strength 0.3 (pH 4.19) and the other of ionic strength 0.1 (pH 3.68)). 2 ml of the enzyme solution was added to each dextran solution (5 ml) and the breakdown was followed viscosimetrically at 25.0° C as before. The quantity $1/\eta_{sp}$ was plotted against time, and the p-values were calculated. In Fig. 5 the obtained p-values have been plotted against the corresponding pH-values. It follows that the enzyme breaking down dextran has its optimum activity at a pH of 5.2—5.3.

SUMMARY

An enzyme extract has been prepared from cultures of the bacterium *Cellvibrio fulva*. This enzyme is capable of breaking down the polysaccharide dextran. The breakdown has been studied mainly by means of viscosity measurements. The experiments indicate that during the first stage of the breakdown the enzyme chiefly splits the dextran into comparatively large

fragments. Glucose or disaccharides originating from the ends of the molecules do not seem to be formed during this phase. The optimum pH of the enzyme has been determined to be pH 5.2—5.3.

I wish to express my gratitude to Prof. T. Svedberg and Prof. A. Tiselius, for giving me the opportunity to carry out this work in their laboratories. The author is grateful for the valuable technical assistance rendered by Miss A. Karlsson and Mrs. M. Hårsta. The investigation was supported financially by grants from A. B. Pharmacia, Stockholm, and Svenska Sockerfabriks A. B., Malmö.

REFERENCES

1. Hehre, E. *J. Biol. Chem.* **163** (1946) 221.
2. Evans, T., and Hibbert, H. *Adv. Carbohydrate Chem.* **2** (1946) 203.
3. Levi, J., Hawkins, L., and Hibbert, H. *J. Am. Chem. Soc.* **64** (1942) 1959.
4. Georges, L. W., Miller, I. L., and Wolfrom, M. L. *J. Am. Chem. Soc.* **69** (1946) 473.
5. Grönwall, A., and Ingelman, B. *Acta Physiol. Scand.* **7** (1944) 97.
6. Ingelman, B., and Halling, M. S. In press.
7. Ingelman, B., and Siegbahn, K. *Arkiv Kemi, Mineral. Geol.* **B 18** (1944) no 1.
8. Ingelman, B., and Siegbahn, K. *Nature* **154** (1944) 237.
9. Ingelman, B. Not yet published.
10. Swanson, M. A. *J. Biol. Chem.* **172** (1948) 805.
11. Proehl, E. C., and Day, H. G. *J. Biol. Chem.* **163** (1946) 667.
12. Swanson, M. A., and Cori, C. F. *J. Biol. Chem.* **172** (1948) 815.
13. Grönwall, A., and Ingelman, B. *Acta Physiol. Scand.* **9** (1945) 1.
14. Ingelman, B. *Acta Chem. Scand.* **1** (1947) 731.
15. Myrbäck, K. *Adv. Carbohydrate Chem.* **3** (1948) 251.
16. Fåhraeus, G. *Symbolae Botanicae Upsalienses* **IX : 2** (1947).
17. Hultin, E. *Acta Chem. Scand.* **1** (1947) 269.
18. Hultin, E. *Svensk Kem. Tid.* **60** (1948) 40.
19. Hultin, E. *Svensk Kem. Tid.* **60** (1948) 131.
20. Ingelman, B., and Malmgren, H. *Acta Chem. Scand.* **2** (1948) 365.

Received October 30, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 813—827

Subtraction of Atom Images from a Fourier Synthesis of a Crystal Lattice

CHR. FINBAK and N. NORMAN

Department of Physics, University of Oslo, Blindern - Oslo, Norway

In the last few years a considerable amount of work has been spent at The University of Oslo on the investigation of the structure of gases and liquids by electron and x-ray diffraction methods. One of the more complicated problems which we had to cope with, was the diffraction error in the Fourier synthesis, and a method which permits a certain control of this error was therefore developed. The principles of this procedure are in fact very simple. The maxima of the experimental distribution curves are removed one by one together with their diffraction maxima and minima. The only difficulty is to find the correct form and magnitude of the diffraction effect relative to a peak representing an interatomic distance in the Fourier synthesis [1,2].

A similar method may be applied to the different types of Fourier syntheses of crystal lattices from x-ray data. In the present paper it shall be demonstrated how the images of the different atoms may be removed from the density function determined by a two-dimentional Fourier synthesis of a crystal lattice.

THE REPRESENTATION OF THE ATOMS BY A FOURIER SYNTHESIS

The electron density $\varrho(x, y, z)$ of a crystal may be expressed by the following three-dimentional Fourier series:

$$\varrho\ (x,\ y,\ z) = \frac{1}{V} \sum_{h,\ k,\ l\ =\ -\infty}^{+\infty} \sum \sum \sum F\ (h,\ k,\ l)\ e^{2\pi i\,(hx+ky+lz)} \qquad (1)$$

where the unities for x, y, and z are the lattice constants a, b and c respectively, V represents the volume of the unit cell, and $F\ (h,\ k,\ l)$ is the crystal structure factor.

However, experimentally it is only possible to determine the numerical value of $F(h, k, l)$ within certain limits, and the expression (1) requires summations from $-\infty$ to $+\infty$. Systematical errors are thus introduced in the density function, $\varrho'(x, y, z)$, determined from experimental intensity data:

$$\varrho'(x, y, z) = \frac{1}{V} \sum_{h,} \sum_{k,} \sum_{l} F(h, k, l)\, e^{2\pi i (hx+ky+lz)} \qquad (2)$$

Bragg and West [3] who first observed this effect, proved that from a theoretical point of wiev, this is analogous to the diffraction phenomenon in optics, and therefore they termed it diffraction effect. Accordingly we may regard the result of a Fourier synthesis as an image of the atoms in the lattice produced by optical diffraction. For atoms situated sufficiently close together or especially in one- or two-dimentional projections, the images of the single atoms may overlap. The diffraction waves belonging to one atom may therefore distort the image of another atom considerably. Already Bragg and West [3] proposed a method which should make it possible to minimize the influence of the diffraction effect. Their method, which implies the introduction of an artificial temperature factor in the intensity expression, has been thoroughly investigated by Grimm, Brill, Hermann and Peters [4-9] and others. The method alters the image of an atom considerably, as it reduces the magnitude of its central peak and at the same time increases its width to a certain extent. This means in fact a reduction of the resolving power of the synthesis.

In a series of publications [10-14] Booth has undertaken a comprehensive investigation of the different errors which are of importance in the application of the Fourier synthesis to structural problems. He has determined expressions which give the approximate magnitude of the shift in the parameter values caused by the different types of errors. Booth also treats the diffraction effect and describes a method for a more correct determination of the parameters. As a practical example Booth applies his method to a two-dimentional projection of oxalic acid dihydrate [14]. For our investigation, we also have chosen this substance, Booths results not being published at the time when our work was started. A comparison between Booths results and ours are therefore very easy.

SUBTRACTION OF THE IMAGE OF SINGLE ATOMS

Two independent experimental intensity measurements are used as a basis for our computation of a projection in the xz-plane of the oxalic acid dihydrate crystal. The first one is published by Robertson and Woodward [15], and the second one by Brill, Hermann and Peters [9].

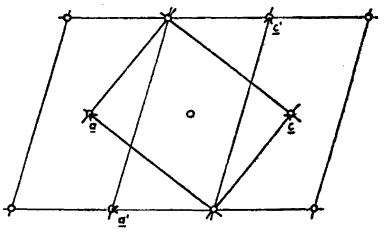


Fig. 1. The x z-plane of the unit cell.

The a and the c-axis used in the computation are derived from the real a- and c-axes by the expression (Fig. 1):

$$\mathbf{a} = \tfrac{1}{2}\ (3\ \mathbf{a}' + \mathbf{c}') \qquad \mathbf{c} = \tfrac{1}{2}\ (-\mathbf{a}' + \mathbf{c}') \tag{3}$$

Fig. 2 represents the AC-plane in the resiprocal lattice, and F (h, k, l) is equal to F (h, k, l) as the crystal has a center of symmetry. The intensities which are measured, are represented by small circles. The part of the diagram above the A-axis corresponds to the results of Brill, Hermann and Peters' measurements, and the part below the A-axis to those of Robertson and Woodward. For reasons which shall be explained later, the intensities which have been measured equal to zero are marked by double circles.

From these experimental intensities three different density functions were now determined. The first one was derived from Robertson and Woodwards

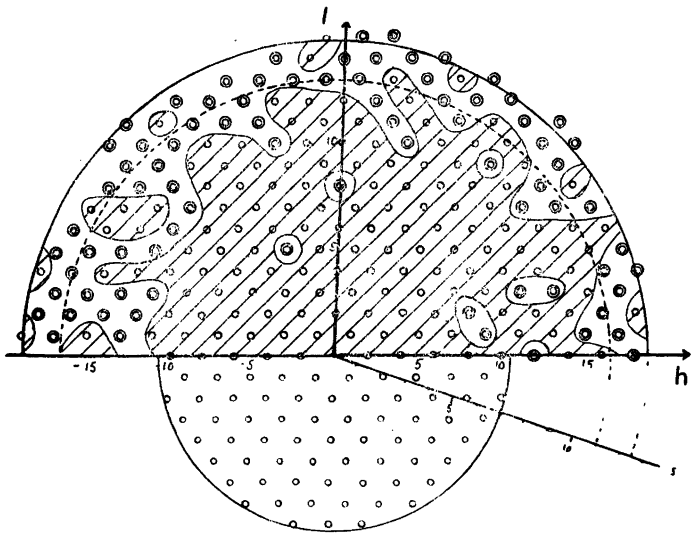


Fig. 2. The AC plane of the resiprocal lattice.

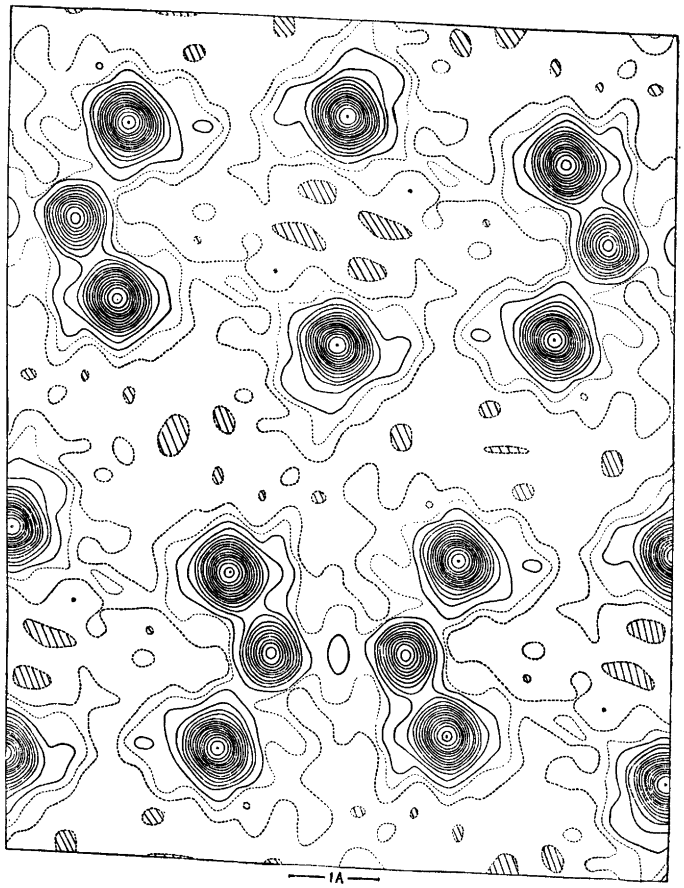


Fig. 3. Electron density map of oxalic acid dihydrate. The equidistance is 1 electron per A^2. *1 electron per* A^2 *is given by the heavy broken lines whereas the light broken lines correspond to* $1\frac{1}{2}$ *electron per* A^2. *The shaded areas represent negative density.*

data, the second from Brill, Hermann and Peters' intensities taking only those points in the resiprocal lattice into consideration which were also found in Robertson and Woodwards determination, and the third using all the points given by Brill, Hermann and Peters. The oxygen atom in the water molecules and the oxygen atom in the carboxyl group situated farthest away from the carbon atom in the projection, proved to have parameters which were in the very best accordance in the three different diagrams. The parameters of the oxygen atom which is closest to the carbon atom and especially those of the carbon atom itself, showed greater deviations in the three cases. The

magnitude of these deviations proved that they are certainly due to differences in the experimental data. In Fig. 3 the xz-projection based on Brill, Hermann and Peters' intensities is given.

If we want to remove the images of the atoms one by one from the projection, it should be most correct to start with the atoms whose positions are determined with the greatest accuracy. In the case of oxalic acid dihydrate this means the oxygen atoms. On the other hand it is also most important that the image removed is as correct as possible in its form and magnitude. The simplest way in which such an image of the oxygen atom may be obtained, is to choose the best possible model for the electron density in the oxygen atom, to place this model atom in accordance with the determined parameters, and to carry out a Fourier synthesis of the resiprocal lattice corresponding to the single atom. The problem is thus simplified to finding the correct model for the electron distribution in the atom, which again may be reduced to a determination of the correct atomic form factor, $f(s)$, of the atom in question. This determination may, however, be accomplished by means of the observed F (h, k, l) values, if the parameters of the atoms are known with a sufficient degree of accuracy. The structure factor, F (h, k, l), can always be written as a linear function of the atomic form factors. In our special case the structure factor may be expressed by:

$$\begin{aligned} \mathrm{F}(h,k,l) = 4\,[&f_{0_I}\cos 2\pi\,(hx_{0_I} + ky_{0_I} + lz_{0_I}) + f_{0_{II}}\cos 2\pi\,(hx_{0_{II}} + ky_{0_{II}} + lz_{0_{II}}) \\ &+ f_{0_{H_2O}}\cos 2\pi\,(hx_{0_{H_2O}} + ky_{0_{H_2O}} + lz_{0_{H_2O}}) + f_{C}\cos 2\pi\,(hx_{C} + ky_{C} + lz_{C}) \end{aligned} \tag{4}$$

leaving out the scattering from the hydrogen atoms. This omission of the influence of the lighter atoms represents in this case a satisfactory approximation, and as the atomic form factor of the hydrogen is decreasing very rapidly with increasing $s = \frac{4\pi \sin \frac{\Theta}{2}}{\lambda}$, this approximation is especially good for greater values of s. If now the parameters x, y and z of the atoms are determined with sufficient accuracy, a system of equations may be derived from expression (4), and from these equations the unknown atomic form factors may be determined. If now the atomic form factor, $f(s)$, is a function of s alone, it should be possible to determine the different atomic form factors for greater values of s. In our special example, we may assume that:

$$f_{0_I} = f_{0_{II}} = f_{0_{H_2O}} \tag{5}$$

Points on the atomic form factor curve for oxygen from Brill, Hermann and Peters' experimental values for $F(h, k, l)$ are shown in Fig. 4. In Fig. 5 the

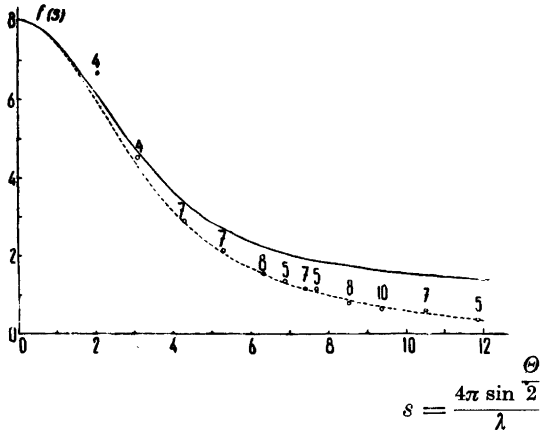


Fig. 4. Atomic form factor for oxygen. O *Calculated points. The figures indicate the number of points in the resiprocal lattice applied by each computation.* —— *$f(s)$ determined from Hartrees atom model,* – – – *$f(s)\ e^{-0.01s^2}$.*

observed values used in the determination of the different points of Fig. 4 are marked by small circles. These points on the atomic form factor curve may be utilized for different purposes. We have chosen to multiply an atomic form factor, $f(s)$, obtained from Hartrees atom model by a function e^{-Bs^2}. The constant B is given a value which brings the function $f(s)\ e^{-Bs^2}$ to fit as closely as possible for greater values of s to the atomic form factor values as derived from the experimental F (h, k, l).

In general it is the outer part of the atom image which is the most important and the most difficult to determine correctly. At greater distances from the center of the atom, the image depends in the first place on the value of

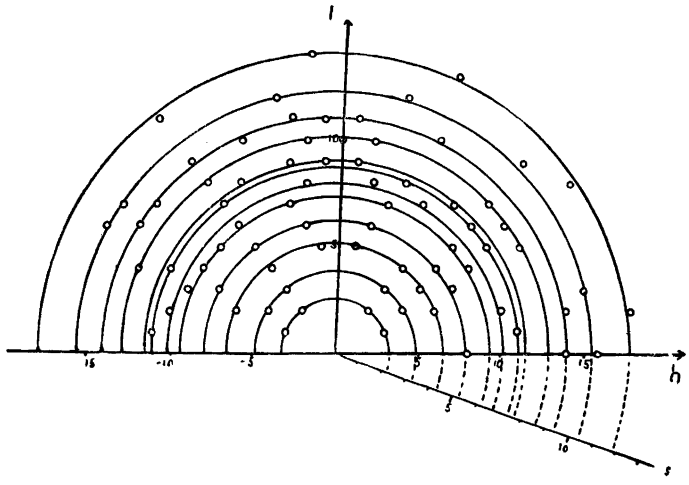


Fig. 5. Points in the reciprocal lattice used for the determination of Fig. 4.

$f(s_0)$ and in the second place on $\frac{df(s)}{d(s^2)}$ for $s = s_0$, where s_0 is the greatest value of s for which any intensity is observed.

A more comprehensive theoretical investigation proves that the image corresponding to a spherical atom in a two dimentional Fourier synthesis may be expressed by the converging series:

$$\varrho(r) = \frac{1}{4\pi} \sum_{k=0}^{\infty} (-1)^k f_{s^2}^{(k)}(s_0) \frac{{s_0}^{2k+2}}{(k+l)!} \Lambda_{k+l}(rs_0) \qquad (6)$$

r is given in Ångstrøm units, s_0 in resiprocal Ångstrøm units, $f_{s^2}^{(k)}(s)$ represents the k^{th} derivate of $f(s)$ with regard to s^2, and Λ_k are functions closely related to the Bessel functions [16]. In general the significance of the terms is decreasing rapidly as k is increasing, and for greater values of rs_0, the series is dominated by the first few terms. It is therefore of the greatest importance that the atomic form factor, used, has a correct form in the neighbourhood of s_0, and one of the greater advantages of this method is that it leads to an atomic form factor curve which is more reliable for greater values of s.

When a Fourier synthesis based on the corrected atomic form factor curve is carried through for the determination of an atom image, the influence of the limits of summation in the resiprocal lattice should also be taken into consideration. In order to illustate this special problem, $F(h, 0, l)$ values have been computed using the corrected atomic form factor curve, $f(s)\ e^{-0.01\,s^2}$, shown in Fig. 4 for the oxygen atoms, and a correspondingly corrected atomic form factor curve, $f(s)\ e^{-0{,}008\,s^2}$ for the carbon atom. The result is given in Table 1. The influence of the hydrogen atoms is also in this case left out. For comparison the corresponding experimental values for F $(h, 0, l)$ are given below the theoretical ones in the table. The theoretical and experimental values are, as it is seen, in the very best agreement, and this is, as should be expected, especially the case for greater values of s. It is of special interest to notice that the table seems to indicate the existence of a certain threshold value for the observed intensities at approximately 1.0 in the units used in the table. The most correct way of fixing the limit of summation in the resiprocal lattice, when the image of an atom is determined, should therefore be to leave out all the F $(h, 0, l)$ values which experimentally are found equal to zero.

The procedure by subtraction of atom images from a Fourier synthesis of a crystal lattice should therefore be: First the correct atomic form factors are determined for the atoms whose parameters are best known. Then these atoms are placed in the lattice according to their parameters, and their images are

Table 1. $\frac{F(h, 0, l)}{2}$ *for oxalic acid dihydrate. The upper figures represent the theoretical values, the lower ones the corresponding experimental values.*

l / h	$\bar{15}$	$\bar{14}$	$\bar{13}$	$\bar{12}$	$\bar{11}$	$\bar{10}$	$\bar{9}$	$\bar{8}$	$\bar{7}$	$\bar{6}$	$\bar{5}$	$\bar{4}$	$\bar{3}$	$\bar{2}$	$\bar{1}$	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	l / h
0																60.0		0.9		$\bar{1.5}$		10.2		$\bar{0.3}$		2.8		3.1		$\bar{1.0}$		0
																66.0		2.7		$\bar{2.4}$		10.5		0		3.0		3.5		0		
1	$\bar{2.1}$		$\bar{0.9}$		1.9		$\bar{4.2}$		$\bar{1.3}$		$\bar{1.8}$		$\bar{26.3}$		11.6		7.6		$\bar{21.1}$		$\bar{1.4}$		$\bar{1.6}$		$\bar{1.2}$		1.5		$\bar{0.6}$		1
	$\bar{2.3}$		0		2.2		$\bar{4.6}$		$\bar{1.3}$		$\bar{3.3}$		$\bar{21.9}$		8.3		6.0		$\bar{16.6}$		$\bar{1.8}$		$\bar{1.6}$		$\bar{1.5}$		1.5		0		
2		$\bar{1.3}$		2.2		1.2		$\bar{0.9}$		2.2		$\bar{6.4}$		$\bar{2.1}$		14.2		$\bar{3.0}$		$\bar{5.3}$		$\bar{3.7}$		0.3		0.9		$\bar{0.8}$		$\bar{0.5}$		2
		$\bar{1.2}$		2.2		1.4		$\bar{1.4}$		2.5		$\bar{6.8}$		3.0		11.1		$\bar{4.4}$		$\bar{6.8}$		$\bar{4.8}$		0.8		1.2		0		0		
3			0.3		2.1		2.7		1.2		0.2		9.2		9.6		12.3		13.2		$\bar{3.5}$		2.0		5.9		0.4		0.7		3
			0		2.5		3.0		1.4		0		8.7		10.5		12.4		13.2		$\bar{4.2}$		2.7		7.4		0		1.2		
4		$\bar{0.9}$		$\bar{1.6}$		0.8		$\bar{0.4}$		$\bar{10.9}$		$\bar{2.8}$		$\bar{3.3}$		$\bar{20.1}$		5.2		$\bar{7.2}$		$\bar{11.1}$		2.8		$\bar{0.2}$		$\bar{2.0}$		0.1		4
		0		$\bar{3.0}$		0		$\bar{0.9}$		$\bar{10.2}$		$\bar{3.4}$		$\bar{2.4}$		$\bar{19.2}$		6.9		$\bar{7.2}$		$\bar{11.2}$		3.6		0		$\bar{2.7}$		0		
5			$\bar{0.3}$		0.8		6.1		$\bar{2.4}$		$\bar{2.9}$		13.6		$\bar{4.6}$		4.9		10.4		$\bar{8.1}$		0.6		4.0		$\bar{0.7}$		0.1		5
			0		0		5.9		$\bar{2.7}$		$\bar{3.8}$		11.7		$\bar{5.0}$		6.4		10.7		$\bar{8.8}$		1.1		4.8		$\bar{1.0}$		0		
6				$\bar{1.4}$		3.2		2.0		$\bar{6.2}$		4.3		4.2		$\bar{1.7}$		9.5		$\bar{0.8}$		$\bar{2.3}$		3.4		2.0		0.6				6
				0		2.9		2.0		$\bar{6.1}$		4.8		5.6		$\bar{2.1}$		9.4		$\bar{1.3}$		$\bar{2.5}$		4.1		2.1		0				
7			$\bar{1.3}$		$\bar{0.4}$		1.5		$\bar{4.1}$		$\bar{5.6}$		$\bar{2.5}$		$\bar{3.5}$		$\bar{2.1}$		$\bar{5.0}$		$\bar{4.7}$		$\bar{2.8}$		$\bar{0.8}$		0.5		$\bar{1.2}$		7
			0		0		1.0		$\bar{4.0}$		$\bar{6.1}$		$\bar{2.0}$		$\bar{3.4}$		$\bar{1.9}$		$\bar{6.2}$		$\bar{5.2}$		$\bar{2.8}$		$\bar{1.4}$		0		$\bar{1.5}$		
8				1.1		2.3		2.0		1.4		$\bar{0.7}$		4.6		9.5		$\bar{0.4}$		1.2		2.3		$\bar{0.7}$		2.6		1.3				8
				0		2.0		1.9		1.2		$\bar{0.9}$		4.9		9.5		0		1.6		3.2		$\bar{1.0}$		3.2		0				
9					0.6		$\bar{1.2}$		0.9		$\bar{2.9}$		$\bar{5.4}$		7.0		0.1		$\bar{4.9}$		2.3		$\bar{1.7}$		0.0		2.5						9
					0		$\bar{0.9}$		0		$\bar{2.9}$		$\bar{5.0}$		7.7		0		$\bar{5.6}$		2.5		$\bar{1.9}$		0		1.6						
10				1.3		$\bar{0.9}$		$\bar{0.3}$		0.7		$\bar{6.8}$		0.3		4.3		$\bar{6.0}$		$\bar{1.7}$		$\bar{0.9}$		$\bar{2.5}$		1.0						10
				0		0		0		0		$\bar{5.8}$		1.0		4.4		$\bar{6.0}$		$\bar{1.4}$		$\bar{1.1}$		$\bar{3.0}$		1.2						
11					1.5		0.2		2.9		$\bar{0.3}$		$\bar{0.5}$		6.9		2.8		0.1		1.4		0.6		1.7		1.4						11
					1.2		0		2.6		0		0		6.7		2.6		0		1.5		0		2.1		0						
12						$\bar{0.5}$		$\bar{0.6}$		$\bar{1.8}$		$\bar{3.1}$		$\bar{0.5}$		0.5		$\bar{0.5}$		$\bar{2.6}$		$\bar{1.9}$		0.7		0.0						12
						0		0		$\bar{1.7}$		$\bar{2.0}$		0		0		$\bar{1.0}$		$\bar{2.4}$		$\bar{2.3}$		0		0						
13							1.1		$\bar{1.3}$		$\bar{0.9}$		0.6		$\bar{0.5}$		2.0		$\bar{0.1}$		$\bar{3.5}$		0.6		0.9								13
							0		$\bar{1.1}$		0		0		0		1.5		0		$\bar{3.6}$		0		0								
14								0.3		$\bar{1.3}$		2.1		0.4		1.3		3.9		$\bar{1.6}$		$\bar{0.3}$		2.5								14
								0		$\bar{1.3}$		1.6		0		1.2		3.6		$\bar{1.7}$		$\bar{2.0}$		2.6								
15									$\bar{2.7}$		$\bar{1.1}$		$\bar{0.3}$		$\bar{2.2}$		1.1		$\bar{1.0}$		$\bar{3.2}$		0.1										15
									$\bar{1.3}$		0		0		$\bar{1.6}$		0		$\bar{1.1}$		$\bar{3.0}$		0										
16										$\bar{0.8}$		1.0		0.3		1.2		1.3		$\bar{0.9}$		$\bar{0.2}$										16
										0		0		0		1.1		0		0		0										
17											$\bar{0.5}$		0.1		1.2		0.7		$\bar{0.1}$														17
											0		0		0		0		0														
18												$\bar{1.7}$		0.4		0.3		$\bar{1.4}$														18
												$\bar{1.1}$		0		0		$\bar{1.4}$														
19															2.0																	19
															1.7																	

determined by a Fourier synthesis. In this synthesis it is of the greatest importance to use the same limits of summation as in the Fourier synthesis of the crystal lattice from the experimental data. The images of the atoms in question are then subtracted from the synthesis based on the experimental intensities. In general it should now be possible to determine the parameters more correctly for the atoms whose images are not yet subtracted. The atom images which are not subtracted in the first step, may now be removed in their turn and so on. This procedure should in general lead to a more correct determination of the parameters, especially for the lighter atoms in the lattice.

It may occur, however, that the images of two or more similar or nearly similar atoms are overlapping more or less in the synthesis based on experimental intensities. In one- or two-dimentional projections this is very often the case. These circumstances make the determination of the parameters of the single atoms very difficult. The problem may, however, be overcome in the following manner. First one of the atoms is placed in an approximately correct position and its image subtracted. Then the next atom is placed as correctly as possible in the lattice and its image subtracted. In general it is now possible to determine more accurate parameters for the atom first subtracted, and the procedure is repeated, if necessary several times. In this way it is always possible to improve the parameter values considerably provided the atom images have the correct form.

In the next part of the present paper, the application of the method shall be elucidated by a practical example.

THE APPLICATION OF THE METHOD

The upper part of Fig. 6 represents the remainder of a Fourier synthesis based on the experimental structure factors, F $(h, 0, l)$ given in Table 1, when the images corresponding to all the oxygen atoms in the oxalic acid dihydrate have been removed. The atomic form factor used for the oxygen atoms is represented by the broken line in Fig. 4, and all the F $(h, 0, l)$'s which, according to the experimental intensity data are equal to zero, have also been left out in the Fourier synthesis of the atom images. The parameters of the carbon atom before and after the subtraction of the oxygen atoms, were determined to be respectively: $x = 0.0025$, $z = 0.1011$ and $x = 0.0033$, $z = 0.1006$. This corresponds to a shift in the xz-projection of the carbon atom equal to 0.0085 Å.

In Fig. 7 the images of both the carbon and the oxygen atoms are removed from the experimental synthesis. The atomic form factor for the carbon atom is determined from Hartrees atom model and multiplied by the function

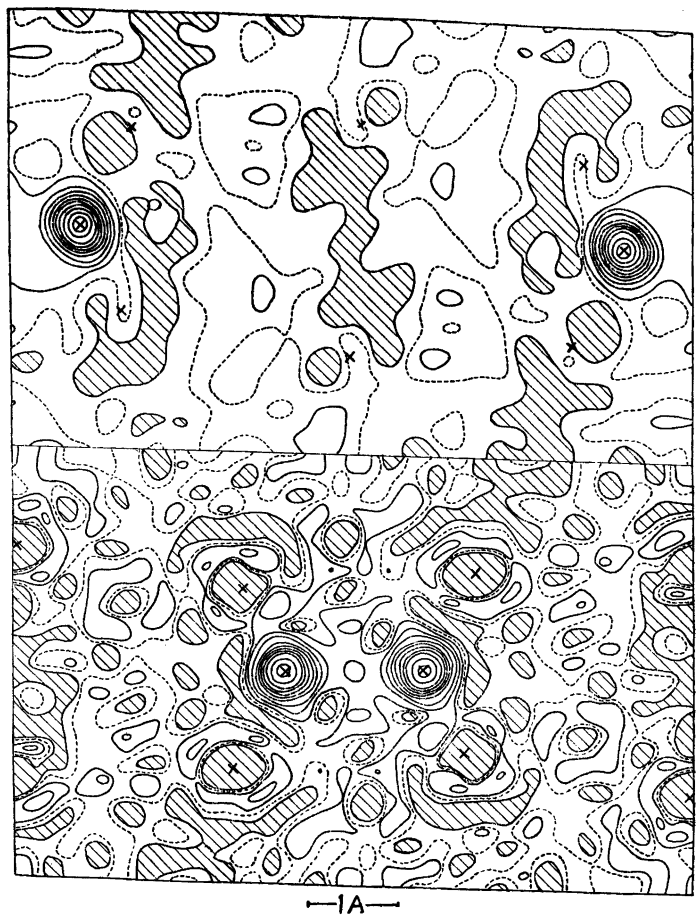


Fig. 6. Electron density map of oxalic acid dihydrate from which the oxygen peaks are removed. The equidistance is 1 electron per A^2. The broken lines correspond to $\frac{1}{2}$ electron per A^2. Negative density is given as shaded areas.

$e^{-0{,}008\,s^2}$. The figure also illustrates the importance of using the correct limit of summation for the determination of the atom images. In the upper half of the figure, all the F (h, 0, l) values which are found equal to zero in the experimental intensities also are left out in the Fourier synthesis of the atom images. In the lower half all the theoretically determined F (h, 0, l) values for the different atoms are used in the summation, provided they are found within the part of the resiprocal lattice for which observations of intensities are available.

It will be seen that this method indicates some rather interesting possibilities. If the intensity measurements are sufficiently accurate, then Fig. 7

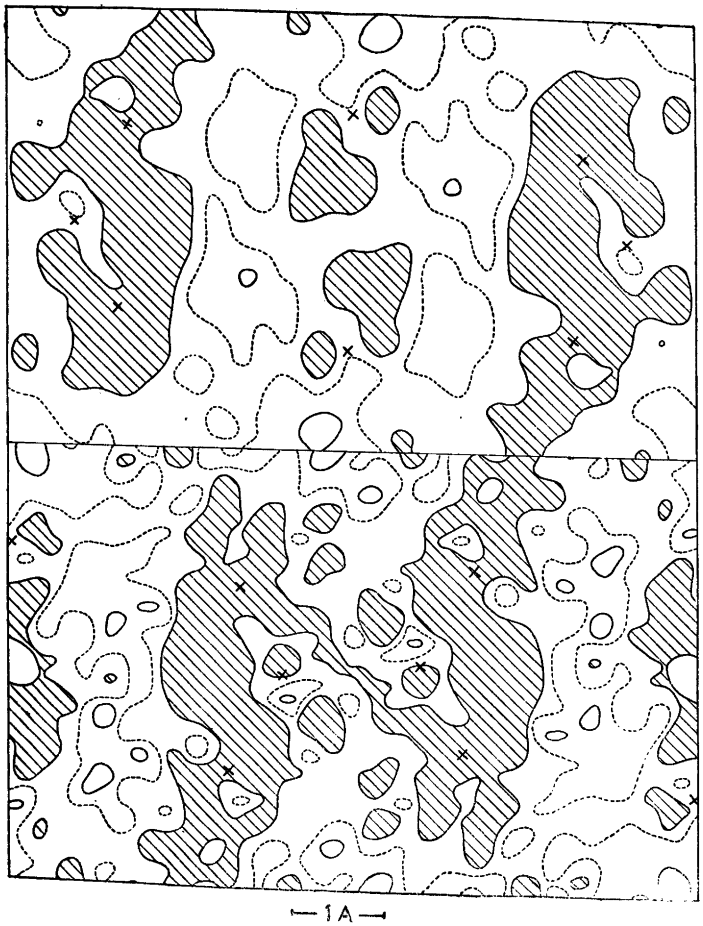


Fig. 7. Electron density map of oxalic acid dihydrate the oxygen and carbon peaks being removed. The equidistance is 1 electron per A^2. The broken lines correspond to $\frac{1}{2}$ electron per A^2. Negative density is marked out by shading.

should represent a projection in the *xz*-plane of the hydrogen atoms and an eventual distortion of the carbon and oxygen atoms. The position of the maxima in Fig. 7 are in the very best accordance with the hydrogen bonds predicted by Brill, Hermann and Peters [9] from the observed interatomic distances between the oxygen atoms. The crystalline oxalic acid dihydrate should therefore rather be described as the oxonium salt of oxalic acid:

$$\begin{matrix} COO \cdot H_3O \\ | \\ COO \cdot H_3O \end{matrix}$$

As mentioned above, this results depend completely on the reliability of Brill, Hermann and Peters' intensity measurements. If we therefore should want to give a better proof of the existence of the hydrogen bonds by this method, it should be necessary to verify experimentally the results of Brill, Hermann and Peters.

Finally, the lower part of Fig. 6 shows the result of a subtraction in which the atom images are obtained by using atomic form factor curves determined directly from Hartrees atom model. Again the image of the oxygen atoms contains diffraction rings, the maxima and minima of which are found at the same distance from the center of the image as in the Fourier synthesis from the experimental F (h, 0, l) values. The magnitude of the diffraction maxima and minima in the image are, however, about four times as great as in the experimental synthesis. The subtraction of such images accordingly must introduce an enlarged negative diffraction effect into the results.

Allready the results illustrated in Fig. 4 proves that the correct atomic form factor curve approaches the s-axis more rapidly with increasing s value than the atomic form factor determined from Hartrees atom model, and Fig. 6 demonstrates quite plainly that an uncritical application of Hartrees atom model in the subtraction may lead to an increased negative diffraction effect, which again may distort the experimental image considerably more than the original diffraction effect. A correction of the parameter values by the subtraction method may therefore easily lead to a deterioration of the results if a proper choice of atom images is not secured.

OTHER METHODS FOR THE DETERMINATION OF ATOM IMAGES

The atomic form factors used in our subtraction method were derived from a theoretical expression for the structure factor F (h, k, l) (4). Other methods for the determination of the proper atom images were, however, also tried.

If we only want to remove the outer part of the atom image, *i. e.* the diffraction maxima and minima, and are not interested in a total removal of its central peak, then the following method may be applied.

The whole atom image obtained from an atomic form factor corresponding to Hartrees atom model may be reduced in such a proportion that the magnitude of the diffraction waves is in accordance with the magnitude of the diffraction waves of the corresponding atom in the experimental synthesis. The procedure can, however, never lead to a complete removal of the total atom image.

From Fig. 4 we may conclude that the observed structure factor values and consequently the correct atomic form factors are falling more rapidly with

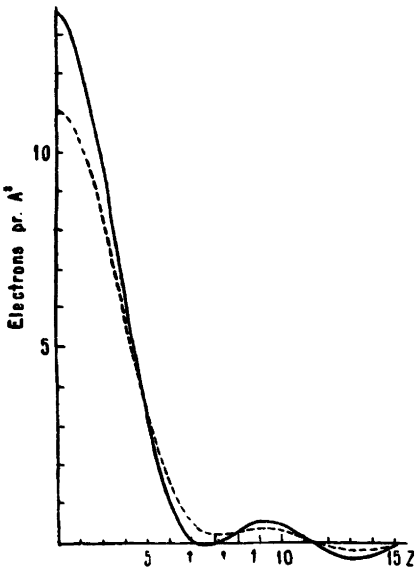


Fig. 8. $s_0 = 7$, —— *f(s) derived from Hartrees atom model,* – – – $f(s)\, e^{-0.011 s^2}$.

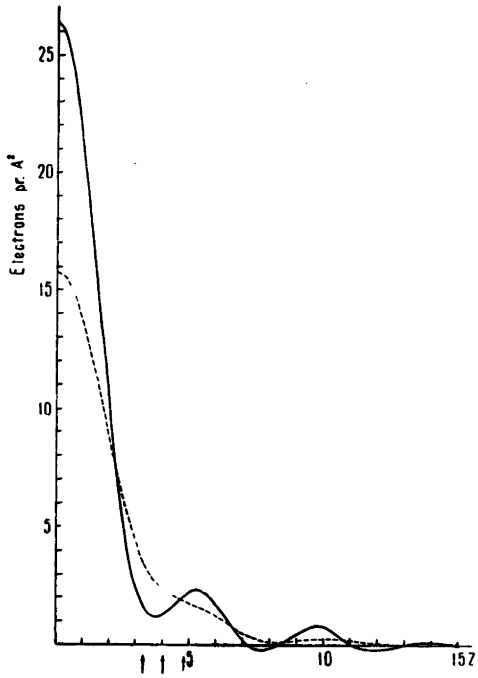


Fig. 9. $s_0 = 12.5$, —— *f(s) from Hartrees atom model,* – – – $f(s)\, e^{-0.011 s^2}$.

increasing s value than the atomic form factors derived from Hartrees atom model. In our subtraction method we attained the correct atomic form factors by introducing the function e^{-Bs^2}, where the constant B is given an appropriate value.

A determination of the constant B may also be attained by a fitting of the image of each atom to the corresponding atom in the experimental synthesis. The Figs. 8 and 9 represent a section through the center of a two-dimentional projection of an oxygen atom, corresponding to a maximum value of $s = s_0$ equal to 7 A^{-1} and 12.5 A^{-1} respectively. The full drawn curves correspond to a Hartree atom model, and the broken curve represents an image obtained by introducing the function e^{-Bs^2} where the constant B is equal to 0.011. In the two figures the position of the first minimum of the functions Λ_1, Λ_2 and Λ_3 [16] are marked by small arrows. A multiplication of the atomic form factor by the function e^{-Bs^2} increases the width and decreases the magnitude of the central peak in the atom image, and both these effects might perhaps be used for the determination of the constant B. A variation in B has, however, a

small influence on the width but a comparatively great influence on the magnitude of the peak. A more detailed investigation of the connection between the constant B and the elevation of the peak, E, expressed in electrons pr. A^2, leads to the formula:

$$E \approx \frac{1}{2\pi} \int_0^{s_0} e^{-Bs^2} f(s)\, s\, ds \tag{7}$$

where $f(s)$ is the atomic form factor determined from Hartrees atom model. The value $B = 0.009$, which was determined from this formula for the oxygen atoms in oxalic acid dihydrate, is in the best accordance with the previously determined value $B = 0.010$.

As mentioned above, the procedure first described for the determination of B is based directly upon the experimental structure factors, and is certainly the more correct and reliable one, and should therefore in general be preferred.

SUMMARY

It is demonstrated how the images of the single atoms may be removed one by one or in groups from the density functions obtained by Fourier synthesis of a crystal lattice. The method is applied to a two-dimentional projection, but may easily be extended to comprise also the three-dimentional case.

By the subtraction of the atom images it is essential to adjust the atom model in such a way that the image fits to the corresponding image in the experimental synthesis. Different methods for such adjustment are described. One of the methods is based on the intensities in a plane of the resiprocal lattice. It is, however, understood that an application of all the intensities in the whole resiprocal lattice certainly should increase the reliability of the results considerably.

If it is of special importance to remove the outer part of the image, *i. e.* the diffraction effect, then the conformity of the theoretical and experimental structure factors must be the best possible for greater values of s. By our method such a conformity of the atomic form factors of the different atoms is secured in the outer parts of the resiprocal lattice, and this means again a conformity of the theoretical and experimental structure factors.

We have shown that there probably exists a certain threshold value for the experimental intensity measurement, which means that in the Fourier synthesis of the different atom images, all the terms which are not observed in the

experimental intensity measurement, should be left out. It means that the limits of summation must be the same both for the synthesis based on the experimental intensities and for the synthesis of each atom image.

We want to express our gratitude to Dr. Ing. H. L. Lorentzen for financial aid to this work.

REFERENCES

1. Viervoll, H. *Avhand. Norske Videnskaps-Akad. Oslo Mat. Naturv. Klasse* (1944) no. 5.
2. Viervoll, H. *Acta Chem. Scand.* **1** (1947) 120.
3. Bragg, W. L., and West, J. *Phil. Mag.* **10** (1930) 823.
4. Grimm, H. G., Brill, R., Hermann, C., and Peters, Cl. *Naturwissenschaften* **26** (1938) 479.
5. Brill, R., Hermann, C., and Peters, Cl. *Naturwissenschaften* **27** (1939) 676.
6. Brill, R., Grimm, H. G., Hermann, C., and Peters, Cl. *Ann. Physik.* **34** (1939) 393.
7. Peters, Cl. *Z. Elektrochem.* **46** (1940) 436.
8. Hermann, C. *Z. Elektrochem.* **46** (1940) 425.
9. Brill, R., Hermann, C., and Peters, Cl. *Ann. Physik.* **42** (1942) 357.
10. Booth, A. D. *Proc. Roy. Soc.* **A 188** (1946) 77.
11. Booth, A. D. *Trans. Far. Soc.* **42** (1946) 444.
12. Booth, A. D. *Trans. Far. Soc.* **42** (1946) 617.
13. Booth, A. D., *Proc. Roy. Soc.* **A 190** (1947) 482.
14. Booth, A. D. *Proc. Roy. Soc.* **A 190** (1947) 490.
15. Robertson, J. M., and Woodward, J. *J. Chem. Soc. II* (1936) 1817.
16. Jahnke, E., and Emde, F. *Tables of functions* Berlin and Leipzig, (1933) 250.

Received August 15, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 828—838

Electrometric Investigation of Equilibria between Mercury and Halogen Ions

VI. Complexes between Hg^{2+} and Br^- and some Equilibria involving Solid Mercury(I)bromide

PER OLOF BETHGE, INGA JONEVALL-WESTÖÖ, and LARS GUNNAR SILLÉN

Institute of Inorganic and Physical Chemistry, University of Stockholm, Sweden

In continuation of the work at this Institute on mercury-halogen equilibria [1-5] we have undertaken an investigation of the complexes Hg^{2+}—Br^-.

By analogy with the Hg^{2+}—Cl^- complexes it might be expected that in aqueous solution Hg^{2+} and Br^- form the complexes $HgBr^+$, $HgBr_2$, $HgBr_3^-$, and $HgBr_4^{2-}$. Actually, in none of the papers hitherto published on equilibria of Hg^{2+}—Br^- complexes, has the existence of all four types of complex been taken into account, and the existing literature data are still more scanty and contradictory than in the case of the chloride complexes.

PREVIOUS WORK

Morse [6] in 1902 measured the solubility of AgBr in Hg^{2+} solutions. With the assumption that only $HgBr^+$ was formed, he found $(Hg^{2+})\,(Br^-)\,(HgBr^+)^{-1} = \varkappa_1^{-1} = 0.4 \cdot 10^{-9}$. (The equilibrium constants $\varkappa_1$, $\varkappa_2$, $\varkappa_3$, $\varkappa_4$, q_1 and q_2 have the same meaning as in previous papers in this series; they are moreover listed together with the corresponding reaction formulae in Table 4 of this paper.) From measurements of the solubility of $HgBr_2$ in $Hg(NO_3)_2$ solutions, Morse estimated $(HgBr^+)^2\,(Hg^{2+})^{-1}\,(HgBr_2)^{-1}$ $[= k_{12} = \varkappa_1^2 \varkappa_2^{-1}] = 12$, which value he considered to be rather uncertain. This would give $\varkappa_2^{-1} = 2.10^{-18}$.

Abegg, Immerwahr (Mrs Haber), and Jander [7] found in 1902 that Hg_2Br_2 is decomposed by solutions of KBr, metallic mercury being formed and bivalent mercury being dissolved. They explained this phenomenon qualita-

tively by assuming that $HgBr_3^-$ was formed, but could not make their results (for 1—3 C KBr) agree quantitatively with the law of mass action. Sherrill [8], in 1903, showed that this was possible if it was assumed that only $HgBr_4^{2-}$ was formed; he moreover measured the emf of cells with a Hg electrode in a solution containing 1.0 or 2.0 C KBr and 0.88—28.3 mC $HgBr_2$. From this he concluded $(HgBr_4^{2-})\,(Hg^{2+})^{-1}\,(Br^-)^{-4} = \varkappa_4 = 4.3 \cdot 10^{21}$.

Sherrill also studied the partition of $HgBr_2$ between benzene and aqueous solutions of KBr (0.05—0.50 C). On assuming that only $HgBr_4^{2-}$, and no $HgBr_3^-$, was formed, he obtained values of $q_2 = (HgBr_4^{2-})\,(HgBr_2)^{-1}\,(Br^-)^{-2}$ that varied very widely. He chose the value $q_2 = 2.8 \cdot 10^4$ but concluded that other complexes are formed too. To these he ascribed the general formula $(HgBr_2)_x\,(Br^-)_2$; from freezing and solubility measurements he concluded that x is 2, thus that the principal ionic species besides $HgBr_4^{2-}$ is $Hg_2Br_6^{2-}$.

In 1907, Sand and Breest [9] showed that Sherrill's analyses could be much better explained if it was assumed that $HgBr_3^-$ was the only complex formed. In this case, $q_1 = (HgBr_3^-)\,(HgBr_2)^{-1}\,(Br^-)^{-1}$ was found to be rather constant, varying only between 164 and 307 in 12 experiments. By combining $\varkappa_4$ and q_2 Sherrill had calculated $(Hg^{2+})\,(Br^-)^2\,(HgBr_2)^{-1} = \varkappa_2^{-1} = q_2\,\varkappa_4^{-1} = 6.5 \cdot 10^{-18}$; since his q_2 is questionable, this value should not be trusted too much either.

Abegg and Sherrill [10] showed later on that the solubility of Hg_2Br_2 in strong KBr solutions could be explained by means of Sherrill's values of k_s for Hg_2Br_2, $\varkappa_2$ and q_2, and the equilibrium constant of Ogg and Abel (quoted by Jonsson *et al.*[3]) $k_0 = (Hg_2^{2+})\,(Hg^{2+})^{-1} = 120$. At equilibrium with Hg and Hg_2Br_2, they found $(HgBr_4^{2-})\,(Br^-)^{-2}$ $[= q_2\varkappa_2 k_s k_0^{-1} = \varkappa_4 k_s k_0^{-1}] = 0.088$. By combining this value with their values of $\varkappa_2$, k_s, and k_0^{-1}, they found $q_2 = 2.9 \cdot 10^4$ in close agreement with the value picked out from Sherrill's partition experiments. These calculations may be a check on their values for $\varkappa_4$, k_s and k_0, and also prove that at the high (Br^-) employed (1—3 C) the mercury is present chiefly as $HgBr_4^{2-}$, However, since $\varkappa_2$ was calculated from q_2 and $\varkappa_4$, they do not, as Abegg and Sherrill seem to think, give an independent proof of the value of q_2 picked out from the partition experiments at much lower (Br^-), where, as we now know, the amount of $HgBr_3^-$ cannot be neglected.

Since 1903, no measurements of $\varkappa_1$ or $\varkappa_2$ for Hg^{2+}—Br^- has been attempted, as far as we know. There is thus no reliable value of $\varkappa_2$, and only one sound determination of $\varkappa_1$, that of Morse [6], giving $\varkappa_1 = 2.5 \cdot 10^9$.

The investigators who have applied the law of mass action to equilibria of $HgBr_2$ with Br^- and higher complexes can be divided into two groups: those who neglected the concentration of $(HgBr_3^-)^{-1}$ and tried to calculate $q_2 = (HgBr_4^{2-})\,(HgBr_2)^{-1}\,(Br^-)^{-2}$, and those who calculated $q_1 = (HgBr_3^-)\,(HgBr_2)^{-1}\,(Br^-)^{-1}$, neglecting the concentration of $HgBr_4^{2-}$.

The former group consists of Sherrill [8] ($q_2 = 2.8 \cdot 10^4$, *vide supra*) Job [11] (spectrographic method, $q_2^{-1} \approx 1.6 \cdot 10^{-6}$, thus $q_2 \approx 6 \cdot 10^5$ at 16° C) and Fromherz and Lih [12] (spectrographic measurements, $q_2 = 1.4 \cdot 10^4$). In the latter group are Sand and Breest [9] $q_1 \approx 200$, *vide supra*) and Garrett [13], who measured the solubility of $HgBr_2$ in 0.003—1 m KBr. His results »suggest that the $HgBr_3^-$ is the main ion formed with the possibility of only a very small amount of $HgBr_4^{2-}$ ion». On this assumption Garrett found $(HgBr_3^-)\,(Br^-)^{-1} = 1.85$, which in combination with the solubility of $HgBr_2$, 0.0170 m, gives $q_1 = 109$.

Of all these measurements, those of Garrett would seem to be the most reliable. However, we shall see that his conclusions are quite at variance with our own.

PLAN OF THE EXPERIMENTS

As in our previous work, the equilibria were studied in solutions of constant acidity ($(H^+) = 10$ mC) and practically constant ionic strength ($(ClO_4^-) = 500$ mC or, for $X_e > 0$, $(ClO_4^-) + X_e = 500$ mC). The apparatus was the same as that described previously.

The equilibrium constant $k_{12} = \varkappa_1^2 \varkappa_2^{-1} = (HgBr^+)^2\,(Hg^{2+})^{-1}\,(HgBr_2)^{-1}$ has already been measured by Sillén and Infeldt [2] who found $k_{12} = 5.8 \pm 0.3$.

The determination of $\varkappa_1$ and $\varkappa_2$ for $Hg^{2+} - Br^-$ was analogous to that for $Hg^{2+} - Cl^-$ [5]. First the solubility product k_s of Hg_2Br_2 was determined by titrations of Hg_2^{2+} with Br^-, using Hg electrodes. Then the maximum redox emf during titrations of Hg_2^{2+}, Hg^{2+} mixtures with Br^-, together with the known k_{12} and k_s, gave $\varkappa_1$ and $\varkappa_2$.

For the chloride complexes, q_1 and q_2 had been calculated from redox titrations of Hg^{2+}, Hg_2^{2+} mixtures with excess of Cl^-. For Br^-, this method proved impracticable. Instead the free concentration of (Hg^{2+}) in $HgBr_2 - Br^-$ mixtures was measured by a Hg electrode. These measurements, which offered certain experimental difficulties, gave directly $\varkappa_3$ and $\varkappa_4$, from which q_1, q_2, and a number of other constants (Table 4) were obtained.

DETERMINATION OF k_s

A titration of a Hg_2^{2+} solution with Br^- will, as was previously described for Cl^- in Part III, give the two quantities E_{10} and E_{1X} defined by

$-\,CE//Hg_2^{2+}/Hg\,+$	$E = E_{10} + 29.58 \log (Hg_2^{2+})$	$(1 = III,1)$
$-\,CE//X^-,Hg_2X_2/Hg\,+$	$E = E_{1X} - 59.16 \log (X^-)$	$(2 = III,2)$

By combining E_{10} and E_{1X}, the solubility product k_s of Hg_2X_2 can be found:

$$E_{1X}—E_{10} = 29.58 \log k_s \qquad (3 = \text{III},6)$$

By X_e we denote the concentration of halogen in excess over that needed to form Hg_2X_2 and HgX_2. In the first part of the titration curve, X_e is negative, and

$$E_{10} = E — 29.58 \log (Hg_2^{2+}) = E — 29.58 \log (-\tfrac{1}{2} X_e) + \delta E \qquad (4 = \text{III}, 12)$$

$$\delta E \approx 12.85\ k_0^{-1} + 6.42\ \varkappa_1 k_0^{-1} \sqrt{k_s}\ (Hg_2^{2+})^{-\frac{1}{2}} \qquad (5 = \text{III}, 13)$$

The term δE is a correction for the amounts of Hg^{2+} and HgX^+ present in equilibrium. By inserting the values of $\varkappa_1$ and k_s for Br, given later in this paper, and the previously known $k_0 = 129.2 \pm 1.0$ (III, 18), we find

$$\begin{array}{l} \delta E = 0.10 + 0.040\ (Hg_2^{2+})^{-\frac{1}{2}}\ \text{mV} \\ \text{thus } (Hg_2^{2+}),\ \delta E/4\text{mC}\ \ 0.12\,\text{mV}/1\,\text{mC}\ \ 0.14\text{mV}/0.25\,\text{mC}\ \ 0.18\ \text{mV} \end{array} \qquad (6)$$

These values are only insignificantly higher than those calculated from preliminary values and given in Part III. Before any halogen has been added, $\delta E = 0.10$ mV.

After the equivalence point, there should be a correction, $\delta'' E$, for the presence of $HgBr_3^-$ and $HgBr_4^{2-}$, since

$$X_e = (X^-) + (HgX_3^-) + 2(HgX_4^{2-}) = X(1 + \varkappa_3 k_s k_0^{-1} + 2\ \varkappa_4 k_s k_0^{-1} X) \qquad (7 = \text{III}, 21)$$

which, in combination with (2), gives

$$E_{1X} = E + 59.16 \log X = E + 59.16 \log X_e — \delta'' E \qquad (8)$$

$$\delta'' E = 59.16 \log (1 + k_s \varkappa_3 k_0^{-1} + 2\ k_s \varkappa_4 k_0^{-1} X) \approx 25.7\ k_s \varkappa_3 k_0^{-1} + \\ + 51.4\ k_s \varkappa_4 k_0^{-1}\ X \qquad (9)$$

By inserting the numerical values

$$\delta'' E = 0.006 + 0.0002_1\ X\ \text{mV} \qquad (10)$$

This is negligible in comparison to the experimental errors.

In Table 1 are given the results of a number of titrations for E_{10} and E_{1X} for Br^-, using concentrations similar to those in Table 3, Part III. The figure $E_{10}—E_{1X}$, which ought to be constant, is seen to vary widely; in the first 10 titrations the average was 362.3 ± 2.3 mV. (Within each titration E_{1X} for different points generally differed only by one or a few 0.1 mV, and

Table 1. Measurements of E_{10} and E_{1X}.

Date	E_{10}	E_{1X}	E_{10}—E_{1X}		Electrode
6. 4. 44	434.5	73.4 ± 0.3	361.1		I
22. 4. 44	434.4	69.2 ± 0.2	365.2		I
17. 6. 44	434.4	73.7 ± 0.3	360.7		I
21. 6. 44	434.4	73.5 ± 0.1	360.9		I
27. 6. 44	434.4	73.3 ± 0.2	361.1	362.3	I
28. 6. 44	434.4	72.4 ± 0.1	362.0	± 2.3	I
2. 8. 45	434.8	73.0 ± 0.4	361.8		A
8. 1. 46	434.4	71.1 ± 0.2	363.3		G
11. 1. 46	434.2	70.0 ± 0.1	363.3		G
10. 9. 46	434.6	70.7 ± 0.2	363.9		G
16. 2. 48	433.6	70.0 ± 0.1	363.6		G
2. 3. 48	434.8	72.4 ± 0.1	362.4		G
10. 3. 48	435.2	72.3 ± 0.3	362.9	363.4	A
21. 4. 48	435.0	72.7 ± 0.3	362.3	± 1.1	A
21. 4. 48	434.6	(73.6 ± 0.2	361.0)		G
21. 4. 48	435.3	70.8 ± 0.4	364.5		W
20. 5. 48	435.3	70.3 ± 0.2	365.0		W

only in one case by as much as ± 0.4 mV). In order to avoid possible disturbances from impurities in the reagents or from slow attainment of equilibrium, a new series of titrations was made with new reagents, where the precipitate of Hg_2Br_2 was allowed to stand for at least two days. In these experiments, 7 titrations gave a somewhat higher average value than before. A possible explanation is that in some of the previous experiments the Hg_2Br_2 was in a fine-grained and thus more soluble state. The average of all seven values is 363.1 ± 1.4. However, we prefer to exclude the value 361.0, which differs widely from all the others, and then obtain:

$$E_{10}—E_{1X} = -29.58 \log k_s = 363.4 \pm 1.1 \text{ mV} \quad (11)$$

$$\log k_s = -12.285 \pm 0.037$$

$$k_s = (5.2 \pm 0.5)10^{-13} \text{ mC}^3 = (5.2 \pm 0.5)10^{-22}\text{C}^3 \quad (12)$$

$$E^{\text{st}}_{1X} = 71.1 \pm 1.1 \text{ mV} \quad (13)$$

The activity product of Hg_2Br_2 has been given by Brodsky and Scherschewer [14] as $K_s = 5.50 \cdot 10^{-23}$. From the standard potentials at 25° C Br^-, Hg_2Br_2/Hg : 139.7 mV (Larson [15]) and Hg_2^{2+}/Hg : 797.5 ± 1.0 mV (Bray and Hershey [16]) we find $K_s = 5.78 \cdot 10^{-23}$ m³. A combination of these values

Table 2. Titrations for E_{max}.

Date	a	59.16 log a	E_{max}	Corr	E^{st}_{max} — 59.16 log a
31. 1. 48	10.00	59.2	603.0	+ 0.8	(544.6)
25. 2. 48	5.00	41.4	591.8	— 0.9	549.5
26. 2. 48	5.00	41.4	594.0	— 0.4	(552.2)
4. 3. 48	5.00	41.4	592.6	— 1.6	549.6
10. 3. 48	5.00	41.4	591.9	— 0.9	549.6
10. 3. 48	5.00	41.4	592.5	— 1.6	549.5
11. 3. 48	10.00	59.2	609.3	— 0.9	549.2
				Average	549.5 ± 0.3

with ours would give about 0.1 for the product of the activity factors in our solutions, which seems reasonable and, also agrees well with our results[3] for Hg_2Cl_2. Sherrill[8] found $k_s = 1.27 \cdot 10^{-21}$ C^3 in a rather indirect way.

TITRATIONS FOR E_{max}

Just as for Cl^-, a solution containing Hg^{2+} and Hg_2^{2+} was titrated with Br^-, and the maximum emf, using a Pt electrode, was observed. In the first experiments each titration was performed within two or three hours. They gave rather wide variations in the quantity $E_{max} - 59.16 \log a$ (a = total concentration of Hg^{II}) which ought to be constant. Most values were between 547 and 550 mV, but stray values were observed all over the range from 542 to 552 mV.

In later experiments the volume of T to be added to obtain the maximum point could be calculated approximately from the preliminary values. Then only a few points in the neighbourhood of the maximum were studied, and the equilibrium potential was waited for for several days. Out of seven titrations performed in this way (Table 2), five gave values within the limits 549.5 ± 0.3 mV, whereas the other two, which differed by + 2.7 and — 4.9 mV, were disregarded. We thus accepted the value

$$E_{max} - 59.16 \log a = 549.5 \pm 0.3 \text{ mV} \qquad (14)$$

The inaccuracy may, of course, be larger because of systematic errors.

From (IV,6) we find, using the value $k_{12} = 5.8 \pm 0.3$ (Sillén and Infeldt[2]):

$549.5 \pm 0.3 = E_{20} + E_{10} - E_{1X} - 59.16 \log (2 + \sqrt{k_{12}}) - 29.58 \log \varkappa_2$
$= 559.4 \pm 0.2 + 363.4 \pm 1.1 - 38.1 \pm 0.4 - 29.58 \log \varkappa_2$

which leads to

$$29.58 \log \varkappa_2 = 335.2 \pm 1.3$$
$$\log \varkappa_2 = 11.332 \pm 0.044$$
$$\varkappa_2 = (2.1_5 \pm 0.2)10^{11}\ \mathrm{mC^{-2}} = (2.1_5 \pm 0.2)10^{17}\ \mathrm{C^{-2}} \qquad (15)$$

By combination with k_{12} we find

$$2 \log \varkappa_1 = \log \varkappa_2 + \log k_{12} = 11.332 \pm 0.044 + 0.763 \pm 0.022 = 12.095 \pm 0.049$$
$$\log \varkappa_1 = 6.048 \pm 0.025$$
$$\varkappa_1 = (1.12 \pm 0.06)10^6\ \mathrm{mC^{-1}} = (1.12 \pm 0.06)10^9\mathrm{C^{-1}} \qquad (16)$$

It thus seems that Morse's determination of $\varkappa_1$ and Morse's and Sherrill's estimations of $\varkappa_2$ give about the right order of magnitude.

TITRATIONS FOR $\varkappa_3$ AND $\varkappa_4$

We tried at first to determine q_1 and q_2 for Br^- in the same way as with Cl^-, by titrations of Hg(I,II) mixtures with excess halogen, using platinum electrodes. The electrodes proved to be easily polarized so that a valve potentiometer had to be used. Even then it was difficult to obtain reproducible emfs. With two different Pt electrodes in the same solution the emfs often varied by several tens of mV. Later on, when the equilibrium constants were known, it was found that the measured emfs had sometimes been slightly lower but had generally been higher than the calculated equilibrium emfs.

For studying the equilibria involving $HgBr_3^-$ and $HgBr_4^{2-}$, we had to resort to the same method as had previously been used by Qvarfort and Sillén (Part VII, to be published) for studying the corresponding equilibria with I^-, namely measuring with a Hg electrode the concentration of free Hg^{2+} in solutions containing HgX_2 and X^-.

In these titrations, the concentrations of all molecular species except X^-, HgX_2, HgX_3^-, and HgX_4^{2-}, could be neglected, thus

$$(Hg^{II})_{\mathrm{total}} = a = (HgX_2) + (HgX_3^-) + (HgX_4^{2-}) = (Hg^{2+})\, X^2\, (\varkappa_2 + \varkappa_3 X + \varkappa_4 X^2) \qquad (17)$$

$$X_c = (X^-) + (HgX_3^-) + 2(HgX_4^{2-}) = X + (Hg^{2+})X^3\, (\varkappa_3 + 2\,\varkappa_4 X) \qquad (18)$$

It is necessary to choose a and the range of X in such a way that Hg_2X_2 will not precipitate. In equilibrium with Hg and Hg_2X_2, $(HgX_2) = k_s k_0^{-1} \varkappa_2$; $(HgX_3^-) = k_s k_0^{-1} \varkappa_3 X$; $(HgX_4^{2-}) = k_s k_0^{-1} \varkappa_4 X^2$

Table 3. Titrations for $\varkappa_3$ and $\varkappa_4$. Only every third point is given.

S = 0.1 mC Hg^{2+}, 406.5 mC Br^-
T = 0.1 mC Hg^{2+} ($= a$)

v ml	X mC	$-E$ mV		$-\log(Hg^{2+})$		$a(Hg^{2+})^{-1}X^{-3}mC^{-3} \cdot 10^{-11}$	
		A	G	A	G	A	G
0	406.8	108.5	108.3	20.469	20.461	4.38	4.29
6	328.1	98.3_5	97.7	20.125	20.102	3.79	3.59
12	274.9	89.7	89.1	19.834	19.813	3.28	3.13
18	236.5	82.7	81.8	19.597	19.565	2.99	2.78
24	207.6	76.5	74.9_5	19.387	19.334	2.72	2.41
30	184.9	70.6_5	69.2	19.189	19.140	2.44	2.18
36	166.7	65.6	64.4_5	19.017_5	18.979	2.25	2.06
42	151.8	61.1_5	59.8	18.867	18.821	2.12	1.91
48	139.9	57.1	54.3	18.730	18.678	1.99	1.76

S = 0.25 mC Hg^{2+}, 336.9 mC Br^-
T = 0.25 mC Hg^{2+} ($= a$)

v ml	X mC	$-E$ mV		$-\log(Hg^{2+})$		$a(Hg^{2+})^{-1}X^{-3}mC^{-3} \cdot 10^{-11}$	
		A	G	A	G	A	G
0	336.9	88.9_5	88.5	19.807	19.793	4.19	4.06
6	271.7	78.0	77.9	19.438	19.434	3.42	3.38_5
12	227.6	69.3	69.1	19.144	19.136	2.95	2.90
18	195.9	61.7	61.7	18.887	18.885	2.49	2.48
24	171.9	55.6	55.8	18.679	18.682_5	2.35	2.37
30	153.1	51.1	52.5	18.527	18.542	2.34	2.62

thus $a = k_s k_0^{-1}(\varkappa_2 + \varkappa_3 X + \varkappa_4 X^2)$ (19)

as can be shown from the definitions of the equilibrium constants. Equation (19) defines for every a a certain minimum value of X (or for every X a maximum value of a) beyond which Hg_2X_2 will tend to precipitate.

Preliminary experiments showed that it was necessary to keep a rather low. With the final values $\varkappa_2$, $\varkappa_3$, and $\varkappa_4$ we can calculate /X mC, a_{max} mC/ 10 0.0035/50 0.022/100 0.063/200 0.20/.

Table 3 gives the results of two titrations with $a = 0.10$ mC and two with $a = 0.25$ mC. At such low a and at the high X used no perceptible error is involved by using X_e instead of X.

In Fig. 1 the function

$$a(Hg^{2+})^{-1}X^{-3} = \varkappa_2 X^{-1} + \varkappa_3 + \varkappa_4 X \approx \varkappa_3 + \varkappa_4 X \quad (20)$$

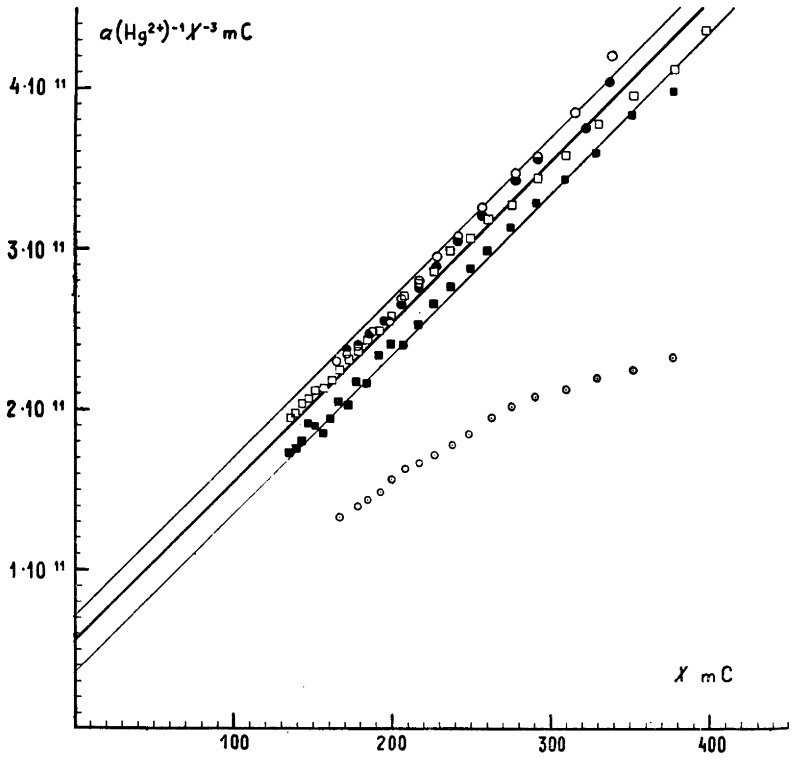


Fig. 1. Titrations for $\varkappa_3$ and $\varkappa_4$. (See Table 3.) Five different titrations. □ *Electrode A, $a = 0.1$.* ■ *Electrode G, $a = 0.1$.* ○ *Electrode A, $a = 0.25$.* ● *Electrode G, $a = 0.25$.* ⊙ *(rejected) A titration where the oxygen was removed from the solution only after mercury had been added.*

is plotted against X. The first term, $\varkappa_2 X^{-1}$, is negligible in comparison with the others. The points are found to form roughly a straight line; there is no tendency to bending upwards for high X as would be expected if HgX_5^{-3} and higher complexes were to be formed in appreciable amounts.

In these experiments it is very important that air is perfectly excluded, since otherwise quite appreciable quantities of Hg^{II} can be formed by oxidation of the metal. For instance, the lower points in Fig. 1 belong to a rejected titration where the oxygen was removed from the solution only after the electrode mercury had been added with the effect that the actual $(Hg^{II})_{tot}$ was higher than assumed in the calculations. The titrations were broken when Hg_2Br_2 was obtained in the solution. (The present titrations, unlike those in Part VII were performed with decreasing X.) If the titration was performed rather rapidly (a new addition of T every 10 min) the formation of Hg_2Br_2

Table 4. Summary of equilibrium constants.

Reaction	Equilibrium constant	log of equil. const. (C scale)
$Hg^{2+} + Br^- \rightleftharpoons HgBr^+$	$\varkappa_1 = \sqrt{k_{12}\varkappa_2} = (1.12 \pm 0.06)10^9 C^{-1}$	9.048 ± 0.025
$Hg^{2+} + 2Br^- \rightleftharpoons HgBr_2$	$\varkappa_2 = (2.1_5 \pm 0.2)10^{17} C^{-2}$	17.332 ± 0.044
$Hg^{2+} + HgBr_2 \rightleftharpoons 2HgBr^+$	$k_{12} = 5.8 \pm 0.3$	0.763 ± 0.022
$Hg^{2+} + 3Br^- \rightleftharpoons HgBr_3^-$	$\varkappa_3 = (0.55 \pm 0.15)10^{20} C^{-3}$	19.740 ± 0.105
$Hg^{2+} + 4Br^- \rightleftharpoons HgBr_4^{2-}$	$\varkappa_4 = (1.00 \pm 0.08)10^{21} C^{-4}$	21.000 ± 0.033
$HgBr_2 + Br^- \rightleftharpoons HgBr_3^-$	$q_1 = \varkappa_3\varkappa_2^{-1} = (260 \pm 70) C^{-1}$	2.408 ± 0.114
$HgBr_2 + 2Br^- \rightleftharpoons HgBr_4^{2-}$	$q_2 = \varkappa_4\varkappa_2^{-1} = (4700 \pm 600) C^{-2}$	3.668 ± 0.055
$2HgBr_2 \rightleftharpoons HgBr^+ + HgBr_3^-$	$\varkappa_3 k_{12}^{\frac{1}{2}} \varkappa_2^{-\frac{3}{2}} = (1.3 \pm 0.5)10^{-6}$	-5.880 ± 0.125
$2HgBr_3^- \rightleftharpoons HgBr_2 + HgBr_4^{2-}$	$\varkappa_2\varkappa_4\varkappa_3^{-2} = 0.07 \pm 0.04$	-1.148 ± 0.217
$Hg_2Br_2(s) \rightleftharpoons Hg_2^{2+} + 2Br^-$	$k_s = (5.2 \pm 0.5)10^{-22} C^3$	-21.285 ± 0.037
$Hg_2Br_2 + 2Hg^{2+} \rightleftharpoons Hg_2^{2+} + 2HgBr^+$	$k_s k_{12}\varkappa_2 = (6.4_5 \pm 1.0)10^{-4} C$	-3.190 ± 0.062
$Hg + Hg^{2+} \rightleftharpoons Hg_2^{2+}$	$k_0 = 129.2 \pm 1.0$	$2.111_2 \pm 0.003_4$
$Hg_2Br_2 \rightleftharpoons Hg + HgBr_2$	$k_s\varkappa_2 k_0^{-1} = (8.6 \pm 1.2)10^{-7} C$	-6.064 ± 0.058
$Hg_2Br_2 + Br^- \rightleftharpoons Hg + HgBr_3^-$	$k_s\varkappa_3 k_0^{-1} = (2.2 \pm 0.7)10^{-4}$	-3.656 ± 0.111
$Hg_2Br_2 + 2Br^- \rightleftharpoons Hg + HgBr_4^{2-}$	$k_s\varkappa_4 k_0^{-1} = (4.0 \pm 0.5)10^{-3} C^{-1}$	-2.396 ± 0.050

was delayed so that measurements could be performed even at lower X than the limit value calculated, as seen from the X values in Table 3. The formation of Hg_2Br_2 was made apparent first by a bend in the E(v) curve, and soon afterwards by an opalescence in the solution.

In Fig. 1, the middle line corresponds to $\varkappa_3 = 0.55 \cdot 10^{11}$ (intercept) and $\varkappa_4 = 1.00 \cdot 10^9$ (slope). The two thin lines have the same slope but intercepts of $0.35 \cdot 10^{11}$ and $0.70 \cdot 10^{11}$. From the experimental points we concluded the following values for $\varkappa_3$ and $\varkappa_4$:

$$\begin{aligned} &\varkappa_3 = (0.55 \pm 0.15)10^{11}\ mC^{-3} = (0.55 \pm 0.15)10^{20}\ C^{-3} \\ &\log \varkappa_3 = 10.740 \pm 0.105 \end{aligned} \tag{21}$$

$$\begin{aligned} &\varkappa_4 = (1.00 \pm 0.08)10^{9}\ mC^{-4} = (1.00 \pm 0.08)10^{21}\ C^{-4} \\ &\log \varkappa_4 = 9.000 \pm 0.033 \end{aligned} \tag{22}$$

By combining with $\varkappa_2$, we find for q_1 and q_2

$$\begin{aligned} &q_1 = 0.26 \pm 0.07\ mC^{-1} = 260 \pm 70\ C^{-1} \\ &q_2 = (4.7 \pm 0.6)10^{-3}\ mC^{-2} = 4700 \pm 600\ C^{-2} \end{aligned} \tag{23}$$

We can thus see that, contrary to the assumptions made by previous workers, both $HgBr_3^-$ and $HgBr_4^{2-}$ are formed in appreciable amounts.

In Table 4 the equilibrium constants already mentioned are listed together with a few others which can be derived from them.

SUMMARY

By electrometric measurements, a number of equilibrium constants, involving Hg^{2+}, $HgBr^+$, $HgBr_2$, $HgBr_3^-$, $HgBr_4^{2-}$, Hg_2^{2+}, Hg_2Br_2(s) and Hg(1) have been determined. The constants are listed in Table 4. They are valid under the special experimental conditions: 25° C, $(H^+) = 10$ mC, ionic strength 500 mC.

Our thanks are due to Magister Sirkka Hietanen, to Miss Ingegerd Qvarfort, and to Mr Erik Ekedahl for their valuable aid.

REFERENCES

1. Sillén, L. G. *Svensk Kem. Tid.* **58** (1946) 52 (Part I).
2. Sillén, L. G., and Infeldt, G. *Svensk Kem. Tid.* **58** (1946) 61 (Part II).
3. Jonsson, A., Qvarfort, I., and Sillén, L. G. *Acta Chem. Scand.* **1** (1947) 461 (Part III).
4. Sillén, L. G. *Acta Chem. Scand.* **1** (1947) 473 (Part IV).
5. Lindgren, B., Jonsson, A., and Sillén, L. G. *Acta Chem. Scand.* **1** (1947) 479 (Part V).
6. Morse, H. *Z. physik. Chem.* **41** (1902) 709.
7. Abegg, R., Immerwahr, C., and Jander, *Z. Elektrochem.* **8** (1902) 688.
8. Sherrill, M. S. *Z. physik. Chem.* **43** (1903) 705.
9. Sand, J., and Breest, F. *Z. physik. Chem.* **59** (1907) 429.
10. Abegg, R., and Sherrill, M. S. *Z. Elektrochem.* **9** (1903) 549.
11. Job, P., *Ann. Chim.* (10) **9** (1928) 162.
12. Fromherz, H., and Lih, K. H. *Z. physik. Chem.* **A 167** (1933) 126.
13. Garrett, A. B. *J. Am. Chem. Soc.* **61** (1939) 2744.
14. Brodsky, A. E., and Scherschewer, J. M. *Z. Elektrochem.* **32** (1926) 1; **35** (1929) 836.
15. Larson, W. D. *J. Am. Chem. Soc.* **62** (1940) 765.
16. Bray, W. C., and Hershey, A. V. *J. Am. Chem. Soc.* **56** (1934) 1892.

Received October 13, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 839—840

Short Communication

Coniferylaldehydgruppen im Holz und in isolierten Ligninpräparaten

E. ADLER UND L. ELLMER

Holzchemische Abteilung des Schwedischen Instituts für Holzforschung, Stockholm

In einer vorangehenden kurzen Mitteilung [1] ist gezeigt worden, dass die Farbreaktionen des Holzes mit Phenolen oder aromatischen Aminen auf das Vorhandensein von Coniferylaldehydresten im Lignin zurückzuführen sind. Die Beweisführung war eine indirekte; sie stützte sich auf den Vergleich mit Modellsubstanzen und insbesondere auf die Tatsache, dass die Farbstoffe, die aus gelöstem, sog. nativem, Lignin sowie aus kurz alkalisierter Lignosulfonsäure mit Phloroglucin-HCl oder *p*-Aminobenzoesäure gebildet werden, hinsichtlich ihrer Absorptionskurven mit den entsprechenden Farbstoffen aus Coniferylaldehyd gut übereinstimmen.

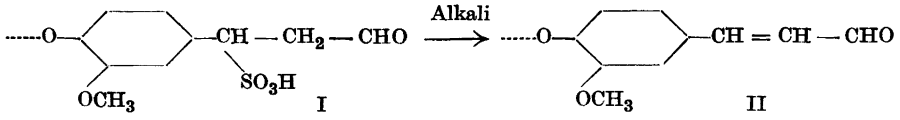
Wir haben in der weiteren Verfolgung dieser Frage das »Hadromal» von Czapek [2] einer näheren Untersuchung unterzogen. Als Hadromal wird ein Gemisch ätherlöslicher Substanzen bezeichnet, das durch mehrstündiges Extrahieren von entharztem Holz mittels Zinnchlorürlösung bei 70° in geringer Ausbeute erhalten wird. Es zeigt in Lösung die typischen Farbreaktionen des Holzes. Die chemische Natur der »chromogenen» Komponente des Hadromals war bisher ungeklärt. Eine Angabe von Hoffmeister [3], wonach das Produkt Coniferylaldehyd enthalten sollte, war von Pauly und Feuerstein [4] entschieden abgelehnt worden. Wir fanden nun, dass die aus Hadromal und Phloroglucin-HCl oder *p*-Aminobenzoesäure erzeugten Farbstoffe die gleichen Absorptionskurven besitzen wie die entsprechenden Farbstoffe aus Coniferylaldehyd (Max. bei 548 mμ für den Phloroglucinfarbstoff, bei 456 mμ für den *p*-Aminobenzoesäurefarbstoff). Durch Umsetzung von Hadromal mit 2,4-Dinitrophenylhydrazin wurde ein Hydrazongemisch erhalten, aus dem sich die 2,4-Dinitrophenylhydrazone des Coniferylaldehyds (Schmp. 265—266°, keine Depression mit synthetischem Präparat) und des Vanillins abtrennen liessen. Damit ist zum ersten Mal Coniferylaldehyd aus Holz einwandfrei isoliert worden.

Die Menge des in Hadromalpräparaten enthaltenen Coniferylaldehyds wurde durch spektrophotometrische Messung der Farbintensität bei der Phloroglucin- oder *p*-Aminobenzoesäurereaktion und Vergleich mit reinem Coniferylaldehyd ermittelt. Es ergab sich, dass die mittels der Czapek'schen Methode aus entharztem Fichtenholz freilegbare Menge Coniferylaldehyd etwa 0,02 % vom Gewicht des Holzes beträgt. Daneben enthalten die Präparate Vanillin in einer Menge, die etwa 0,05 % des Holzes entspricht.

Der wirkliche Gehalt des Holzes an gebundenem — nicht mittels Alkohol-Benzol extrahierbarem — Coniferylaldehyd konnte mit Hilfe einer später zu beschreibenden Methode auf 0,5—0,75 % geschätzt werden. Unter der Annahme, dass diese Coniferylaldehydgruppen im wesentlichen in das Lignin eingebaut sind, berechnet sich hieraus, dass das Lignin des Fichtenholzes 1 Coniferylaldehydgruppe auf durchschnittlich 40—60 Phenylpropaneinheiten besitzt.

In sog. nativem, d.h. mittels Alkohol extrahierbarem Lignin aus Fichte konnten wir spektrophotometrisch 1 Coniferylaldehydgruppe auf je 40 Methoxylgruppen nachweisen.

In Lignosulfonsäure liegen die Coniferylaldehydreste in der sulfonierten Form (I) vor; sie können daraus durch kurzes Alkalisieren der Lösung bei gewöhnlicher Temperatur regeneriert werden und sind dann, z. B. nach Umsetzung mit *p*-Aminobenzoesäure, der kolorimetrischen Bestimmung zugänglich [1].



(Die Verknüpfung der Coniferylaldehydgruppe mit dem Rest des Moleküls ist in den voranstehenden Formeln über die phenolische OH-Gruppe angegeben; eine andere Art der Verknüpfung ist jedoch ebenfalls möglich.) In Lignosulfonsäuren, die durch Dialyse von Hartkochungsablaugen, Kationenaustausch und Fällung als Bariumsalz gewonnen waren, erhielten wir so Werte von 1 Coniferylaldehydgruppe auf rund 50 Methoxylgruppen. Zum gleichen Werte gelangt man, wenn man das an Aldehydgruppen der Lignosulfonsäure gebundene Sulfit entfernt — was durch Titrieren mit Jod in Gegenwart von Calciumcarbonat gelingt — und hierauf das nach dem voranstehenden Schema mittels Alkali abspaltbare Sulfit jodometrisch bestimmt [5].

Die den Coniferylaldehydresten (II) zugehörenden Aldehydgruppen machen nur einen kleineren Teil der Gesamtmenge von Carbonylgruppen im Lignin aus. Durch Umsetzung von »nativem» Lignin mit Hydroxylaminhydrochlorid und potentiometrische Titration der freiwerdenden HCl bestimmten wir den Carbonylgruppengehalt zu 1 >C=O auf je 8 Methoxylgruppen. Die oben erwähnten Lignosulfonsäuren enthielten 1 >C=O auf je 10 Methoxylgruppen. Im letzteren Falle konnte durch Bisulfitbindungsversuche [5] wahrscheinlich gemacht werden, dass der grösste Teil der Carbonylgruppen aus Aldehydgruppen besteht. Die Anwesenheit von Aldehydgruppen in Lignosulfonsäuren war bereits von Hägglund, Johnson und Trygg [6] gezeigt worden.

Wir sehen in den oben mitgeteilten Ergebnissen eine experimentelle Stütze für die zuerst von Klason [7] ausgesprochene Hypothese, wonach das Lignin aus Coniferylaldehydresten aufgebaut sei.

1. Adler, E., Björkqvist, K. J., und Häggroth, S. *Acta Chem. Scand.* **2** (1948) 93.
2. Czapek, F. *Z. physiol. Chem.* **27** (1899) 141.
3. Hoffmeister, C. *Ber.* **60** (1927) 2062.
4. Pauly, H., und Feuerstein, K. *Ber.* **62** (1929) 297.
5. Adler, E., und Häggroth, S. Unveröffentlicht.
6. Hägglund, E., Johnson, T., und Trygg, L. H. *Svensk Papperstidn.* **32** (1929) 815.
7. Klason, P. *Ber.* **55** (1922) 448; **56** (1923) 300.

Eingegangen am 4. Dezember 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 841—855

The Behaviour of Weak Electrolytes in Moving Boundary Systems

I. The Moving Boundary Equation

HARRY SVENSSON

Institutes of Physical Chemistry and Biochemistry, Uppsala, Sweden

After the introduction of the moving boundary method as a tool for the measurement of ionic mobilities by Lodge [1] and Whetham [2–7], a thorough theoretical treatment of the method was given by Kohlrausch [8] and Weber [9]. In these publications we find the differential equation governing the motion of ions in an electric field. Moreover, a complete solution of the differential equation was given for the case of three ion species, which has been of the greatest importance in all applications of the method. Another result of great consequence was Kohlrausch's finding that the function:

$$\sum \frac{c_i}{u_i} \tag{1}$$

named by Kohlrausch *die beharrliche Funktion*, and nowadays generally called the Kohlrausch function or the regulating function (MacInnes and Longsworth [10]), is independent of time if the ionic mobilities u_i can be regarded as constants (c_i = the equivalent concentrations, taken with signs). Simplified theoretical treatments of the moving boundary method have later been presented by MacInnes and Longsworth (*l. c.*) and by Hartley and Moilliet [11]. MacInnes and his collaborators have collected a great number of transference numbers by the moving boundary method, which data are the most accurate hitherto available.

Picton and Linder [12], Whitney and Blake [13], and Hardy [14] were the first who applied the method to colloids. During thirty years, the method was gradually developed, but great difficulties were encountered in localizing the

diffuse boundaries, and another obstacle was the fact that moving colloid boundaries always showed different rates of migration on the rising and descending sides. Tiselius [15] obtained accurate and reproducible mobility data for proteins by using low protein and fairly high salt concentrations. The assymmetry between the two limbs of the U-tube was depressed in this way, and the processes going on in the two limbs were nearly mirror image processes.

Henry and Brittain [16] attacked the problem in another way. They applied the Kohlrausch and Weber differential equation to the colloidal ions and came to the conclusion that the descending boundary, although more diffuse, gave the correct mobility, which was also in agreement with that obtained by the transference method.

When Tiselius [17] developed the moving boundary method into a quantitative analytical method for colloids seven years later, an extension of the theory to multicomponent systems became desirable. A first attempt in this direction was made by Svensson [18] in 1943. An equation giving the concentration change for an arbitrary ion across a moving boundary was given, and by summing this equation over all ions, an expression for the conductivity change at the boundary was derived. By applying the equation to the colloidal ions in the mixture, the conclusion was drawn that every ion changes its concentration at every boundary. The importance of this in the interpretation of electrophoresis patterns was discussed.

The moving boundary equation was given a more general form by Longsworth [19] and Dole [20] two years later. Dole's ingeneous mathematical treatment of the whole moving boundary system revealed not only the number of boundaries but also a complete solution of the complicated system of equations. The solution was based upon the assumption of constant relative mobilities, as was also Svensson's treatment. The experimental test given simultaneously by Longsworth showed that the theory yielded a good quantitative agreement with experiments under the conditions defined in the theory. Longsworth and Dole clearly understood and expressed the fact that the assumption of constant relative mobilities restricted the validity of the theory to strong electrolytes.

However, a bulk of experimental evidence has also been collected giving strong support to the theoretical predictions even for colloidal systems. Svensson [18] showed that the apparent composition of pig serum varied considerably with the salt concentration in the way that could be expected, and Perlmann and Kaufman [21] found the same independently for human serum. Continued experimental research in this field has been reported by Koenig and Hogness [22], Koenig, Perrings, and Hogness [23], and by Armstrong, Budka, and

Morrison [24]. These works give a qualitative confirmation of the theories, but a quantitative verification for proteins is still lacking.

Thus we have now a complete understanding of moving boundary systems of strong electrolytes, but we miss the corresponding knowledge of the behaviour of weak electrolytes. An extended treatment of moving boundary systems is therefore desirable already for the interpretation of simple systems with only a small number of weak electrolytes. Moreover, Tiselius' electrophoresis technique involves multi-component systems where weak electrolytes cannot be avoided: the buffer contains at least one weak electrolyte, and the colloids under investigation also belong to this class.

In the general case, with an arbitrary number of components of weak or strong electrolytes, the complete solution of the mathematical problem, which would allow us to predict the number and velocities of the boundaries and the composition of each inter-boundary layer from the known compositions of the two end solutions, or *vice versa*, is not available. If it were given, it would probably be too complicated to be of any practical significance. There is, however, another way of attacking the problem, namely to apply the moving boundary equation to each constituent and to each boundary in moving boundary systems that are actually observed experimentally.

THE CHARACTERISTICS OF WEAK ELECTROLYTES

By definition, a weak electrolyte is an electrolyte which is incompletely dissociated. One could also say that the ions of weak electrolytes react chemically with each other to form complex ions or uncharged molecules. Strong electrolytes are completely dissociated, and the interaction between positive and negative ions is restricted to that described by the Debye-Hückel theory. There is no reason to assume that there is a sharp boundary between the two groups, but there is strong experimental evidence that many salts, acids, and bases are pratically completely dissociated in dilute solutions in polar solvents.

A very important group within the weak electrolytes are the weak acids, bases, and ampholytes. In these substances, we have complexes where one of the water ions is a partner. According to Brønsted's views, however, the hydroxyl ion is dispensed with as a complex partner; Brønsted's protolytes are proton-donors or proton-acceptors.

Ions of weak electrolytes are thus characterized of being complex-formers. It should be noted in this connection that the partner in a complex formation need not be another ion, it may also be a non-electrolyte. The hydrated ions, the metal-ammonia complexes, the ammonium ion, and the iodide-iodine complex are examples of this type of particles.

The term »ion» means nowadays a free ion. To denote the sum of free ions and those bound in different complexes, MacInnes [25] uses the term »ion constituent», and Hartley and Moilliet [11] have proposed the term »radical». An ion constituent or a radical of a weak electrolyte is thus capable of existence in a number of different forms which we will subsequently call *subspecies*. The difference between an ion and a subspecies is then that a subspecies may be uncharged. In a solution, the relative concentrations of the different subspecies depend upon the concentrations of those ions or molecules with which they enter into complex formation. For protolytes, pH is the chief factor. The acetic acid radical exists partly as undissociated acid, HAc, and partly as the acetate ion, Ac^- (the hydration being neglected); it has two subspecies. The oxalic acid radical has three subspecies, one uncharged, one monovalent, and one bivalent. The citric acid radical has six subspecies because there are two isomeric forms of the monovalent, and two of the bivalent citric acid anions. With an increased number of ionizable groups in the molecule, the number of subspecies increases very rapidly. All subspecies are in equilibrium with each other, the equilibria being quantitatively described by a number of mass-action equations equal to one less than the number of subspecies.

From the point of view of moving boundary systems, it is not essential to distinguish between strong and weak electrolytes. The only matter of importance is to decide whether the mobilities can be regarded as constant or not. It is true that complex formation may cause a mobility to vary in a much more radical way than other influences (ionic interaction, viscosity, temperature, etc.), but nevertheless it is well possible to design moving boundary systems containing typical weak electrolytes where the assumption of constant mobilities can be used more successfully than in other systems with only strong electrolytes.

THE MOVING BOUNDARY EQUATION

This equation is the corner-stone in the theory for the migration of strong electrolytes, and it must be so even for weak electrolytes. It is now available in a number of different forms. As a matter of fact, it was given already by Weber [9] for a three-ion system in the case where no ion disappeared at the boundary. Svensson [18], being primarily interested in colloidal systems with small changes across the boundaries, gave the equation in terms of increments in concentration, mobility, and conductivity. The forms now most generally used are those given by Longsworth [19] and Dole [20]:

$$V^{\alpha\beta}\ (C_j^{\alpha} - C_j^{\beta}) = T_j^{\alpha} - T_j^{\beta} \qquad (2)$$

$$\left(\frac{r_j}{\sigma^\alpha} - V^{\alpha\beta}\right) C_j^\alpha = \left(\frac{r_j}{\sigma^\beta} - V^{\alpha\beta}\right) C_j^\beta \tag{3}$$

$$(r_j - V^{\alpha\beta}\sigma^{\alpha})\ T_j^\alpha = (r_j - V^{\alpha\beta}\sigma^{\beta})\ T_j^\beta \tag{4}$$

Here V is the velocity of the moving boundary in cm³ per faraday, C is the concentration of the component in faradays per cm³, T are transference numbers, r are relative mobilities assumed to be constant, σ is the »relative conductivity» $= \Sigma\, C_j r_j$, the superscripts $^\alpha$ and $^\beta$ refer to the two phases on either side of the boundary, and the subscript j refers to the component.

Svensson [25] gave the equation in the following forms in 1946:

$$\frac{c_{i1}(u_{i1} - U')}{\varkappa_1} = \frac{c_{i2}(u_{i2} - U'')}{\varkappa_2} \tag{5}$$

$$(c_{i1} - c_{i2})(u_{i1} - U') = \left(\frac{\varkappa_1}{\varkappa_2} - \frac{u_{i1}}{u_{i2}}\right) c_{i2}\, u_{i2} \tag{6}$$

$$(c_{i2} - c_{i1})(u_{i2} - U'') = \left(\frac{\varkappa_2}{\varkappa_1} - \frac{u_{i2}}{u_{i1}}\right) c_{i1}\, u_{i1} \tag{7}$$

In these equations, c is the concentration of the constituent in equivalents per unit volume, and u its mobility. U' and U'' are the velocities of the boundaries divided by the field strengths prevailing above and below it, respectively; they have the dimension of a mobility and may be called the mobilities of the boundaries. $\varkappa$ is the conductivity. The first subscript, i, denotes the number of the constituent, and the second, 1 or 2, refers to the two solutions above and below the boundary. Between the absolute velocity, V, in cm. per sec., and the quantities U' and U'' we have the relations:

$$V = \frac{U'I}{\varkappa_1} = \frac{U''I}{\varkappa_2} \tag{8}$$

where I is the current density.

We will restrict this discussion of the moving boundary equation to the forms (5) — (7) above and to (3), which is very nearly identical. The equations (2) and (4), containing the transference numbers, will be discussed in a forthcoming paper in connection with a discussion on the concept of transference number.

The validity of the moving boundary equation in the forms (2)—(4) has been demonstrated by Longsworth [19] for strong electrolytes. If we want

to extend its applicability to arbitrary systems, however, we have to give three definitions. First, it is necessary to define the constituents for which the equation can be used. Second, we must define the concentration scale or scales that can be used. Third, we must give an extended definition of the concept of mobility.

THE CONSTITUENTS TO WHICH THE MOVING BOUNDARY EQUATION MAY BE APPLIED

In systems of strong electrolytes, the equation may be used for every ion species. All but one of the ionic concentrations can be chosen at will, the concentration of the last ion being given by the electroneutrality condition. In systems of weak and non-electrolytes, the situation is not so simple. First, to describe the behaviour of the whole system, it is necessary to consider not only ions, but also non-electrolytes that may be electrically transferred due to complex formation with ions. We must also consider the fact that a radical of a weak electrolyte exists in several forms, subspecies, and decide whether we shall use the equation for the radicals or for the subspecies.

What concerns non-electrolytes, the most natural view-point is to regard them as parts of a mixed solvent and not to include them into the general treatment of the moving boundary system or to treat the non-electrolytes together as one component. However, if the non-electrolytes enter into complex formation with ions selectively, a relative motion between the different non-electrolytes will take place, and we cannot any longer describe the phenomena from the point of view of a mixed solvent. In the general case, therefore, we must regard each non-electrolyte as an independent constituent and apply the moving boundary equation to it in the same way as to other constituents. For some non-electrolytes, this is immediately evident: ammonia is formally a non-electrolyte, but it has a strong electrical transference due to complex formation with protons and other cations. For the same reason, nitrogen monoxide can be expected to be electrically transported in the presence of ferrous ions, and iodine in the presence of iodides. It is quite evident that certain mobility values may be ascribed to these non-electrolytes and, consequently, that they will have concentration increments at moving boundaries. The affinity between the ions and the solvent in the solvated ions is far less specific, but the effect upon the electrical migration of the solvent is quite analogous. Thus even the solvent will possess a certain mobility. However, this mobility is equal to zero by definition as long as we choose the solvent as the frame of reference.

The electrical behaviour of ion species existing as two or several subspecies has been considered by a number of authors. When introducing the mobilities of the ions, Kohlrausch stated: »Gemeint ist hier natürlich die empirisch bestimmte mittlere Beweglichkeit aller ortsanwesenden Ionen eines Elementes, nicht diejenige der dissociierten Ionen, die man im allgemeinen nicht kennt.» At that time, all electrolytes were believed to be incompletely dissociated. Miller [27] pointed out that the transference numbers determined by means of the Hittorf method are identical with those from moving boundary experiments whether the ions of the solution be complex or simple. Tiselius [15] wrote that a substance that in a certain medium contains the fractions α_1, α_2, α_3 . . . . . of the forms 1, 2, 3, ... (all in equilibrium) with the mobilities u_1, u_2, u_3, ... will migrate as a uniform substance with a mobility u given by:

$$u = \alpha_1 u_1 + \alpha_2 u_2 + \alpha_3 u_3 + \ldots . \tag{9}$$

provided that the time of existence of each subspecies is small in comparison with the time of migration (momentaneous equilibrium reactions).

Already from this historical survey, it is evident that the application of the moving boundary equation to subspecies is not justified. The equation is not valid for ions that can be formed or disappear in chemical reactions when the ion traverses the boundary. Let us assume that we are considering a moving boundary across which there is a considerable pH change. When acetate ions traverse this boundary from the alkaline side, a certain fraction of them will disappear due to the chemical reaction:

$$H^+ + Ac^- = HAc$$

Thus the moving boundary equation cannot be applied to the acetate ion if acetic acid is present on one or on both sides of the boundary. For the same reason, it cannot be applied to the undissociated acetic acid in the presence of acetate ions.

The incorrectness of using the moving boundary equation for subspecies is also evident from the following discussion. Let us assume that we know the composition of the solution above a certain moving boundary, and that we know the conductivity in both solutions. With the aid of the moving boundary equation, we can then compute the composition of the solution below the boundary if the assumption of constant relative mobilities is valid. Let us assume that we apply the equation to HAc, to Ac^-, to $H_2PO_4^-$, and to HPO_4^{--}. From the values obtained for the concentrations of HAc and Ac^- below the boundary, we would be able to compute the pH of this solution. We could also

compute pH from the ratio of the concentrations obtained for the two phosphate ions. These two pH values cannot be proven to be identical, thus there must be something wrong. We can obtain a third value of pH by computing the concentration of free hydrogen ions below the boundary with the aid of the moving boundary equation, and a fourth value by doing the same for the hydroxyl ions. All these pH values are wrong and not identical, and the reason is that the moving boundary equation is not valid for subspecies.*

There are two ways to escape from this difficulty. One is to modify the moving boundary equation and to take into account the chemical reactions with the aid of the mass-action law. The other way is to give up the treatment of subspecies altogether and to apply the equation to the radicals, which include the specific chemical groupings wherever they are found, in free or complex form (*cf.* Hartley and Moilliet [11]). There is no doubt that the latter procedure is the more rational one. The treatment of individual subspecies is on the whole not possible if there are more than two or three, and even in these simple cases, the computation of their concentrations involves non-thermodynamical assumptions.

A protein is a protolyte with a large number of ionizable groups and an innumerable number of subspecies. We would never consider the possibility of using the moving boundary equation for all these subspecies because it is impossible; we know nothing about them. But since we know to a high degree of accuracy both the concentrations and mobilities of HAc, Ac^-, $H_2PO_4^-$, and HPO_4^{--} in buffer mixtures, we are inclined to apply the equation to each of them. This is not logical, and unless we take into account chemical reactions between them and modify the moving boundary equation accordingly, it is also incorrect.

As already pointed out, no sharp distinction can be made between strong and weak electrolytes, and the situation is the same between weak and non-electrolytes. Even the alkali metal cations, being characterized by a very small tendency to complex formation, are well known to be strongly solvated in polar solvents, although the degree of solvation is very difficult to measure. Moreover, alkali salts of high-molecular poly-acids have been found to be poorly dissociated, probably due to non-specific electrostatic forces. This suggests that we must try to find a mode of treatment that is not restricted to any group of substances but applicable to strong as well as to weak and non-electrolytes. In other words, as far as the question is how to define a constituent, we have to neglect the difference between strong and weak electrolytes.

---

* The failure of the moving boundary equation for ions of weak acids in the presence of the acid itself has recently been discovered by Alberty and Nichol [33] in independent research.

The only modification that is necessary to introduce in the definition of an electrical constituent for strong electrolytes to make it adaptable for arbitrary substances is thus to replace the word »ion» by »radical», and to include non-electrolytes; thus: *the moving boundary equation may be applied to every radical, charged or uncharged, that cannot be dissociated into simpler radicals or transformed into any other radical under the conditions to which the investigation is restricted.*

All these constituents are not mutually independent as to their concentrations. Since some of them are positive or negative radicals, we have here, as in the case of strong electrolytes, the restriction that the sum of all equivalent concentrations, taken with signs, must add up to zero. Furthermore, since we have now taken the solvent or its radicals as electrical constituents, we have also another restriction, namely that the sum of all partial volumes (that fraction of the total volume that is occupied by the constituent) must be equal to unity.

The requirement that the radical must be non-dissociable is obvious since its migration cannot be described if it can dissociate into two parts with different mobilities. On the other hand, the property of being stable or dissociable depends upon the conditions to which the investigation is restricted. For instance, in a system containing ammonium phosphate, the radicals of this salt can be defined as NH_4^+ and HPO_4^{--} if the investigation is restricted to pH:es below 7, for in this pH range these subspecies can be regarded as stable. However, if the investigation is extended to the entire pH range, we have to define the radicals as NH_3 and PO_4^{---}.

The situation is similar for water. In many cases we can regard the water as non-dissociable and thus define H_2O as a constituent. However, in a solution of tri-sodium phosphate, it is obviously erroneous to say that we have the constituents Na^+, PO_4^{---}, and H_2O, since we also have a quite appreciable concentration of hydroxyl ions; in this case the water cannot be treated as nondissociable. Instead of the water, we have two smaller radicals: the hydrogen radical and the hydroxyl radical.

It should be noted that, according to the above definition, the hydrogen radical is a constituent as soon as a protolyte is present, not only in acid, but also in neutral and alkaline solutions, and that all ions containing the hydrogen radical must be considered as subspecies, not only of the protolyte, but also of hydrogen. The concentration of the hydrogen radical stands in no relation to pH. pH is only related to one subspecies of the hydrogen radical, namely to the free hydrogen ions.

In water solutions containing no other weak electrolytes than protolytes, two constituents have an exceptional position: the hydrogen radical as being

the only positive complex-partner, and the water as being the solvent (or the hydroxyl radical in the case that the water must be treated as dissociable). It is therefore convenient, in these systems, to calculate these two concentrations, if they are of interest, from the two restrictive conditions and to apply the moving boundary equation to all the other constituents. Thus it is never necessary to use the moving boundary equation for the hydrogen and hydroxyl radicals. In the absence of protolytes and in mixed solvents, the exceptional positions of the hydrogen radical and the water disappears.

THE CONCENTRATION

In all earlier contributions to the theory of the moving boundary method, the concentrations of the constituents have been expressed in equivalents per unit volume, in later works even with the signs of the charges. This is extremely convenient for many reasons. The valences of the components need not be introduced, the same equations apply to both cations and anions, and the electroneutrality condition takes the simple form: $\Sigma\, c_i = 0$.

Since the equations (3), (5), (6), and (7) contain the concentrations as factors on both sides, the concentration scale seems to be arbitrary. However, it is evident for dimensional reasons that the concentration must be given *on a volume basis.* The equation cannot be derived and does not hold if the concentrations are expressed in mols per 1000 grams of solvent, in grams per 1000 grams of solvent, or in percent. *The concentration unit must be mols, equivalents, or grams per unit volume.*

The equations (2) and (4), containing the transference numbers, require that the concentrations be expressed in equivalents per unit volume. Thus these equations cannot be used in cases where the concepts of equivalent concentration and transference number are not well defined. It is inapplicable to non-electrolytes, to amino acids, to proteins and to most other colloids. The question how to define equivalent concentrations and transference numbers for these substances will be dealt with in a separate communication.

THE MOBILITY

If we look in a physico-chemical table-work (International Critical Tables; Landolt-Börnstein, Tabellen) for mobility data of the acetate and phosphate radicals, we find nothing but the data for the subspecies Ac^-, $H_2PO_4^-$ and HPO_4^{--}. For other weak electrolytes, the situation is similar, or there is no information at all. It is obvious that these data refer to a certain pH range (for Ac^-: above pH 8) or to a single pH value (for $H_2PO_4^-$: pH 4.5; for HPO_4^{--}:

pH 9), where these subspecies exist alone. Mobility data under conditions where both subspecies exist together are not available. Now, we have come to the conclusion that, under such circumstances, we cannot treat the subspecies as independent constituents, thus mobility data are required even in pH ranges where two or more subspecies exist simultaneously. An extended definition of a mobility is apparently necessary, a definition according to which it is possible to measure the required data experimentally. This definition must be given in terms of moving boundary or transference experiments. We are allowed to base the definition on both types of experiments since it is now well established that they give the same result if the plane of reference is the same (Miller [27]).

In terms of a moving boundary experiment, the definition is as follows. *The mobility in a certain medium of a constituent as defined above, comprising a radical in different subspecies, is the velocity relative to some plane of reference or other of a moving boundary at which the constituent with all its subspecies disappears, divided by the field strength next to the boundary in the solution containing the constituent in question* (*cf.* Longsworth [19], Dole [20], and Svensson [25 p. 13]).

Sometimes it is very difficult or impossible to arrange a moving boundary experiment satisfying the three necessary conditions: 1) sharp boundary; 2) gravitationally stable boundary; 3) the conductivity next to the boundary known. In such cases a transference experiment must be made instead, and the mobility derived from the following definition: *The mobility in a certain medium of a constituent in the sense given above is the mass transported relative to some plane of reference or other per unit time, unit cross section of the tube, unit field strength, and unit concentration of the constituent* (*cf.* Hartley and Moilliet [11]):

$$u = \frac{M}{c\ q\ t\ E} = \frac{M\,\varkappa}{c\,Q} \tag{10}$$

In the above equations, M is the transported mass of the radical, c its concentration, q the cross-sectional area of the tube, t the time, E the field strength, Q the amount of electricity passed, and $\varkappa$ the conductivity. The second expression is more useful than the first.

The classical frame of reference is the solvent, but this is not generally useful. Thus, in the case of mixed solvents, the components of which are in relative motion, it is evident that not the solvent as a whole, but well one component of it, can serve as the frame of reference. There are also other cases where it is not suitable to have the solvent as the plane of reference. Therefore, in the definitions given above, we have left the plane of reference

open to free choice, but evidently it is necessary to state to which plane of reference a given mobility refers.

Early workers in the field of moving boundaries proposed to use an electrically inert substance as the frame of reference instead of water. Transference numbers thus obtained were called »true transference numbers», and from the difference between them and the Hittorf transference numbers relative degrees of hydration between cations and anions could be estimated (Lewis [27], Buchböck [28], Washburn [29, 30]). A recent investigation by Longsworth [31] shows, however, that we have no means to demonstrate that there is any substance that is more electrically inert than water. The migration of water relative to the added non-electrolytes turned out to be different for different reference substances. Thus the concept of a true transference number involves an assumption, the validity of which is difficult to demonstrate.

It should be pointed out that the planes of reference used for the mobilities above and below a moving boundary must not be in relative motion towards each other if the moving boundary equation shall retain its absolute validity. This condition is never fulfilled by the solvent, nor by any other constituent in the mixture. It is satisfied by the apparatus and by any moving or stationary boundary in the system. The frame of reference was discussed at length by Dole [20], who gave a modified moving boundary equation with exact validity for the case that the solvent is chosen as the frame of reference in every interboundary layer.

Special difficulties are encountered in applying the definitions to the hydrogen and hydroxyl radicals in water solutions because these are the solvent radicals. As already pointed out, however, the moving boundary equation need not be applied to these radicals.

If we are concerned with chemical equilibria which are not instantaneously established, the above definitions will in general be inapplicable. It is evident that the observed mobility will be a function of time before the equilibrium is reached. After reaching equilibrium, the transference method may give consistent and reproducible mobilities, whereas the moving boundary method requires that the equilibrium is established momentaneously.

If the highest accuracy is not necessary, and if the number of subspecies is small, it is sometimes possible to omit the moving boundary or transference experiment and to calculate the desired mobility with the aid of equation (9). The use of this equation, however, necessarily involves some assumption that cannot be tested. For instance, in an acetate buffer with known amounts of sodium acetate and acetic acid, we can neglect the dissociation of the latter, so we know very accurately the concentrations of the two subspecies. Their mobilities, however, cannot be measured in the mixture where they coexist.

The mobility of the acetate ion must be measured in the absence of acetic acid (above pH 8), and *vice versa*, and to compute the mobility of the acetate radical in the mixture, one must assume that the mobility of one subspecies is independent of the presence of the other. This assumption is, however, justified to a high degree of accuracy in many cases.

DISCUSSION

Again considering the special case that the only weak electrolytes present are protolytes, the abundance of the different subspecies will essentially depend upon pH, and the mobilities of the constituents will be pH-functions. This explains why it is possible to avoid the treatment of a number of mass-action equations and the reformulation of the moving boundary equation by adding new terms to make allowance for chemical reactions. The law of mass-action is inherent in the function $u = f(\mathrm{pH})$ in an operational manner.

In solutions of one single weak electrolyte, the necessity of treating the radicals instead of the subspecies is easily understood and has been so for a very long time. In mixtures of a weak acid and its salt, however, this point of view is not similarly self-evident. The dissociation of the acid being very much depressed in such a mixture, it lies close at hand to regard it as a non-electrolyte and to treat the salt as a strong electrolyte. This treatment is completely satisfactory in many cases, whereas in other cases it is quite untenable, which has been shown experimentally both in this laboratory and by Alberty and Nichol [33].

Moving boundary experiments with buffer mixtures designed according to the definition of a mobility given above have been carried out in this laboratory in great number during the last year. Thus the mobility of the acetate radical can be measured at arbitrary pH values by placing the acetate buffer in the bottom of the Tiselius U-tube and by using a propionate buffer as supernatant. Moving boundaries develop at which the acetate radical disappears from the bottom and the propionate radical from the top. These boundaries may be given any desired mobility between that of the free acetate ion and zero by varying the proportion between sodium acetate and free acetic acid (pH). This new type of moving boundary experiments showed the additional peculiarity that the moving boundary, although gravitationally stable and very sharp, had a negative refractive index increment.

Two special cases are of interest in showing the connection between viewpoints restricted to strong electrolytes and such of general validity. First, if the number of subspecies reduces to one, we have the case of strong electrolytes. This is very nearly the case for salts such as sodium acetate, ammonium

chloride, disodium phosphate, where the hydrolysis is very slight. Second, if pH is essentially constant in the system *after the boundaries have developed*, the relative mobilities of the radicals will also be constant. Chemical reactions will occur only to a small extent when subspecies traverse the boundaries or *vice versa*. Under such circumstances, the application of the moving boundary equation to subspecies will be approximately justified. This has been shown experimentally by Longsworth [34] who computed the small pH change across the moving boundary by applying the moving boundary equation to the acetate ions. In spite of the presence of undissociated acetic acid, the pH change thus computed showed a good agreement with experimental observations. The same result has been obtained in this laboratory.

The case mentioned above, with a constant pH throughout the moving boundary system, is of considerable importance in the field of electrophoresis. Electrophoretic analyses are nowadays carried out in well-buffered solutions, and thus we can conclude that the results may be interpreted with the aid of the theory for strong electrolytes to a very high accuracy. This was realized and pointed out by Longsworth [19].

SUMMARY

The fact that hitherto available theories for moving boundary systems are strictly valid only for strong electrolytes, as pointed out by Dole, made it evident that a generalization of the theory is required. This article is the first step in such a generalization in that it gives the moving boundary equation in a form of quite unrestricted validity. Formally, the equation is the same as earlier. The constituents to which it can be applied have been defined as non-dissociable radicals. It has been pointed out that not only equivalent concentrations, but also molar and other concentrations on a volume basis may be used, which is of importance for substances such as amino acids, proteins, other colloids, and non-electrolytes. A generalized definition of the mobility of a radical has been given in terms of moving boundary or transference experiments. A new type of moving boundary experiments according to this definition were briefly mentioned.

The author wishes to express his gratitude to Professor A. Tiselius for his kind interest in this work and for reviewing the manuscript. His thanks are also due to to Professor T. Svedberg for facilities put at his disposal. The manuscript was finished at the Rockefeller Institute for Medical Research, New York, and the author has the pleasure of expressing his best thanks to Drs. D. A. MacInnes, L. G. Longsworth, and V. P. Dole for their interest in these questions and for very helpful discussions.

The study of weak electrolytes in electrophoresis is being financially supported by the Swedish Natural Science Research Council, Stockholm.

REFERENCES

1. Lodge, O. *Brit. Assoc. Advancement Sci. Rept.* (1886) 389.
2. Whetham, W. C. D. *Proc. Roy. Soc. London* **52** (1892—93) 283.
3. Whetham, W. C. D. *Phil. Trans. A* **184** (1893) 337.
4. Whetham, W. C. D. *Z. physik. Chem.* **11** (1893) 220.
5. Wetham, W. C. D. *Phil. Mag.* **38** (1894) 392.
6. Whetham, W. C. D. *Proc. Roy. Soc. London* **58** (1895) 182.
7. Whetham, W. C. D. *Phil. Trans.* **186** (1895) 507.
8. Kohlrausch, F. *Ann. Physik* **62** (1897) 209.
9. Weber, H. *Sitzungsber. Akad. Wiss. Berlin* (1897) 936.
10. MacInnes, D. A., and Longsworth, L. G. *Chem. Rev.* **11** (1932) 171.
11. Hartley, G. S., and Mcilliet, J. L. *Proc. Roy. Soc. London A* **140** (1933) 141.
12. Picton, H., and Linder, S. E. *J. Chem. Soc. London* **71** (1897) 568.
13. Whitney, W. R., and Blake, J. C. *J. Am. Chem. Soc.* **26** (1904) 1339.
14. Hardy, W. B. *J. Physiol.* **33** (1905).
15. Tiselius, A. *Nova Acta Regiae Soc. Sci. Upsaliensis IV*, **7** (1930) no. 4.
16. Henry, D. C., and Brittain, J. *Trans. Faraday Soc.* **29** (1933) 798.
17. Tiselius, A. *Trans. Faraday Soc.* **33** (1937) 524.
18. Svensson, H. *Arkiv Kemi, Mineral. Geol.* **A 17** (1943) no. 14.
19. Longsworth, L. G. *J. Am. Chem. Soc.* **67** (1945) 1109.
20. Dole, V. P. *J. Am. Chem. Soc.* **67** (1945) 1119.
21. Perlmann, G. E., and Kaufman, D. *J. Am. Chem. Soc.* **67** (1945) 638.
22. Koenig, V. L., and Hogness, K. R. *Arch. Biochem.* **9** (1946) 119.
23. Koenig, V. L., Perrings, J. D., and Hogness, K. R. *Arch. Biochem.* **11** (1946) 345.
24. Armstrong, S. H., Budka, M. J. E., and Morrison, K. C. *J. Am. Chem. Soc.* **69** (1947) 416.
25. Svensson, H. *Arkiv Kemi, Mineral. Geol.* **A 22** (1946) no. 10.
26. MacInnes, D. A. *The principles of electrochemistry* New York (1939).
27. Miller, W. L. *Z. physik. Chem.* **69** (1909) 436.
28. Lewis, G. N. *J. Am. Chem. Soc.* **32** (1910) 862.
29. Buchböck, G. *Z. Physik. Chem.* **55** (1906) 563.
30. Washburn, E. W. *J. Am. Chem. Soc.* **31** (1909) 322.
31. Washburn, E. W. *J. Am. Chem. Soc.* **37** (1915) 694.
32. Longsworth, L. G. *J. Am. Chem. Soc.* **69** (1947) 1288.
33. Alberty, R., and Nichol, J. C. *J. Am. Chem. Soc.* **70** (1948) 2297.
34. Longsworth, L. G. *J. Phys. Colloid Chem.* **51** (1947) 171.

Received April 1, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 856—860

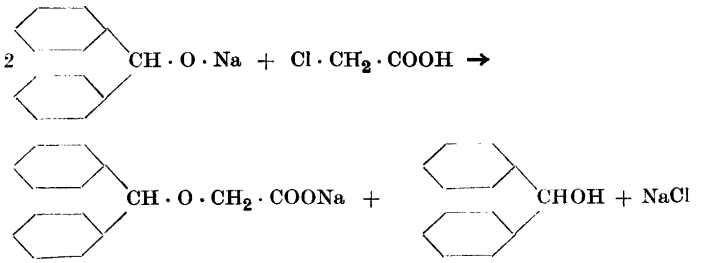
Diphenylmethoxyacetic Acid and Some Related Compounds

RICHARD DAHLBOM and LARS-ERIC ÖSTERBERG

Central Laboratories, Astra, Södertälje, Sweden

As starting materials for certain syntheses we required diphenylmethoxyacetic acid, diphenylmethylthioglycolic acid and the amides and ethyl esters of these acids. Among these compounds only diphenylmethylthioglycolic acid appears to have been reported in the literature [1]. This paper describes the syntheses of these new compounds and their properties.

Diphenylmethoxyacetic acid was prepared by treating the sodium salt of benzohydrol with chloroacetic acid.



The ethyl ester could be obtained by esterification with ethanol and sulfuric acid. It was, however, prepared more conveniently from ethyl bromoacetate and the sodium salt of benzohydrol.

In attempting to obtain the amide via the acid chloride, it was found that the acid was disintegrated by treatment with chlorinating agents such as thionyl chloride, phosphorus pentachloride and phosphorus oxychloride and only benzohydrol or diphenylmethyl chloride could be isolated.

By reacting the acid with equimolar amounts of thionyl chloride and pyridine and treating the reaction product with ammonia by the method of Human and Mills [2], the acid amide was certainly obtained, but it was synthesized more conveniently and with a better yield by heating the ethyl ester with alcoholic ammonia in a sealed vessel at 100°.

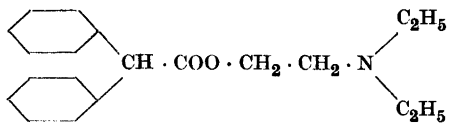
As a starting material for heterocyclic compounds, especially imidazolines, we required diphenylmethoxyacetonitrile too. It proved, however, impossible

to obtain this compound by dehydration of the amide. Distillation with phosphorus pentoxide caused cleavage of the molecule at the ether linkage, benzohydrol being formed. Treatment with thionyl chloride or phosphorus chlorides yielded diphenylmethyl chloride. We succeded, however, in producing the desired imidazoline in another way, by the action of 2-(chloromethyl)-imidazoline on the sodium salt of benzohydrol [3].

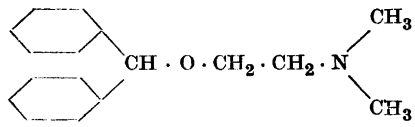
Diphenylmethylthioglycolic acid was prepared by the procedure of Holmberg [1]. The ethyl ester was obtained by esterification with ethanol and sulfuric acid in the usual way. The amide of this acid was prepared by heating the ester with alcoholic ammonia in a sealed vessel.

In this connection it seemed to us to be worth while investigating whether the β-dialkylaminoethyl esters of these two acids possessed any interesting pharmacological properties. These compounds bear a certain structural resemblance to Trasentin (I) and Benadryl (II), which are well known as an antispasmodic and an antihistamine agent, respectively.

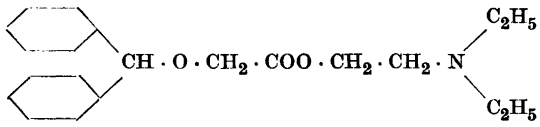
We therefore prepared the β-diethylaminoethyl esters of these acids (III, IV) by treating the acids with β-diethylaminoethyl chloride by the method of Miescher and Hoffmann [4].



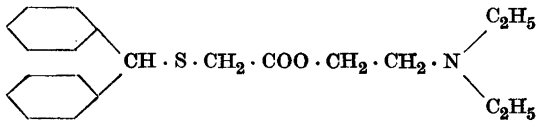
I. Trasentin.



II. Benadryl.



III.



IV.

The compounds III and IV have been directly compared with Benadryl on isolated guinea-pig ileum *. They exerted a slight antihistamine action, and

* The pharmacological investigations have been carried out at Astra's Biological Department by Dr. S. Wiedling.

their antispasmodic effect against barium chloride and acetylcholine was comparatively weak.

EXPERIMENTAL

Diphenylmethoxyacetic acid

184 g of benzohydrol was dissolved in 200 ml of dry toluene, and 23 g of powdered sodium added. The mixture was allowed to stand at room temperature overnight, and was then warmed with stirring for two hours at 100°. After cooling, a solution of 47 g of chloroacetic acid in 200 ml of toluene was added in portions, and the mixture was then refluxed for 7 hours. After cooling to room temperature, the mixture was extracted with 600 ml of 0.2 *N* sodium hydroxide. The alkaline solution was acidified with 5 *N* hydrochloric acid, whereupon the crude acid separated as an oil, which crystallized in a couple of days. The yield was 76 g. Extraction of the acidified solution with ether and evaporation of the ether yielded another 1.5 g of the acid. The total yield amounted to 64 %. From the toluene 85 g of benzohydrol could be recovered.

The crude acid was recrystallized from 50 % ethanol. M. p. 74—75°.

$C_{15}H_{14}O_3$ (242.3)	Calc.	C 74.5	H 5.78	Eq. wt. 242
	Found	» 75.0	» 5.67	» 242

For characterizing of the acid, its phenacyl ester was prepared.

A mixture of 3.1 g of the acid, 2.0 g of phenacyl bromide, 0.5 g of sodium carbonate and 30 ml of 66 % ethanol was refluxed for 1 hour. The mixture was then poured out into ice water, whereupon the ester separated as an oil, which soon crystallized. After recrystallization from ethanol it melted at 92—94°.

$C_{23}H_{20}O_4$ (360.4)	Calc.	C 76.7	H 5.58
	Found	» 76.7	» 5.60

Ethyl diphenylmethoxyacetate

A solution of the sodium salt of benzohydrol was prepared from 50 g of benzohydrol, 5.75 g of sodium and 225 ml of toluene along the lines of the preceding experiment. 42 g of ethyl bromoacetate was added, and the mixture was refluxed for 7 hours. After cooling, the mixture was washed with water in order to remove the separated sodium bromide. The toluene layer was dried over calcium chloride, and the toluene was distilled off on a water bath at 15 mm. The residue, 72 g, was distilled in high vacuum. The fraction boiling at 105—115° bath temperature at 0.002 mm was collected. Yield 30.0 g (41 %) of a colourless oil.

$C_{17}H_{18}O_3$ (270.3)	Calc.	C 75.6	H 6.71
	Found	» 76.0	» 6.70

This ester was very sensitive to alkaline hydrolysis. Thus it was saponified almost instantaneously at room temperature by means of ethanolic sodium hydroxide.

7.5 g of the ethyl ester was dissolved in 7.5 ml of ethanol, and 1.9 g of sodium hydroxide dissolved in 30 ml of ethanol was added. A vigorous reaction took place, and in a couple of minutes the solution had solidified. The precipitate was filtered off, dissolved in 200 ml of water and acidified with 2 N hydrochloric acid, whereupon 4.0 g of white crystals separated. After recrystallization from 50 % ethanol, m. p. 74—75° and eq. wt. 242.

Diphenylmethoxyacetamide

A. It proved advantageous to prepare this compound from the crude ethyl ester and not from the distilled product. 75.0 g of the crude ester, prepared as described above, was dissolved in 115 ml of ethanol, and the solution was saturated with dry ammonia gas in the cold. The mixture was heated in a sealed steel tube at 100° for sixteen hours. On cooling, 31 g of white crystals separated. The yield was 47 % calculated upon benzohydrol. The amide was recrystallized from 66 % ethanol. M. p. 134—135°.

$C_{15}H_{15}NO_2$ (241.3)	Calc.	C 74.7	H 6.28	N 5.81
	Found	» 74.7	» 6.27	» 5.79

B. To a solution of 7.3 g of diphenylmethoxyacetic acid and 2.4 g of pyridine in 75 ml of ether, 3.58 g of thionyl chloride was added, drop-wise with stirring. The mixture was left at room temperature for an hour, 75 ml of pyridine was then added, and the mixture was saturated with dry ammonia gas and was allowed to stand overnight. The mixture was then poured out into ice water, and the precipitate was collected. Yield 3.1 g (42 %). After recrystallization from 66 % ethanol m. p. 134—135°.

β-Diethylaminoethyl diphenylmethoxyacetate

12.1 g of diphenylmethoxyacetic acid, 8.6 g of β-diethylaminoethyl chloride hydrochloride [5] and 13.5 g of dried potassium carbonate were suspended in 50 ml of acetone and refluxed with stirring for 24 hours. The mixture was filtered, and the chilled filtrate acidified with a solution of dry hydrogen chloride in acetone. The resulting precipitate was collected, the yield was 6.7 g. After recrystallization from ethyl acetate the pure hydrochloride melted at 143—144°.

$C_{21}H_{27}NO_3 \cdot HCl$ (377.9)	Calc.	Cl 9.39	N 3.70
	Found	» 9.31	» 3.65

The free base could be obtained by dissolving the hydrochloride in water and precipitating with sodium carbonate solution. This yielded an oil, which was extracted with ether. The ether was died over calcium chloride and distilled off. The residue distilled at 0.002 mm at 160° in the bath giving a colourless oil.

$C_{21}H_{27}NO_3$ (341.4) Calc. N 4.12 Found N 4.11

Diphenylmethylthioglycolic acid

Holmberg [1] prepared this compound by heating equivalent parts of benzohydrol and thioglycolic acid for 4 hours on the water bath either suspended in 2 *N* hydrochloric acid or without any diluent. We prepared it by a slight modification of the latter method.

If a drop of conc. hydrochloric acid was added to the reaction mixture, a vigorous reaction commenced, and in a few minutes the crude acid crystallized.

Ethyl diphenylmethylthioglycolate

A mixture of 5.0 g of diphenylmethylthioglycolic acid, 5 ml of conc. sulfuric acid and 50 ml of ethanol was refluxed for 5 hours. The solution was poured out into 300 ml of ice water, and the crude ester was then extracted with ether. The ether layer was washed with sodium carbonate solution and water, dried over calcium chloride and evaporated. Distillation of the residue at 0.001 mm at 125° in the bath yielded 4.4 g of a colourless oil (79.5 %).

$C_{17}H_{18}O_2S$ (286.4)	Calc.	C 71.3	H 6.33	S 11.2
	Found	» 71.0	» 6.26	» 11.2

Diphenylmethylthioglycolamide

28.8 g of the ethyl ester was dissolved in 100 ml of ethanol, and the solution was saturated, at a low temperature with ammonia gas. It was then heated in a sealed steel tube at 100° for 16 hours. On cooling, 17.1 g of the amide separated. Recrystallization from ethanol gave white crystals with the m. p. 110—112°.

$C_{15}H_{15}NOS$ (257.3)	Calc.	N 5.45	S 12.5
	Found	» 5.42	» 12.3

β-Diethylaminoethyl diphenylmethylthioglycolate

A mixture of 13.0 g of diphenylmethylthioglycolic acid, 8.6 g of β-diethylaminoethyl chloride hydrochloride, 13.5 g of potassium carbonate and 50 ml of acetone was refluxed with stirring for 24 hours. The solution was filtered and acidified with a solution of dry hydrogen chloride in acetone. The precipitated hydrochloride amounted to 6.1 g. After recrystallization from acetone it melted at 164—165°.

$C_{21}H_{27}NO_2S \cdot HCl$ (394.0)	Calc.	Cl 9.02	N 3.56
	Found	» 9.03	» 3.56

SUMMARY

The preparation of diphenylmethoxyacetic acid and a number of derivatives of this acid and diphenylmethylthioglycolic acid has been described.

REFERENCES

1. Holmberg, B. *J. prakt. Chem.* **141** (1934) 93.
2. Human, J. P. E., and Mills, J. A. *Nature* **158** (1946) 877.
3. Dahlbom, R., and Sjögren, B. *Acta Chem. Scand.* **1** (1947) 777.
4. Miescher, K., and Hoffmann, K. *Helv. Chim. Acta* **24** (1941) 458.
5. Slotta, K. H., and Behnisch, R. *Ber.* **68** (1935) 754.

Received June 29, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 861—871

The Crystal Structure of Mo_4O_{11} (γ-Molybdenum Oxide)

ARNE MAGNÉLI

Institute of Chemistry, University of Uppsala, Uppsala, Sweden

In a paper from this Institute [1] on X-ray studies of the products obtained by heating mixtures of molybdenum and molybdenum trioxide *in vacuo* a phase (γ-molybdenum oxide) was described which formed at about 650—700° C from preparations of compositions between $MoO_{2.85}$ and $MoO_{2.72}$. Mo_4O_{11} was given as a probable formula of this oxide. In a recent notice Glemser and Lutz [2] report a molybdenum oxide (β-phase according to their notation) evidently identical with the phase mentioned above. Their statement of a homogeneity range from $MoO_{2.75}$ to $MoO_{2.65}$, derived from X-ray data and measurements of electrical resistance, disagrees with our observation that no displacements of the powder photograph reflections, indicating an extended homogeneity range, could be detected.

Crystals of γ-molybdenum oxide suitable for single-crystal investigations with X-rays have now been obtained by prolonged heating of the oxide. They form small reddish-violet thin tablets of orthorhombic symmetry. The crystal actually used for this purpose was picked out from a preparation $MoO_{2.80}$ heated at about 700° C for 45 days. From Laue photographs the Laue symmetry was found to be D_{2h}—*m m m*. Rotation and Weissenberg photographs were taken around the axes [0 1 0] (layer lines 0—6) and [0 0 1] (the equator layer line) with Mo-*K* radiation. Correlation between the intensity scales of the former photographs was brought about by means of the reflections *h k* 0 of the latter. The intensities were estimated visually, 4 : 2 : 1 being adopted as the intensity ratio of Mo-$K\alpha_1$, Mo-$K\alpha_2$, and Mo-$K\beta$ radiation. Relative values of F^2_{hkl} were obtained by multiplying the intensity values with the factor $\cos^2\mu \cdot \sin \Upsilon\ (1 + \cos^2 2\Theta)$ utilizing the curves given by Chia-Si Lu [3]. The temperature factor was neglected.

The dimensions of the orthorhombic unit cell as calculated from the single crystal photographs are:

$$a = 24.4 \text{ Å},\ b = 5.45 \text{ Å},\ c = 6.70 \text{ Å},\ V = 890 \text{ Å}^3.$$

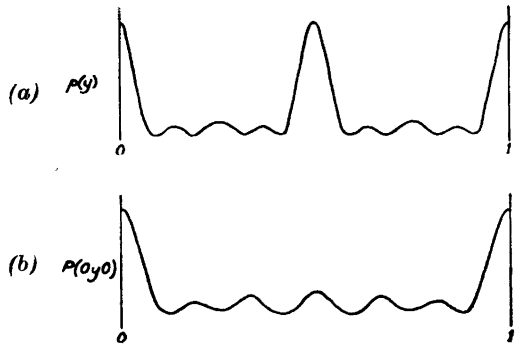


Fig. 1. a. *Projection of the Patterson function on the b axis, p(y).*
b. *The Patterson function along the b axis, P(0 y 0).*

For a preparation $MoO_{2.75}$, heated at 700° C for 100 hours, a density of 4.18 was measured. This corresponds to 16.0 $MoO_{2.75}$ units (4 Mo_4O_{11}) per cell.

Reflections $h\ k\ 0$ only appear when h is even and reflections $0\ k\ l$ only when $k + l$ is even. This is characteristic of the space-groups D_{2h}^{16}-*Pnma* and C_{2v}^{9}-$Pn2_1a$. The fact that the number of points corresponding to the point positions of these space-groups are all four or multiples of four gives support to the above assumption concerning the cell content.

MOLYBDENUM POSITIONS

The projection of the Patterson function * on the b axis, p(y), Fig. 1 a, only showing strong maxima at $y = 0$ and ½, indicates that all the heavy metal atoms are arranged in or close to two planes normal to [0 1 0] and at a distance of $b/2$. However, the only strong maximum of the Patterson function along the b axis, P(0 y 0), Fig. 1 b, is the peak at the origin, which excludes the presence of metal atoms related by mirror planes. If *Pnma* is the actual space-group the Mo atoms thus must be situated in the mirror planes and occupy fourfold positions 4(*c*) of that space-group. The conception of the Mo atoms being arranged in this way is supported by the appearance of the

* As far as possible all Fourier summations were carried out by means of the rapidly working Hägg-Laurent machine [4]. However, in the actual performance the machine only permits a maximum wave number of 16 and determination of the sum at intervals of 6° (and of course at intervals of 3° for even wave numbers up to 32), while h indices up to 34 were observed in the photographs. The calculations were then performed either by means of the Robertson strips [5] (maximum wave number 22) or without special calculating means. In the x direction the final summations were carried out at intervals of 3°.

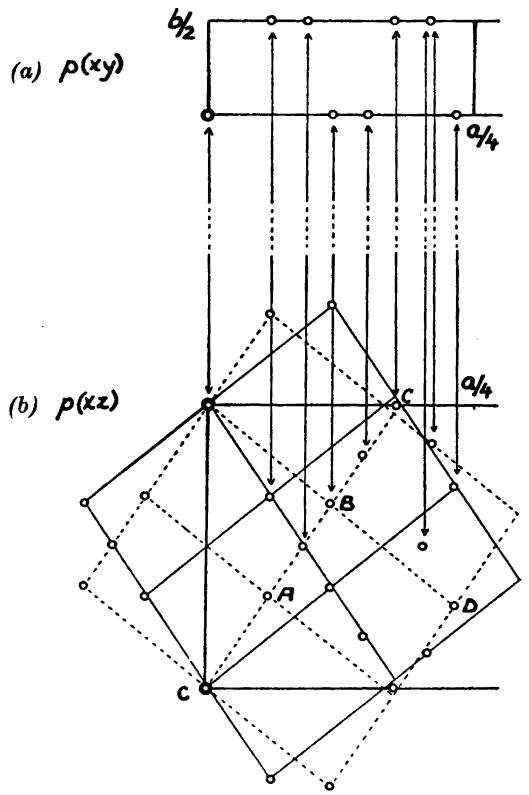


Fig. 2. a. Projection of the Patterson function parallel to [0 0 1], p(xy). b. Projection of the Patterson function parallel to [0 1 0], p(xz). Only the heavy maxima within the region $0 < x < \frac{a}{4}$ are indicated. There is an obvious tendency of the maxima of this projection to reduce their heights and blur out when x approaches $\frac{1}{8}$. Arrows connect identical maxima of the two projections. Each of the two systems of networks connects maxima due to interaction between Mo atoms of the same layer of the structure.

Patterson projection parallel to [0 0 1], p(*xy*), Fig. 2 a, where the *y* coordinates of the heavy maxima do not show any deviations from the values 0 and ½. It may thus be supposed that *Pnma* is the actual space-group and the consistency of the structure with that symmetry will be examined.

The projection of the Patterson function parallel to [0 1 0], p(*xz*), is represented in Fig. 2 b. Most maxima of this function occur at points forming two congruent interpenetrating networks, indicated in the figure by full and dotted lines. The side lengths of the unit mesh are about 3.8 and 2.6 Å. These values agree with the probable lengths of the space diagonal and the side of a regular MoO_6 octahedron. The following structure scheme based on these observations is consistent with the heavy maxima of p(*xz*) and p(*xy*) *:

* Notations according to *International tables for the determination of crystal structures* Berlin (1935).

4 Mo_1 in 4(c):	$\frac{x_A}{2}$	$\frac{3}{4}$	$\frac{1+z_A}{2}$
4 Mo_2 in 4(c):	$\frac{x_A+x_B}{2}$	$\frac{1}{4}$	$\frac{z_A+z_B}{2}$
4 Mo_3 in 4(c):	$\frac{x_B+x_C}{2}$	$\frac{3}{4}$	$\frac{z_B+z_C}{2}$
4 Mo_4 in 4(c):	$\frac{x_C+x_D}{2}$	$\frac{1}{4}$	$\frac{z_C+(z_D-1)}{2}$

For moderately high values of h and l these metal positions give F^2_{h0l} values in harmony with the observed ones. The Mo coordinates could be improved from a few successive calculations of the electron density projection $\varrho(xz)$. It was then possible to obtain the sign of the structure factor of most reflections $h\ k\ l$ with a high degree of certainty, the influence of the O atoms in general being too small to be of any importance compared with the contribution of the metal atoms. The positions of the Mo atoms were definitely settled from the cut $\varrho(x\ \frac{1}{4}\ z)$ in the electron density function, utilizing almost all of the observed reflections. In that way the following arrangement of the Mo atoms, consistent with the symmetry *Pnma*, was obtained:

4 Mo_1 in 4(c):	0.0285	$\frac{3}{4}$	0.840
4 Mo_2 in 4(c):	0.087	$\frac{1}{4}$	0.518
4 Mo_3 in 4(c):	0.1465	$\frac{3}{4}$	0.196
4 Mo_4 in 4(c):	0.2055	$\frac{1}{4}$	0.869

The accuracy is estimated to be $\pm$ 0.001 for the x coordinates and $\pm$ 0.003 for the z coordinates.

OXYGEN POSITIONS

The projections of the Patterson function p(xy) and p(xz) contain a number of diffuse maxima that might be due to Mo-O interaction, but it was not thought possible to make any conclusions from them as to the arrangement of the O atoms. However from the disposition of the Mo atoms indexed 1—3 it seems reasonable that these atoms coordinate six O atoms in an octahedral way, the MoO_6 octahedra thus formed being joined by corners. Then each Mo_4 atom will be surrounded by four O atoms forming a tetrahedron.

If *Pnma* is the actual space-group space reasons require the O atoms to be situated in the mirror planes ($y = \frac{1}{4}$ and $\frac{3}{4}$) or approximately midway between them. The cuts $\varrho(x\ \frac{1}{4}\ z)$ and $\varrho(x\ 0\ z)$ in the electron density function might thus give information about the x and z coordinates of the O atoms.

The former cut, besides the heavy Mo maxima, shows a number of minor peaks. All those required by the proposed O arrangement are found with quite

reasonable distances mutually and to the Mo positions. However, as there are also other maxima of almost the same magnitude, not explicable as being due to a possible structure and evidently caused by cutting off effects, the accuracy of the following O positions cannot be very high.

4 O_5 in 4(c):	0.028	$\frac{1}{4}$	0.34
4 O_6 in 4(c):	0.088	$\frac{3}{4}$	0.03
4 O_7 in 4(c):	0.163	$\frac{1}{4}$	0.67
4 O_8 in 4(c):	0.222	$\frac{3}{4}$	0.37

In $\varrho(x\ 0\ z)$ maxima occur which can be interpreted as due to O atoms completing the octahedra around Mo_1, Mo_2, and Mo_3 and the tetrahedron around Mo_4 and giving quite reasonable interatomic distances. A maximum at $x = 0$, $z = 0$ must correspond to O atoms occupying a fourfold position 4(a), as the possibility of its connection with an eightfold position 8(d) is excluded for space reasons. The x and z coordinates of the remaining O atoms — situated in three eightfold positions 8(d) — cannot be very accurate as a few rather strong accessory peaks occur in the cut. The y coordinates of these atoms must be close to 0 (and $\frac{1}{2}$) for space and functional reasons.

4 O_1 in 4(a):	0	0	0
8 O_2 in 8(d):	0.063	0.00	0.69
8 O_3 in 8(d):	0.123	0.00	0.35
8 O_4 in 8(d):	0.183	0.00	0.01

DESCRIPTION OF THE STRUCTURE

The following structure is thus arrived at:

Cell content: 4 Mo_4O_{11}
Space-group: D_{2h}^{16} — *Pnma*

4 Mo_1 in 4(c):	0.0285	$\frac{3}{4}$	0.840
4 Mo_2 in 4(c):	0.087	$\frac{1}{4}$	0.518
4 Mo_3 in 4(c):	0.1465	$\frac{3}{4}$	0.196
4 Mo_4 in 4(c):	0.2055	$\frac{1}{4}$	0.869
4 O_1 in 4(a):	0	0	0
8 O_2 in 8(d):	0.063	0.00	0.69
8 O_3 in 8(d):	0.123	0.00	0.35
8 O_4 in 8(d):	0.183	0.00	0.01
4 O_5 in 4(c):	0.028	$\frac{1}{4}$	0.34
4 O_6 in 4(c):	0.088	$\frac{3}{4}$	0.03
4 O_7 in 4(c):	0.163	$\frac{1}{4}$	0.67
4 O_8 in 4(c):	0.222	$\frac{3}{4}$	0.37

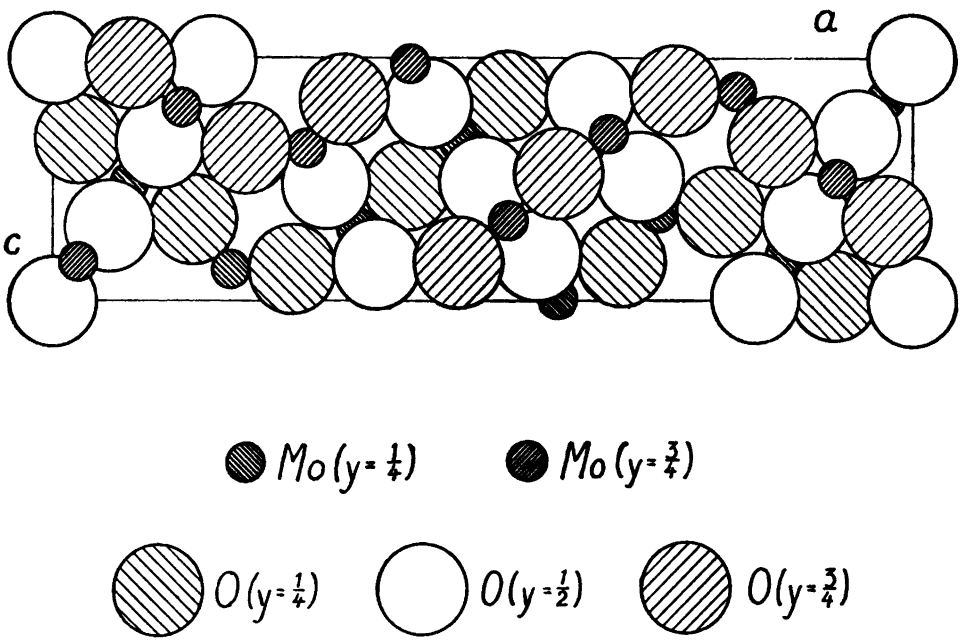


Fig. 3. Projection of the unit cell of Mo_4O_{11} parallel to [0 1 0]. The O atoms at y = 1 are omitted.

A projection of the elementary cell parallel to [0 1 0] is given in Fig. 3. In Table 1 a number of observed and calculated structure factor values are compared. Their agreement may be considered rather satisfactory.

The lattice may be described as consisting of layers of infinite extension normal to [1 0 0], *cf.* Fig. 4. Three fourths of the Mo atoms (Mo_1, Mo_2, and Mo_3) coordinate six O atoms to form distorted MoO_6 octahedra, which are mutually joined by corners. The rest of the Mo atoms (Mo_4) coordinate three O atoms of adjacent MoO_6 octahedra of the same layer and one O atom of the neighbouring layer to form distorted MoO_4 tetrahedra, thus bringing about the connection between the layers. The finite extension of the layers corresponds to three MoO_6 octahedra and one MoO_4 tetrahedron joined by corners, the Mo atoms of these polyhedra being situated close to a straight line. The directions of these lines are different for adjacent layers.

No difference can be observed between the Mo-Mo distances of adjacent octahedra or the corresponding distances of an octahedron and a tetrahedron. They all vary about 3.75 Å, the limiting values being 3.70 and 3.79 Å. These figures are in agreement with the Mo-Mo distances of octahedra joined by

Table 1. Weissenberg photographs with Mo-K radiation. Comparison between observed and calculated $|F|$ values.

$h\ k\ l$	$\|F\|$ obs.	$\|F\|$ calc.	$h\ k\ l$	$\|F\|$ obs.	$\|F\|$ calc.	$h\ k\ l$	$\|F\|$ obs.	$\|F\|$ calc.
4 0 0	50	70	13 0 2	—	10	22 0 7	100	130
6 0 0	70	80	14 0 2	50	50	23 0 7	—	60
8 0 0	70	70	15 0 2	50	30	24 0 7	—	0
10 0 0	70	70	16 0 2	—	20	16 1 0	—	40
12 0 0	60	40	17 0 2	—	10	16 2 0	520	380
14 0 0	130	120	18 0 2	—	20	16 3 0	—	40
16 0 0	250	220	19 0 2	—	10	16 4 0	310	200
18 0 0	300	250	20 0 2	—	20	16 5 0	—	30
20 0 0	—	30	21 0 2	—	10	16 6 0	350	380
22 0 0	—	50	22 0 2	140	140	16 7 0	—	30
24 0 0	—	30	23 0 2	180	200	16 8 0	240	160
26 0 0	—	20	24 0 2	140	150	26 1 0	370	300
28 0 0	—	20	25 0 2	—	20	26 2 0	—	30
30 0 0	—	30	26 0 2	—	10	26 3 0	320	290
32 0 0	—	40	27 0 2	120	180	26 4 0	—	20
34 0 0	260	280	28 0 2	160	110	26 5 0	230	260
36 0 0	—	50	29 0 2	110	170	26 6 0	—	20
0 2 0	760	530	30 0 2	—	30	26 7 0	200	230
0 4 0	720	970	1 0 7	—	10	26 8 0	—	10
0 6 0	660	420	2 0 7	—	30	34 1 0	—	0
0 8 0	480	610	3 0 7	90	90	34 2 0	330	230
0 0 2	—	20	4 0 7	210	220	34 3 0	—	0
0 0 4	70	90	5 0 7	200	220	34 4 0	330	250
0 0 6	330	470	6 0 7	—	80	34 5 0	—	0
0 0 8	—	80	7 0 7	70	100	34 6 0	270	180
0 0 10	—	30	8 0 7	—	0	34 7 0	—	0
0 0 12	—	60	9 0 7	—	10	34 8 0	260	210
1 0 2	160	100	10 0 7	—	20	1 2 2	120	80
2 0 2	—	10	11 0 7	—	20	2 2 2	120	100
3 0 2	200	120	12 0 7	120	130	3 2 2	120	130
4 0 2	—	20	13 0 7	160	170	4 2 2	120	100
5 0 2	240	180	14 0 7	70	140	5 2 2	430	320
6 0 2	240	180	15 0 7	—	20	6 2 2	440	360
7 0 2	150	120	16 0 7	—	40	7 2 2	370	220
8 0 2	120	110	17 0 7	—	10	8 2 2	100	90
9 0 2	50	40	18 0 7	—	40	9 2 2	—	10
10 0 2	300	260	19 0 7	—	30	10 2 2	210	150
11 0 2	330	360	20 0 7	—	70	11 2 2	280	250
12 0 2	150	190	21 0 7	140	110	12 2 2	150	110

Table 1 (contd.).

h k l	F		h k l	F		h k l	F	
	obs.	calc.		obs.	calc.		obs.	calc.
13 2 2	—	10	19 2 2	—	10	25 2 2	—	10
14 2 2	—	10	20 2 2	—	40	26 2 2	—	30
15 2 2	—	10	21 2 2	—	30	27 2 2	150	220
16 2 2	—	0	22 2 2	70	60	28 2 2	150	140
17 2 2	—	40	23 2 2	170	160	29 2 2	110	160
18 2 2	—	0	24 2 2	190	140	30 2 2	—	40

corners observed for Mo_9O_{26} and Mo_8O_{23}, the average values being 3.70 and 3.77 Å respectively [6].

The Mo-O and O-O distances cannot be very exact due to the relative inaccuracy of the O positions. The Mo-O distances within the MoO_6 octahedra vary between 1.8 and 2.2 Å with an average value of 1.91 Å. (For Mo_9O_{26} and Mo_8O_{23} the average Mo-O distances are 1.91 and 1.94 Å respectively[6].) However, there seems to be some difference between the Mo-O distances depending on whether the O atom is common to two octahedra or to one octahedron and one tetrahedron. In the former case the distances are between 1.8 and 2.0 Å, in the latter they fall in the interval 2.05 to 2.2 Å. In this way the O atoms will be closer to the Mo atoms of the tetrahedra showing Mo-O distances of about 1.75 Å. Similar values have been calculated by Sillén and Nylander [7] for the Mo-O distance of the MoO_4 group of $SrMoO_4$.

The O-O distances of the MoO polyhedra are of reasonable lengths, with values from 2.55 to 3.1 Å.

DISCUSSION OF THE STRUCTURE

The structure of Mo_4O_{11} is rather different from those of the other molybdenum oxides hitherto investigated. The occurence of MoO_4 tetrahedra in Mo_4O_{11} totally lacks analogy in these oxides which are exclusively built up by MoO_6 octahedra. The deficit in oxygen content of the lower molybdenum oxides in relation to MoO_3 is thus maintained in two quite different ways: by the presence of MoO_4 tetrahedra in Mo_4O_{11} and by the MoO_6 octahedra to a minor or greater extent sharing edges in Mo_9O_{26}, Mo_8O_{23} [6], and MoO_2 [8]. (In the latter oxide every O atom is in this way common to three octahedra.)

The structure of Mo_4O_{11} shows planes sparingly occupied by atoms, viz. the joints between the layers mentioned above. This causes a rather weak

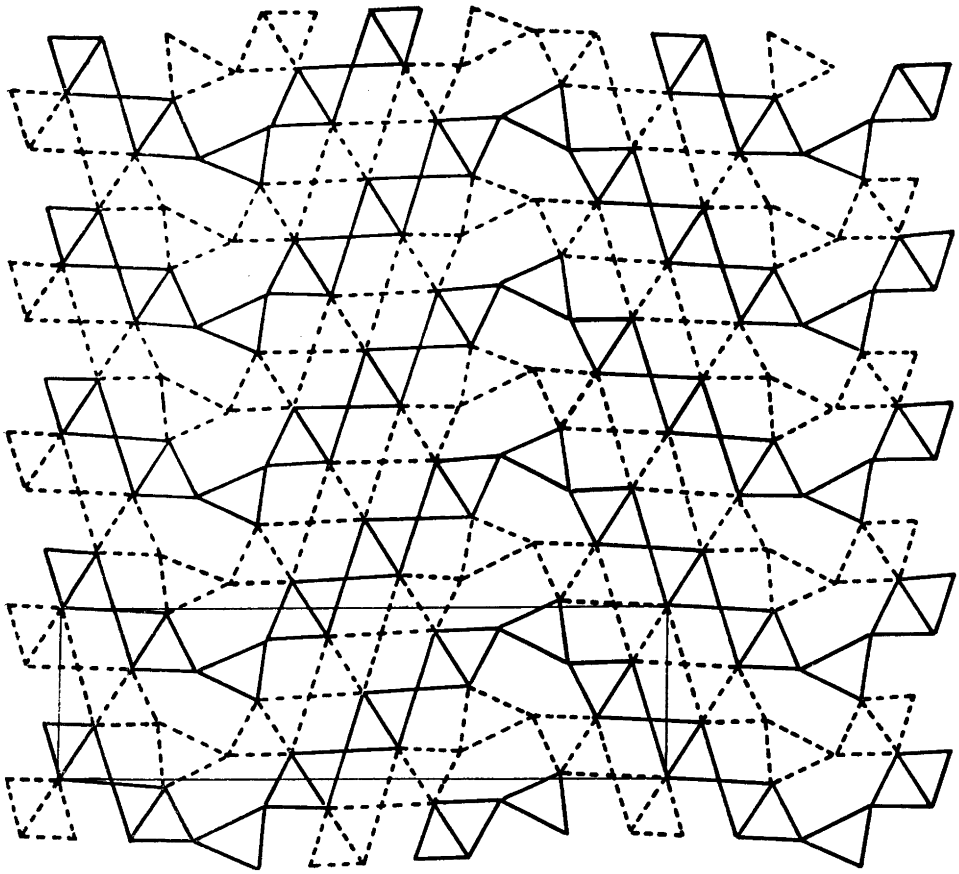


Fig. 4. Projection of the structure parallel to [0 1 0]. The extension of one unit cell is indicated. MoO_6 octahedra and MoO_4 tetrahedra, the Mo atoms of which are situated at $y = \frac{1}{4}$ and $y = \frac{3}{4}$, are indicated by dotted and full lines respectively.

cohesion in the direction of the *a* axis, which is reflected in the shape of the crystals, showing thin plates parallel to (1 0 0).

The structure of Mo_4O_{11} is a very spacious one compared with those of the other molybdenum oxides. The quotient between the volume of the unit cell and the number per cell of the bulky O atoms may to some extent be used as a measure of this property. The average space available per O atom (the volume of the small Mo atoms being neglected) for the different molybdenum oxides is listed in Table 2 as well as the corresponding figure for a hypothetical compound RO_3 of $D0_9$-type (ReO_3-type)[9] with a R-R distance of 3.75 Å. It is

Table 2. The average space available per O atom for different molybdenum oxides.

	Number of formula units per cell	Volume of the unit cell, V	V divided by the number of O atoms per cell
MoO_2	4	131.3 * Å^3	16.4 Å^3
Mo_4O_{11}	4	890	20.2
Mo_8O_{23}	2	870	18.9
Mo_9O_{26}	2	970	18.7
MoO_3	4	203.0 *	16.9
RO_3 of $D0_9$-type (ReO_3-type) R—R = 3.75 Å	1	52.7	17.6

evident that the lattices of Mo_9O_{26}, Mo_8O_{23}, and especially of Mo_4O_{11} are much more loosely packed than those of MoO_2 and MoO_3, which are even more compact than the hypothetical RO_3 lattice. However, while the bulkiness of the structures of Mo_9O_{26} and Mo_8O_{23} is due to the deformation of the MoO_6 octahedra, being lengthened parallel to [0 1 0], the lattice of Mo_4O_{11} contains irregularly shaped holes of appreciable size, situated in the layer joint regions. It may be that extra atoms or molecules can be inserted in these interstices. The catalytic action of »molybdenum oxides» on divers chemical reactions may be connected with these cavities.

SUMMARY

The γ-molybdenum oxide has been shown to have the composition Mo_4O_{11}. A complete structure determination has been carried out. The lattice is constructed of MoO_6 octahedra and MoO_4 tetrahedra coupled together by sharing corners. The oxide may be described as being composed of layers infinitely extended in two dimensions. The MoO_4 tetrahedra connect adjacent layers.

The author wishes to thank Professor G. Hägg for his encouraging interest in the investigation. He is also indebted to Mr. Arne Hansson for his valuable assistance with the calculations. The research has been financially supported by the *Swedish Natural Science Research Council*,, which support is gratefully acknowledged.

* The figures given in [1] and [8], in fact expressed in kX units, are here converted into Å units.

REFERENCES

1. Hägg, G., and Magnéli, A. *Arkiv Kemi, Mineral. Geol.* **A 19** (1944) no. 2.
2. Glemser, O., and Lutz, G. *Naturwissenschaften* **34** (1947) 215.
3. Chia-Si Lu *Rev. Sci. Instr.* **14** (1943) 331.
4. Hägg, G., and Laurent, T. *J. Sci. Instr.* **23** (1946) 155.
5. Robertson, J. M. *J. Sci. Instr.* **25** (1948) 28.
6. Magnéli, A. *Acta Chem. Scand.* **2** (1948) 501.
7. Sillén, L. G., and Nylander, A.-L. *Arkiv Kemi, Mineral. Geol.* **A 17** (1943) no. 4.
8. Magnéli, A. *Arkiv Kemi, Mineral. Geol.* **A 24** (1946) no. 2.
9. *Z. Krist.* Strukturbericht 1928—1932 (1937).

Received October 8, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 872—877

Glucosides of Pinosylvin and its Monomethyl Ether

BENGT LINDBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

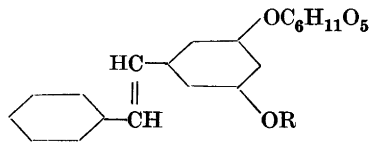
The heart wood of Scots pine *(Pinus silvestris)* and most, perhaps all, members of the genus *Pinus* contain fungicidic stilbene derivatives, pinosylvin and its monomethyl ether [1]. Glucosides of these phenols have not been isolated from natural sources but the rhizome of the rhubarb *(Rheum rhaponticum)* contains a similar stilbene derivative in the form of a glucoside (rhapontin) [2]. If glucosides of pinosylvin and its monomethyl ether occur naturally the isolation, undoubtedly, would be facilitated by a knowledge of their physical properties.

The presence of pinosylvin phenols in the heart wood can be demonstrated by illumination with ultraviolet light, when the heart wood shows a more or less brilliant violet fluorescence. The deposition of pinosylvin in the heart wood is the result of the activity of the cambium. It could well be conceived that this activity is displayed not only in a centripetal direction but also in a centrifugal. As a matter of fact the inner part of the bark adjoining the cambium generally exhibits the same fluorescence as the heart wood. The cause of this fluorescence is at present being studied at this institute.

Pinosylvin, and still more its monomethyl ether are very sparingly soluble in water and this is a complicating factor in the measurement of their toxicity to wood destroying fungi. The glucosides would presumably be more readily soluble and since it may be anticipitated that these fungi have glucosidases at their disposal, the mycological investigation of pinosylvin glucosides is a matter of interest.

The syntheses of the glucosides followed conventional lines. The acetylated derivatives were prepared from the hydroxy stilbenes employing the silver oxide-quinoline method and then deacetylated. In the case of the pinosylvin monoglucoside it was necessary to protect one of the hydroxyl groups. This was accomplished by benzoylation.

It was not possible, however, to obtain the glucosides β-pinosylvin monomethyl ether glucoside I,β,β'-pinosylvin diglucoside II and β-pinosylvin monoglucoside III in a distinctly crystalline form, but the analytical results, including catalytic hydrogenation, indicated a fair degree of purity.



I R = CH_3

II R = $C_6H_{11}O_5$

III R = H

Docent Rennerfelt, Statens Skogsforskningsinstitut, Stockholm, has been kind enough to test the biological activity of these preparations and reports that neither pinosylvin monomethyl ether glucoside (slightly soluble in water) nor pinosylvin diglucoside (easily soluble in water) possesses any fungicidic activity to wood destroying fungi *(Pullularia pullulans* and *Polyporus annosus)*.

EXPERIMENTAL

Pinosylvin and pinosylvin monomethyl ether were isolated by the method given by Erdtman [3].

Tetraacetyl-β-pinosylvin monomethyl ether glucoside

Pinosylvin monomethyl ether (2.26 g) and acetobromoglucose (5.36 g) were dissolved in anhydrous quinoline (6 ml) in a small beaker. Active silver oxide (2.8 g) was added and the mixture was cooled with ice and stirred for 20 minutes. It was then allowed to stand in a desiccator for an hour. Acetic acid (20 + 10 ml) was then added and the mixture was well stirred, centrifuged and filtered into water (600 ml). The precipitate was filtered, washed with water, dissolved in benzene and dried over calcium chloride. The brown benzene solution was filtered through a column of aluminium oxide (Brockmann). Most of the colour was removed and the solution obtained was now slightly yellow. It was concentrated under reduced pressure and precipitated with petrol ether. An oil was obtained which slowly solidified. Yield 5.2 g (93 %). From hot methanolic solution it precipitated as a voluminous gel, which could be dried to an amorphous powder of m. p. 89—90°. When a dilute methanolic solution was allowed to evaporate slowly at room temperature, the substance was obtained as colourless needles. It was also obtained in this form when a dilute solution in benzene was precipitated with petrol ether. M. p. 106—107° $[\alpha]_D^{20}$—24° in chloroform *.

$C_{29}H_{32}O_{11}$ (556.3)	Calc.	OCH_3	5.58	$OCCH_3$	30.9
	Found	»	5.64	»	31.1

* All melting points uncorrected. All rotations in about 2 % solutions.

β-Pinosylvin monomethyl ether glucoside

Tetraacetyl-β-pinosylvin monomethyl ether glucoside (1 g) was dissolved in ethanol (25 ml) and a small piece of potassium (about 30 mg) was added. The solution was boiled up and allowed to stand for two hours. It was then diluted with water (40 ml), acidified with acetic acid and the ethanol distilled off. On cooling a voluminous precipitate was formed, which was filtered and dried, first on porous pot, then over sulphuric acid in a vacuum. Yield 0.59 g (84 %). After one further recrystallization from ethanol and two from ethyl acetate it had m. p. 168—169°. When dried for several days over phosphorus pentoxide at 70° in a vacuum it had m. p. 177—178° and $[\alpha]_D^{20}$—58° in acetone-water, 1 : 1. The substance forms a white powder which appears to be amorphous even under the microscope. The m. p. however is sharp and different preparations had the same melting point, so the substance might be microcrystalline.

$C_{21}H_{24}O_7$ (388.2)	Calc.	C 64.9	H 6.23	OCH_3 7.99
	Found	» 64.7	» 6.21	» 7.91

On catalytic hydrogenation in ethanol with palladium on animal charcoal as catalyst 1 mole β-pinosylvine monomethyl ether glucoside consumed 1.00 mole hydrogen.

A small amount of the glucoside was acetylated with acetic anhydride in pyridine, the solution poured into water and the precipitate recrystallized from benzene-petrol ether. M. p. 106—107°. The mixed m. p. with the acetate described above was 106—107°.

Octaacetyl-β,β′-pinosylvin diglucoside

Octaacetyl-β,β′-pinosylvin diglucoside was prepared in exactly the same way as tetraacetyl-β-pinosylvin monomethyl ether glucoside. With pinosylvine (1.06 g), acetobromoglucose (5.36 g), quinoline (6 ml) and silver oxide (2.8 g) a sample of slightly discoloured crystals of m. p. 173—174° was obtained after one recrystallization from ethanol. Yield 2.7 g (62 %). After three further recrystallizations from ethanol the material had m. p. 174—175° and $[\alpha]_D^{20}$—25° in chloroform. It forms colourless needles, slightly soluble in ethanol.

$C_{42}H_{48}O_{20}$ (872.4)	Calc.	C 57.8	H 5.65	$OCCH_3$ 39.5
	Found	» 57.9	» 5.79	» 39.3

β,β-Pinosylvin diglucoside

Octaacetyl-β,β′-pinosylvin diglucoside (2.7 g) was deacetylated with potassium ethoxide in ethanol and the solution titrated with perchloric acid to pH 7.55 in order to remove the potassium [4]. The potassium perchlorate was filtered off and the ethanol evaporated under reduced pressure. The residue, a slight yellow amorphous powder, could not be recrystallized. Yield 1.65 g (100 %). When dried in a vacuum it became somewhat darker, if the temperature was raised to 70° it became dark brown. It is easily soluble in water and acetic acid, very slightly soluble in ethanol and practically insoluble in anhydrous organic solvents such as ether and benzene. It has no melting point but slowly decomposes at 140°. $[\alpha]_D^{20}$—87° in acetone-water, 1 : 1.

$C_{26}H_{32}O_{12}$ (536.3)	Calc.	C 58.2	H 6.02
	Found	» 57.9	» 6.15

On catalytic hydrogenation 1 mole glucoside consumed 0.97 mole hydrogen.

A small amount of the glucoside was acetylated with acetic anhydride in pyridine and the resulting product recrystallized from ethanol. M. p. 174—175°. Mixed m. p. with the octaacetate described above 174—175°.

Pinosylvin monobenzoate

Pinosylvin (5.8 g) was dissolved in a mixture of absolute ether (100 ml) and absolute pyridine (10 ml). Benzoyl chloride (3.16 ml) in ether (50 ml) was slowly added with vigourous mechanical stirring. The ether was washed with 2 *N* hydrochloric acid (200 ml) and with water (200 ml). Ether was added to a final volume of 1000 ml and the solution extracted with 0.3 % sodium hydroxide. From this alkaline solution pinosylvin (1.6 g) was recovered. The ether was distilled and the residue recrystallized from ethanol. Crude pinosylvin dibenzoate (2.0 g) separated. The mother liquor was evaporated to dryness. When the residue was recrystallized from xylene, a second crop of crude pinosylvin monobenzoate (3.2 g), m. p. 110—115°, was obtained. After two recrystallizations from xylene the m. p. was 118—120°. From the mother liquors further amounts of pure monobenzoate could be recovered. Total yield 2.7 g, m. p. 118—120°.

Pinosylvin monobenzoate (207.7 mg) was acetylated with acetic anhydride in pyridine (2 ml), the amount equivalent to 45.65 ml 0.0996 *N* sodium hydroxide. After the acetylation the mixture was poured into water and titrated. 39.22 ml 0.0996 *N* sodium hydroxide were consumed.

The difference, 6.43 · 0.0996 m. equivalents, has been consumed by the pinosylvin monobenzoate.

$C_{21}H_{16}O_3$ (316.1) Found equiv. wt. 324

Pinosylvin monobenzoate (100 mg) was methylated with diazomethane in ether. The excess of diazomethane was destroyed with acetic acid and the ether evaporated. The yield of pinosylvin monomethyl ether benzoate was quantitative. The substance was recrystallized from acetone-methanol. M. p. 82—84°. The substance prepared by Erdtman [1] by benzoylation of pinosylvin monomethyl ether had m. p. 84.5—86° and the mixed m. p. of the two preparations was 83—85°.

Pinosylvin monobenzoate acetate

Pinosylvin monobenzoate was acetylated with acetic anhydride in pyridine (see above). When the mixture was poured into water, colourless crystals appeared. The suspension was extracted with ether, the ether solution shaken with dilute hydrochloric acid, sodium bicarbonate and water, dried over sodium sulphate and finally filtered through a column of aluminium oxide. The ether was distilled and the remaining oil soon solidified, yielding colourless crystals. They were recrystallized twice from ethanol. M. p. 131—132.5°.

$C_{23}H_{18}O_4$ (358.1)	Calc.	C 77.1	H 5.07
	Found	» 77.0	» 5.01

Tetraacetyl-β-pinosylvin monobenzoate glucoside

Pinosylvin monobenzoate (1.64 g) and acetobromoglucose (5.36 g) were dissolved in quinoline and silver oxide (2.8 g) was added. The mixture was stirred for 20 minutes and then allowed to stand in a desiccator for 1 hour. Acetic acid (20 + 10 ml) was then added and the mixture was well stirred and centrifuged. The liquid phase was filtered into 600 ml water. The precipitate was filtered, washed with water and dissolved in benzene. The benzene solution was dried over calcium chloride, filtered through a column of aluminium oxide and concentrated in a vacuum. The residue could not be recrystallized but was obtained as a white, amorphous powder by precipitating an ethanolic solution with water. Yield 2.8 g (83 %). M. p. 63—65° (unsharp). $[\alpha]_D^{20}$—20° (chloroform).

$C_{35}H_{34}O_{12}$ (646.3)	Calc. C 65.0	H 5.31	Acyl groups 5	
	Found » 64.1	» 5.40	» »	5.15

The substance is evidently not pure.

β-Pinosylvin monoglucoside

The above tetraacetyl-β-pinosylvin monobenzoate glucoside was treated with potassium ethoxide in ethanol and freed from potassium salts with perchloric acid in the ordinary way. The ethanol was evaporated and the residue washed with ether to remove ethyl benzoate. When dried over phosphorus pentoxide a slightly brown, amorphous powder was obtained. The material was soluble in acetic acid, slightly soluble in water and ethanol and insoluble in ether. On catalytic hydrogenation 0.93 mols of hydrogen were consumed. All attempts to obtain this substance in a definitely crystalline state or in a state of higher purity failed. It was therefore acetylated, but yielded an amorphous product.

A small sample of the phenol glucoside preparation was methylated with diazomethane in methanol. Part of the methylated product was acetylated with acetic anhydride in pyridine. M. p. of the obtained glucoside 174—175°. Mixed m. p. with β-pinosylvin monomethyl ether glucoside 175—176°. M. p. of the obtained acetate 106—107°. Mixed m. p. with tetraacethyl-β-pinosylvin monomethyl ether glucoside 106—107°. The glucoside and its acetate from the above experiments proved, then, to be identical with the previously described β-pinosylvin monomethyl ether glucoside and its acetate.

β-Dihydropinosylvin monoglucoside

A small sample of crude pinosylvin monoglucoside was hydrogenated in ethanol with palladium on animal charcoal as catalyst. The solution was filtered and the ethanol evaporated. The residue was dissolved in methanol. When the methanol was allowed to evaporate at room temperature the glucoside crystallized in colourless needles.

The crystalline mass was washed with water and ether and recrystallized once more from methanol. M. p. of the thoroughly dried preparation 168—170°.

$C_{20}H_{24}O_7$ (376.2)	Calc. C 63.8	H 6.43	
	Found » 62.5	» 6.50	

The substance evidently is not pure. The amount available, however, was too small to permit further purification.

SUMMARY

The mono- and di-β-glucosides of pinosylvin and the β-glucoside of pinosylvin monomethyl ether have been prepared. The monoglucoside, however, could not be obtained in a state of purity.

I am indebted to Professor Holger Erdtman for suggesting this work and for helpful advice.

REFERENCES

1. Erdtman, H. *Ann.* **539** (1939) 116.
2. Kawamura, S. *J. Pharm. Soc. Japan* **58** (1938) 83 (Chem. Zentr. 1939, I, 130).
3. Erdtman, H. *Svensk Papperstidn.* **48** (1945) 217.
4. Bonner, W. A., and Koehler, W. L. *J. Am. Chem. Soc.* **70** (1948) 314.

Received November 8, 1948.

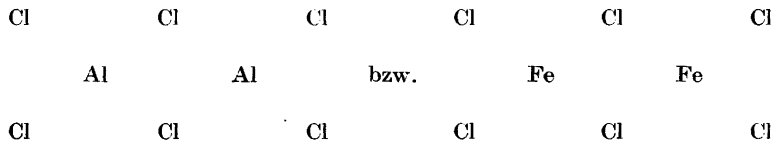
ACTA CHEMICA SCANDINAVICA 2 (1948) 878—882

Über die Flüchtigkeit des Aluminiumchlorids und des Eisen(III)-chlorids

WALTER HÜCKEL

Chemisches Institut der Universität Lund, Schweden

Aluminiumchlorid und Eisen(III)-chlorid bilden bekanntlich bei nicht zu hoch über dem Siedepunkt liegenden Temperaturen Doppelmoleküle, deren Struktur auf Grund von Elektronenbeugungsversuchen [1] und des Ramanspektrums [2] feststeht; es sind zwei mit einer Kante zusammenstossende $MeCl_4$-Tetraeder:



Im Gitter sind solche Moleküle nicht vorgebildet [3]; dem Gittertypus nach gehören beide Verbindungen zum $CrCl_3$-Schichtgittertyp, $FeCl_3$ mit einer anderen Aufeinanderfolge der Schichten als $CrCl_3$, $AlCl_3$ ist überdies monoklin deformiert. In den Schichten sind die Metallatome oktaëdrisch von 6 Chloratomen umgeben, die sich in dichtester oder nahezu dichtester Kugelpackung befinden. Bis vor kurzem war das Gitter des $AlCl_3$ als Molekülgitter mit Molekülen Al_2Cl_6 beschrieben, wodurch es in Parallelle zum Al_2Br_6 und Al_2J_6 zu stehen schien; die Leichtflüchtigkeit der 3 Aluminium-halogenide — $AlCl_3$ Schmp. unter Druck 193°, Subl.pkt 183°; $AlBr_3$ Schmp. 93°, Sdp. 260°; AlJ_3 Schmp. 125°, Sdp. 350° — war danach in denselben einfachen Zusammenhang mit ihrer Molekülgitterstruktur zu bringen. Nun hat aber Ketelaar [3] seine ältere Strukturbestimmung [4] berichtigt, so dass nunmehr das Gitter des $AlCl_3$ ganz anders gebaut erscheint als das Gitter von Al_2Br_6 und Al_2J_6, und damit entfällt die Möglichkeit, die Leichtflüchtigkeit des $AlCl_3$ ohne weiteres durch seine Gitterstruktur zu erklären, wie das bisher geschehen ist [5].

Dieser neuerdings festgestellte Gittertyp des $AlCl_3$, der bereits vor längerer Zeit von Nowacki [6] vermutet worden war, macht aber Beobachtungen von Biltz verständlich, welche auf Unterschiede beim kristallisierten $AlCl_3$ einerseits, $AlBr_3$ und AlJ_3 andrerseits hinweisen. Das Aluminiumchlorid vergrössert beim Schmelzen unter Druck sein Volumen ungewöhnlich stark, fast auf das Doppelte, während Bromid und Jodid dies nicht tun; weiter leitet das kristalline Chlorid den elektrischen Strom elektrolytisch, wenn auch nicht gut, im Gegensatz zu seiner Schmelze, die ein Nichtleiter ist, und zu dem kristallisierten wie geschmolzenen Bromid und Jodid. Biltz schloss daraus, dass das Gitter des Aluminiumchlorids ein Ionengitter sei. Nun ist aber die elektrolytische Leitfähigkeit kristalliner Stoffe an Lockerstellen oder Störstellen im Gitter gebunden, und Ionengitter, die solche nicht enthalten, sind Nichtleiter; überdies gibt es Gitter mit einseitiger elektrolytischer Leitung, die nicht mehr annähernd als Ionengitter angesprochen werden können, wie z. B. das Silberjodidgitter. Der Schluss von Biltz ist also nicht bindend; es ist dazu zu bemerken, das kristallisiertes $FeCl_3$ den elektrischen Strom nicht leitet. Doch darf man aus seiner Beobachtung über die Leitfähigkeit so viel entnehmen, dass das Gitter des $AlCl_3$ Störstellen oder Lockerstellen besitzt, an denen relativ leicht Ionen gebildet werden können. Das weist, ebenso wie der niedrige Schmelz- bzw. Sublimationspunkt, auf eine Instabilität des Gitters, die auch Ketelaar [8] hervorhebt. Da das Eisen(III)-chlorid nicht elektrisch leitet, auch sein Sublimationspunkt mit 317° wesentlich höher liegt, muss man auf eine grössere Stabilität seines Gitters schliessen, obwohl auch aus diesem Gitter beim Verdampfen Doppelmoleküle entstehen. Vollends ganz stabil sind die Gitter der um 1200° sublimierenden Chloride $CrCl_3$ und VCl_3.

Die unterschiedliche Stabilität der Gitter muss, da der Gittertypus sehr ähnlich ist, auf die verschiedenen Bindungstärken der Atome im Gitter zurückgeführt werden. Um die Bindungsverhältnisse zu beschreiben, kann man von den beiden Grenzfällen ausgehen, von dem aus Ionen aufgebauten Gitter oder von einem homöopolaren Chloridmolekül, das man dann ins Gitter eintreten lässt.

In dem Ionengitter unterscheiden sich die Metallionen durch ihre Grösse und ihre Elektronenhülle. Die Unterschiede in den Radien sind nicht so gross, als dass man daraus die Reihenfolge der Stabilität herleiten könnte. Zwar hat $Al^{\cdots}$ den kleinsten Radius und wäre danach am wenigsten geneigt, die Koordinationszahl 6 gegenüber dem Chlor im Gitter anzunehmen, aber Eisen und Chrom, zwischen denen der grosse Sprung in der Flüchtigkeit liegt, haben als dreiwertige Kationen praktisch den gleichen Radius, so dass also eine Erklärung durch die Radienverhältnisse versagt:

	$Al^{\cdots}$	$Fe^{\cdots}$	$Cr^{\cdots}$
Radius nach Goldschmidt	0,57	0,67	0,65
» » Pauling	0,50	0,60	0,64
Radienverhältnis (G.)	0,315	0,37	0,36
» (P.)	0,277	0,33	0,355

Überhaupt wird beim Radienverhältnis der für oktaëdrische Konfiguration erforderliche Grenzwert 0,414 beim Aluminium nur dann erreicht, wenn man den von Zachariasen auf Einwertigkeit reduzierten Ionenradius 0,79 benutzt; bei Eisen und Chrom ist eine solche Reduktion nicht möglich, da diese das Vorhandensein einer Aussenschale von 8 Elektronen voraussetzt.

Die Betrachtung der verschiedenen Elektronenhüllen von $Al^{\cdots}$, $Fe^{\cdots}$ und $Cr^{\cdots}$ ist also wesentlich. Für diese ergibt sich nun aber genau die umgekehrte Reihenfolge wie bei der Stabilität der Gitter der Chloride:

$Al^{\cdots}$ hat die stabile Achterschale. $Fe^{\cdots}$ besitzt eine M-Schale, in der die d-Elektronen gerade zur Hälfte aufgefüllt sind, was wie beim $Mn^{\cdot\cdot}$-Ion [9] eine bevorzugte Stabilität bedeutet, die aber hinter der der Achterschale merklich zurücksteht. $Cr^{\cdots}$ besitzt nur 3, $V^{\cdots}$ nur 2 Elektronen in der unvollständigen d-Gruppe von Elektronen über einer Achterschale; beide heben sich also durch keine besonders Stabilität heraus.

Daraus muss man schliessen, dass die betrachteten Gitter keine reinen Ionengitter sind. Auch mit der Annahme einer Ionendeformation durch Polarisation kommt man nicht weiter, da auch hier das $Al^{\cdots}$-Ion als am wenigsten beeinflussbar erscheinen muss.

Da man auf diesem Wege nicht weiter kommt, muss man von dem andern Grenzfall eines homöopolaren Moleküls ausgehen, dessen an sich schon polare Bindungen dann durch Eintritt ins Gitter stärker polar werden, wie das auch sonst der Fall ist. Die Valenzelektronen werden bei einer solchen homöopolaren Bindung nicht von der unter ihr liegenden Schale abgehoben, sondern bilden zusammen mit je einem Elektron von jedem Liganden eine neue Schale. Bei den Trichloriden enthält diese also ein Sextett von Elektronen, ist also unvollständig. Deshalb ist sie bestrebt, sich zum Oktett aufzufüllen. Das kann entweder durch Hineinnehmen von 2 Elektronen, die von neuen Liganden stammen, geschehen, oder es können auch Elektronen aus der darunter liegenden Schale bei einer solcher Auffüllung mit hineinspielen. Hierbei müssen sich nun Aluminium, Eisen und Chrom wegen der unterschiedlichen Stabilität der untergelagerten Schale charakteristisch verschieden verhalten.

Beim Aluminium ist diese Schale eine Achterschale, die Neonschale, die festgefügt ist und nicht mehr angetastet werden kann; sie kann also nicht mitspielen. Bei der zur Hälfte aufgefüllten d-Elektronengruppe der M-Schale des

Eisens ist dies möglich, aber es besteht wegen ihrer verhältnismässigen Stabilität nur geringe Neigung der d-Elektronen, sich zu betätigen; beim Chrom und Vanadin schliesslich liegen keine durch Stabilität ausgezeichneten Schalen unter dem Elektronensextett.

Das $AlCl_3$ muss also die grösste Neigung haben, ein Doppelmolekül mit vollen Oktetten unter Verknüpfung durch Halogenobrücken zu bilden, beim $FeCl_3$ wird sie geringer und noch geringer beim $CrCl_3$ und VCl_3 sein.

Weiter wird man annehmen dürfen, dass das bei hoher Temperatur existenzfähige $AlCl_3$-Molekül wegen des Elektronensextetts ebenso wie andere Verbindungen mit Elektronensextett, z. B. BF_3 und BCl_3, eben sein wird; ein $FeCl_3$-Molekül dürfte wegen des zumal bei höherer Temperatur merklichen Hineinspielens von d-Elektronen in die Bindungsverhältnisse zwischen Fe und Cl schwach pyramidal gebaut sein und das $CrCl_3$-Molekül eine ausgesprochene Pyramide bilden. Betrachtet man nun die Änderungen, die diese Moleküle beim Eintritt in ein Gitter vom $CrCl_3$-Typ erleiden, abgesehen von der durch Polarisation bedingten Erhöhung der Polarität der Me-Cl-Bindung, so muss das $AlCl_3$ seine Form am meisten, das $CrCl_3$ am wenigsten, vielleicht auch gar nicht ändern.

So wirken beim Zustandekommen des Gitters beim Aluminiumchlorid zwei Faktoren erniedrigend auf dessen Stabilität, nämlich die starke Deformation des Einzelmoleküls beim Eintritt in das Gitter und die grosse Neigung, Doppelmoleküle zu bilden. Dadurch wird also die grosse Flüchtigkeit des Aluminiumchlorids trotz Bildung eines im Typus den Ionengittern nahestehenden Gittertyps verständlich.

Es bleibt noch der Vergleich des Aluminiumchlorids mit den übrigen Halogeniden des Aluminiums. Dass Bromid und Jodid kein Gitter des $CrCl_3$-Typus bilden, sondern Molekülgitter, ist einfach eine Raumfrage; die Koordinationszahl 6, die das Koordinationsgitter verlangt, ist hier aus räumlichen Gründen unmöglich.

Das festgefügte Gitter des erst bei 1260° sublimierenden Aluminiumfluorids [10] ist wegen der grösseren Elektronenaffinität des Fluors zweifellos viel stärker polar als das des Aluminiumchlorids, entspricht auch einem ganz anderen Gittertyp. Aber auch in diesem hat das Aluminium die Koordinationszahl 6, freilich genauer $3 + 3$.

Das Koordinationsoktaëder ist hier ziemlich stark deformiert, so dass sich ein AlF_3-Molekül mit kleineren Al-F-Abständen 1,71 Å (gegen 1,89 Å) als sonst in Al F_6''' -Komplexen im Gitter abzuheben beginnt. Deshalb und weil die Fluoratome keine dichteste Kugelpackung bilden, dürfte auch hier nicht ein rein heteropolarer Bindungstypus vorliegen. Die Stabilität des AlF_6-Komplexes ist aber im Vergleich mit den $AlCl_6$-Komplexen einmal wegen

der günstigeren räumlichen Verhältnisse, dann wahrscheinlich auch wegen der stärkeren Durchdringung der Elektronenhüllen von Al''' und F', die sich zumal in den 3 kleineren Al-F-Abständen von 1,71 Å abzeichnet, so gross, dass demgegenüber die Neigung zur Bildung von Al_2F_6-Molekülen mit F-Brücken, wie sie auch sonst nirgends in Molekülen bekannt geworden sind, keine Rolle spielt.

ZUSAMMENFASSUNG

Die unterschiedliche Flüchtigkeit von Aluminium-, Eisen- und Chromtrichlorid wird auf den verschiedenen Schalenbau der Atome bzw. der Ionen zurückgeführt. Durch diesen ist eine in der Reihenfolge Al-Fe-Cr abnehmende Neigung, Doppelmoleküle zu bilden, abnehmende Deformation des Einzelmoleküls beim Eintritt in das Gitter und zunehmende Stabilität des Koordinationsgitters bedingt.

LITERATUR

1. Palmer, K. J., und Elliot, N. *J. Am. Chem. Soc.* **60** (1938) 1852.
2. Gerding, H., und Smit, E. *Z. physik. Chem.* **B 50** (1941) 171.
   Kohlrausch, K. W. F., und Wagner, J. *Ibid.* **B 52** (1942) 185.
3. Ketelaar, J. A. A. *Rec. trav. chim.* **66** (1947) 503.
4. Ketelaar, J. A. A. *Z. Krist.* **A 90** (1935) 237.
5. Hückel, W. *Anorganische Strukturchemie* Stuttgart (1948) S. 170 und 470.
6. Nowacki, W. *Z. Krist.* **82** (1932) 360.
7. Biltz, W., und Voigt, A. *Z. anorg. Chem.* **126** (1923) 39.
8. a. a. O. S. 512.
9. Biltz, W., und Klemm, W. *Z. Elektrochem.* **39** (1933) 597.
10. Siehe darüber auch: Hückel, W. *Anorganische Strukturchemie* Stuttgart (1948) S. 605—606.

Eingegangen am 11. Oktober 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 883—889

Die Oberflächenspannungscorrection des Capillarviscosimeters. II

CARL DRUCKER

Physikalisch-Chemisches Institut, Uppsala, Schweden

Die Gestalt des oberen Gefässes eines Capillarviscosimeters verursacht bekanntlich, dass bei jeder Höhenlage ein von der Oberflächenspannung der untersuchten Flüssigkeit abhängiger, ständig variirender Zug nach oben auftritt, welcher dem Auslaufdrucke entgegenwirkt. Für die dadurch bedingte Correction gab Applebey [1] eine Näherungsformel an. Es wurde dann vor einigen Jahren [2] versucht, die Correction direct experimentell dadurch zu bestimmen, dass relative — auf die einer Standardflüssigkeit bezogene — Auslaufzeiten sowohl für den Totalauslauf des Instrumentes gemessen wurden (z_t) wie für den Auslauf des rein cylindrisch gestalteten mittleren Teiles (z_m), für welchen sie, wenn die Radien r_o und r_u beider Schenkel gleichgross sind, den Wert Null annimmt. Das Verhältnis $z_m : z_t$ ist dann der Correctionsfactor für die totale relative Auslaufzeit.

Eine nähere Betrachtung ergibt jedoch, dass bei diesem Verfahren noch eine zweite Correction angebracht werden muss, die als »Verdrängungscorrection» bezeichnet werden möge und die selbst im Falle $r_o = r_u$ von Null verschieden ist.

DIE VERDRÄNGUNGSCORRECTION

Wenn in die communicirenden Rohre des Viscosimeters mit den Radien r_o und $r_u > r_o$ ein bestimmtes Volumen v der Standardflüssigkeit (Wasser) von der Capillaritätsconstanten a^2 eingefüllt ist und der untere Meniscusrand im oberen Gefässe bei A, im unteren bei B steht, so ist bekanntlich die wahre Druckhöhe an dieser Stelle nicht gleich dem Abstande H_o zwischen diesen beiden Linien, sondern

$$H = H_0 - a^2\left(\frac{1}{r_o} - \frac{1}{r_u}\right) = H_0\left[1 - \frac{a^2}{H_0}\left(\frac{1}{r_o} - \frac{1}{r_u}\right)\right] = H_0\,(1 - \lambda_1) \qquad (1)$$

Ersetzt man die Flüssigkeit durch eine andere mit der Capillaritätsconstanten $a'^2 < a^2$ *und stellt den Meniscus wieder auf A ein*, so geht die Druckhöhe über in

$$H' = H_0 - \alpha'^2\left(\frac{1}{r_o} - \frac{1}{r_u}\right) = H_0\left[1 - \frac{\alpha'^2}{H_0}\left(\frac{1}{r_0} - \frac{1}{r_u}\right)\right] = H_0\,(1 - \lambda_1') \qquad (1a)$$

und die Combination beider Formeln ergibt die Applebeysche Correction.

Zugleich aber haben sich die »mittleren Höhen» — Volumen durch Querschnitt — der Menisken geändert, auf der einen Seite von μ_o in $\mu_o' < \mu_o$, auf der anderen von μ_u in $\mu_u' < \mu_u$, und es ist infolge dessen ein Teil des constanten Volumens v von der einen Seite nach der anderen verdrängt worden. Dadurch hat die Druckhöhe eine weitere Verkleinerung um

$$(\mu_0 - \mu_0') - (\mu_u - \mu_u') + (\mu_0 - \mu_0')\frac{r_o^2}{r_u^2}$$

erfahren und beträgt nun

$$H' = H_0 - \alpha'^2\left(\frac{1}{r_o} - \frac{1}{r_u}\right) - \left[\left(\mu_o - \mu_0'\right)\left(1 + \frac{r_o^2}{r_u^2}\right) - \left(\mu_u - \mu_u'\right)\right] \qquad (1b)$$

Sind beide Gefässe durchweg cylindrisch, d. h. ist sowohl r_o wie r_u constant, so hat man die gemessene relative — auf die der Standardflüssigkeit bezogene- Auslaufzeit mit

$$\frac{H'}{H} = \frac{1 - \frac{\alpha'^2}{H_0}\left(\frac{1}{r_o} - \frac{1}{r_u}\right) - \frac{1}{H_0}\left[\left(\mu_o - \mu_o'\right)\left(1 + \frac{r_0^2}{r_u^2}\right) - \left(\mu_u - \mu_u'\right)\right]}{1 - \frac{\alpha^2}{H_0}\left(\frac{1}{r_o} - \frac{1}{r_u}\right)} = \frac{1 - \lambda'_1 - \lambda_2}{1 - \lambda_1}$$

zu multipliciren, wählt man aber, wie früher [2] geschehen, $r_o = r_u$, so reducirt sich diese Formel auf

$$\frac{H'}{H} = 1 - \lambda_2 = 1 - \frac{1}{H_0}(\mu_o - \mu_o') \qquad (2a)$$

da dann λ_1 und λ'_1 wegfallen und $\mu_o = \mu_u$ wird.

Statt der im cylindrischen Teile eines solchen Instrumentes gefundenen relativen Auslaufzeit z_m ist deshalb $z'_m = z_m\ (1 - \lambda_2)$ als wahrer Wert anzusetzen, und will man einen Correctionsfactor für die gemessene Relativzeit des Totalauslaufes einführen, so beträgt dieser nicht, wie früher angegeben, $F = z_m : z_t$ sondern $F' = z'_m : z_t$.

Diese Correctionsgrössen können die Grössenordnung von einigen Tausendsteln erreichen und müssen also bei Präcisionsmessungen in Betracht gezogen werden, wie aus dem folgenden Zahlenbeispiele hervorgeht.

VERSUCHSERGEBNISSE

In einem Viscosimeter (XI) mit den Radien $r_o = r_u = 5{,}01$ mm — r_o gilt für den cylindrischen Mittelteil — der mittleren Druckhöhe $H_0 = 187$ mm des Mittelteiles und möglichst kurzen conischen Teilen, dessen Wasserzeiten bei 20,3° im cylindrischen Teile $t_m = 1929{,}6$ sec und bei Totalauslauf $t = 2336{,}5$ sec betrugen, wurden ausser Wasser (W, $a^2 = 14{,}70$) eine Dichloressigsäurelösung (D, $a^2 = 11{,}66$), *o*-Xylol (X, $a^2 = 6{,}95$) und Äthylenbromid (Ä, $a^2 = 3{,}82$) untersucht. Es ergaben sich die relativen Zeiten z_m und z_t. Aus den mittleren Meniscushöhen (Berechnung s. 3) μ_o in mm folgte λ_2 nach Formel (2a) und weiter damit die corrigirte Relativzeit z_m'.

Tabelle 1. Correctionsfactoren F und F' für Viscosimeter XI.

	z_m	z_t	μ_0	λ_2	z'_m	F	F'
W	—	—	0,986	—	—	—	—
D	1,1327	1,1318	0,915	0,00038	1,1322	1,0008	1,0004
X	0,8620	0,8594	0,744	0,00130	0,8609	1,0030	1,0016
Ä	0,8037	0,7994	0,528	0,00246	0,8017	1,0053	1,0029

$F' - 1$ ist also nur etwa halb so gross wie $F - 1$ und bei diesem Instrumente überhaupt sehr klein, was jedenfalls auf die besonders niedrige Form der conischen Teile zurückzuführen ist. Bei solcher Bauart des Apparates wird man selbst für Präcisionsmessungen oft auf Correction der relativen Totalauslaufzeit verzichten dürfen, wenn die relative Steighöhe ($h = a'^2 : a^2$) nicht kleiner ist als etwa 0,95, d. h. wenn es sich um den Vergleich einer verdünnten Lösung eines schwach capillaractiven Stoffes mit ihrem Lösungsmittel handelt (s. auch Drucker [2]).

In anderen Fällen wird dies nicht zulässig sein, besonders wenn die Radien stark verschieden sind, also die allgemeine Formel (2) angewendet werden muss. Es seien dafür einige Beispiele angeführt.

Ein Viscosimeter (XIV) mit cylindrischen Gefässen, jedoch stark verschiedenen Radien $r_o = 4{,}01$ und $r_u = 11{,}4$ mm, der mittleren Druckhöhe $H_0 = 176$ mm des Mittelteiles und einer Totalauslaufzeit des Wassers von etwa 1100 sec bei 25° gab die in Tabelle 2 verzeichneten Werte z_m und z_t. Aus μ_o und μ_u (s. o.) folgten die Correctionsterme λ_1 und λ_2. Wegen der Verschiedenheit der Radien ist hier λ_1 nicht gleich Null, sondern sehr gross, und λ_2 wird im Gegensatze zu oben negativ. Ferner wird z'_m grösser als z_m statt kleiner, und die wahren Correctionsfactoren F' des Totalauslaufes erhalten viel grössere Werte als im Falle $r_o = r_u$.

Tabelle 2. Correctionsfactoren F und F' für Viscosimeter XIV.

	z_m	z_t	μ_0	μ_u	λ_1	λ_2	z'_m	F	F'
W	—	—	0,898	0,908	0,0134	—	—	—	—
X	0,8988	0,8963	0,715	0,584	0,0063	0,0007	0,9058	1,0029	1,0106
Ä	0,8268	0,8256	0,540	0,411	0,0035	0,0005	0,8355	1,0015	1,0120

Daraus folgt, dass für Präcisionsbestimmungen der Fall $r_o < r_u$ möglichst vermieden werden soll.

Für einige andere Viscosimeter sind in Tabelle 3 die Factoren F' verzeichnet, wie sie sich aus der Totalauslaufzeit und dem an dem Instrumente XI gefundenen Werte z'_m bei 20,3° (s. o.) ergaben. XIV ist bereits beschrieben, III hatte im cylindrischen Mittelteile $r_o = r_u = 6{,}00$ mm; die beiden anderen hatten die gewöhnliche Form mit zwei ungleich grossen Kugeln.

Tabelle 3. Correctionsfactoren der Totalauslaufzeiten von Viscosimetern verschiedener Construction.

	III	XIV	II	I
D	1,0010	—	1,002	1,004
X	1,0030	1,0090	1,005	1,007
Ä	1,0050	1,0136	1,009	1,013

Tabelle 4. Wahre relative Auslaufzeiten z'_m für drei Normalinstrumente.

	X	XI	18	Mittel
D	1,1333	1,1322	1,1329	1,1326
X	0,8615	0,8609	0,8602	0,8609
Ä	0,8017	0,8017	0,8008	0,8015

Das Instrument mit der günstigsten Form (III) zeigt die kleinsten Werte für F'.

Um nun zu prüfen, ob die corrigirten relativen Auslaufzeiten z'_m wirklich die richtigen Werte sind, bestimmt man sie am besten an mehreren Instrumen-

ten ähnlicher Bauart aber ungleicher Dimensionen. Deshalb wurde die oben für das Viscosimeter XI beschriebene Messungsserie noch an einigen anderen durchgeführt; Nr X mit $r_o = r_u = 5{,}01$ mm und $H_0 = 165$ mm und Nr 18 mit $r_o = r_u = 4{,}20$ mm und $H_0 = 158$ mm. In Tabelle 4 sind die Werte z'_m zusammengestellt.

Dâ Nr XI etwas genauer arbeitete als die beiden anderen Rohre, so wurde es bei der Bildung des Mittelwertes mit doppeltem Gewichte in Rechnung gesetzt. Die Abweichungen erreichen nicht 1 Promille und werden sich in Zukunft noch vermindern lassen.

Auf diese Weise können also die wahren relativen Auslaufzeiten einer geeignet gewählten Serie von Flüssigkeiten ermittelt werden. Bestimmt man dann an denselben Flüssigkeiten die relativen Totalauslaufzeiten eines beliebigen anderen Instrumentes, so erhält man die für dieses einzuführenden Correctionsfactoren.

DIE VERHÄLTNISSE IN DEN ÜBERGANGSTEILEN

Die Verschiedenheit der mittleren Meniscushöhen, welche den Formeln (1) und (2) zu Grunde liegt, bildet auch die Hauptursache dafür, dass die relativen Zeiten z_o und z_u der conischen Teile des Viscosimeters von der im cylindrischen Teile gefundenen relativen Zeit z_m (resp. dem corrigirten Werte z'_m) stark abweichen.

Während der Cylinderteil von allen Flüssigkeiten stets das gleiche Volumen v_m aufnimmt, gilt für den oberen Conus v_o bei Wasser, dagegen bei anderen Stoffen $v'_o = v_o + (\mu - \mu')\,\pi\, r^2$, worin r den Cylinderradius bedeutet, und für den unteren v_u resp. $v'_u = v_u - (\mu - \mu')\,\pi r^2$, oder

$$\frac{v'_o}{v_o} = 1 + \frac{(\mu - \mu')\,\pi\, r^2}{v_o} = 1 + \xi_o \;, \quad \frac{v'_u}{v_u} = 1 - \frac{(\mu - \mu')\,\pi r^2}{v_u} = 1 - \xi_u \qquad (3)$$

Fasst man alle constanten Grössen der Viscositätsgleichung in einen Factor A zusammen, so gilt im Cylinderteile für die kinematische Viscosität des Wassers

$$\frac{\eta}{s} \cdot A = \frac{1}{v_m} \cdot H_m \cdot t_m$$

und für eine andere Flüssigkeit

$$\frac{\eta'}{s'} \cdot A = \frac{1}{v_m} H'_m \cdot t'_m$$

wonach (s. oben)

$$\frac{\eta'}{s'}\Big/\frac{\eta}{s} = \frac{H'_m}{H_m}\cdot\frac{t'_m}{t_m} = z'_m$$

Für die beiden conischen Teile ist dagegen zu schreiben

$$\frac{\eta'}{s'}\Big/\frac{\eta}{s} = \frac{1}{1+\xi_o}\cdot\frac{H_o'\cdot t_o'}{H_o\cdot t_o} = \frac{1}{1-\xi_u}\cdot\frac{H_u'}{H_u}\cdot\frac{t_u'}{t_u}$$

so dass aus der folgenden Doppelbeziehung

$$z'_m = \frac{z_o}{1+\xi_o}\cdot\frac{H_o'}{H_o} = \frac{z_u}{1-\xi_u}\cdot\frac{H'_u}{H_u} \tag{4}$$

die dem oben berechneten Factor H'_m/H_m des mittleren Teiles entsprechenden Verhältnisse H'_o/H_u und H'_u/H_u berechnet werden können, welche die Capillaritätscorrectionen dieser beiden Teile in der Form eines Druckhöhenverhältnisses darstellen.

Eine solche Berechnung für das Instrument XI (vgl. Tabelle 5) sei hier beigefügt.

Tabelle 5. Volumencorrection der conischen Teile (Instrument XI).

$v_0 = 429$ mm^3 $v_m = 3186$ mm^3 $v_u = 240$ mm^3

	t_o	t_u	z_o	z_u	z_m	μ	$1+\xi_o$	$1-\xi_u$	$\frac{H_o'}{H_o}$	$\frac{H_u'}{H_u}$
W	206,2	200,5	—	—	—	0,986	—	—	—	—
D	238,4	220,2	1,156	1,097	1,1322	0,915	1,013	0,977	0,992	1,009
X	187,5	157,0	0,909	0,783	0,8609	0,744	1,044	0,921	0,988	1,012
Ä	181,7	135,7	0,881	0,677	0,8017	0,528	1,084	0,850	0,987	1,006

Die Werte der beiden letzten Columnen liegen erwartungsgemäss sehr nahe bei 1. Man könnte sie auch nach Gl. (2) unabhängig gewinnen, doch wären dazu willkürliche Annahmen über »mittlere Radien» der conischen Teile erforderlich, so dass eine solche Rechnung bedeutungslos wäre.

Mit anderen Instrumenten ergab sich qualitativ dasselbe Resultat.

ZUSAMMENFASSUNG

Das in einer früheren Untersuchung befolgte Verfahren zur Ermittlung wahrer relativer Auslaufzeiten von Capillarviscosimetern wurde durch Ein-

führung einer oft geringfügigen aber theoretisch notwendigen Correction verbessert.

Auf Grund der so an Präcisionsinstrumenten gefundenen Zahlenwerte können die nicht selten beträchtlichen Correcturen bestimmt werden, welche an den Totalauslaufzeiten beliebiger für laufende Arbeit verwendeter Viscosimeter anzubringen sind.

LITTERATUR

1. Applebey, M. P. *J. Chem. Soc.* **97** (1910) 2000.
2. Drucker, C. *Arkiv Kemi, Mineral. Geol.* **A 22** (1946) Nr 20.
3. Drucker, C. *The Svedberg* (1945) S. 94.

Eingegangen am 18. Oktober 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 890—899

Form und Volumen von Flüssigkeitsmenisken

CARL DRUCKER

Physikalisch-Chemisches Institut, Uppsala, Schweden

Die Frage, welche Form ein Flüssigkeitsmeniskus in einem cylindrischen Rohr annimmt, gehört zu den alten Problemen der Kapillaritätstheorie. Ihre Lösung wird durch den Umstand erschwert, dass für die Integration der allgemeinen Differentialgleichung (s. unten) eine weitere Relation erforderlich ist, welche nicht aus der Theorie selbst folgt und deshalb auf anderem Wege gewonnen werden muss.

Steigt eine benetzende Flüssigkeit in einem vertikalen Rohr vom Radius R auf, so hängt die Form des gebildeten Meniskus von R und der Kapillaritätskonstanten * a^2 der Flüssigkeit ab. Sein meridionaler Vertikalschnitt ist in Fig. 1 dargestellt, wo CA seine horizontale Tangentebene ist und die Strecke AB — die Randlinienhöhe — in der Rohrwand liegt und mit H bezeichnet werden soll. Der Meniskus tangiert AB vertikal in B und AC im Symmetriecentrum C. Eine Vertikale von einem Punkte P schneidet CA in P'. Wird die Ordinate PP' mit y, die Abscisse CP' mit x und der Winkel zwischen CA und der Tangenten in P mit φ bezeichnet, so gilt

$$\tan \varphi = \frac{dy}{dx} \tag{1}$$

Gemäss den Grundformeln der Kapillaritätstheorie ist dann

$$\frac{2}{a^2}[h + y] = \frac{1}{x}\frac{d}{dx} \cdot \left[\frac{x}{\sqrt{1 + \left(\frac{dx}{dy}\right)^2}}\right] = \frac{1}{x}\frac{d}{dx}[x \cdot \sin \varphi] \tag{2}$$

* Definitionsgemäss ist $a^2 = 2 \cdot \gamma/s$ die korrigierte Steighöhe in einem Rohr vom Radius 1 mm, wenn γ die Oberflächenspannung und s das specifische Gewicht bedeuten.

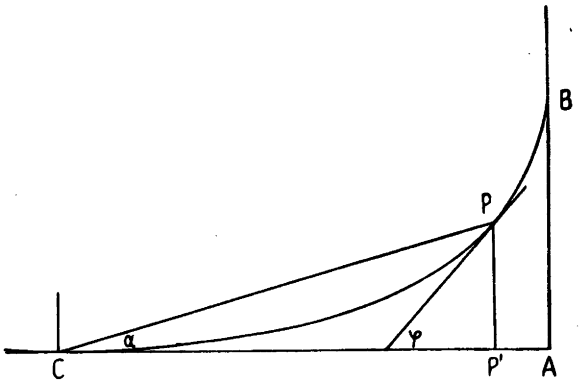


Fig. 1. Meridionalschnitt eines Meniskus. CA Berührungsebene, AB Rohrwand.

oder nach Integration

$$x \cdot \sin \varphi = h \cdot \frac{x^2}{a^2} + \frac{u}{\pi a^2} \tag{2a}$$

h ist die Höhe der Flüssigkeitssäule zwischen dem Nullniveau und CA und u dasjenige Volumen

$$u = 2\pi \int_{0,0}^{x = R,\ y = H} y \cdot x \cdot dx \tag{3}$$

welches entsteht, wenn die Fläche CP'P unter der Meniskuskurve um die Symmetrieaxe rotiert.

Man hat lange nach einer allgemeinen Beziehung zwischen x und y gesucht, welche gestatten würde die Gleichungen (2) und (3) zu integrieren. In Ermangelung einer solchen hat man sich bisher mit Näherungsformeln begnügen müssen, welche zu recht verwickelten Ausdrücken führen.

Es ist daher hier versucht worden, den entgegengesetzten Weg einzuschlagen, d. h. auf Grund der vorliegenden Versuchsresultate eine empirische Formel zu gewinnen.

TOTALVOLUMEN UND MITTLERE HÖHE DES MENISKUS

Bezeichnet man das Gesamtvolumen des Meniskus (in mm³), welches experimentell bestimmt werden kann, mit V, so ist die zugehörige »mittlere Höhe»

$$\mu = \frac{V}{\pi \cdot R^2} \tag{4}$$

und es folgt aus der Definition

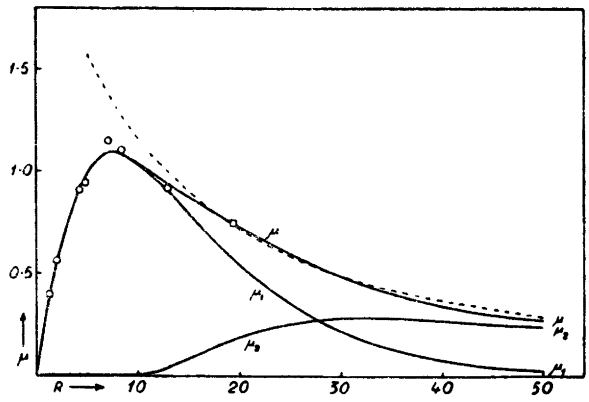


Fig. 2. Mittlere Höhe von Wassermenisken bei 10° für Radien bis zu 50 mm. μ_1, μ_2 und μ berechnet nach Formel (6a), die gestrichelte Linie nach Verschaffelt. Kreise nach Winkler, Quadrat nach Th. W. Richards.

$$\frac{a^2}{R} = h + \mu \tag{5}$$

Vor einigen Jahren [1] wurde gezeigt, dass die bisher experimentell gefundenen Werte von μ dem Ausdrucke

$$\mu = \frac{R}{3} \cdot e^{\frac{\mu - R}{2a}} \tag{6}$$

folgen, sofern R nicht grösser ist als etwa 10 mm. Übersteigt es diesen Betrag, so gilt die nachstehende Beziehung [2] (2)

$$\mu = \mu_1 + \mu_2 = \frac{R}{3} \cdot e^{\frac{\mu - R}{2a}} + \frac{a^2}{R} \cdot e^{\frac{a^2}{R} \cdot \frac{3\pi^2}{3\mu - R}} \tag{6a}$$

Als Beispiel für diese beiden Ausdrücke sei hier ein Diagramm angeführt, das sich auf Wasser bei 20° bezieht. Die direkt gefundenen Werte von μ wurden teils von Winkler bestimmt [3] (Kreise) teils von Richards [4] (Quadrat). Im Gebiete bis $R \approx 10$ genügt Formel (6) für μ_1, während dort μ_2 vernachlässigt werden darf. Bei grösseren Radien fällt μ_1 merklich zu klein aus, wogegen die Summe μ sich dem experimentellen Verlaufe, soweit er bekannt ist, gut anschliesst und schliesslich in die gestrichelte Linie * über-

* Diese wurde berechnet mittels der von Verschaffelt [6] abgeleiteten Formel für sehr grosse Radien

$$h = \frac{a}{\sqrt{2}} \cdot 0{,}924 \cdot \sqrt{\frac{2\pi \cdot R \cdot \sqrt{2}}{a}} \cdot e^{-\frac{R\sqrt{2}}{a}}$$

geht, welche gegen den theoretischen Grenzwert $\mu = \frac{a^2}{R} = 0$ verläuft. Der Fehler von μ dürfte etwa 0,02 mm nicht übersteigen. Da die Formel (6a) hiernach als ausreichend bestätigt gelten darf, soll sie den folgenden Berechnungen zu Grunde gelegt werden.

RANDLINIENHÖHE DES MENISKUS

Verschaffelt und van der Noot [5] haben die Endordinate H (s. Fig. 1) bei etwa 10° für Radien zwischen 1 und 15 mm an vier Flüssigkeiten: Wasser, Benzol, Äther und Schwefelkohlenstoff mikrometrisch bestimmt. Da diese wichtigen Ergebnisse nicht allgemein bekannt zu sein scheinen, werden sie unten ausführlich wiedergegeben.

Sie lassen sich mit guter Näherung darstellen durch

$$H = a\left(1 - e^{-\frac{R}{a}\cdot Q}\right) \tag{7}$$

wo

$$Q = e^{\frac{H}{a}\cdot e^{-\frac{R}{2H}}} \tag{7a}$$

In Tabelle 1 ist der exponentielle Faktor Q mit angeführt worden, um eine Vorstellung seines Verlaufes und seines Einflusses auf H zu geben. Er steigt in allen Fällen von 1 bis zu einem Maximum und fällt danach wieder auf 1. Formel (7) ergibt $H = 0$ für $R = 0$ und $H = a$ für $R = \infty$.

Tabelle 1. Randlinienhöhen der Menisken. H_f gefunden, H_b berechnet nach Formel (7). a: Wasser, b: Benzol, c: Äther, d: Schwefelkohlenstoff.

R	a			b			c			d		
	H_f	H_b	Q	H_f	H_b	Q	H_f	H_b	Q	H_f	H_b	Q
0,94	0,95	0,95	1,16	0,91	0,92	1.22	0,94	0,93	1,28	0,92	0,91	1,24
1,95	1,86	1,89	1,32	1,62	1,69	1,49	1,54	1,60	1,43	1,56	1,60	1,45
2,89	2,46	2,52	1,42	2.06	2,10	1,47	1.96	1,93	1,51	1,96	1,92	1,52
3,99	3.02	3,04	1,48	2,34	2,34	1,46	2,04	2,07	1,40	2,13	2,05	1,43
5,94	3,64(?)	3,50	1,48	2,41	2.49	1,31	2,15	2,18	1,27	2,20	2,16	1,27
9,56	3,74	3,73	1,31	2,50	2,59	1,15	2,23	2,24	1,11	2,22	2,22	1,12
15,6	3,83	3,84	1,14	2,62	2,62	1,05	2,26	2,26	—	2,22	2,24	—
∞	3,89			2,63			2,26			2,24		

Tabelle 2. Korrespondierende Werte von x und y nach Formel (8). a: Wasser, b: Benzol.

a

R	2,89		5,00		10,0		12,0		15,6	
H	2,46		3,40		3,76		3,80		3,83	
h	4,49		2,03		0,44		0,26		0,07	
y	x	G	x	G	x	G	x	G	x	G
0,05	—	—	—	—	3,19	1,017	3,85	1,029	5,29	1,109
0,15	1,38	1,011	2,20	1,040	4,35	1,037	5,22	1,063	7,45	1,215
0,30	1,71	1,008	2,71	1,035	5,32	1,051	6,48	1,083	9,28	1,264
0,50	2.02	1,007	3,18	1,025	6,27	1,055	7,60	1,088	10,74	1,261
0.70	2,29	1,004	3,53	1,019	6,95	1,051	8,45	1,080	11,72	1,230
1,00	2.49	1,002	3,95	1,010	7,73	1,040	9,35	1,064	12,70	1,179
1,50	2,74	1,000	4,42	1,004	8,60	1,002	10,42	1,034	13,80	1,100
2,00	2,85	1,000	4,71	1,001	9,25	1,001	11,09	1,015	14,52	1,047
2,50	—	—	4,90	1,000	9,65	1,000	11,45	1,004	15,02	1,016
3,00	—	—	—	—	9.89	1,000	11,85	1,001	15,46	1,001

b

R	2,89		7,00		10,0	
H	2,08		2,47		2,50	
h	1,77		0,29		0,07	
y	x	G	x	G	x	G
0,05	—	—	—	—	3,74	1,103
0,15	1,47	1,027	3,64	1,159	5,27	1,182
0,30	1,82	1,019	4,42	1,128	6,53	1,198
0,50	2,14	1,017	5,08	1,084	7,50	1,169
0,70	2,37	1,006	5,55	1,059	8,15	1,127
1,00	2,60	1,002	6,08	1,025	8,80	1,075
1,50	2,82	1,000	6,65	1,002	9,58	1,064
2,00	2,88	1,000	6,95	1,000	9,89	1,000

EIN AUSDRUCK FÜR DEN VERTIKALSCHNITT DES MENISKUS

Totalvolumen und mittlere Höhe des Meniskus, sowie seine Randlinienhöhe, welche hiernach als bekannte Grössen betrachtet werden dürfen, treten in der nachfolgenden neuen Formel für den Zusammenhang zwischen den Koordinaten x und y des Meniskuskurve auf. An Stelle von φ (s. oben) wird der Winkel $\alpha = \widehat{PCP'}$ (s. Fig. 1) eingeführt, dessen Tangens $\tan \alpha = y/x$ ist, und es wird weiter gesetzt

$$\tan \alpha = \frac{y}{x} = \frac{H}{R} \cdot e^{\left(\frac{y}{H} - \frac{R}{x}\right) \cdot G} \tag{8}$$

wo

$$G = e^{\left(\frac{R-x}{x}\right)^2 \cdot \frac{y}{h}} \tag{8a}$$

(Die Grössen R, H und h wurden bereits definiert.)

Der exponentielle Faktor G spielt eine entscheidende Rolle lediglich dann, wenn R gross, somit h relativ klein ist (vgl. Formel (5)).

Tabelle 2 enthält zusammengehörige Werte von x und y, welche der Formel (8) genügen und mittels der oben berechneten Werte für a, H und μ (resp. h) gefunden wurden. Wie oben Q ist hier der Faktor G mit aufgenommen worden, um seinen Verlauf zu zeigen *.

ANWENDUNG DER FORMEL (8) AUF TOTALVOLUMEN UND MITTLERE HÖHE

Das Totalvolumen ergibt sich durch Integration der Formel (3) als

$$V = 2\pi \cdot \int_{0,0}^{H,\,R} y \cdot x \cdot dx$$

Zeichnet man die Kurve $y = f(x)$ gemäss Tabelle 2 und bestimmt mittels Planimeter die zugehörige Fläche F — entsprechend CAB in Fig. 1 — sowie in derselben Weise die Abscisse X der diese Fläche halbierenden Ordinate, so wird

$$V = 2\pi \cdot F \cdot X \qquad \text{und} \qquad \mu = \frac{2 \cdot F \cdot X}{R^2}$$

Die derart gefundene mittlere Höhe ist in Tabelle 3 unter μ_a verzeichnet, während μ_b aus Formel (6a) folgt. Alle Grössen sind in mm resp. mm² ausgedrückt und gelten natürlich für 10°. μ_a und μ_b stimmen gut überein. Der Faktor G kommt bei kleinen Radien nicht zur Geltung und kann für diese

* Für die Berechnung eignet sich die logarithmische Form

$$\ln g + \ln \frac{R}{H} = \ln x + \left(\frac{y}{H} - \frac{R}{x}\right) \cdot G \tag{8b}$$

in welcher, bei willkürlich gewähltem y, x so lange verändert wird, bis beide Seiten übereinstimmen.

Tabelle 3. Mittlere Höhe der Menisken bei 10°, berechnet nach Formel (8) (μ_b) resp. Formel (6^a) (μ_a); a: Wasser, b: Benzol.

	a						b			
R	2,89	5,00	10,0	12,0	15,6		2,89	4,00	7,00	10,0
F	1,25	2,88	6,05	6,72	8,07		1,02	1,60	2,70	3,38
X	2,43	4,30	8,60	10,50	13,80		2,50	3,43	6,05	8,70
μ_a	0,73	0,99	1,04	0,98	0,92	(0,81)	0,61	0,69	0,67	0,59
μ_b	0,73	0,99	1,06	0,96	0,90		0,63	0,71	0,67	0,61

gleich 1 gesetzt werden; bei grossen Radien dagegen gewinnt er an Bedeutung *.

EINZELNE PUNKTE DER MENISKUSKURVE

Die vorstehende Berechnung bezieht sich auf den Fall, dass in Formel (2^a) $x = R$, $y = H$ und $\sin\varphi = 1$. Ist aber für einen beliebigen anderen Punkt $\sin\varphi$ bekannt, so kann dieselbe Berechnung auch auf diesen angewendet werden. Man erhält nun diesen Winkel einfach durch Differentiation der Gleichung (8) wie folgt **

$$\frac{dy}{dx} = \tan\varphi = \frac{y}{x} \cdot \frac{1 + G\left[\frac{R}{x} - \frac{y^2 \cdot x \cdot 2\,(R-x)}{H \cdot 3 \cdot h \cdot R^2} + \frac{R \cdot y \cdot 2 \cdot (R-x)}{3 \cdot h \cdot R^2}\right]}{1 - G\left[\frac{y}{H} + \frac{y^2 \cdot (R-x)^2}{H \cdot 3 \cdot h \cdot R^2} - \frac{R \cdot y \cdot (R-x)^2}{x \cdot 3 \cdot R^2}\right]} \tag{9}$$

Wird der hieraus sich ergebende Wert von $\sin\varphi$ in Formel (2^a) eingesetzt, erhält man für den gewählten Punkt (x, y) das seinen Koordinaten entsprechende Teilvolumen des Meniskus

$$\left(\frac{u}{\pi}\right)_1 = a^2 \cdot x \sin\varphi - h \cdot x^2 \tag{10}$$

---

* Wollte man z. B. bei $R = 15{,}6$ für Wasser G gleich 1 annehmen, so würde sich μ_a zu 1,06 statt 0,92 ergeben. Andrerseits erhält man bei sehr grossen Radien recht unsichere Werte für h, weil dann a^2/R fast gleich μ wird. Es wurde als Beispiel für den Einfluss dieser Unsicherheit in einem Falle (Tabelle 3, Wasser, $R = 15{,}6$) der wie angegeben berechnete Wert von $h = 0{,}07$ durch $h = 0{,}036$ ersetzt, wie er sich nach Verschaffelt [6] ergibt. Damit wurde $\mu_a = 0{,}81$ erhalten (vgl. Tabelle 3, eingeklammert). Wegen dieses Umstandes sind die Rechnungen für Benzol nur bis $R = 10$ ausgeführt worden.

** Wird in Formel (8) durchweg $G = 1$ gesetzt, reduciert sich (9) auf

$$\frac{dy}{dx} = \tan\varphi = \frac{x}{y} \cdot \frac{1 + R}{1 - H} \tag{9a}$$

Tabelle 4. Teilvolumina von Menisken für ausgewählte Einzelpunkte, nach Formel (2a) und (10) ($(\frac{u}{\pi})_1$), resp. Formel (11) ($(\frac{u}{\pi})_2$). $R = 10$. a: Wasser, b: Benzol.

y	x	$\sin\varphi$	$(\frac{u}{\pi})_1$	F_u	X_u	$(\frac{u}{\pi})_2$
			a			
1,00	7,73	0,455	27	1,95	6,40	25
2,00	9,25	0,712	62	4,15	7,85	65
			b			
1,00	8,80	0,470	24	1,72	7,55	26
2,00	9,89	0,881	50	3,15	8,65	54

Ermittelt man andrerseits die zugehörige Fläche F_u sowie die Abscisse X_u, deren Ordinate F_u in zwei gleiche Teile scheidet, mit Hilfe des Planimeters in gleicher Weise wie oben die Grössen F und X, so muss

$$\left(\frac{u}{\pi}\right)_2 = 2 \cdot F_u \cdot X_u \tag{11}$$

eben so gross gefunden werden wie $\left(\frac{u}{\pi}\right)_1$.

Das Ergebnis einer solchen Prüfung der Formeln wird hier in Tabelle 4 für Wasser und Benzol angeführt, wobei in beiden Fällen $R = 10$.

Die beiden Werte stimmen danach innerhalb der Fehlergrenze überein.

ANWENDUNG AUF DIREKT AUSGEMESSENE MENISKUSKURVEN

Wenn für beliebig viele Punkte (x, y) einer Meniskuskurve sowohl x wie y direkt bestimmt worden sind, können die Formeln über den gesamten Verlauf der Kurve geprüft werden.

Brauchbare Messungen dieser Art liegen wohl nur für Quecksilbermenisken vor, die leider, wie bekannt, sehr empfindlich gegen die Zusammensetzung der Atmosphäre sowie das Wandmaterial sind.

Palacios [7], der etwa drei Dutzend solche Kurven mittels Mikrometer bei Radien zwischen 5 und 12 mm aufgenommen hat, fand in einem Falle ($R =$ 5,10 mm) die unten in Tabelle 5 wiedergegebenen Werte für x und y. Nach seiner Berechnung ergibt sich daraus als Oberflächenspannung des Quecksilbers $\gamma = 264 \frac{\text{dyn}}{\text{cm}}$ oder $26{,}9 \frac{\text{mg}}{\text{mm}}$, d. h. $a^2 = 3{,}99$ mm^2. Demnach sollte die

Tabelle 5. Koordinaten eines Quecksilbermeniskus. x Abscisse, Ordinaten y (experimentell gefunden), y_1 berechnet nach Formel (8), y_2 berechnet von Palacios.

x	y_1	y	y_2	x	y_1	y	y_2
4,90	1,30	1,301	1,296	4,30	0,76	0,764	0,757
4,80	1,18	1,172	1,175	4,20	0, 70	0,703	0,697
4,70	1,08	1,076	1,085	4,10	0,64	0,648	0,655
4,60	0,98	0,980	0,981	4,00	0,58	0,598	0,597
4,50	0,90	0,901	0,903	3,80	0,48	0,506	0,506
4,40	0,83	0,829	0,828	3,60	0,40	0,430	0,414

Randlinienhöhe kleiner sein als $a = 2{,}00$, während Palacios $H = 2{,}43$ findet. Trotz dieses Widerspruches * stimmen seine berechneten Werte gut mit den direkt gemessenen. Man darf danach wohl annehmen, dass auch bei anders gewählten Konstanten die Uebereinstimmung bestehen bleibt.

Da der Exponent G (Formel 8) nur unwesentlich von 1 abweicht, falls x nahe gleich R ist, so setzen wir in diesem Falle näherungsweise bei den grössten x-Werten $G = 1$. Dann ergibt sich aus Gl. (8) mit guter Näherung $H = y_{x=R}$. Es folgt weiter a aus Gl. (7), ferner ein Wert für μ aus Gl. (6a) und endlich ist $h = \frac{a^2}{R} - \mu$. Auf diese Weise wurde gefunden $H = 2{,}10$, $a = 2{,}20$, $\mu = 0{,}61$ und $h = 0{,}33$. Setzt man diese Zahlen in Gl. (8) ein, so ergeben sich die in Tabelle 5 stehenden Werte y_1, während Palacios y fand und y_2 berechnete.

Der Unterschied zwischen y_1 und y erscheint unerheblich, wenn man beachtet, dass kleine Fehler von H und h stark zur Geltung kommen und diese Grössen nicht mit der wünschenswerten Genauigkeit berechnet werden können.

Demnach zeigen alle oben durchgeführten Prüfungen, dass die Formeln (6), (7) und (8) die experimentell gefundenen Verhältnisse befriedigend wiedergeben. Die in diesen Formeln auftretenden Exponenten bedürfen vermutlich in Zukunft gewisser sekundärer Verbesserungen, doch sind Versuche in dieser Richtung wohl so lange zwecklos, bis Volumen und Randlinienhöhe mit wesentlich grösserer Genauigkeit als bisher bestimmt werden können.

Wichtiger als dies erscheint es in principieller Hinsicht, dass das Volumen des Meniskus, seine Randlinienhöhe und der Zusammenhang zwischen Ordinate und Abscisse durch exponentielle, resp. mehrfach-exponentielle Formeln dargestellt werden können. Die Aufmerksamkeit auf diesen Umstand zu richten, ist der Zweck der vorstehenden Mitteilung.

* In einem anderen Falle ($R = 10{,}11$) findet Palacios $\gamma = 339 \frac{\text{dyn}}{\text{cm}}$ oder $a^2 = 5{,}12$ und $H = 2{,}50$, also auch hier $H > a$.

ZUSAMMENFASSUNG

Es werden drei Formeln mitgeteilt, deren erste das Volumen eines Flüssigkeitsmeniskus, die zweite die Höhe seiner Randlinie und die dritte den Zusammenhang zwischen Abscisse und Ordinate seines Meridionalschnittes zu berechnen gestattet.

Die Berechnung setzt lediglich die Kenntnis von Rohrradius und Kapillaritätskonstante der Flüssigkeit voraus.

Die Formeln wurden an Versuchsresultaten der Litteratur bestätigt.

Der Verfasser benutzt gern die Gelegenheit, Herrn Professor Svedberg für die Ermöglichung der Durchführung dieser und der vorstehenden Untersuchung und sein dauerndes Interesse zu danken.

LITTERATUR

1. Drucker, C. *The Svedberg-Festschrift* (1944) S. 94.
2. Drucker, C. Mitteilung auf dem 6. Skandinaviska Kemistmöte i Lund (Aug. 1947).
3. Winkler, L. *Z. angew. Chem.* **16** (1903) 718.
4. Richards, Th. W., und Carver, E. K. *J. Am. Chem. Soc.* **43** (1921) 827.
5. Verschaffelt, J. E., und van der Noot, L. *Bull. Ac. Belg. (Sciences)* (1911) 831.
6. Verschaffelt, J. E. *Comm. Phys. Lab. Univ. Leiden* Suppl. **42** (1917).
7. Palacios, J. *Trab. Lab. Invest. Fis.* Nr. **62** (1919), **63** (1920). S. auch Scheel, F., und Heuse, W. *Ann. Physik.* **33** (1910) 291.

Eingegangen am 26. Oktober 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 900—913

Chemical Studies on the Jelly Coat of the Sea-Urchin Egg

ERIK VASSEUR

Wenner-Grens Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden

In normal sea-water the jelly coat of the sea-urchin egg slowly increases in volume and goes into solution. This process was demonstrated by Herbst[1] to be greatly accelerated when the pH of the sea-water is lowered sufficiently. Lillie [2] made the interesting observation that sea-urchin sperm are activated and agglutinated by homologous egg-water, *i. e.*, sea-water which has been in contact with eggs of the same species. While he was of the opinion that the agglutinating agent, fertilisin, was continuously secreted by the egg, subsequent investigations did not support this view. Instead, fertilisin has been proved to arise from the jelly coat of the egg. The reader is referred to the excellent review by Tyler [3]. This evidence and the importance which has been attributed to fertilisin in the process of fertilisation [2—4] has aroused great interest in the chemical composition of the jelly coat substance [5—7] and of fertilisin [8].

In order to obtain more precise analytical data on the nature of the carbohydrate and protein components, as well as elementary analyses, of the jelly coat substance from different sea-urchin species, the following study was undertaken.

EXPERIMENTAL

Material and preparation

For the present investigation, the jelly coat of the following sea-urchin species was used: from the Swedish west coast, Kristineberg Zoological Station, *Strongylocentrotus droebachiensis* (O. F. Müller), *Echinus esculentus* (Linné), *Psammechinus miliaris* (Gmelin), *Echinocardium cordatum* (Pennant), and *Brissopsis lyrifera* (Forbes); from Station Biologique, Roscoff, *Paracentrotus lividus* (Lamarck), and from Naples, frozen-dried egg-water of *Arbacia lixula* (Linné)*.

* Generously supplied by Fil. mag. Tore Hultin.

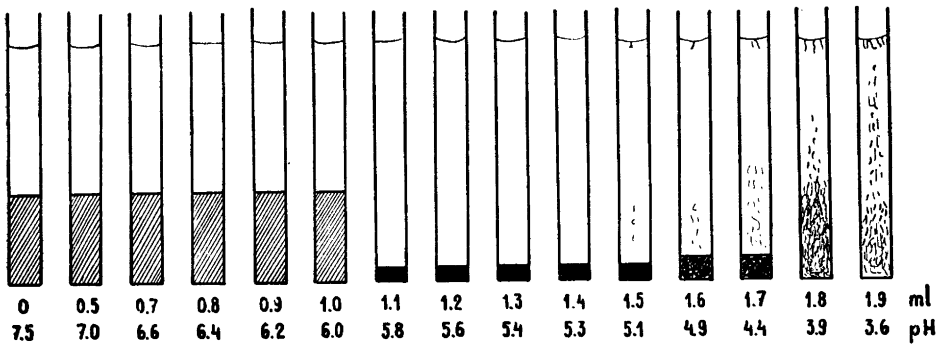


Figure 1. A diagram of the test for removal of the jelly coat of Echinus esculentus eggs at different pH. Each tube contains 2 ml egg suspension and different amounts of 0.01 N HCl (upper row); total volyme 6 ml. For further explanation see text.

The sea-urchin eggs were washed several times by sedimentation in sea-water and their jelly coat was removed by treatment with acid sea-water; the appropriate pH for this was investigated for each sea-urchin species. Into small flat-bottomed test tubes (10 × 90 mm), 0—2.0 ml of 0.01 *N* HCl in sea-water was pipetted. The volume was adjusted to 4 ml with sea-water and the solution was mixed, whereafter 2 ml of egg suspension was added to each tube in rapid succession. In order to obtain homogenous suspensions, the tubes were inverted several times and then allowed to stand undisturbed. An inspection of the tubes clearly showed in which tubes the eggs were deprived of their jelly coats, since these eggs sedimented more rapidly and, when packed on the bottom, occupied less space than those with jelly coats, as illustrated in Fig. 1. The pH was determined in the clear supernatant, after centrifuging when necessary, with a Beckman pH-meter. For *Echinus esculentus*, the jelly coat was completely dissolved without injury to the eggs between pH 5.0—5.8. In the pH interval 4.0—5.0, the jelly dissolved, but simultaneously some of the eggs were destroyed. Below pH 4.0 even more eggs cytolysed. Above pH 6.0, there was no difference from the eggs in normal sea-water. A pH near the upper limit was used for the preparative work in order to avoid degradation of the jelly coat substance (*cf.* below). This limit is for *Strongylocentrotus droebachiensis* at pH 5.6, *Echinus esculentus* 5.8, *Echinocardium cordatum* 5.1, and *Psammechinus miliaris* 5.3.

The egg suspension was centrifuged after some minutes acid treatment and the supernatant jelly coat solution decanted off and cleared by repeated strong centrifugings. When necessary, the solution was also filtered. In some experiments, this clear colourless jelly solution was directly dialysed without prior precipitation. Concentration by freezing and thawing was also employed. Usually the gelatinous substance was best precipitated with either ammonium sulphate or alcohol and ether. For the complete precipitation of *Echinus esculentus* jelly substance with ammonium sulphate, an almost saturated solution was required and resulted in a very viscid precipitate, whereas only about 70 per cent saturation was necessary for *Strongylocentrotus droebachiensis* giving, in this case, a firm fibrous precipitate which could easily be collected with a spoon. On the

other hand, precipitation with 1 volume of alcohol and 0.2 volumes of ether was most convenient for *Echinus esculentus*, since a coherent fibrous mat formed on the surface. The *Strongylocentrotus droebachiensis* jelly substance, precipitated under these conditions, did not accumulate at the surface but was distributed through the whole solution and settled only slowly. To precipitate the *Paracentrotus lividus* jelly substance, two volumes of 96 per cent alcohol were necessary to obtain a clear solution; with one volume, the supernatant remained opalescent due to incomplete precipitation of calcium sulphate from the sea-water. One volume of alcohol added to *Psammechinus miliaris* jelly solution however gave a clear supernatant. Obviously, these differences in precipitation indicate differences in the composition of the various jelly substances.

The precipitates were collected with a spoon or glass rod and most of the solution was pressed out of the fibrous mass. In this state, the gelatinous substance may be preserved in the cold for long periods.

For further purification, the fibrous precipitate was dialysed in a cellophane tube against running tap-water and finally against distilled water until completely free of chloride and sulphate ions. During this process, the substance dissolved and a very viscous solution was obtained. The ammonium sulphate precipitated substance dissolved more readily than the alcohol and ether precipitates and gave a more viscous solution. The jelly coat substance was obtained in a very suitable dry form by freeze-drying this concentrated solution. All samples of jelly substance used in this investigation were frozen-dried unless otherwise stated. They contained 4—15 per cent moisture.

In the tables the following abbrevations are used for the different methods of preparation: Am_2SO_4 = precipitation with ammonium sulphate; Alc. = precipitation with 96 per cent alcohol; Alc. + Et_2O = precipitation with alcohol and ether; Thawing = the jelly solution is frozen in cubes, the cubes crushed in a mortar and poured in a funnel; only the more viscous portions were kept for further treatment. All these preparations were completed by dialysis and freeze-drying. Not pptd. = direct dialysis, followed by freeze-drying. Alc. + Et_2O, eldial. + = precipitation with alcohol and ether, dialysis, electro-dialysis, plus-fraction, freeze-drying.

Chemical analyses

1. Carbohydrate

A. Orcinol and sulphuric acid reaction

The chemical nature of the carbohydrate in the jelly coat[6] was investigated with the orcinol and sulphuric acid method of Vasseur[9]. The colour curves obtained with the jelly coat solutions of *Strongylocentrotus droebachiensis*, *Echinocardium cordatum*, and *Paracentrotus lividus* did not correspond to the colour curves for hexoses or pentoses, but very good agreement was found with the colour curves for the methylpentose, rhamnose. On the other hand, the jelly coat solution of *Echinus esculentus* gave colour curves corresponding well with those of the aldohexoses. It was difficult to decide, however, with this method which particular aldohexose it was. Immers, in collaboration

with the author, recently has studied the carbohydrate component of *Echinus esculentus* jelly with the aid of paper chromatography and has shown that it is most probably identical with galactose and not with mannose or glucose.

With *Strongylocentrotus droebachiensis* jelly solution, more detailed studies were carried out with the orcinol colour reaction. An extra heating time of 5 minutes was found necessary to give colour curves comparable with those of rhamnose. Thus, 10 and 25 minutes heating gave colour curves of the same form as the 5 and 20 minute curves of rhamnose, respectively. This retardation of colour development depends upon the extra time required to release from the polysaccharide molecule, the furfural derivatives which react with the orcinol. Quantitative estimations by this colorimetric method showed a carbohydrate content of approximately 39 per cent, calculated as polyrhamnose.

On different preparations of *Echinus esculentus* jelly solutions, colour curves for 20 minutes heating were made. These were identical regardless of whether the jelly solution was only dialysed, was precipitated by ammonium sulphate or alcohol and ether and then dialysed, or was electrodialysed after precipitation, and the plus- or minus-fractions tested.

B. Chromic acid oxidation

In order to obtain more reliable values of carbohydrate content, two determinations * with the quantitative chromic acid oxidation of Lemieux and Purves [10] were made on the *Strongylocentrotus droebachiensis* jelly substance.

Analysis: Two lots of L(+)rhamnose (Hoffmann-LaRoche, 20 mg each) gave 34.60 and 34.85 per cent acetic acid; calculated 36.59 per cent. This corresponds to 95 per cent yield.

Two lots of *Strongylocentrotus droebachiensis* jelly substance (50 mg each) gave 16.63 and 18.76 per cent acetic acid; assuming 95 per cent yield, 17.90 and 19.75 per cent, respectively. This corresponds to 42.6 and 48.1 (mean 45.3) per cent poly-methylpentose in the jelly coat substance.

A further identification of the carbohydrate component is under investigation.

C. Hexosamine and hexuronic acid

Hexosamine was estimated in *Echinocardium cordatum* jelly substance ** using the modification of Nilsson [11] of the method of Elson and Morgan.

* These analyses were generously performed by Mrs. Eva Borenstein at Professor Erik Jorpes' laboratory.

** This analysis was generously performed by Mr. Evert Lindberg at Professor Gunnar Blix' Laboratory.

Hexuronic acid was determined in *Echinocardium cordatum* * and in *Strongylocentrotus droebachiensis* ** jelly substances according to Burkhart, Baur, and Link [12]. No hexuronic acid was detectable, and the hexosamine content was very small, perhaps insignificant.

D. »Glycogen»

One analysis of «glycogen» according to Meyerhof and Lohmann [13] was carried out on *Echinocardium cordatum* jelly coat solution and 4 analyses according to Lindberg [14] on *Echinus esculentus* jelly solution.

Analysis: 1.5 g KOH was dissolved in 5 ml of 0.045 per cent *Echinocardium cordatum* jelly solution. The sample was hydrolysed 2 hours at 100°, chilled, precipitated with 2 volumes of absolute alcohol, and allowed to stand overnight. The precipitate was centrifuged, washed twice with absolute alcohol, once with ether, and dried in a desiccator over blue silica gel. The dry residue was suspended in 10 ml of 2.5 *N* HCl, and hydrolysed 3 hours at 100°, chilled, and neutralised to phenolphthalein with NaOH. The volume was adjusted to 20 ml and the reducing value in two aliquots, 3.0 and 5.5 ml, was determined according to the method of Hagedorn and Jensen [15]. Found: 76 γ, and 115 γ, respectively, as glucose, corresponding to 22.5 and 18.6 (mean 20.0) per cent of the jelly substance.

Echinus esculentus jelly: two samples were hydrolysed in 30 per cent KOH and two samples in 40 per cent KOH and treated according to Lindberg [14] for total glycogen content. Result: 14.6, 14.3, 13.0, and 14.0 per cent of the jelly substance as »glucose». Treatment of the alcohol precipitate with half-saturated sodium sulphate did not change these figures.

2. *Elementary analysis*

A. Carbon, hydrogen, and ash

The automatic semi-micro elementary analysis apparatus of Reihlen and Weinbrenner [16] was used, and the results are summarised in Tables 1 and 2.

Table 1. Carbon, hydrogen, and ash content of Echinocardium cordatum jelly coat substance.

Sample		C	H	Ash	
Preparation	mg	per cent	per cent	mg	per cent
Am_2SO_4	39.80	23.95	5.70	8.60	21.60
	22.60	25.1	5.4	4.7	20.8
Not pptd.	25.65	12.5	4.40	11.85	46.2
	36.40	11.6	4.40	17.00	46.7

* This analysis was generously performed by Mr. Evert Lindberg at Professor Gunnar Blix' laboratory.

** These analyses were generously performed by Mrs. Eva Borenstein at Professor Erik Jorpes' laboratory.

Table 2. Elementary composition of Strongylocentrotus droebachiensis jelly coat substance:

C, H, ash, S, Ca, and N.

Analysis	Sample Preparation	mg	C per cent	H per cent	Ash per cent	S in ash per cent of whole sample	Total S per cent	Ca per cent	N per cent
Ea 46 : 1	Alc. + Et_2O	31.46	22.7	4.32	39.8	8.5			
Ea 46 : 7		26.98	20.6	4.27	44.0				
GK 47 : 3		25.04			41.5	9.2	11.2		
Kj 48 : 14		26.6							4.02
Ea 46 : 2a	Alc. + Et_2O	32.38	26.48	5.07	34.4			9.1	
Ea 46 : 2b		36.46	25.62	5.23	35.8			7.5	

Ea = elementary analysis [16], GK = total sulphur determination [19], Kj = total nitrogen determination [21].

Sulphate was determined on the ash of elementary analysis Ea 46 : 1 according to the benzidine sulphate method of McKittrick and Schmidt [17]. The figure obtained is certainly too low to represent the total sulphur content of the sample. Determinations with the Grote and Krekeler apparatus (*cf.* below) show that some of the sulphur is always carried away from the ship by the air-stream.

On the ash of analyses Ea 46 : 2a and b (*cf.* Table 2) calcium determinations were made according to the method of de Loureiro and Janz [18]. The data show that in this preparation which was precipitated with alcohol, the major share (70—90 per cent) of the cations is calcium. It is however probable that alcohol precipitation promotes an enrichment of the calcium in the preparation since calcium sulphate is the first salt to be precipitated from sea-water upon addition of alcohol. Upon dialysis of the jelly substance together with the calcium sulphate, the calcium ions may replace other cations in the substance to some extent.

B. Sulphur. a. Total sulphur

For total sulphur determinations, the jelly coat substance was combusted in the Grote and Krekeler apparatus [19], and the sulphate determined as benzidine sulphate by titration with 0.01 *N* NaOH [17], both in the ash and in the absorption liquid. The results are given in Table 3.

Table 3. Sulphur analyses on the jelly coat substance of different sea-urchin species; cf. also Tables 2 and 7.

Sample			S in ash	S in abs. liquid	Total S	Ash
Species	Preparation	mg	per cent of whole sample		per cent	per cent
Echinus esculentus	Alc. + Et_2O	32.68	3.42	5.36	8.77	15.9
Echinocardium cordatum	Not pptd.	28.03	3.03	4.73	7.76	24.0
Paracentrotus lividus	Alc.	34.03	4.70	3.54	8.24	22.0
Arbacia lixula	Not pptd.	24.33	4.1	2.4	6.5	29.2
Brissopsis lyrifera	Am_2SO_4	34.25	1.14	1.54	2.68	8.47

b. Acid-hydrolysable sulphate

Acid-hydrolysable sulphate was determined by hydrolysis with 0.05 *N* HCl in an autoclave at 2.5 atmospheres, followed by precipitation and titration as benzidine sulphate (*cf.* Vasseur [7]). Autoclaving for 30 minutes at 2.5 atm. was sufficient for an almost complete hydrolysis of the sulphate in *Echinus esculentus* jelly substance, as shown in Table 4.

For this investigation, an alcohol and ether precipitated sample was dialysed until free of chloride and sulphate ions. An accurately weighed sample of about 5 g of the resultant viscous solution was measured into a 100 ml volumetric flask, mixed with an equal volume of 0.1 *N* HCl, and then placed in an autoclave. Fifteen to twenty minutes was allowed for the steam to displace the air, the vent was then closed, and a pressure of 2.5 atmospheres was reached within 5—10 minutes. For 0 hours autoclaving, the gas was switched off at this moment, and within 10 minutes the autoclave cooled to a pressure of 1 atmosphere after which the sample was removed. The dry weight of this jelly

Table 4. Hydrolysis of bound sulphate with 0.05 N HCl in an autoclave at 2.5 atm. pressure; Echinus esculentus jelly coat solution, precipitated with alcohol and ether, and dialysed.

Time of heating at 2.5 atm. hours	Per cent free sulphate				
	Analysis no.				Mean
	1	2	3	4	
0	24.4	22.2	25.7	25.9	24.5
0.5	30.4	32.5	30.9	31.4	31.3
3	29.3	33.0	33.6	33.1	32.2
4	31.5	32.5	32.2	33.1	32.4

solution was determined by heating in an oven to 105° until constant weight was attained. The obtained sulphate values are therefore higher than those for the frozen-dried samples which contained 4—15 per cent moisture. Two determinations of total sulphur gave 11.1 and 11.0 per cent (33.2 and 33.1 per cent SO_4). Total nitrogen was 2.17 per cent.

C. Total phosphorus

About 0.5 per cent total phosphorus in *Echinocardium cordatum* jelly and about 0.4 per cent in *Strongylocentrotus droebachiensis* jelly were obtained by the method of Briggs [20].

D. Total nitrogen

Total nitrogen was determined according to the procedure of Parnas [21] using the combustion fluid of Arnold and Wedemeyer (*cf.* Parnas). The ammonia was driven off by 10 ml of 30 per cent NaOH containing 30 g of $Na_2S_2O_3$ per liter, absorbed in 4 ml of 2 per cent H_3BO_3, and titrated with 0.01 *N* HCl using the Tashiro indicator (100 ml 0.03 per cent methyl red solution and 15 ml 0.1 per cent methylene blue solution) adjusted to the proper pH. The results are summarised in Table 5 *.

3. *Amino acids*

Microbiological assay * was performed for threonine, valine, *iso*-leucine, histidine, arginine, and lysine, using *Streptococcus faecalis* growing on the basal

Table 5. Total nitrogen in different jelly coat preparations; cf. also Tables 2 and 8.

Sample		Total N per cent	Sample		Total N per cent
Preparation	mg		Preparation	mg	
Strongylocentrotus droebachiensis			*Paracentrotus lividus*		
Thawing	27.4	5.30	Alc.	32.4	4.79
	26.9	5.24			
Echinocardium cordatum			*Echinus esculentus*		
Not pptd.	27.0	2.77	Am_2SO_4**	35.9	4.54
	36.7	2.92		18.8	4.42
Am_2SO_4	48.5	3.47		20.8	4.42
	28.2	3.52	Am_2SO_4	27.6	3.01
	28.8	3.82		25.9	3.02
			Alc. + Et_2O	31.4	2.77
			Alc. + Et_2O, eldial. +	36.1	2.09
				21.5	2.03

* I am indebted to Mag. Janis Immers for these analyses.

** Two ammonia-nitrogen determinations on this sample gave 1.61 and 1.50 per cent; this leaves 2.9 per cent as not-ammonia nitrogen.

medium of Stokes and Gunness [22], and for phenylalanine, tyrosine, proline, and lysine, using *Leuconostoc mesenteroides* growing on the basal medium of Henderson and Snell [23]. The results are summarised in Table 6, but may only be considered as approximate, owing to the small number of analyses. With paper chromatography aspartic acid and glutamic acid have been shown to be present. A rough estimation based on the colour intensities indicates an approximate amount of 1 or 2 per cent.

Table 6. Microbiological assay of some amino acids in the jelly coat substance of different sea-urchin species.

Species:	*Paracentrotus lividus*	*Echinus esculentus*		*Strongylocentrotus droebachiensis*		*Echinocardium cordatum*	
Preparation:	Alc.	Am_2SO_4	Alc. + Et_2O	Alc. + Et_2O	Alc. + Et_2O	Am_2SO_4	
Amino acid	per cent	per cent	per cent	per cent	per cent	per cent	Microorganism
Lysine	1.6	1.7	1.1	1.5	1.4	0.3	*Streptococcus faecalis*
	1.4	1.4	0.5	0.9	1.2	0.3	*Leuconostoc mesenteroides*
Arginine	2.0	1.8	1.9	1.9	1.9		*Streptococcus faecalis*
Histidine		0.2					*Streptococcus faecalis*
Valine	6.5	8.7	6.7	8.3	7.7	3.2	*Streptococcus faecalis*
Iso-leucine		1.8		1.0			*Streptococcus faecalis*
Threonine	2.0	1.6	1.2	2.1	1.8	2.0	*Streptococcus faecalis*
Phenyl-alanine	1.0		0.7	0.9	0.9	0.6	*Leuconostoc mesenteroides*
Tyrosine	0.7		0.5	0.7	0.7	0.3	*Leuconostoc mesenteroides*
Proline	0.7		0.6	0.9	0.7	0.3	*Leuconostoc mesenteroides*
Total N	4.79	4.46	2.77	3.75	4.02	3.60	

Table 7. Analyses after electrodialysis of Echinus esculentus jelly coat solution (precipitated with alcohol and ether).

Sample Analysis	Fraction	mg	S in ash	S in abs. liquid	Total S	Ash	Total N
			per cent of whole sample		per cent	per cent	per cent
GK 48 : 3	Minus	23.27	2.41	6.23	8.63	17.75	
Kj 46 : 8	Minus	12.0					2.20
GK 48 : 4	Plus	30.49	(7.87)	4.20	(12.07)	33.45	
Kj 46 : 3	Plus	24.5					2.11

4. Chemical analyses after electrodialysis

Some of the preparations were further purified by electrodialysis. According to its acidic character [6], the jelly coat substance migrated towards the anodic membrane. But whereas the *Strongylocentrotus droebachiensis* jelly formed a compact mass on the membrane, the *Echinus esculentus* jelly was concentrated only at the anodic compartment; plus- and minus-fractions of this latter jelly were therefore collected.

On the plus- and minus-fractions of one of these, namely an alcohol and ether precipitated *Echinus esculentus* preparation, determinations of total nitrogen (Kj) and total sulphur (GK) were performed, as seen in Table 7. The ash of the plus-fraction was unfortunately insoluble, probably because of high calcium sulphate content. Sulphur determination was therefore carried out only on the contents of the absorption vessel. However, if the assumption is made that the ash consists entirely of calcium sulphate, the values in the brackets are obtained. They are at least approximately valid since the calcium content is then 9.9 per cent, a fairly satisfactory agreement with the calcium content of *Strongylocentrotus droebachiensis* jelly substance precipitated with alcohol and ether.

5. Chemical analyses after dialysis at pH 3—4

A carefully dialysed sample of *Strongylocentrotus droebachiensis* jelly solution was redialysed against dilute hydrochloric acid of pH 3.9 for one day, and then against dilute hydrochloric acid of pH 3.6 for two more days. The dialysis was then continued against distilled water until free of chloride ions. After freeze-drying, the sample was analysed for total nitrogen (Kj), total sulphur (GK), carbon, hydrogen, and ash (Ea). The values so obtained are compared with those for the original dialysed material (also frozen-dried), as shown in Table 8. The sulphur analysis S 46 : 8 is according to Vasseur [7].

Table 8. Analyses after dialysis against distilled water and against dilute hydrochloric acid at pH 3—4 of Strongylocentrotus droebachiensis jelly coat solution (precipitated with alcohol and ether).

Analysis	Sample: Dialysis	Sample: mg	Total S per cent	Ash per cent	C per cent	H per cent	Total N per cent
GK 46 : 1	Distilled water	25.72	9.71	41.7			
Ea 48 : 1		25.85		43.0	20.0	4.40	
Kj 48 : 4		30.2					3.77
Kj 48 : 10		29.0					3.78
Kj 48 : 11		24.2					3.70
S 46 : 8	pH 3—4	26.43	2.40				
Ea 48 : 2		20.07		6.33	40.5	9.35	
Kj 48 : 15		20.2					7.24
Kj 48 : 16		22.2					7.20

DISCUSSION

The values obtained for total sulphur and acid-hydrolysable sulphate (Tables 3 and 4) support the view that all or nearly all of the sulphur in the jelly coat substance exists in the form of ester-linked sulphate. The analyses of total nitrogen (Table 5) and of the amino acids thus far conducted (Table 6) show that about 20—25 per cent of the jelly substance is composed of protein- or peptide-like material. All attempts to liberate this protein fraction or isolate the carbohydrate fraction have hitherto been fruitless. Electrodialysis removes some of the nitrogen, but the major part seems to be firmly bound (*cf.* Table 5). The strong linkage between the amino acids and the carbohydrate portion of the jelly substance, and the observation that the basic amino acids constitute only a small fraction of the total amino acids suggests that the amino acids are possibly bound in another fashion than by salt-linkage between basic groups of a protein and the acid polysaccharide. The analyses of the electrodialysed substance (Table 7) show that the cations are firmly bound and are preferentially carried to the anodic side.

The figures reported for carbohydrate, sulphate, total nitrogen, etc. are in accordance with the hypothesis of a protein-carbohydrate complex consisting of 20—25 per cent protein and 75—80 per cent polysaccharide esterified with one sulphate group per monosaccharide residue, the polysaccharide being a poly-methylpentose in the jelly coat substance of *Strongylocentrotus droebachiensis*, *Paracentrotus lividus*, and *Echinocardium cordatum*, and a polyhexose (probably a polygalactose) in the *Echinus esculentus* jelly. Since the

methylpentose, fucose, is the most widely distributed in different marine algae, *e. g.*, *Fucus*, *Laminaria*, the methylpentose in these jelly coats probably is also fucose.

The observation that the jelly coat in situ and in solution exhibits a strong metachromatic staining with toluidine blue, and the striking analogies between jelly coat substance and heparin [24] constitute additional evidence for the view that the polysaccharide portion contains ester-linked sulphate.

The analyses given in Table 8 for jelly dialysed at pH 3—4 show that the sulphate groups begin to hydrolyse even at this pH. Tyler and Fox [8] state that the agglutinin (the jelly coat solution) of *Strongylocentrotus purpuratus* is inactivated rapidly at pH 2; this probably depends on the loss of sulphate groups. Since it has been shown [25, 26] that *Brissopsis lyrifera* egg-water and jelly solution do not exhibit any agglutination of sperms from the same species, it is interesting to note that the sulphur content of *Brissopsis* jelly is very low (Table 3). The presence of a sufficient number of sulphate groups may thus have a decisive rôle for the appearance of this reaction. The finding that sodium periodate destroys not only the blood coagulation inhibiting effect [24] but also the agglutinating activity of a jelly solution indicates further that the carbohydrate part is of utmost importance for the fertilisin mechanism [23], whereas the protein fraction may be of significance for the species specificity of this reaction.

The positive reaction for glycogen given by *Echinocardium cordatum* and *Echinus esculentus* jelly certainly does not imply that the »glycogen» in the sea-urchin egg or in other organisms actually consists of this polysaccharide alone. A greater or lesser part of it instead may be of acidic character just as is the jelly coat substance. Monné [27] recently demonstrated that the yolk fixed in Bouin's fluid may be stained only by certain acid dyes and by means of Best's carmine, Bauer's, and McManus and Hotchkiss' methods for detecting carbohydrates. From these facts, it is inferred that the yolk contains basic proteins and carbohydrates which cannot be glycogen because they are not attacked by saliva; possibly it is a mucous substance. The decrease of glycogen content after fertilisation found by Örström and Lindberg [28] may represent a change in the content of a mucopolysaccharide which perhaps originated from the yolk. Lindberg [14] showed furthermore that the major part of this »glycogen» is combined with protein, *cf.* also Hutchens, Keltch, Krahl, and Clowes [29]. Runnström [30] demonstrated that there is a formation of acid immediately after fertilisation and that this acid is not lactic acid. If the »glycogen» is truly a mucopolysaccharide, there exists the possibility that the acid formed upon fertilisation originates from this mucopolysaccharide. This aspect of the problem deserves further study.

SUMMARY

Chemical analyses are presented on the jelly coat substance of the sea-urchin egg, particularly from *Strongylocentrotus droebachiensis*, *Echinus esculentus*, and *Echinocardium cordatum*. Furthermore, some data are given on the jelly substance from *Paracentrotus lividus*, *Brissopsis lyrifera*, *Arbacia lixula*, and *Psammechinus miliaris*.

The gelatinous substance consists of about 20—25 per cent protein- or peptide-like material and 75—80 per cent polysaccharide esterified with one sulphate group per monosaccharide residue, the polysaccharide being a polymethylpentose (probably polyfucose) for *Strongylocentrotus droebachiensis*, *Echinocardium cordatum*, and *Paracentrotus lividus*, and a polyhexose (probably polygalactose) for *Echinus esculentus*.

Different methods of preparation of the jelly coat substance are described.

The work has been supported by grants from the *Rockefeller Foundation*, the *Hierta-Retzius stipendiefond*, *Wilhelm Leches stipendiefond*, *E. W. Smitt's stipendiefond*, *H. S. och Emmy Josephsons stipendiefond*, and the doktorandstipendium of the Swedish Government. I wish to express my warmest thanks to Professor John Runnström for the interest he has shown throughout this investigation and for the unfailing support he has always given me. I wish to especially thank Mr. Juhan Kuslap and Dr. med. Ivan Resch for valuable assistance during the study.

REFERENCES

1. *Cf.* Loeb, J. *J. Exptl. Zool.* **17** (1914) 132; *Die chemische Entwicklungserregung des tierischen Eies (Künstliche Parthenogenese)* Berlin (1909) 116.
2. Lillie, F. R. *Science* **36** (1912) 527; *Problems of fertilization* Chicago (1919).
3. Tyler, A. *Physiol. Revs.* 28 (1948) 180.
4. Runnström, J. *Proc. VI. Intern. Congr. Exptl. Cytology.* Stockholm (1947).
5. Kuhn, R., and Wallenfels, K. *Ber.* **73** (1940) 458; Wallenfels, K. *Österr. Chem.-Ztg.* **43** (1940) 187.
6. Runnström, J., Tiselius, A., and Vasseur, E. *Arkiv Kemi, Mineral. Geol.* **A 15** (1942) no. 16.
7. Vasseur, E. *Arkiv Kemi, Mineral. Geol.* **B 25** (1947) no. 6.
8. Tyler, A., and Fox, S. *Science* **90** (1939) 516; *Biol. Bull.* **79** (1940) 153.
9. Vasseur, E. *Acta Chem. Scand.* **2** (1948) 693.
10. Lemieux, R. U., and Purves, C. B. *Can. J. Research* **B 25** (1947) 485.
11. Nilsson, I. *Biochem. Z.* **285** (1936) 386.
12. Burkhart, B., Baur, L., and Link, K. P. *J. Biol. Chem.* **104** (1934) 171.
13. Meyerhof, O., and Lohmann, K. *Biochem. Z.* **171** (1926) 381.
14. Lindberg, O. *Arkiv Kemi, Mineral. Geol.* **B 20** (1945) no. 1.
15. Cf. Rappaport, F. *Mikrochemie des Blutes* Wien and Leipzig (1935) 135; Barrenscheen, H. K., and Pany, J. *Bestimmung von Kohlehydraten.* In Bamann, E., and Myrbäck, K. *Die Methoden der Fermentforschung*, Leipzig. (1941) p. 1074.

16. Reihlen and Weinbrenner *D. R. P.* 660105 8/10 (1933).
17. McKittrick, D. S., and Schmidt, C. L. A. *Arch. Biochem.* **6** (1945) 411.
18. de Loureiro, J. A., and Janz, G. J. *Biochem. J.* **38** (1944) 16.
19. Grote, W., and Krekeler, H. *Angew. Chem.* **46** (1933) 106; Schöberl, A. *Angew. Chem.* **50** (1937) 334.
20. Briggs, A. P. *J. Biol. Chem.* **53** (1922) 13.
21. Parnas, J. K. *Z. anal. Chem.* **114** (1938) 261.
22. Stokes, J. L., and Gunness, M. *J. Biol. Chem.* **157** (1945) 651.
23. Henderson, L. M., and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
24. Immers, J., and Vasseur, E. *Experientia* (in press).
25. Vasseur, E., and Hagström, B. *Arkiv Zool.* **A 37** (1946) no. 17.
26. Vasseur, E., and Carlsen, I. *Arkiv Zool.* **A 41** (1948) no. 16.
27. Monné, L. Unpublished results.
28. Örström, Å., and Lindberg, O. *Enzymologia* **8** (1940) 367.
29. Hutchens, J. O., Keltch, A. K., Krahl, M. E., and Clowes, G. H. A. *J. Gen. Physiol.* **25** (1942) 717.
30. Runnström, J. *Biochem. Z.* **258** (1933) 257.

Received October 9, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 914—920

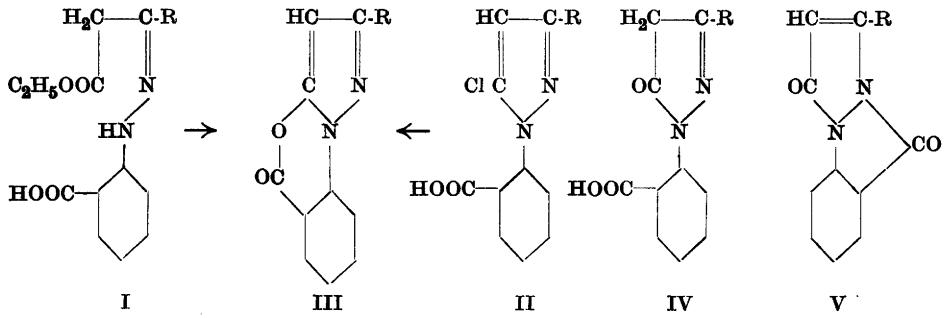
Pyrazole Studies

I. On the Pyrazo-*iso*coumarazones of A. Michaelis

STIG VEIBEL and N. H. ARNFRED

Department of Organic Chemistry, Technical University of Denmark, Copenhagen, Denmark

In the years 1904—1910 Michaelis and his pupils [1-7] investigated some substances obtained either by condensation of *o*-carboxyphenylhydrazine with esters of acylacetic acids and distillation of the *o*-carboxyphenylhydrazones primarily formed (I) or by heating of 1-(2'-carboxyphenyl)-3-alkyl-(or aryl-)5-chloropyrazole (II) to some 200°.



I III II IV V

The unsubstituted substance (III), R = H, Michaelis termed pyrazo-*iso*-coumarazone.

With R = CH_3 Michaelis and collaborators claim to have isolated 3 different substances, α, β and γ, all of them having the empirical formula corresponding to (III) and regarded by the authors as 3 modifications of one compound. Treated with bromine they yielded 3 different monobromosubstituted substances which too were regarded as modifications of one compound.

The γ-compound was obtained by distillation of the *o*-carboxyphenylhydrazone of ethylacetoacetate. The β- and the γ-compound could be transformed into the α-compound by heating them for some time to 160° with an-

hydrous zink chloride or by boiling them for some hours with water, the α- and the γ-compounds could be transformed into the β-compound by dissolving them in 10 parts of concentrated sulphuric acid and after 20 hours at room temperature pouring them slowly into 100 parts of water.

All 3 modifications were soluble in sodium hydroxide. By acidification of the alkaline solutions one and the same 1-(2'carboxyphenyl)-3-methyl-pyrazolone-(5), (IV), R = CH_3), with m. p. 195° could be isolated in all 3 instances.

Michaelis *et al.* were not able to propose structural formulas for the 3 modifications mentioned, but into their opinion polymorphism could be excluded. A closer study of these compounds, therefore, seemed useful.

In the course of our investigations we could show that the γ-compound of Michaelis *et al.* is not a pure compound but a mixture of the α- and the β-compound. The same holds good for the γ-modification of the monobromo-substituted compounds. We, no more than Michaelis, so far have been able to put forward proposals for structural formulas for the 2 series of compound but the investigation is continued.

Both modifications are showing weakly acidic properties. An alcoholic solution of the β-compound may be titrated with standard sodium- or barium-hydroxide as a monobasic acid. The equiv. wt. is found to be some 2—3 % lower than that calculated for the lactone (III). From the neutralized solution the β-compound may be isolated after acidification.

The α-compound, too, may be titrated, but only very slowly, and the equiv. wt. found is some 5 % lower than that calculated for (III). When the neutralized solution of the α-compound is acidified the β-compound and not the α-compound may be isolated. The neutralisation of the α-compound thus seems to be preceeded by, or accompanied by, a rearrangement into the β-compound.

The alcoholic solutions of both modifications are colourless. Enolisation does not seem to occur, as none of the solutions give colour-reaction with ferric chloride. When titrated with sodium hydroxide, however, the solutions assume a faint greenish-yellow colour during the titration.

Michaelis [7] proposed as structural formulas for two of the three modifications found by him the lactone-formula (III) and the lactim-formula (V). This proposal cannot, however, be maintained on account of the results of the titrations. A rearrangement of (III) to (V) or vice-versa cannot possibly occur without the opening of the lactone- or the lactim-ring and by this opening of the ring the acid (IV) is formed. This acid, as mentioned above, is a stable compound, and in alcoholic solution it is titrated with standard alcali as a dibasic acid, the solution of which remains colourless during the titration. Moreover, the alcoholic solution of (IV) gives a typical reddish colour-reaction

with ferric chloride, evidently due to an enolisation of the 5-keto-group in the pyrazolonering.

It is possible that during the titration an opening of the lactone- or lactimring occurs to a very limited extent. This would explain that the equivalent weights are found a little too low by titration. As a matter of fact, however, neither of the formulas (III) and (V) seem to contain a hydrogen atom able to be split off as a proton.

EXPERIMENTAL PART

Ethylacetoacetate-*o*-carboxyphenylhydrazone

(Formula I, R = CH_3), $C_{13}H_{16}O_4N_2$ = 264.3

This compound is easily precipitated if the ethylacetoacetate is added to an aqueous solution of *o*-carboxyphenylhydrazine hydrochloride. The purification of the substance is, however, facilitated by following procedure:

25 g *o*-carboxyphenylhydrazine hydrochloride are dissolved in 1000 ml of hot water. The solution is stirred mechanically and 1 ml of ethylacetoacetate is added. A yellow to brown precipitate is formed which after some minutes is filtered off and discarded. To the filtrate is added, under efficient mechanical stirring, 13 ml ethylacetoacetate and the stirring is continued for 2 hours. The cream-coloured precipitate is then filtered off by suction, washed free of chlorine with water and dried, first at room temperature, then at 95—100°. Yield 22.8 g = 87 %.

Prepared in this way the substance is pure. Michaelis [7] indicates m. p. 125°, Blockmann [1] 135°. We found in different preparations m. p. between 123 and 136° and were able to show that the substance exists in two dimorphous forms, with m. p. 123.5 and 136° respectively. The two forms may be transformed mutually by recristallisation from ethanol-water mixtures and inoculation with the form desired. By titration of ethanolic solutions of both forms with standard barium hydroxide the equiv. wt. calculated for a monobasic acid $C_{13}H_{16}O_4N_2$ is found within the limits of error.

0.2610 g claim 9.52 ml 0.1048 *N* $Ba(OH)_2$ (indicator phenolphthalein). *M* found 261.7, calc. 264.3.

3-Methyl-pyrazoisocoumarazone or 1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5-lactone

(Formula III or V ?, R = CH_3) $C_{11}H_8O_2N_2$ = 200.2.

»*γ-Modification.*» According to Michaelis [7] the γ-modification of the lactone (with m. p. 112°) is formed by distillation of the phenylhydrazone mentioned above at atmospheric pressure, whereas distillation under reduced pressure leads to the β-modification with m. p. 132°. These results we have not been able to reproduce. By distillation at atmospheric pressure we obtained distillates which melted very unsharply, with intervals from 125 to 145°, and by distillation in vacuum we only once obtained a distillate with m. p. 109—110°, corresponding to the γ-modification of Michaelis, in other cases the interval of melting was from 115 to 150°. As, however, the substances obtained could be

transformed into the pure α- and β-modifications we did not continue the investigations on the preparation of the γ-modification, which later could be shown to be a mixture of the α- and the β-modifications.

β-Modification. According to Michaelis [7] and to Ziesel [6] the β-modification is obtained by treatment of the γ-modification with concentrated sulphuric acid. Our distillates, too, treated in this manner, yielded the β-lactone, but as the yield of distillate is not very great the overall yield of β-lactone is poor. It may be considerably improved by treating the carboxyphenylhydrazone with sulphuric acid without previous distillation. Boiling with glacial acetic acid, too, leads, as indicated by Michaelis [7] and Brockmann [1], to the formation of β-lactone, but the yield is better when treated with sulphuric acid.

10 g ethylacetoacetate-*o*-carboxyphenylhydrazone are heated on the steam bath for 15 minutes with 12 ml conc. sulphuric acid. The mixture is cooled and poured into 120 ml of ice-water. The white, flocculous precipitate is filtered with suction, washed with water and recrystallised from ethanol, the ethanolic solution being decolorized with adsorption coal. Yield 3.1 g = 41 % of colourles needles with m. p. 135°, unaltered by further recrystallisation. (Michaelis indicates m. p. 132° for the pure β-compound).

Micro-determination of N *: Calc. 14.00 %, found 14.08 %.

0.2056 g dissolved in ethanol were titrated with 0.1234 *N* $Ba(OH)_2$ (indicator phenolphthalein). During the titration the original colourless solution assumed a faint yellow-green colour which somewhat disturbed the observation of the end point, reached when 8.60 ml had been added. When the substance is regarded as a monobasic acid *M* is found to 194, calculated 200.2.

α-Modification. Michaelis [7] obtained the α-modification by melting the γ-modification with anhydrous zink chloride. On treatment of the β-modification with zink chloride we obtained a substance which evidently was identical with the α-modification of Michaelis.

5 g of β-lactone are mixed with 5 g anhydrous zink chloride in a conical flask equipped with a cork stopper with a glass tube as air condenser. The mixture is heated in an oil bath to 165° for 4 hours. After cooling the solidified mixture is dissolved in ethanol, forming a dark brown solution, which is poured into water acidified with HCl. The brown precipitate is filtered off and recristallised from ethanol, the ethanolic solution being decoloured with adsorption coal. Yield 2 g = 40 % of a light yellow substance with m. p. 166—168°. Michaelis indicates for the α-lactone m. p. 165°.

Micro-determination of N: Calc. 14.00 %, found 13.97 %.

An ethanolic solution of 0.0319 g was titrated with 0.1000 *N* $Ba(OH)_2$ (indicator phenolphthalein). After the addition of some few drops of $Ba(OH)_2$ the solution assumed a pink colour, but the colour disappeared after few minutes, even if the admission of CO_2 was prevented by a stream of CO_2-free nitrogen. In this way the titration could be continued till 1.68 ml had been added. The red colour which till then had disappeared in few minutes after the addition of one drop of $Ba(OH)_2$ now persisted for at least ½ hour. During the titration the solution had assumed the same faint yellow-green colour as the solution of the β-lactone. From the titration result *M* is found to be 190 (monobasic acid), calculated 200.2.

---

* We are indebted to Mr. O. Rosenlund Hansen, Civil engineer, for the carrying out of the micro-N-determinations.

Identification of the γ-modification as a mixture of the α- and the β-modification

The velocity with which the α- and the β-modification were neutralised by standard alkali was found so different that it was natural to investigate the velocity of neutralisation of the γ-modification too. 0.2614 g were dissolved in ethanol and titrated with 0.1000 *N* $Ba(OH)_2$, a stream of CO_2-free nitrogen being passed through the solution in order to exclude the action of carbon dioxide from the air. When 9.0 ml had been added the end point was apparently reached, but the colour faded and only after the addition of 13.0 ml the red colour persisted for ½ hour. As to 13.0 ml corresponds an equiv. wt. of 201, the mol. wt. of the lactones, this result may be explained by the assumption that the γ-modification is a mixture of 65—70 % β-lactone and 35—30 % α-lactone. We prepared a series of mixtures of β- and α-lactone with different content of the two components and determined their melting points. Table 1 shows that a mixture with 65—70 β-lactone and only such a mixture shows an approximately sharp m. p., concordant with the m. p. indicated for the γ-modification. This corroborates the impression gained by the titration.

Table 1.

α-β-Mixture	M. p.	M. p. after solidification
5 mg α— + 1.5 mg β—	112—125°	113—137°
5 α— + 2.0 β—	112—130°	112—120°
5 α— + 2.5 β—	112—113°	112—117°
5 α— + 3.0 β—	112—114°	110—113°
5 α— + 3.5 β—	112—125°	—
»γ-Modification«	109—110°	110—111°

Michaelis [7] indicates that the γ-modification by prolonged boiling with water with continuous renewal of the water evaporated is transformed to the α-lactone. We are now able to give another explanation of the phenomenon mentioned by Michaelis. Incidentally we observed that the β-modification is somewhat volatile with water vapour. The prolonged boiling, which actually is a distillation with steam, thus eliminates the β-modification, and only the α-modification which is not volatile remains and may be isolated.

Usually the β-lactone is not observed, even if the distillate is collected. This is due to the fact that the lactone-ring is opened by boiling with water, and the 1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5 (IV, R = CH_3) thus formed is more soluble in water than either of the lactone-modifications. The following experiment demonstrates clearly the opening of the lactone-ring during the boiling with water.

1-(2′-Carboxyphenyl)-3-methyl-pyrazolone-5
$C_{11}H_{10}O_3N_2 = 218.2$ (IV, R = CH_3)

0.2 g of the β-lactone were dissolved by refluxing the lactone for 1 ½ hour with 60 ml of water. The clear solution was left over night at room temperature. 0.01 g had separated out and were filtered off. The filtrate was concentrated to a small volume. From the distillate 0.01 g crystallised. M. p. of both crops of crystals was 134—135°, *i. e.* they consist of unaltered β-lactone.

The residue from the distillation was cooled in ice. 0.13 g crystals were filtered off. M. p. 189—191°. Michaelis [7] indicates m. p. 191° for the substance IV. As control we prepared IV from both the α- and the β-modification by dissolving the lactone in con-

centrated sodium hydroxide followed by acidification with hydrochloric acid (Michaelis, *l. c.*). The crystals melted about 191—193°, recrystallised from 30 % ethanol at 200°, somewhat higher than indicated by Michaelis.

Both the product obtained by boiling the β-lactone with water and the products obtained from the α- and β-modifications after solution in sodium hydroxide assumed a red colour during the melting process which was accompanied by an evolution of gas (vapour). Aqueous solutions of all 3 preparations gave a red colour reaction with ferric chloride.

The titration of an ethanolic solution of the pyrazolone with $Ba(OH)_2$ (indicator phenolphthalein) gave an equivalent weight of 110.2. (0.0476 g, 3.50 ml 0.1234 *N* Ba $(OH)_2$). This means that the pyrazolone is titrated as a dibasic acid whereas the neutralisation of the lactones claims only one equivalent of base. The transformation of the α-modification into the β-modification under the influence of barium hydroxide cannot, then, pass through the pyrazolone-carbonic acid IV as intermediate.

Bromination of the lactones

Michaelis indicates that all 3 modifications of the lactone may be brominated and that 3 different monobromolactones were isolated by him. As the γ-modification has been proved to be a mixture of the α- and the β-lactones we thought it necessary to investigate the bromination products too.

Bromination of the β-modification. The bromination was carried out as a titration with bromine as indicated by Meyer [8]. 0.1669 g claimed 18.5 ml 0.0864 *N* bromine solution. The equiv. wt. is 96, *i. e.* a double bond or an easily substituted hydrogen atom is found in 192 g lactone. In another experiment the equiv. wt. 101 was found. As the mol. wt. of the lactone is 200 it may be concluded that 1 mol. of β-lactone reacts with 1 mol. of bromine at room temperature. The bromination product was isolated. The m. p. was 152—153°, in accordance with the indication of Michaelis, 151°.

The α-lactone was brominated very slowly at room temperature so that a titration could not be carried out. To a sample of α-lactone the amount calculated (1 mol. bromine pr. mol. lactone) of a bromine solution was added and the mixture heated on steam bath. The colour of bromine disappeared very fast and by evaporation the bromination product was isolated. It showed m. p. 190—193° whereas Michaelis indicates m. p. 187° for the bromination product of the α-lactone.

The »γ-modification» was brominated according to Michaelis (*l. c.*) with bromine in glacial acetic acid. The bromination product started to cristallise in the acetic acid solution and was precipitated completely by addition of water. It melted unsharply at 118—125°, whereas Michaelis for the brominated γ-modification indicates m. p. 135—137°. By repeated recrystallisations, first from ethanol, then from acetone-water mixtures, we succeeded in separating the product into two fractions, one melting sharply at 151—152°, identical with the bromination product of the β-lactone, the other, more soluble, melting very unsharply, starting at 134° but completely melted only at 175°. We were not able to isolate the pure brominated α-lactone with m. p. 190—193°, but it is likely that the more soluble fraction contains this substance as main product.

1-(2′-Carboxyphenyl)-3-methyl-pyrazolone-5-hydrochloride

$C_{11}H_{11}O_3N_2Cl = 254.7$

Michaelis [7] indicates that the β-lactone when boiled with concentrated hydrochloric acid for 5 hours in an open vessel forms a chlorhydrate with m. p. 220°.

We boiled 1 g β-lactone with 100 ml conc. hydrochloric acid. After 3 hours so much water had disappeared that further boiling was impossible. The solution was cooled and a salt separated out in rather great crystals. The crystals were filtered off, washed with diluted hydrochloric acid and with alcohol. Yield 0.70 g with m. p. 218—221°. A determination of chlorine after Volhard showed $M = 260$. (0.1097 g, 4.22 ml 0.1000 N $AgNO_3$).

The chlorhydrate was recrystallised from ethanol and titrated in ethanolic solution with barium hydroxide (indicator phenolphthalein). 0.1848 g claim 17.7 ml 0.1234 N $Ba(OH)_2$. Equiv. wt. = 84.5 or 1/3 of that found by the chlorine-determination.

For the chlorhydrate of the lactone is calculated $M = 236.7$, for the chlorhydrate of the pyrazolone-carbonic acid IV (R = CH_3) $M = 254.7$. Evidently the chlorhydrate is titrated as a tribasic acid, whereas a chlorhydrate of the lactone could use only 2 equivalents of base.

The solution of the chlorhydrate remained colourless during the titration. An aqueous solution gave a red colour reaction with ferric chloride. This too is to be expected if it is the chlorhydrate of the open acid, but not if it is the chlorhydrate of the lactone.

Most of the experiments described here were carried out in the Laboratory of Organic Chemistry, the University of Copenhagen.

SUMMARY

The anhydrides of 1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5 previously examined by Michaelis and collaborators were reexamined. Michaelis claims that 3 modifications of the anhydrides may be isolated. We have been able to show that only 2 modifications exist, one of Michaelis' modifications being a mixture of the two others.

Structural formulas for the remaining two modifications have not been proposed. One, the β-modification, may be titrated directly as a monobasic acid with standard base, the other, the α-modification, only after rearrangement to the β-modification, this rearrangement taking place during the titration.

1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5 is titrated as a dibasic acid.

Michaelis claims that the anhydride forms a chlorhydrate by treatment with hydrochloric acid. We have been able to show that the chlorhydrate is formed not by the anhydride but by the acid 1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5.

REFERENCES

1. Blockmann, R. *Dissertation* Rostock (1902).
2. Eisenschmidt, C. *Dissertation* Rostock (1904).
3. Michaelis, A., and Eisenschmidt, C. *Ber.* **37** (1904) 2228.
4. Krug, C. *Dissertation* Rostock (1906).
5. Leo, J. *Dissertation* Rostock (1908).
6. Ziesel, M. *Dissertation* Rostock (1909).
7. Michaelis, A. *Ann.* **373** (1910) 129.
8. Meyer, K. H. *Ann.* **380** (1911) 212.

Received October 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 921—926

Pyrazole Studies

II. Anhydrides of 1-(2′-Carboxyphenyl)-3-phenyl-pyrazolone-5 and 1-(2′-Carboxyphenyl)-3-methyl-4-ethyl-pyrazolone-5

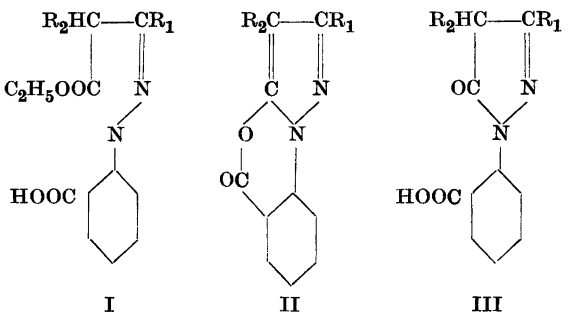
STIG VEIBEL and N. H. ARNFRED

Department of Organic Chemistry, Technical University of Denmark, Copenhagen, Denmark

In the first paper of this series [1] we were able to show that from the 3 modifications of the anhydride of 1-(2′-carboxyphenyl)-3-methyl-pyrazolone-5 postulated by Michaelis [2] only 2 of them are pure compounds, the third being a mixture of the two others. Michaelis mentions that the anhydride of 1-(2′-carboxyphenyl)-3-phenyl-pyrazolone-5, by him named 3-phenyl-pyrazo*iso*-coumarazone, seems to exist in one modification only.

Pyrazolones substituted both in position 3 and in position 4 were not examined by him.

We now have examined the formation of anhydrides of the *o*-carboxyphenylhydrazones of ethyl benzoylacetate (I, $R_1 = C_6H_5$, $R_2 = H$) and of ethyl ethylacetoacetate (I, $R_1 = CH_3$, $R_2 = C_2H_5$). In both cases the anhydrides, possibly of structure as II, are formed by heating the *o*-carboxy-

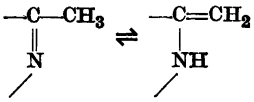


I II III

phenylhydrazones above their melting points. After treatment with conc. sulphuric acid these anhydrides may be isolated in modifications similar to the

β-modification of 1-(2'-carboxyphenyl)-3-methyl-pyrazolone-5-anhydride (the 3-methyl-pyrazo*iso*coumarazone of Michaelis), whereas treatment with anhydrous zink chloride at elevated temperature transforms the anhydrides to modifications with properties similar to the α-modification of the previously investigated anhydride. In all 3 cases we thus have 2 series of anhydrides, one with weakly acidic properties, the other neutral. Both modifications may be titrated in ethanolic solution with standard base as monobasic acids, the α-modification, however, only after rearrangement to the β-modification. The acidic properties are, therefore, not due to an opening of the lactone ring, as the pyrazolone-carbonic acids III by titration claim 2 equivalents of base to their neutralisation. (An exception is III with $R_1 = CH_3$, $R_2 = C_2H_5$, this acid claiming only one equivalent of base).

We still are not able to indicate at what place of the molecule the acidic properties are located or which hydrogen atom may be split off as a proton. With $R_1 = CH_3$ the possibility seems to exist that a transformation



might take place and that the imino-hydrogen was the one which showed acidic properties but with $R_1 = C_6H_5$ this explanation seems impossible. When $R_2 = H$ the acidic properties might be located at the carbon atom 4 in the pyrazolone-ring, if the bond to the hydrogen atom is sufficiently loosened, thus allowing the hydrogen atom to be removed as a proton, but with $R_2 = C_2H_5$ no such possibility exists. And even if the acidic properties might be explained in such ways there would be no explanation of the existence of two different modifications of the lactones, at all events not if the acidic properties were located at C_4, as the difference of the two modifications would be only the place of an electron and this difference may cause a case of mesomerism, not one of tautomerism or isomerism.

In pyrazolones of simpler structure, too, we have found similar cases of isomerism between an acidic and a neutral modification. Possibly the explanation of the isomerism mentioned in this paper may be understandable when the explanation of the simpler case has been found. The investigations on the simpler case will be published later.

EXPERIMENTAL PART

1. Ethyl benzoylacetate-o-carboxyphenylhydrazone (I, $R_1 = C_6H_5$, $R_2 = H$). $C_{18}H_{18}O_4N_2 = 326.3$. 13 g *o*-carboxyphenylhydrazine hydrochloride were dissolved in 500 ml water. The solution was stirred mechanically and 0.9 g ethyl benzoylacetate were added. The precipitate formed was filtered off and discarded. The filtrate was stirred mechanically and 9.6 g ethyl benzoylacetate were added and well emulgated in the

solution. A precipitate was formed soon, but to complete the reaction the mixture was heated on steam bath for some two hours. After refrigeration the precipitate was filtered off, washed chlorine-free and dried. Yield 11 g = 67 %. M. p. 165° (on a metal block, heated with the block), but the instantaneous m. p. (block Maquenne) was 175°. Michaelis (*l. c.* p. 179) indicates m. p. 166—167°. Recrystallised from 10 parts of ethanol the m. p. was raised to 173—175° when the substance was slowly heated or 184—185° (block Maquenne). Further recrystallisation did not alter the m. p.

0.4111 g were dissolved in ethanol and titrated with 0.0927 *N* $Ba(OH)_2$. After addition of 16 ml the end point seemed to be reached as the solution turned red, but the colour faded very fast and only after the addition of 26.9 ml $Ba(OH)_2$ the colour persisted for 1 hour (CO_2-free nitrogen athmosphere) and with further 0.3 ml the colour persisted for 1 ½ hour. The equivalent weight is thus between 164.8 (26.9 ml) and 163.0 (27.2 ml), *i. e.* the *o*-carboxyphenylhydrazone is titrated as a dibasic acid (*M* calculated 326.3, found $329.6 > M > 326.0$.

The *o*-carboxyphenylhydrazone of ethyl acetoacetate was titrated as a monobasic acid [1] and we therefore examined if the present *o*-carboxyphenylhydrazone had been transformed into another substance during the titration.

1 g of the *o*-carboxyphenylhydrazone was dissolved in ethanol and neutralised with the calculated amount of $Ba(OH)_2$ (2 equivalents of base pr. mol of *o*-carboxyphenylhydrazone). The barium salt which separated out as a precipitate during the addition of base, was filtered off and dissolved in water. By acidification with hydrochloric acid a precipitate was formed, which was filtered off, washed Cl^--free with water and dried. Yield 0.8 g of a substance with m. p. about 200°, *i. e.* different from the starting material. By titration 0.1732 g claimed 13.47 ml 0.0927 *N* $Ba(OH)_2$, which corresponds to an equiv. wt. of 138.7 or a mol. wt. of 277.4. Both the m. p. and the equiv. wt. is concordant with m. p. and equiv. wt. of 1-(2'-carboxyphenyl)-3-phenyl-pyrazolone-5 described below. During the titration of the *o*-carboxyphenylhydrazone, therefore, a saponification of the ester and ring-closure of the acid to a pyrazolone has taken place.

2. *1-(2'carboxyphenyl)-3-phenyl-pyrazolone-5-lactone* (II?, $R_1 = C_6H_5$, $R_2 = H$). $C_{16}H_{10}O_2N_2 = 262.2$. Michaelis [2] indicates that the ethyl benzoylacetate-*o*-carboxyphenyl-hydrazone by heating above the melting point over direct flame is transformed into a lactone with the composition $C_{16}H_{10}O_2N_2$, named by him 3 phenyl-pyrazo*iso*coumarazone. M. p. of the lactone is 198—200°, dependent on the medium from which it is recrystallised. Michaelis states that neither by treatment with sulphuric acid nor by melting with anhydrous zink chloride is the lactone transformed into other modifications as was the case with the 3-methyl-pyrazo*iso*coumarazone.

We prepared the lactone after the indications of Michaelis and obtained a substance with m. p. 201—202°, very sparingly soluble in ethanol. It may, however, be recrystallised from ethyl acetate. The substance was titrated in hot ethanolic solution with standard $Ba(OH)_2$ in a stream of CO_2-free nitrogen. 0.1196 g claimed 9.80 ml 0.0927 *N* $Ba(OH)_2$. To this corresponds an equiv. wt. 131.7. Under the circumstances of the experiment the lactone ring is opened during the titration, the lactone thus claiming 2 equivalents of $Ba(OH_2)$.

When titrated at room temperature only a little more than 1 equivalent of base is used to neutralisation. 0.1614 g claimed 6.92 ml 0.0962 *N* $Ba(OH)_2$. *M* found 243.1, calc. 262.2. As was the case with the β-3-methyl-pyrazo*iso*coumarazone [1] the mol. wt. is found to be 6—7 % lower than calculated, most likely due to a partial opening of the

lactone ring. When more $Ba(OH)_2$ is added the colour fades very slowly, an excess of 0.5 ml base claiming some 6 hours to be used. At room temperature, therefore, the opening of the lactone ring is a very slow proces.

β-Modification. 20 g of the lactone were heated on the steam bath for 1 ¼ hours with 100 ml conc. sulphuric acid. The solution was cooled and poured into 1 l of water. The precipitate was filtered off, washed with water and dried. Yield 19 g with m. p. 192—193°, recrystallised from ethyl acetate m. p. 201—202°. The lactone prepared by direct heating of the ethyl benzoylacetate-*o*-carboxy-phenylhydrazone, therefore, is the β-modification.

The β-modification is not volatile with steam and its solution gives no colour reaction with ferric chloride.

α-Modification. 13 g β-lactone were mixed with 13 g anhydrous zink chloride and heated for 3 hours in a metal bath to 250°, protected against the humidity of the air. A sublimate of β-modification was from time to time pushed back in the melt. When cooled the dark coloured solidified melt was dissolved in 100 ml absolute ethanole. The ethanolic solution was poured into 500 ml *N*/1 HCl. A dark tar was precipitated which solidified to an amorphous pitchy mass. The pitch was repeatedly boiled with 1 liter portions of water. When the solution was filtered as hot as possible through a funnel with a plate of sintered glass 0.2 g yellowish crystals separated out from each portion on cooling. 1.6 g of these crystals were gained, which recrystallised from 35 ml ethanol gave 1.2 g yellow crystals with m. p. 140° (on a metal block) or 144—145° (in capillary tube). Further recrystallisation with treatment of the solution with absorption coal did not alter the colour or the m. p. Yield 9—10 %.

0.0925 g were dissolved in ethanol and titrated (in CO_2-free nitrogen atmosphere) with 0.1234 *N* $Ba(OH)_2$ (indicator phenolphthalein). Each drop of $Ba(OH)_2$ provoked a colour which faded slowly. 2.00 ml were used in 20 minutes, further 0.55 ml in 10 min, 0.30 ml in 10 min, 0.05 ml in 20 min, 0.03 ml in 50 min. When heated the colour faded very fast.

To 2.95 ml corresponds an equivalent weight of 254.1, calculated 262.2. The α-modification is thus titrated at room temperature as a monobasic acid, but as was the case with the α-3-methyl-pyrazo*iso*coumarazone, not directly. In warm solution the lactone ring is opened during the titration.

The ethanolic solution does not give any colour reaction with ferric chloride.

3. *1-(2'-Carboxyphenyl)-3-phenyl-pyrazolone 5* (III, $R_1 = C_6H_5$, $R_2 = H$). $C_{16}H_{12}O_3N_2$ = 280.3. As mentioned above the β-lactone may be titrated in warm ethanolic solution as a dibasic acid. From the neutralised solution the open phenyl-pyrazolone-carbonic acid may be isolated after acidification. The α-lactone behaves itself in the same manner.

The 1-(2'-carboxyphenyl)-3-phenyl-pyrazolone-5 is more soluble in ethanol than the lactones. The ethanolic solution is coloured brown by ferric chloride. Heated on a metal block it melts at 196—197° with evolution of gas (vapour?), then solidifies and melts by further heating at 201°. This means that it is very easily transformed into the β-lactone.

0.0894 g dissolved in ethanol claims 5.17 ml 0.1234 *N* $Ba(OH)_2$. The titration is easy and the end-point is sharply determined. The equiv. wt. is 140.1.

Melting point and titration show that this substance is identical with the substance isolated after complete neutralisation of the ethyl benzoylacetate-*o*-carboxyphenyl-hydrazone.

4. *Ethyl ethylacetoacetate-o-carboxyphenylhydrazone* (I, $R_1 = CH_3$, $R_2 = C_2H_5$). $C_{15}H_{20}O_4N_2 = 292.3$. The preparation of this substance was completely analogous to the preparation of the ethyl benzoylacetate-*o*-carboxyphenylhydrazone. The raw product (m. p. 141—145°) was purified by dissolving it in ethanol, treating the ethanolic solution with adsorption coal and precipitating the hydrazone by addition of water. The pure ethyl ethylacetoacetate-*o*-carboxyphenylhydrazone melts at 148—149°. Yield of pure substance 16.5 g = 57 %.

0.3712 g dissolved in ethanol claim 12.97 ml 0.1000 *N* $Ba(OH)_2$. Equiv. wt. found 289.8, calc. 292.3.

5. *1-(2'-Carboxyphenyl)-3-methyl-4-ethyl-pyrazolone-5-lactone* (II?, $R_1 = CH_3$, $R_2 = C_2H_5$. $C_{13}H_{12}O_2N_2 = 228.2$.

β-Modification. From our experience with the preparation of the β-3-methyl-pyrazo-*iso*coumarazone [1] we prepared the β-lactone in the following way.

17 g ethyl ethylacetoacetate-*o*-carboxyphenylhydrazone were placed in a fractionating flask and heated in oil bath for 3 hours to 160—180°. 3.1 g distillate were collected (calculated for loss of 1 mol ethanol and 1 mol water 3.7 g). The residue in the flask was heated on steam bath for 2 hours with 15 ml conc. sulphuric acid. When cooled the sulphuric acid solution was poured into 200 ml of water. The white precipitate was filtered off, washed with water and dried. Yield 12.7 g with m. p. 98—100° or 95 %.

By repeated recrystallisations from ethanol the m. p. was raised to 107—108°.

Dissolved in ethanol 0.2171 g claimed 10.18 ml 0.0927 *N* $Ba(OH)_2$. Equiv. wt. found 230.2, mol. wt. calculated 228.2, *i. e.* the β-lactone is titrated as a monobasic acid. The originally colourless solution assumes as the solution of the β-3-methyl-pyrazo*iso*coumarazone a faint yellow-green colour during the titration.

The lactone-ring in 3-methyl-4-ethyl-pyrazo*iso*coumarazone seems to be more resistant than the lactone-ring in the lower homologue. The loss of substance during the treatment with sulphuric acid is not as great in the former as in the latter case. The 4-ethyl-lactone, like its lower homologue, is volatile with water-vapour, and the lactone separates out from the distillate on cooling. The acid which would be formed from the lactone if the lactone-ring was opened during the distillation cannot be isolated from the distillate, whereas in the case of the lower homologue most of the lactone was transformed into the acid during the distillation with water-vapour [1].

The ethanolic solution gives no colour-reaction with ferric chloride.

α-Modification. 5 g β-lactone were mixed with 5 g of anhydrous zink chloride. The mixture was heated to 170° for 1 hour in an oil bath. When cool, the solidified melt was dissolved in 50 ml ethanol. The solution was poured into 400 ml of water acidified with 5 ml conc. hydrochloric acid. The brown precipitate was filtered off and dried. Yield 3.6 g with m. p. 130—133°. Recrystallised from 20 parts of ethanol (treatment of the ethanolic solution with adsorption coal) the yield was 2.0 g faint yellow crystals with m. p. 144—145° or 40 %. Neither the colour nor the m. p. was altered by further recrystallisation.

As in the case of the other α-lactones, the titration of the ethanolic solution of the α-lactone with standard $Ba(OH)_2$ was very delayed. A solution of 0.2021 g used 9.50 ml 0.0927 *N* $Ba(OH)_2$ in 90 minutes. Further 0.10 ml were used in 20 minutes and still further 0.10 ml were not used in 30 minutes. From this is found 229.5 > equiv. wt. > 227.1. The calculated mol. wt. is 228.2, which shows that the α-lactone is titrated with delay as a monobasic acid. Its solutions do not give colour reaction with ferric chloride.

6. *1-(2'-carboxyphenyl)-3-methyl-4-ethyl-pyrazolone-5* (III, $R_1 = CH_3$, $R_2 = C_2H_5$). $C_{13}H_{14}O_3N_2 = 246.2$. By treatment with excess alcali both the *α*-and the *β*-lactone are transformed into the open acid. 0.5 g *α*-lactone were treated with an excess of NaOH. When dissolved the solution was acidified with hydrochloric acid. A crystalline precipitate was filtered off. Yield 0.26 g with m. p. 153—155°. By titration of its ethanolic solution with 0.1234 *N* $Ba(OH)_2$ 0.0711 g claimed 2.33—2.40 ml, *i. e.* 247 > equiv. wt. > 240. The calculated mol. wt. being 246.2 this means that the acid is titrated as a monobasic acid, in disagreement with the two other pyrazolones investigated, which both of them were titrated as dibasic acids. Another difference from the two other pyrazolones is that the pyrazolone examined here does not give a colour reaction with ferric chloride, at least at room temperature, whereas the two others give a colour reaction. The hot aqueous solution does give a faint colour reaction, but it disappears by addition of hydrochloric acid. The tendency of enolisation of the pyrazolone is evidently diminished by the substitution of a hydrogen atom for an ethyl group at C_4.

Most of the experiments described here were carried out in the Laboratory of Organic Chemistry, the University of Copenhagen.

SUMMARY

It has been shown that contrary to the statement of Michaelis the anhydride of 1-(2'-carboxyphenyl)-3-phenyl-pyrazolone-5 does exist in 2 modifications from which one may be titrated directly with standard base, the other only after rearrangement.

From 1-(2'-carboxyphenyl)-3-methyl-4-ethyl-pyrazolone-5 too, not examined previously, two modifications of an anhydride may be prepared.

REFERENCES

1. Veibel, S., and Arnfred, N. H. *Acta Chem. Scand.* **2** (1948) 914.
2. Michaelis, A. *Ann.* **373** (1910) 129.

Received October 16, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 927—932

Pyrazole Studies

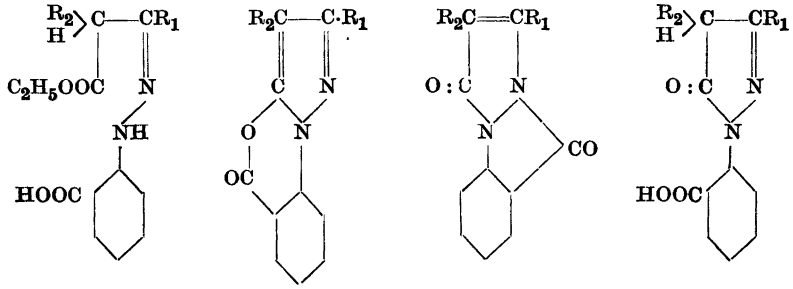
III. Further Investigations on the α- and β-Lactones of some 1-(2′-Carboxyphenyl)-pyrazolones-5 (Pyrazo*iso*coumarazones)

STIG VEIBEL, IB REFN and ARTHUR FRIEDIGER

Department of Organic Chemistry, Technical University of Denmark, Copenhagen, Denmark

In two previous papers [1,2] one of us have described the preparation of some *o*-carboxyphenylhydrazones of esters of β-ketoacids and has shown that such *o*-carboxyphenylhydrazones (I), as first described by Michaelis [3], by heating above their melting point not only, as other phenylhydrazones of β-ketoacids, form pyrazolones (IV) by ring closure, but that the 1-(2′-carboxyphenyl)-pyrazolones-5 thus formed by further ring closure loose 1 mol of water and form anhydrides which by Michaelis and by us were considered as lactones of the enolised form of the pyrazolones (II) and by Michaelis were termed pyrazo-*iso*coumarazones.

So far nothing unusually is to be seen in the formation of these lactones. But Michaelis was able to show that with $R_1 = CH_3$, and $R_2 = H$ (II) exists in two different modifications, α and β, which are mutually interconvertible



I II III IV

and are not dimorphous forms of the lactone, as bromination leads to two different monobromocompounds. With $R_1 = C_6H_5$, $R_2 = H$ Michaelis did

not succeed in preparing two modifications of the lactone, but in one of the above mentioned papers [2] we were able to show that not only with $R_1 = C_6H_5$, $R_2 = H$, but also with $R_1 = CH_3$, $R_2 = C_2H_5$ two modifications of the lactones may be prepared.

A simple explanation of the existence of two substances with the composition as an anhydride of IV would be that one is the lactone II, the other the lactim III. This explanation, however, we found it necessary to abandon as we found that the β-series of the anhydrides may be titrated in ethanolic solution as monobasic acids, whereas the α-series cannot be directly titrated, but only with delay, caused by a previous rearrangement of the α-modifications to the β-modifications.

The natural explanation that the different velocity with which the two modifications is titrated is due to a difference in velocity of opening of the lactone-ring and the lactim-ring had to be abandoned as we were able to show that the acids IV resulting from such a ring-opening are titrated as dibasic acids (with the exception of the acid with $R_1 = CH_3$, $R_2 = C_2H_5$ which is titrated as a monobasic acid).

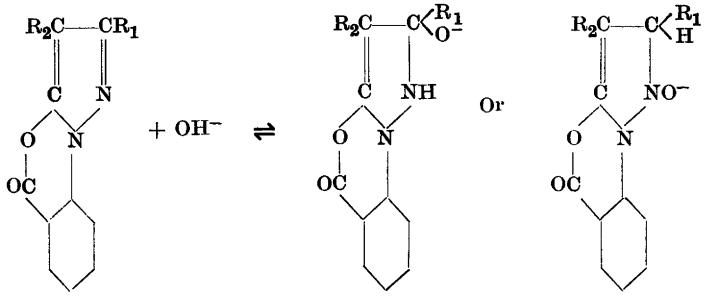
So far, the proof that the α-modifications are rearranged to the β modifications before or during the titrations has been only indirect, but now we think that we have been able to produce a direct proof of this transformation. We have neutralised the two modifications with 1 equivalent of base and then added the equivalent amount of hydrochloric acid, whereupon we have isolated the substances resulting from these operations. In both series it is the β-modification which may be isolated after these treatments.

We then examined the possibility that the two series of anhydrides were due to polymerism so that the α-lactones were dimeres or polymeres of the β-lactones. But cryoscopic determinations of the molecular weights of the α- and the β-lactones proved that both series are monomeres with molecular weights corresponding to the formulas II or III so that this possibility, too, had to be abandoned. With $R_1 = CH_3$ and $R_2 = H$ or C_2H_5 the α-modification shows a higher melting point than the β-modification whereas with $R_1 = C_6H_5$, $R_2 = H$ the opposite is the case. But in all three cases it is the β-modification which may be titrated directly. In order to find a further proof of the analogous structure of the 3 α-lactones on one hand, the 3 β-lactones on the other hand, we determined the dipole moments of the 6 lactones and found that the 3 α-lactones have relatively small dipole moments ($\mu = 1.33$—1.89), the 3 β-lactones relatively great dipole moments ($\mu = 2.53$—2.80). These results show with practically absolute certainty that the structure of all 3 α-lactones on one hand and the 3 β-lactones on the other hand are analogous. It may further be concluded that the possibility mentioned in one

of the previous papers [2], namely that the acidic properties of the β-lactones was due to a repartition of the electrical charges on the carbon atoms of the pyrazolone ring facilitating the splitting off of the hydrogen atom at C_4 as a proton, cannot be correct as such a repartition of the charges would implicate a dipole moment much greater than the dipole moments found for the β-lactones. These measurements thus corroborate the conclusions arrived at in the previous papers, where it was the fact that even with $R_2 = C_2H_5$ the β-modification may be titrated which was not reconcilable with the assumption, but if the dipole moment of the α-lactone with $R_2 = C_2H_5$ did not fit in with the dipole moments of the other α-lactones the assumption might still be valid if $R_2 = H$, and another explanation had to be found for lactones with R_2 different from H.

From the values of the dipole moments of the two series it may be concluded that the β-modifications correspond to formula II, the dipole moment being determined principally by the carbonyl-groups, from which II contains one, and III two acting in opposite directions.

From the two formulas proposed II is the only one which may possibly be able to neutralise bases. It contains a C = N-double bond to which a hydroxyl ion may be added as O^- and H:



Different mesomeric (or tautomeric) formulas may be considered, too. By addition of acid (hydrogen ion) the hydroxyl ion is eliminated and the β-modification is reformed.

The α-modification, on the other hand, does not contain a C = N-double bond and is, therefore, not able to add a hydroxyl ion. As a delayed titration of the α-modification is possible, accompanied by a rearrangement of the α-modification into the β-modification, the open acid IV not being an intermediate, an intramolecular rearrangement, catalysed by hydroxyl ions, may be anticipated. The mecanism of this rearrangement is not clear yet, but its invegstiation is continued.

EXPERIMENTAL PART

a. Proof that the α-modifications are rearranged to the β-modifications during titration

1. β-Methyl-pyrazo*iso*coumarazone (II, $R_1 = CH_3$, $R_2 = H$). M. p. 136°. 0.5272 g were dissolved in ethanol and neutralised with 27.4 ml 0.0962 *N* $Ba(OH)_2$ (1 equivalent), followed by 30.0 ml 0.0848 n HCl (1 equivalent). The solution was evaporated to drynes in a vacuum, the residue extracted with ethanol and the ethanolic solution evaporated in a vacuum dessiccator. M. p. of the β-lactone regained 125—127°.

2. α-Methyl-pyrazo*iso*coumarazone. M. p. 168°. 0.4225 g dissolved in ethanol were neutralised with 21.4 ml 0.0962 *N* $Ba(OH)_2$ (1 equivalent). Phenolphthalein was added. When the colour had faded (½ hour) 24.3 ml 0.0848 *N* HCl (1 equivalent) were added and the lactone recovered as above.

M. p. of the substance recovered	131—132°
Mixed with equal parts of α-lactone	110—130°
Mixed with equal parts of β-lactone	132—134°

Evidently the α-lactone has been rearranged to the β-lactone during the neutralisation.

3—6. Quite analogous experiments were carried out with the α- and β-modifications of 3-methyl-4-ethyl-pyrazo*iso*coumarazone (II, $R_1 = CH_3$, $R_2 = C_2H_5$) and of 3-phenyl-pyrazo*iso*coumarazone (II, $R_1 = C_6H_5$, $R_2 = H$). The melting points of the substances recovered and, if necessary, of mixtures of these with the authentic α- and β-lactones are indicated below.

	M. p. of pure substance	M. p. of substance recovered	M. p. of mixture with α-lactone	M. p. of mixture with β-lactone	M. p. of mixture of α- and β-lactone
β-3-Methyl-4-ethyl-pyrazo*iso*-coumarazone	107—108°	105—108°	—	—	—
α- —»—	144—145°	95—104°	95—130°	96—105°	94—134°
β-3-Phenyl-pyrazo-*iso*coumarazone	201—202°	197—198°	—	—	—
α- —»—	144—145°	195—198°	—	—	—

As the m. p. of the acid resulting from an opening of the lactonering of 3-phenyl-pyrazo*iso*coumarazone is 196—197°, that of the β-lactone 201—202°, the m. p. 197—198° of the substance recovered might mean that the lactone ring had been opened during the titration. As, however, the acid is titrated as a dibasic acid, the lactone as a monobasic acid, and the substance recovered could use 1 equivalent of base only, we think it quite obvious that in this case, too, the α-lactone is rearranged to the β-lactone during the titration.

b. Cryoscopical determination of the molecular weight

The melting points of solutions of the different lactones in benzene were determined. All determinations are double determinations, the difference between the two determinations for a given solution being 0.001° or less. For the molecular depression of the freezing point of benzene we have used the value 49.0°. The benzene used was a Kahlbaum-preparation, c. p. for molecular weight determinations. The m. p. of the pure benzene on the Beckmann-thermometer used was 1.309°.

	Weight of substance *a* g	Weight of benzene *b* g	M. p. (Beckmann)	*t*	*M*	*M* calc.
			3-Methyl-pyrazo*iso*coumarazone			
α	0.1140	14.431	1.112	0.197	197	200.2
	0.1162	14.101	1.093	0.216	187	200.2
β	0.1565	14.502	1.034	0.275	192	200.2
	0.1565	18.948	1.095	0.214	198	200.2
			3-Methyl-4-ethyl-pyrazo*iso*coumarazone			
α	0.1882	17.743	1.074	0.235	221	228.2
β	0.1807	10.950	0.933	0.376	215	228.2
			3-Phenyl-pyrazo*iso*coumarazone			
α	0.1238	12.566	1.121	0.188	257	262.2
β	0.1945	28.867	1.178	0.131	252	262.2

From these determinations may be concluded with absolute certainty that all the modifications are monomeres.

c. Dipole measurements

The measurements of the dielectric constants were carried out with a precision-apparatus from Kipp and Zonen [4].

The lactones were dissolved in benzene (Kahlbaum, c. p.). The specific gravity of the solutions were determined by means of a flask-pyknometer, enabling determinations with an exactitude of 0.00001, which is sufficiently exact for the present purpose.

	Mol fraction of substance	Sp. gr. of solution	ε	P	
			3-Methyl-pyrazo*iso*coumarazone. $M = 200.190$		
	0.00265	0.87632	2.2851	(104.91)	P_E (calc.) = 44
α	0.00778	0.88049	2.3002	93.92	P_∞ = 97
	0.01550	0.88668	2.3235	90.83	μ = 1.60
	0.00429	0.87733	2.3156	189.28	P_E (calc.) = 44
β	0.00564	0.87837	2.3287	188.44	P_∞ = 190
	0.00980	0.88013	2.3665	187.30	μ = 2.66

3-Methyl-4-ethyl-pyrazo*iso*coumarazone. $M = 228.242$

	0.00198	0.87546	2.2775	88.94	P_E (calc.) = 52.6
α	0.00603	0.87893	2.2873	87.73	P_∞ = 89
	0.00764	0.87997	2.2912	88.96	μ = 1.33
	0.00206	0.87549	2.2912	(186.62)	P_E (calc.) = 52.6
β	0.00472	0.87755	2.3140	181.90	P_∞ = 185
	0.00838	0.88013	2.3462	182.63	μ = 2.53

3-Phenyl-pyrazo*iso*coumarazone. $M = 262.256$

	0.00259	0.87721	2.2850	127.62	P_E (calc.) = 58
α	0.00418	0.87877	2.2926	123.43	P_∞ = 132
	0.00165	0.87608	2.2802	(117.00)	μ = 1.89
	0.00468	0.87807	2.3232	219.52	P_E (calc.) = 58
β	0.00311	0.87715	2.3070	218.53	P_∞ = 220
	0.00337	0.87755	2.3100	217.61	μ = 2.80

Most of the experiments described here were carried out in the Laboratory of Organic Chemistry, the University, Copenhagen.

SUMMARY

By further investigation of the anhydrides of some 3- and 4-substituted 1-(2′carboxyphenyl)-pyrazolones-5, each existing in 2 modifications, α and β, it has been proved that the α-modifications are transformed into the β-modifications by titration with standard base.

Cryoscopical determinations of the molecular weights of the different modifications show that they are all of them monomers.

Measurements of the dipole-moments show that the 3 α-modifications examined on one hand and the 3 β-modifications on the other hand are ranged in two series with different dipole-moments, the moments of the β-modifications (the modifications which may be titrated without rearrangement) being considerably greater than those of the α-modifications.

REFERENCES

1. Veibel, S., and Arnfred, N. H. *Acta Chem. Scand.* **2** (1948) 914.
2. Veibel, S., and Arnfred, N. H. *Acta Chem. Scand.* **2** (1948) 921.
3. Michaelis, A. *Ann.* **373** (1910) 129.
4. See Cohen-Henriques, P. *Rec. Trav. Chim. Pays-Bas* **54** (1935) 327.

Received October 30, 1948.

ACTA CHEMICA SCANDINAVICA 2 (1948) 933—937

Short Communications

Observations on the Formation and Structure of Plastein

(3rd Preliminary Communication)

ARTTURI I. VIRTANEN, HEIKKI K. KERKKONEN AND TERTTU LAAKSONEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Butler *et al.*[1] have recently advanced the idea that in the process of plastein formation new peptide bonds do not arise in the protein hydrolysates. Precipitation would likewise not be caused by enzymatic reaction but by denaturation of some high-molecular products of proteolysis. This concept is based on the findings made with peptic digests of insulin.

The precipitation of the said authors does not correspond to the plastein formation examined in this laboratory during the recent years.

When zein is hydrolyzed with crystalline pepsin at about pH 1 at 37° C, the solution obtained contains 15—21 % α-amino-N estimated by van Slyke's method (5 min. shaking) and approximately the same amount by the Cu method by using the factor 0.14[2]. Taking into account the formation of free amino acids it can be concluded from the free amino groups that the average size of peptides in pepsin hydrolysate varies between hexa and tetrapeptides, depending on the reaction time and the quantity of pepsin. It is probable that at first octapeptides and still higher peptides are formed and from these tetrapeptides. In old pepsin hydrolysates there are still smaller peptides [3].

When pepsin is destroyed by keeping the solution for half an hour at 80—85°, the clear hydrolysate evaporated in a vacuum until the solution contains about 30 mg N per ml, and the pH of the solution adjusted to 4 with sodium hydroxide, the solution remains clear at room's temperature *. Crystalline pepsin admixed to the solution causes a very rapid formation of white precipitate. After standing overnight at 37° C the separated precipitate (»raw plastein») contains about 30 % of the nitrogen of the total hydrolysate. The amount of the precipitate varies greatly depending on the concentration of the hydrolysate as will be seen from Table 1.

During the precipitation of plastein amino N decreases. In our recent experiments the amount of amino N in *raw plastein + filtrate* has been 13.5—18 % lower than in the initial hydrolysate.** Hence, with the formation of plastein new peptide bonds are formed. Hydrazine which prevents the protein hydrolysis with pepsin [5] does not affect plastein formation.

---

* From a highly concentrated hydrolysate a brown sticky mass may be precipitated without pepsin when the pH of the solution is changed to 4. On the basis of the free NH_2 groups this substance is a mixture of open peptides whose average size corresponds to about hexapeptides, consequently, to somewhat larger peptides than those of the hydrolysate on an average. No decrease of amino groups does thereby occur, hence the question is about »denaturation» and precipitation reactions. Such a precipitate perhaps corresponds to the »plastein» of Butler *et al* [1].

** The values regarding the plastein formation from egg albumen given in the first communication [4] signify the decrease of the amino groups in the solution during precipitation of raw plastein and not the loss of the amino groups in the whole system.

Table 1. Dependence of the formation of raw plastein on the concentration of the pepsin hydrolysate of zein.

N mg/ml hydrolysate	N in pepsin mg/ml hydrolysate	Yield of raw plastein mg/40 ml hydrolysate	N of raw plastein, % of N in the hydrolysate
10	0.35	458	13.9
20	»	1658	25.2
30	»	3102	31.5
40	»	4561	34.7
56.5	»	6613	35.7

Table 2. Dependence of the formation of raw plastein on the concentration of pepsin.

N, mg/ml hydrolysate	N in cryst. pepsin, mg/ml hydrolysate	Yield of raw plastein mg/10 ml hydrolysate	N of raw plastein, % of N in the hydrolysate
28.35	0	0	0
»	0.1	859	32.7
»	0.3	996	36.5*
»	0.7	1012	37.2
»	1.0	1034	37.4

* The decrease of amino groups was abt. 17 % of the amino groups in the hydrolysate.

By allowing the raw plastein precipitate to stand for some days in water at room's temperature (toluene as a bactericide) large amount of peptides are brought into the solution. Their average size, on the basis of amino nitrogen, is about that of penta-hexapeptides. After this abundantly peptides still pass to boiling water. These correspond approximately to octadecapeptides on the basis of amino nitrogen. The higher the concentration of the hydrolysate from which raw plastein is precipitated, the more water-soluble parts the precipitate contains. The yield of raw plastein diminishes after extraction with cold and hot water, often with 40—50 % and more.

The peptides extracted from the raw plastein with cold and hot water do not give any precipitate with trichloracetic acid. It seems that they are not synthetical products but peptides formed during peptic hydrolysis.

The plastein left over after a thorough treatment with cold and hot water (»real plastein») still contains some amino nitrogen. The lowest percentage of amino N in our plastein preparations has been 2.2 % of total N. In the previous communication it has been held possible that the small amount of NH_2-N found in van Slyke's estimation may arise from amide groups and from the guanidine group of arginine. Since, however, the Cu-method yields approximately the same result as does van Slyke's method, evidently α-amino nitrogen is in question.

If the molecular weight of the insoluble plastein is less than 1000, as has been supposed, the substance must be a mixture of cyclopeptides [6]. The small amount of α-amino nitrogen may then result either from open peptides still present in the plastein preparation or from side chains attached to the ring. The low molecular weight of raw plastein (abt. 300) found cryoscopically in several estimations in phenol solution is, however, obviously too

low (cf. the corresponding estimations with gramicidin [7] in phenol giving values abt. 900 instead of the probable value abt. 3000). New estimations with thoroughly extracted plastein in formic acid have given values for mol. weight from 600 to 750 (Miettinen). In the peptic digest of plastein about 17—20 % amino nitrogen are formed, accordingly, the average size of cyclopeptides would correspond to tetra-pentapeptides [6], provided that amino nitrogen arises at the opening of the ring. The average mol. weight found cryoscopically in formic acid is in agreement with this opinion. The size of the individual cyclopeptides is naturally unknown.

The observations of Svedberg [8] and Ecker [9] that no sedimentation takes place in alkaline plastein solutions in the ultracentrifuge give evidence for a low molecular weight of plastein. Unfortunately, the preparations used for the estimations have been raw plasteins the amino N of which was unknown.

The observed »denaturation» of plastein during the hot water treatment adds for its part to the difficulties met in the plastein examination. Pepsin hydrolyzes rapidly only cold-water-treated plastein at pH 1—2, whereas after a thorough hot-water-treatment plastein is very slowly and incompletely hydrolyzed. The pepsin-resistant plastein dissolves also very difficultly in an alkaline solution at pH 9 to 13 where raw plastein dissolves very easily without decomposition (no rise in NH_2-N). Clear solution is obtained from the hot-water-treated plastein only by adding alcohol to the alkaline solution up to about 50—60 per cent. In the course of the hot water treatment of long duration amide nitrogen splits almost entirely from the plastein. This may, however, not be the cause to the rise of the pepsin-resistance of plastein and to the lowering of its solubility. We have discussed the possibility that the free carboxyls of the aminodicarboxylic acids would participate in the intramolecular ester formation and that the »denaturation» of plastein would result from such a lactone formation.

In view of the above observations, only the decrease in the amino nitrogen can be taken for a criterion of the formation of plastein. The amount of the precipitate and its nitrogen content do not give a real picture of it.

By using the membraneless electrophoresis apparatus of Spies [10] we are attempting to divide the pepsin hydrolysate of zein into different peptide fractions and to examine the plastein formation from peptides of different size.*

* *Addition to proof.*

In the first experiment on this line Virtanen and Maire Hakala formed plastein precipitates in different peptide fractions the average molecular size of which ranged from 4 to 6 peptides. The amino nitrogen of the plastein preparations formed was 2—3 % of the total nitrogen without cold and hot water extraction. The preparations were soluble in alkaline solution (pH 9). Cryoscopical determination of the molecular weight of a plastein with 2.1 % amino N gave in absolute formic acid a value of 445 which corresponds fairly well to cyclotetrapeptide.

1. Butler, J. A. V., Dodds, E. C., Phillips, D. M. P., and Stephen, J. M. L. *Biochem. J.* **42** (1948) 122.
2. Kerkkonen, H. K. *Acta Chem. Scand.* **2** (1948) 518.
3. Laine, T. *Ann. Acad. Sci. Fennicae, Ser. A. II. Chem.* No. 11 (1944).
4. Virtanen, A. I., and Kerkkonen, H. K. *Acta Chem. Scand.* **1** (1947) 140.
5. Schales, O., Suthon, A. M., Roux, R. M., Lloyd, E., and Schales, S. S. *Arch. Biochem.* **19** (1948) 119.
6. Virtanen, A. I., and Kerkkonen, H. K. *Nature* **161** (1948) 888.
7. Tishler, M., Stokes, J. L., Trenner, N. R., and Conn, J. B. *J. Biol. Chem.* **141** (1941) 197.
8. Folley, S. J. *Biochem. J.* **26** (1932) 99.
9. Ecker, P. G. *J. Gen. Phys.* **30** (1947) 399.
10. Spies, J. R. *J. Am. Chem. Soc.* **63** (1941) 1166.

Received December 20, 1948.

The Nitration of Tetraacetyl-β-Phenyl Glucoside

BENGT LINDBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In experiments dealing with the transformation of β-glucosides into α-glucosides, a glucoside of a nitrophenol was needed. Hurd and Bonner [1] have prepared tetraacetyl-β-*p*-bromphenyl glucoside by direct bromination of the phenyl glucoside in acetic acid. Analogously, tetraacetyl-β-phenyl glucoside could be nitrated with concentrated nitric acid in acetic acid. The yield was almost quantitative but the resulting product was a mixture of the ortho and para derivatives, which could be fractionated by recrystallization. Tetraacetyl-α-phenyl glucoside was treated in the same way, but in this case it was not possible to isolate any crystalline substances from the sirupy mixture obtained.

The structure of the substances obtained on nitration of tetraacetyl-β-phenyl glucoside was proved by comparison with authentic samples of tetraacetyl-β-*o*-nitrophenyl glucoside and the corresponding p-derivative, which were prepared according to Glaser and Wulwek [2]. For tetraacetyl-β-*o*-nitrophenyl glucoside Glaser and Wulwek report the rotation + 53° (chloroform) and the melting point 158—159°*. Montgomery, Richtmeyer and Hudson [3], who prepared the substance by a modification of the Helferich and Schmitz-Hillebrecht fusion procedure, report the rotation + 45° and the melting point 150—152°. The substance prepared by the two independent methods in this laboratory, rotated + 45° and melted at 158—159° (corr). The most probable explanation of this discrepancy is that Montgomery, Richtmeyer and Hudson obtained the pure substance but in a low-melting modification, and that the specific rotation reported by Glaser and Wulwek is incorrect (even the recalculated value). The present author, however, has never been able to isolate a low-melting modification of this compound.

Experimental. Tetraacetyl-β-phenyl glucoside (5 g) was dissolved in a mixture of acetic acid (40 ml) and acetic anhydride (2 ml). Almost dry nitric acid, b. p. 86°, (5 ml) was added and the mixture allowed to stand at room temperature. After an hour it was poured into ice water (1000 ml) containing sodium acetate (20 g). The white precipitate was filtered and dried (5.5 g). Separation of the ortho derivative was effected by recrystallization from hot ethanol. After four recrystallizations pure tetraacetyl-β-*o*-nitrophenyl glucoside (2.7 g) was obtained. $[\alpha]_D^{20}$ + 45°. M. p. 158—159°. It showed no m. p. depression with an authentic sample of tetraacetyl-β-*o*-nitrophenyl glucoside.

The mother liquors were concentrated and the residue recrystallized several times from a mixture of water and acetic acid when pure tetraacetyl-β-*p*-nitrophenyl glucoside (0.3 g) was obtained. $[\alpha]_D^{20}$ − 41°. M. p. 174—175°. It showed no m. p. depression with an authentic sample of tetraacetyl-β-*p*-nitrophenyl glucoside.

The new mother liquors were evaporated and the residue thoroughly mixed and dried. The product (1.8 g) showed a rotation of — 7.5°. Assuming that this mixture consists of the *o*- and *p*-derivatives, the total yield of *ortho* and *para* nitrophenyl glucosides was 70 % and 30 % respectively.

* The rotations reported in this paper had been calculated incorrectly. The above value is recalculated from the data given by the original authors. *Cf.* Helferich and Peters. *J. prakt. Chem.* [2] **138** (1933) 283, footnote 1. *Cf.* also ref. 3.

Equitransferent Solutions

K. V. GROVE-RASMUSSEN

Department of Inorganic Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark

When working with liquid junction potentials the present author found that the terminology is not adequate as there is no term which distinctly indicates that the total cation and the total anion mobilities are the same within a solution, *i. e.* the equation

$$\Sigma\, u_i \cdot c_i = \Sigma\, v_j \cdot c_j$$

is satisfied, u_i and v_j denoting the equivalent-conductances for cations and anions, respectively, and c_i and c_j the corresponding equivalent concentrations.

Acree and co-workers have applied the term isoelectric to the above condition. Their definition of this concept may be seen from the following quotation: »The ideal reference half-cell is one with a stable, reversible electrode (AgCl, Hg_2Cl_2), without hysteresis, with a low emf-temperature coefficient, and whose electrolyte is very concentrated and isoelectric, that is, of such a composition that equal numbers of positive and negative charges diffuse into its junction with another liquid whose pH is to be determined.«

It may be said in passing that it is very questionable whether we are justified in discussing a separate diffusion of positive and negative charges from one phase to another as such a phenomenon can hardly be imagined on account of the electrostatic forces present. The work quoted shows that the said authors have applied the term isoelectric to the quality defined above. Against this may, however, be objected, that the term isoelectric has already for many years been used to denote another quality, namely a certain state of a solution containing amfoions.

The present author therefore suggests the term equitransferent, as the quality we wish to describe involves that the effective cation- and anion-transference are equal in the solution in question.

The author wishes to thank *Statens Naturvetenskapliga Forskningsråd* for financial support and Mr. L. Asp for skilful assistance.

1. Hurd, C. D., and Bonner, W. A. *J. Am. Chem. Soc.* **67** (1945) 1764.
2. Glaser, E., and Wulwek, W. *Biochem. Z.* **145** (1924) 574.
3. Montgomery, E. M., Richtmeyer, N. K., and Hudson, C. S. *J. Am. Chem. Soc.* **64** (1942) 690.
4. Manov, G. G., De Lollis, N. J., and Acree, S. F. *J. Research Nat. Bur. Standards* **33** (1944) 273.

Received December 2, 1948.

Received December 18, 1948.